Entomology Monographs

Hideharu Numata
Kenji Tomioka *Editors*

Insect Chronobiology

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Insects are the most diverse group of organisms, and many biological advances have been derived from this group. Although entomology is considered to be a classic science, recent developments in molecular methods, application of modern theoretical concepts and collaboration with related sciences have opened new directions in entomology. Japanese scientists play a significant role in these fields, and this book series will focus on such developments. The book series Entomology Monographs publishes refereed volumes on all aspects of entomology, including ecology, ethology, physiology, taxonomy, systematics, morphology, evolutionary developmental biology, genetics, biochemistry, and molecular biology in insects and related arthropods. Authors are not restricted to Japanese entomologists, and other international experts will also be considered on the basis of their recent contribution to these fields.

Hideharu Numata • Kenji Tomioka
Editors

Insect Chronobiology

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Editors
Hideharu Numata
Institute for the Future of Human Society
Kyoto University
Kyoto, Japan

Kenji Tomioka
Graduate School of Natural Science and Technology
Okayama University
Okayama, Japan

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This book is dedicated to Yoshihiko Chiba (1931–2022), an insect chronobiologist who contributed greatly to the establishment of chronobiology in Japan. Yoshihiko was trained as an ecologist under Prof. Mutsuo Kato of Tohoku University, a pioneer of the study on insect rhythms in Japan. During this time, he started a chronobiological study on mosquito behaviour. After obtaining his PhD, he worked as an assistant professor at the same university for a while before moving to Yamaguchi University, where he established the Chronobiology Laboratory. His life-long research was aimed at elucidating the mechanism and biological significance of the bimodal activity rhythm of mosquitoes, which peaks in the morning and evening. He also conducted research on the link between the physiological state and behavioural rhythms of female mosquitoes. For his scientific contributions through these series of studies, he was awarded the Zoological Society Prize and the Aschoff and Honma Honorary Prize. During his active academic career, he made significant contributions to societies related to

chronobiology, including serving as the first president of the Japan Society for Chronobiology. After retiring from the university, he started a new carrier as an outstanding painter. He has produced many works that combine art and biology, and he has received many art awards. Yoshihiko became an unforgettable presence in both the scientific and artistic fields.

This photograph was taken at a French restaurant in Yamaguchi, Japan, on 11 March 2003. Left to right: Kenji Tomioka, Hideharu Numata, Yoshihiko Chiba, Shin-ichi Inouye, Katsuhiko Endo. Photo by Shin-ichi Inouye

A partial view of the work “Internal Clock” by Yoshihiko Chiba (2010)

Foreword

Almost seven decades ago, whilst an undergraduate student at King's College, University of London, I first encountered biological rhythms from lectures given by John Cloudsley-Thompson, who was conducting research on locomotor rhythmicity in cockroaches. Apart from a few such pioneers, the prevalent opinion at that time was either that these rhythms were a direct (exogenous) response to environmental conditions or merely trivial—or even regulated by unidentified factors from the cosmos. Since then, however, the true nature and importance of such phenomena have been revealed: the almost universal occurrence as endogenous biological processes in eucaryotes (and in some photosynthetic procaryotes), in which they regulate vital functions at all levels of organisation from molecules to behaviour and ecology. In this, insects have played a major role, not only because they are a dominant group of organisms, but because of their ease of laboratory maintenance, often short life cycles and, in some cases, their importance in agriculture, medicine or as model species in biological research. This last work culminated in the 2017 Nobel Prize awarded to Doctors Hall, Rosbash and Young for their work on circadian rhythmicity in *Drosophila melanogaster.* Today, the field of insect chronobiology generates wide research activity and seemingly countless publications! For this reason alone, books such as the present one are essential for today's researchers.

The book is organised in two main sections. After an opening chapter on historical aspects of insect chronobiology by Hideharu Numata, the first section—on the better-known and overt circadian rhythms—proceeds logically from general aspects through entrainment to their molecular and neural mechanisms, and finishes with rhythms in peripheral tissues, circa-bidian rhythms and rhythms in social insects. The second section then deals with rhythmic phenomena that display endogenous periods close to tidal, lunar or annual cycles and with seasonal photoperiodism, which has a circadian basis, but one that is not overtly expressed. Like the first section, the second proceeds, as far as is known, from general features through to neural and molecular aspects of the phenomena involved. Evolution of insect rhythms and accounts of seasonal timing in aphids and navigation are also described.

There is no doubt that the considerable advances in the field of insect chronobiology described in this book will facilitate further progress—for example in the molecular basis of photoperiodic time measurement and non-circadian periodicities to match that for circadian regulation of activity rhythms in *D. melanogaster*.

Edinburgh, UK
December 2022

David Saunders

Preface

Chronobiology of insects has a long history and has had a great influence on various disciplines: The pioneering work of Karl von Frisch's group from the first half of the twentieth century, followed by the comprehensive work of Colin Pittendrigh in the second half of the twentieth century. Junko Nishiitsutsuji-Uwo, a senior of Hideharu Numata, one of the editors of this book, at the Faculty of Science, Kyoto University, was the first to locate the circadian clock in the nervous system of the cockroach with Pittendrigh. More recently, the first clock gene was discovered in the fruit fly. Chronobiology is not restricted to the study of circadian clocks, which are responsible for the daily rhythm of animals. Clocks with different periods have evolved as adaptations to environmental variations such as tidal, (semi)lunar, and annual cycles. Photoperiodism, which has also evolved to cope with seasonal variations, is strongly related to the circadian clock. Time compensation by a circadian clock is essential for celestial navigation as shown by von Frisch in the honey bee. Insects have played important roles in elucidating these broad mechanisms.

Currently, both of the editors are still employed by the universities but officially retired from their professorships at their Graduate School in March 2021 at the age of 65. They have both been involved in insect chronobiology research since 1980, which means that they have been working in this field for more than 40 years. Their dates of birth are also close, and they have always enjoyed talking about insect chronobiology whenever they meet. They hit it off when they decided to plan a monograph on this field together at their first retirement. In addition to the chapters that the editors themselves have contributed to this book, they have also invited their colleagues to write concise summaries of what is currently known about insect chronobiology in their respective fields. The editors hope that this book will be useful not only to researchers and those who aim to become researchers in the field of insect chronobiology but also to non-insect chronobiologists, non-chronobiology entomologists, and graduate and undergraduate students in biology.

During the development of the book, Fumiko Yamaguchi, Springer Nature, Japan, kindly gave us expert advice. The editors thank their wives, Wakaha Numata and Fumiko Tomioka, for tolerating and supporting the many hours not only during

editing this book but also over the long years of their research in this field. This book is inspired by the vision of the pioneers of insect chronobiology, especially Yoshihiko Chiba, to whom we dedicate it.

Kyoto, Japan — Hideharu Numata
Okayama, Japan — Kenji Tomioka
December 2022

Contents

Chapter 1
Historical Survey of Chronobiology with Reference to Studies in Insects

Hideharu Numata

Abstract Chronobiology is the study of the temporal characteristics of biological phenomena. Humans must have recognized before the establishment of civilization that the activity of organisms has periodicity. Studies in chronobiology have advanced from the folklore to the molecular level by way of natural history and classic experiment levels. During these processes, observations and experiments in insects have made significant contributions. The climax was the discovery of molecular mechanisms of the circadian clock in *Drosophila melanogaster* in the late twentieth century. Currently, chronobiology is expanding further to various aspects, such as circadian clocks in various organisms, molecular and neural mechanisms of photoperiodism, clocks of which the period is different from approximately 24 h, and the ecological aspect of biological clocks.

Keywords Biological clock · Circadian clock · Exogenous timing hypothesis · Photoperiodism · Solar compass · Time memory

1.1 What Is Chronobiology?

Chronobiology is a combination of *chronos*, which means time in Greek, and biology. This word was proposed by Franz Halberg in 1950 and became a common term after his review named "Chronobiology" (Halberg 1969). According to Halberg (1969), chronobiology is defined as the study of the temporal characteristics of biological phenomena. If we understand it as time-related biology, all developmental phenomena should be the objects of chronobiology. However, the discipline of chronobiology actually focuses on the periodic activity of living organisms that repeats over time, rather than phenomena such as development that progresses in one direction along the time axis.

H. Numata (✉)
Institute for the Future of Human Society, Kyoto University, Kyoto, Japan
e-mail: numata.hideharu.8r@kyoto-u.jp

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Almost all organisms on the Earth are subject to environmental changes. Whereas some environmental changes occur completely irregularly, others have a fixed period. There are at least five types of environmental changes with constant periods due to geophysical mechanisms. These cycles are the tidal cycle (12.4 h), daily cycle (24 h), semilunar cycle (spring tide and neap tide cycle, 14.8 days), lunar cycle (29.5 days), and annual cycle (365.2 days). All of them are brought by the positional relationship between the Earth, the moon, and the sun. Changes in the environment with such cycles may directly affect the activity of the organism and produce periodicity, but conversely, to respond to the periodic change in the environment, the organism autonomously becomes periodic. Organisms have acquired a physiological mechanism that indicates the above, the biological clock. The main object of chronobiology is the phenomenon related to the biological clock.

It is common knowledge today that "the clock exists in the body of organisms" and at least those who have learned biology do not doubt its existence. However, when we think about it without knowledge in modern biology, the biological clock seems to be a truly mysterious mechanism. The first clock invented by human beings was the ancient Egyptian sundial. However, the sundial did not keep track of time autonomously but used the movement of the sun in the sky. Later, water clocks and hourglasses were invented, but none of them autonomously showed periodicity. They were one-shot mechanisms that ended when water or sand fell down. Most likely the first clock rotating once a day was made by improving the water clock in China during the Tang dynasty. Eventually, mechanical watches using pendulums, springs, and gears developed, and wristwatches appeared approximately 1800. It has not been easy to believe that organisms have such a mechanism because small and accurate clocks were invented only in modern times in the long history of mankind. In fact, there was fierce controversy before the idea that the periodicity of organisms was produced by the biological clock was established (see Sect. 1.4). In this chapter, I summarize the history of chronobiology, focusing on insect clocks.

1.2 From Folklore to Natural History

Humans must have recognized before the establishment of civilization that the activity of organisms has periodicity. Humans are animals that grow and act by eating other organisms. To acquire food resources efficiently, therefore, it is essential to know when and where prey organisms exist. Especially for human ancestors who began to eat the meat of other animals instead of the fruit diet, it must have become even more important to know when and what activities the prey animals perform. Moreover, it is also important to know the activities of animals that are harmful to humans. Therefore, knowledge of chronobiology must have increased the survival value of humans since prehistoric times. That is, we are born chronobiologists.

Let me give you one famous example. The palolo worm *Palola viridis* (Annelida: Polychaeta) reaching 40 cm in length lives in a cave at a depth of 3–5 m on a coral reef of Fiji and Samoa in the southwest Pacific (Fig. 1.1). When a palolo worm

Fig. 1.1 A sexually matured adult female of the Palolo worm *Palola viridis*. The green posterior region of the body (epitoke) contains mature eggs. Photo by Masa Ushioda, Blue Planet Archive LLC

sexually matures, the posterior region of the body swells with developed gonads (the epitoke). Then, twice at the end of October and the beginning of November, when the dawn overlaps with the high tide on neap tide days, the developed posterior region tears off from the head region and swims, reaching the sea surface, and spawns. This phenomenon is called rising. Thereafter, the head region remaining in the cave regenerates the posterior region. William Burrows, a resident commissioner of Fiji, reported that the developed gonads of palolo worms are very tasty, and people living on these islands board a small boat and scoop up a large number of gonads every year at the time of rising (Burrows 1945). In other words, the people of the islands accurately handed down the day and time when palolo worms swim. Schulze (2006) summarized the knowledge in the phylogeny of palolo worms and showed early references of their rising and its use as a food source by native people of Pacific islands.

In the eighteenth century, Jean-Jacques d'Ortous de Mairan observed that the movement of the leaves of the sensitive plant (probably *Mimosa pudica*) to open during the day and hang down at night continued even when the plant was transferred to dark places. In the current knowledge, this is proof of the existence of a biological clock and an excellent experimental result in chronobiology. However, he interpreted the result that the plant is sensitive to external stimuli showing day and night, even without sunlight (Anonymous 1729).

In the 1900s, observations that the honeybee *Apis mellifera* might learn and memorize the time were reported (see Renner 1960). Hugo von Buttel-Reepen noticed that bees came to the buckwheat field exactly between 10 and 11 am when the buckwheat flowers secreted nectar, as if the bees knew the time. He concluded that such behavior was only possible if the bees had a sense of time (Zeitsinn) (von Buttel-Reepen 1900, 1915). In 1906, when Auguste Forel had breakfast on the terrace facing the garden every day between 7:30 and 9:30 am, he always observed that bee workers came to the jam that he intended to spread to pieces of bread. Even on the day when Forel did not supply jam, the bees came to the terrace. This result means that the bees were not attracted to the color or smell of the jam but remembered the time and place in relation to each other (Forel 1910). Based on this observation, Forel (1910) proposed the term time memory (Zeitgedächtnis).

1.3 From Natural History to Experimental Science

It was the group of Karl von Frisch, one of the winners of the Nobel Prize in Physiology or Medicine 1983, who experimentally proved the time memory in honeybees suggested by Forel (1910). Ingeborg Beling placed sugar solution on a desk a few meters away from a honeybee nest and trained bee workers to come at a fixed time every day, marking each bee to identify the individual. Then, on the last day, she observed which bees came to the desk all day without sugar solution, and most of the bees came to the desk at the time of training. In other words, the bees actually learned the time. Next, she performed the same experiment in the laboratory where the light, temperature, and humidity were kept constant, but the behavior of the bees did not change, so the change in the position and brightness of the sun, temperature, and humidity was not used as a clue. However, the electrical conductivity of air changed during the day, and it was possible that the air was reacting to the bees as it freely moved in and out of the laboratory. Therefore, radium was used to increase the ions in the air to increase the electrical conductivity throughout the day, but the time that the bees remembered was still correct (Beling 1929). In addition, Oskar Wahl, another student of von Frisch, examined the effects of cosmic rays. Cosmic rays fluctuate daily, passing through the roofs and walls of buildings and reaching the inside of the laboratory. Therefore, he brought a honeybee nest into a salt mine that was too deep for cosmic rays to reach. The same experiment was performed, where the bees showed that they knew the time (Wahl 1932). In this way, even if the external cues were eliminated, the bees remembered the time, so it became accepted that there is an endogenous clock.

Before these experiments, Garner and Allard (1920) reported that the Maryland mammoth variety of the tobacco *Nicotiana tabacum* had flower buds when the light period of a day was artificially shortened. They first revealed that an organism reacts to the photoperiod and named the response photoperiodism. Three years later, Marcovitch (1923) showed the first example of photoperiodism in animals. Under natural conditions, the strawberry aphid, *Aphis forbesi*, switches from

parthenogenesis to bisexual reproduction in November in Tennessee. When transferred to a short day in February, the aphid changed the reproductive mode in May, much earlier than under natural conditions. Furthermore, Kogure (1933) showed that the induction of egg diapause depends on the photoperiod and temperature of the maternal egg period in the silk moth *Bombyx mori*. Thereafter, it was shown in many insects that diapause is regulated by photoperiodism (see Chap. 12).

Erwin Bünning proposed the idea that time measurement in photoperiodism is determined by the relationship between the phase of an endogenous rhythm and light, based on the experimental results on the endogenous rhythm in the runner bean, *Phaseolus coccineus* (Bünning 1936; see Bünning 1960 also). He postulated the existence of distinct scotophil (dark-requiring) and photophil (light-requiring) sections of the rhythm, and a photoperiodic effect is triggered according as to whether light falls in the scotophil. In 1936, when Bünning first proposed the idea, the word circadian clock had not yet been coined for a biological clock with a period of approximately a day (see Sect. 1.4), but this idea in current terms that "the circadian clock is involved in the measurement of daylength in photoperiodism" is later called Bünning's hypothesis (Pittendrigh 1960). It is now known that the time measurement in photoperiodism is not as simple as the original Bünning's hypothesis, which cannot explain various responses in photoperiodism. However, it is now believed that the circadian clock is involved in photoperiodism (e.g., Numata et al. 2015; Saunders 2021). Because the involvement of the circadian clock was first presented by Bünning (1936), the monumental value of Bünning's hypothesis is great. Although Bünning was a plant physiologist, he also published some experimental results on the photoperiodic induction of pupal diapause in the large white butterfly, *Pieris brassicae*, which supported Bünning's hypothesis (e.g., Bünning and Joerrens 1959; Bünning 1960).

The first insect shown to have diapause controlled by photoperiod was *B. mori*, which can only live in an artificial environment (Kogure 1933). However, Danilevskii (1961) clarified the implications of photoperiodism in the life cycle of wild insects from the 1940s to the 1960s. He examined geographical variation in the critical daylength, the daylength at the boundary that distinguishes between long days and short days in photoperiodism, within a species and revealed that variation in the critical daylength shows the life cycle adaptation to local climates of the insect. In general, the higher the latitude is, the faster the arrival of winter and the larger the fluctuation of the daylength. Therefore, the higher the latitude is, the longer the critical daylength for inducing winter diapause. By comparing populations in vast areas of the Soviet Union, including current Russia, Ukraine, and Georgia, Danilevskii (1961) concluded that a 5-degree higher latitude would increase the critical daylength by approximately 1.5 h. It is notable that he showed that we can discuss seasonal adaptations of insects based on the experimental results obtained in the laboratory.

I mentioned above the studies by two disciples of von Frisch, but here I show a study by himself. In 1919, von Frisch found that when a honeybee worker returned to the nest after finding food in the field, she was dancing to inform nestmate workers that there was food. In 1948, von Frisch published that honeybees are telling the

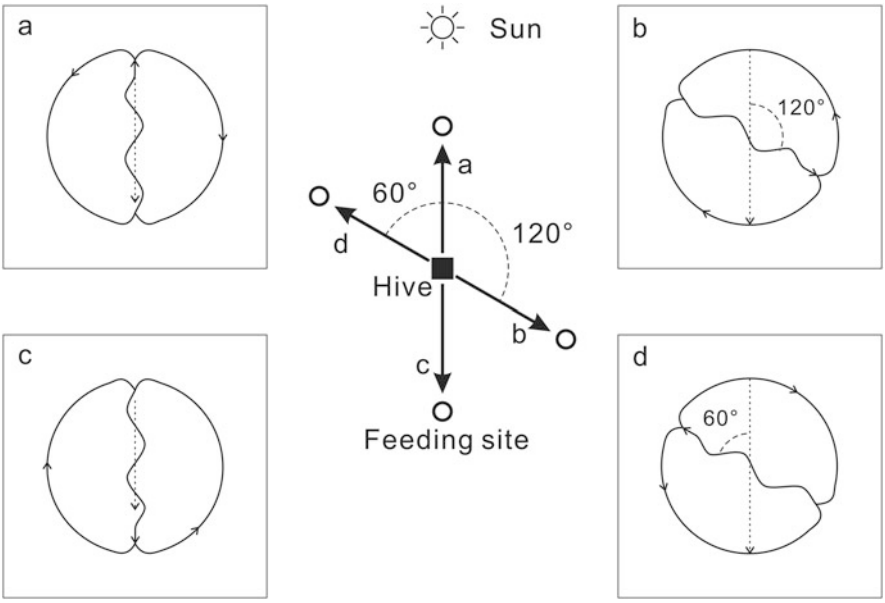

Fig. 1.2 Solar compass of the honeybee *Apis mellifera*. Workers show the direction of the feeding site by waggle dance on the vertical honeycomb according to the position of the sun. Broken arrows show the direction of gravitational force. Based on von Frisch (1948)

direction and distance of the feeding site with this dance, later known as the waggle dance. The angle between the direction of the feeding site and the direction of the sun is expressed as the angle between the straight line in the middle of the waggle dance and the vertical line (Fig. 1.2; von Frisch 1948). This mechanism of orientation based on the position of the sun is called the solar compass. Even more surprisingly, honeybees can know the position of the sun even if they cannot see the sun itself. If honeybees can see the blue sky, they use its polarization pattern. Unlike the magnetic compass, where the north pole always points north and the South Pole points south, in the case of a solar compass, the direction indicated by the sun changes over time. Therefore, the sun compass is useless unless the direction indicated by the sun is corrected according to the time of day. In fact, honeybees know the change in the position of the sun and correct the direction indicated by the solar compass. Von Frisch (1950) observed that honeybees doing a waggle dance change the straight part of the dance counterclockwise over time. Moreover, when the nest was transferred elsewhere during the night when the bees were resting, honeybees searched for food in the same direction as yesterday in the next morning (even if the position of the sun was opposite). The results showed that the solar compass of the honeybee is time-compensated. Kramer (1950) also reported a time-compensated solar compass in the common starling, *Sturnus vulgaris*. It is called celestial navigation that an animal knows the direction based on the position of a celestial body to move, and its typical

example is a solar compass. Thus, time-compensated celestial navigation was discovered almost at the same time by von Frisch and Kramer.

Approximately a quarter century after Beling (1929) and Wahl (1932), Max Renner, also a student of von Frisch, transported honeybees on a passenger plane over the Atlantic and examined what time they arrived for food. Renner (1955a) placed a honeybee nest in a large box of 7 m × 3 m × 3 m to allow humans to enter and feed the bees. Renner (1955b) prepared two boxes in Paris and in New York and first flew bees who learned when to feed in Paris to New York. Because a jet plane entered service on the transatlantic route in 1958, it took more than 16 h to be transported by a propeller plane at this time. The bees, trained to take sugar solution at 8 am in Paris, flew to the feeding place at 3 am on the next day in New York. Considering the time difference of 5 h between Paris and New York, it means that they flew to the feeding place 24 h after the time when they fed the day before. Next, Renner (1955b) conducted an experiment in which other bees were trained in New York and then carried to Paris, but again the bees visited the feeding place at the time of the city where they were first trained. In other words, it was clarified that the honeybee does not respond to any stimulus from the outside world after moving, but the time memory is based on the time indicated by the endogenous clock. Today, every time we travel abroad by jet, we suffer from jet lag as an unintentional experimental result, but the significance of conducting such an experiment in an animal for the first time is great.

1.4 Endogenous or Exogenous?

It came to be recognized that there is an endogenous clock in organisms because the endogenous clock was considered essential for the time memory and the correction of the solar compass of honeybees and the photoperiodic time measurement of many plants and animals. This concept is called the biological clock theory. As mentioned above, the role played by the experiments in insects was great in the establishment of the theory.

However, Frank Brown Jr. raised a strong doubt on the biological clock theory and argued against it (Brown 1960, 1970). The most obvious rationale for those who insist on the biological clock theory is that the activity of organisms is repeated at regular intervals when the environmental conditions are kept constant in the laboratory. In chronobiology, the rhythm observed under constant conditions is called the free-running rhythm, and the cycle length is called the free-running period, abbreviated as τ in the Greek letter.

Brown argued that the researchers kept the environmental conditions constant only by keeping some conditions constant within the range that the researchers could control. In many experiments that apparently have shown a free-running rhythm, light and temperature are kept constant, and food and water are continuously supplied. However, what about other factors? In fact, many factors, such as atmospheric pressure, gravity, magnetic field, electric field, electrical conductivity of air,

and cosmic rays, should change periodically in these experiments. There are some cases where the effects of electrical conductivity and cosmic rays are individually denied (Beling 1929; Wahl 1932), but it is extremely difficult to keep all conditions constant. Brown argued that some factors from the outside world that researchers could not keep constant may affect the activity of organisms, resulting in the appearance of periodicity. In other words, what looks like the nature of an organism's clock is the manifestation of the organism's constant response to geophysical and astrophysical actions. This is called the exogenous timing hypothesis (Brown 1983). In many cases, the fact that the cycle of the free-running rhythm deviates from 24 h, which is the cycle of environmental fluctuations, and that there are slight differences among individuals is the argument of the biological clock theory. However, Brown argued that the combination of responses to various external stimuli could be explained without a biological clock. Then, to prove the exogenous timing hypothesis, various experiments were conducted, such as using cesium-137 to periodically apply weak gamma rays to organisms (Brown 1970, 1983).

Despite Brown's struggle, the biological clock theory is generally accepted today. However, the exogenous timing hypothesis was not denied by a single definitive experiment. In principle, the exogenous timing hypothesis is a hypothesis that cannot be logically denied. This is because even if we conduct an experiment with all possible environmental conditions constant and claim that there is an endogenous clock in the organism, there are many other factors that cannot be controlled by the experiment. However, no biologist currently supports the exogenous timing hypothesis. This is because those who believe in the existence of biological clocks have become more confident by accumulating experimental evidence one after another, and at the same time, Brown's explanation by the influence from the outside world was becoming increasingly complicated. Finally, no one accepts it.

There are numerous experiments conducted by those who support the biological clock theory, but here I show a large-scale experiment that clearly aims to counter Brown's theory. In 1960, Karl Hamner and his collaborators in the United States carried the following five organisms to the South Pole: the golden hamster, *Mesocricetus auratus*, the common bean *Phaseolus vulgaris*, the red bread mold *Neurospora crassa*, the fruit fly *Drosophila pseudoobscura*, and the American cockroach *Periplaneta americana*. It was already known that the locomotor activity of *M. auratus* and *P. americana*, the eclosion of *D. pseudoobscura*, the vertical movement of the leaves of *P. vulgaris*, and the mycelium growth of *N. crassa* show a free-running rhythm. Hamner et al. (1962) placed these organisms on an aluminum turntable that rotates counterclockwise once a day in a garage only approximately 800 m from the South Pole. The temperature was kept at 20–22 °C, and the activity of these organisms was recorded in darkness. As a result, the four species of organisms except *P. americana* showed a free-running rhythm, similar to that observed in the United States (Hamner et al. 1962). Under these conditions, at least the stimuli associated with the rotation of the Earth were denied as the cause of the rhythms because the four organisms showed free-running rhythms for different activities even though all geophysical changes of the day were removed. Curiously, *P. americana* did not show periodicity in its activity in Antarctica, but

when the same strain was examined later in the United States, it did not show periodicity. It seems that Hamner et al. (1962) accidentally brought such a strain to Antarctica.

Halberg (1959) proposed the term circadian rhythm by combining the Latin "approximately" *circa* and "day" *dies* for the endogenous rhythm that controls the diurnal activity of organisms. The term quickly became established and is now widely used.

1.5 Localization of the Circadian Clock

Although it became recognized that the clock was in the body of organisms, it was not clear for a long time where the clock actually existed in the body. Janet Harker was the first to be interested in this question. She concluded that the circadian clock is in the subesophageal ganglion and the light received by the ocellus entrains the clock to light-dark cycles based on the results of a series of experiments conducted on the nocturnal locomotor activity rhythm in *P. americana* (Harker 1956).

Colin Pittendrigh had doubts about these experimental results and their interpretation by Harker (1956). Pittendrigh was studying the circadian eclosion rhythm of *D. pseudoobscura* and clarified the properties of the circadian clocks one after another since the 1950s: the temperature compensation of the free-running rhythm, the phase response curve to light stimuli, and the existence of a transient are all shown first by Pittendrigh and his coworkers (Pittendrigh 1954, Pittendrigh and Bruce 1957, Pittendrigh et al. 1958; see Pittendrigh 1993 also). Pittendrigh conducted a replication study of Harker's experiment with his graduate student Shephard Roberts but could not reproduce the results at all (Roberts 1965, 1966). Therefore, Pittendrigh welcomed Junko Nishiitsutsuji-Uwo (Fig. 1.3), who specializes in surgery of the insect nervous system, to his laboratory and examined the effects of painting and removal of photoreceptors, cutting of nerves and partial removal of the nervous system on the locomotor activity rhythm in the cockroach *Rhyparobia* (*Leucophaea*) *maderae*. The result was quite different from Harker's: the circadian clock that controls the activity rhythm exists in the optic lobe, and the light that entrains this clock to light-dark cycles is received by the compound eye (Nishiitsutuji-Uwo and Pittendrigh 1968a, 1968b). The optic lobe is located between the compound eye and the central brain and processes the optical information that enters the compound eye. It was pointed out that Harker's experiments and analyses had some deficiencies, and the results were completely denied. Nishiitsutuji-Uwo and Pittendrigh (1968a, 1968b) is the first localization of the biological clock before the discovery of the mammalian circadian clock in the suprachiasmatic nucleus (Moore and Eichler 1972; Stephan and Zucker 1972).

Thereafter, in *R. maderae* and the cricket *Gryllus bimaculatus*, it was shown that even if the optic lobe is completely excised from the body and cultured, the electrical activity maintains a periodicity of approximately one day (Collwell and Page 1990; Tomioka and Chiba 1992). Therefore, it was undoubtedly proven that a circadian

Fig. 1.3 Colin Pittendrigh (left) and Junko Nishiitsutsuji-Uwo (right). Pittendrigh visited Kyoto, Japan, for attending the 16th International Congress of Entomology in August 1980. Photo by Hideharu Numata

clock exists in the optic lobe. In addition, from the expression of clock genes and clock proteins, some cell groups in the brain are shown to be the main body of the circadian clock in *D. melanogaster* (Ewer et al. 1992).

1.6 Molecular Mechanism of the Circadian Clock

Because the biological clock is innate, it is naturally expected that genes control it. However, showing the expectation by experiments seemed very difficult even approximately 1970, when the biological clock theory was almost accepted. Konopka and Benzer (1971) obtained three mutants of a single locus on the X chromosome in *D. melanogaster* by treatment with a chemical mutagen. These mutants had abnormal circadian rhythms in eclosion and adult activity, and the gene was named *period*. This is the first study to demonstrate that a gene constitutes a biological clock (Konopka and Benzer 1971).

Beginning with the sequence of *period* of *D. melanogaster*, clock gene structures have been determined one after another in various insects (Bargiello et al. 1984; Reddy et al. 1984; see Tomioka and Matsumoto 2015). Studies on the molecular mechanism of the circadian clock in vertebrates were started to find a homolog of *period* of *D. melanogaster* (Tei et al. 1997; Sun et al. 1997). The Nobel Prize in Physiology or Medicine 2017 was awarded jointly to Jeffrey Hall, Michael Rosbash, and Michael Young for their discoveries of molecular mechanisms controlling the circadian rhythm (https://www.nobelprize.org/prizes/medicine/2017/summary/).

The most important knowledge for this award is that the negative feedback loop between clock genes and their product proteins produces oscillation of the circadian clock. This was also reported first in *D. melanogaster* (Hardin et al. 1990).

1.7 Future Forecast

Insects show various phenomena related to clocks, and many interesting studies have been conducted. I counted the number of research papers on insect clocks indexed in Web of Science™ over the past 73 years (Fig. 1.4). The number of papers in this field has increased drastically since 1990, and more than 200 papers have been published every year since 2007. This increase started just after the discovery of the negative feedback loop (Hardin et al. 1990), and the proportion of *Drosophila* papers also increased in this period (gray bars, Fig. 1.4). Therefore, the increase is apparently due to the discovery of the molecular mechanism controlling the circadian rhythm, for which the Nobel Prize in Physiology or Medicine 2017 was awarded later.

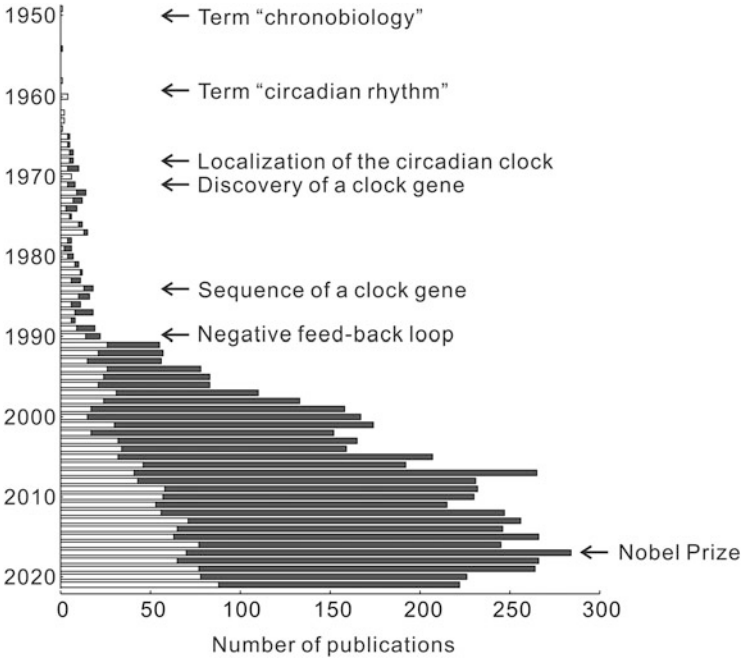

Fig. 1.4 The number of research papers on insect clocks indexed in Web of Science™ over the past 73 years. Historically important events in chronobiology are also shown. Gray and white sections of horizontal bars represent the number of papers in *Drosophila* and that in the other insects, respectively

As shown in this chapter, chronobiology has gone deeper into the elucidation of the molecular mechanism of the circadian clock in *Drosophila*. However, insect chronobiology should not be restricted to the molecular mechanism of the circadian clock nor in *Drosophila*. In the next era, I expect that chronobiology will expand more widely: for example, circadian clocks in various organisms; the molecular and neural mechanisms of photoperiodism, the period of which is different from approximately 24 h; and the ecological aspect of biological clocks will be in the limelight. The fact that the number of chronobiology papers out of *Drosophila* has steadily increased since 2000 is a sign of such expansion (white bars, Fig. 1.4).

References

Anonymous (1729) Observation botanique. In: Histoire de l'Académie Royale des Sciences. Année 1729, pp 35–36. https://www.biodiversitylibrary.org/item/88101#page/49/mode/1up

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. Nature 312:752–754. https://doi.org/10.1038/312752a0

Beling I (1929) Über das Zeitgedähtnis der Bienen. Z Vgl Physiol 9:259–338. https://doi.org/10.1007/BF00340159

Brown FA Jr (1960) Response to pervasive geophysical factors and the biological clock problem. Cold Spring Harb Symp Quant Biol 25:57–71. https://doi.org/10.1101/SQB.1960.025.01.007

Brown FA Jr (1970) Hypothesis of environmental timing of the clock. In: Brown FA Jr, Hastings JW, Palmer JD (eds) The biological clock two views. Academic Press, New York

Brown FA Jr (1983) The biological clock phenomenon: exogenous timing hypothesis. J Interdiscipl Cycle Res 14:137–162. https://doi.org/10.1080/09291018309359807

Bünning E (1936) Die endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion. Ber Dtsch Bot Ges 54:590–607. (cited in Bünning, 1960)

Bünning E (1960) Circadian rhythms and the time measurement in photoperiodism. Cold Spring Harb Symp Quant Biol 25:249–256. https://doi.org/10.1101/SQB.1960.025.01.026

Bünning E, Joerrens G (1959) Versuche zur photoperiodischen Diapause-Induktion bei *Pieris brassicae* L. Naturwissenschaften 46:518–519. https://doi.org/10.1007/BF00703549

Burrows W (1945) Periodic spawning of 'Palolo' worms in Pacific waters. Nature 155:47. https://doi.org/10.1038/155047a0

Collwell CS, Page TL (1990) A circadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. J Comp Physiol A 166:643–649. https://doi.org/10.1007/BF00240014

Danilevskii AS (1961) Photoperiodism and seasonal development of insects. Leningrad University Press, Leningrad (in Russian). English translation by Johnston J. Oliver & Boyd, Edinburgh (1965)

Ewer J, Frisch B, Hamblen-Coyle MJ, Rosbash M, Hall JC (1992) Expression of the period clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. J Neurosci 12:3321–3349. https://doi.org/10.1523/JNEUROSCI.12-09-03321

Forel A (1910) Das Sinnesleben der Insekten. Reinhardt, München

Garner WW, Allard HA (1920) Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. J Agricultural Res 58:553–606

Halberg F (1959) Physiologic 24-hour periodicity; general and procedural considerations with reference to the adrenal cycle. Z Vitamin Hormon Fermentforsch 10:225–296. (cited in Halberg, 1969)

Halberg F (1969) Chronobiology. Annu Rev Physiol 31:675–726. https://doi.org/10.1146/annurev.ph.31.030169.003331

Hamner KC, Flinn JC, Sirohi GS, Hoshizaki T, Carpenter BH (1962) The biological clock at the south pole. Nature 195:476–480. https://doi.org/10.1038/195476a0

Hardin PE, Hall JC, Rosbash M (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature 343:536–540. https://doi.org/10.1038/343536a0

Harker JE (1956) Factors controlling the diurnal rhythm of activity of *Periplaneta americana* L. J Exp Biol 33:224–234. https://doi.org/10.1242/jeb.33.1.224

Kogure M (1933) The influence of light and temperature on certain characters of the silkworm, *Bombyx mori*. J Dept Agric Kyushu Imp Univ 4:1–93. https://doi.org/10.5109/22568

Kramer G (1950) Weitere Analyse der Faktoren, welche die Zugaktivität des gekäfigten Vogels orientieren. Naturwissenschaften 37:377–378. https://doi.org/10.1007/BF00626007

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 68:2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Marcovitch S (1923) Plant lice and light exposure. Science 58:537–538. https://doi.org/10.1126/science.58.1513.537-a

Moore RY, Eichler VB (1972) Loss of circadian adrenal corticosterone rhythm following suprachiasmatic nucleus lesion in the rat. Brain Res 42:201–206. https://doi.org/10.1016/0006-8993(72)90054-6

Nishiitsutuji-Uwo J, Pittendrigh CS (1968a) Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. Z Vergl Physiol 58:1–13. https://doi.org/10.1007/BF00302433

Nishiitsutuji-Uwo J, Pittendrigh CS (1968b) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? Z Vergl Physiol 58:4–46. https://doi.org/10.1007/BF00302434

Numata H, Miyazaki Y, Ikeno T (2015) Common features in diverse insect clocks. Zool Lett 1:10. https://doi.org/10.1186/s40851-014-0003-y

Pittendrigh CS (1954) On temperature independence in the clock system controlling emergence time in *Drosophila*. Proc Natl Acad Sci U S A 40:1018–1029. https://doi.org/10.1073/pnas.40.10.1018

Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. Cold Spring Harb Symp Quant Biol 25:59–184. https://doi.org/10.1101/SQB.1960.025.01.015

Pittendrigh CS (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol 55:17–54. https://doi.org/10.1146/annurev.ph.55.030193.000313

Pittendrigh CS, Bruce VG (1957) An oscillator model for biological clocks. In: Rudnick D (ed) Rhythmic and synthetic processes in growth. Princeton University Press, Princeton, pp 75–109

Pittendrigh CS, Bruce VG, Kaus P (1958) On the significance of transients in daily rhythms. Proc Natl Acad Sci U S A 44:965–973. https://doi.org/10.1073/pnas.44.9.965

Reddy P, Zehling WA, Wheeler DA, Pirrotta V, Hadfield C, Hall JC, Rosbash M (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell 38:701–710. https://doi.org/10.1016/0092-8674(84)90265-4

Renner M (1955a) Über die Haltung von Bienen in geschlossenen, künstich beleuchteten Räumen. Naturwissenschaften 42:539–540. https://doi.org/10.1007/BF00630155

Renner M (1955b) Ein Transozeanversuch zum Zeitsinn der Honigbiene. Naturwissenschaften 42:540–541. https://doi.org/10.1007/BF00630156

Renner M (1960) The contribution of the honey bee to the study of time-sense and astronomical orientation. Cold Spring Harb Symp Quant Biol 25:361–367. https://doi.org/10.1101/SQB.1960.025.01.037

Roberts SK (1965) Photoreception and entrainment of cockroach activity rhythms. Science 148:958–959. https://doi.org/10.1126/science.148.3672.958

Roberts SK (1966) Circadian activity rhythms in cockroaches. III. The role of endocrine and neural factors. J Cell Physiol 67:473–486. https://doi.org/10.1002/jcp.1040670312

Saunders D (2021) Insect photoperiodism: Bünning's hypothesis, the history and development of an idea. Eur J Entomol 118:1–13. https://doi.org/10.14411/eje.2021.001

Schulze A (2006) Phylogeny and genetic diversity of palolo worms (*Palola,* Eunicidae) from the tropical North Pacific and the Caribbean. Biol Bull 210:25–37

Stephan FK, Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity are eliminated by suprachiasmatic lesions. Proc Natl Acad Sci U S A 54:1521–1527. https://doi.org/10.1073/pnas.69.6.1583

Sun ZS, Albrecht U, Zhuchenko O, Bailey J, Eichele G, Lee CC (1997) RIGUI, a putative mammalian ortholog of the *Drosophila period* gene. Cell 90:1003–1011. https://doi.org/10.1016/S0092-8674(00)80366-9

Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, Sakaki Y (1997) Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. Nature 389:512–516. https://doi.org/10.1038/39086

Tomioka K, Chiba Y (1992) Characterization of an optic lobe circadian pacemaker by in situ and in vitro recording of neural activity in the cricket, *Gryllus bimaculatus*. J Comp Physiol A 171:1–7. https://doi.org/10.1007/BF00195955

Tomioka K, Matsumoto A (2015) Circadian molecular clockworks in non-model insects. Curr Opin Insect Sci 7:58–64. https://doi.org/10.1016/j.cois.2014.12.006

von Buttel-Reepen H (1900) Sind die Bienen Refflex-maschinen? Experimental Beiträge zur Biologie der Honigbiene. Biol Zentralbl 20:97–108. (cited in Renner, 1960)

von Buttel-Reepen H (1915) Leben und Wesen der Bienen. Vieweg und Sohn, Braunschweig. (cited in Renner, 1960)

von Frisch K (1948) Gelöste und ungelöste Rätsel der Bienensprache. Naturwissenschaften 35:12–23. https://doi.org/10.1007/BF00626624

von Frisch K (1950) Die Sonne als Kompaß im Leben der Bienen. Experientia 6:210–221. https://doi.org/10.1007/BF02173654

Wahl O (1932) Neue Untersuchungen über das Zeitgedächtnis der Bienen. Z Vergl Physiol 16:529–589. https://doi.org/10.1007/BF00338333

Part I
Insect Circadian Rhythms

Chapter 2
General Feature of Circadian Rhythms

Kenji Tomioka

Abstract The circadian rhythm is an endogenous rhythm with a period of approximately 24 h. Organisms, including insects, possess the rhythm to live with the daily environmental and ecological cycles. This chapter describes and discusses the general characteristics and properties of circadian rhythms. The rhythm is driven by the circadian clock system that often consists of two or more clocks, synchronizing to the environmental cycles to set behavioral and physiological events to occur at an appropriate time of day. Insects use various zeitgebers for synchronization with light, which is the most important and powerful approach. Light not only resets the clock in a phase-dependent manner but also has long-lasting aftereffects that are observed in the free-running period and waveform of the rhythm. Constituent clocks form bilaterally paired or hierarchical structures that are important to adapt to environmental cycles with seasonal or day-to-day changes.

Keywords Aftereffects · Endogenous · Entrainment · Phase response curve · Relative coordination · Temperature compensation

2.1 Introduction: Self-Sustaining Rhythm

After the genesis of our planet, organisms have been exposed to daily cyclic environments. To cope with the cyclic changes in the environment has been the most important challenge for the organisms living on this planet. Today, organisms live in ecosystems that are changing daily in terms of not only geophysical but also biological aspects. Thus, organisms, including insects, possess daily rhythms to adapt to the daily changing environment. The rhythm is called a circadian rhythm because it persists with a period near but not exactly 24 h even after organisms are transferred to constant conditions, i.e., constant darkness (DD) or constant light (LL), and at a constant temperature.

K. Tomioka (✉)
Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan
e-mail: tomioka@cc.okayama-u.ac.jp

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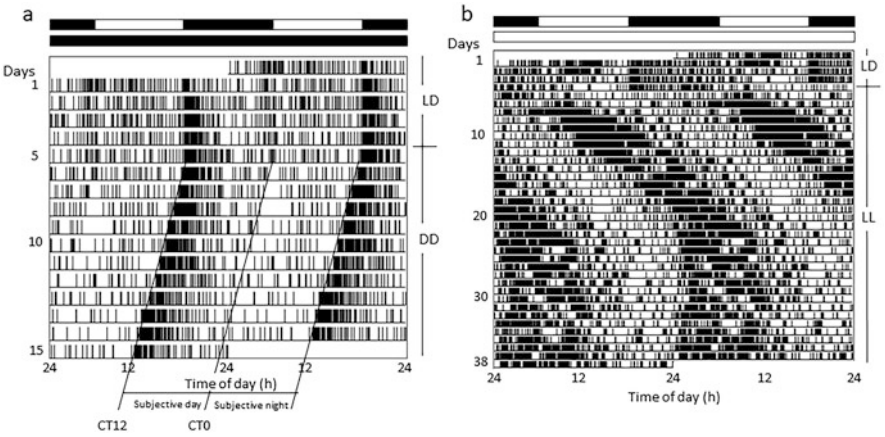

Fig. 2.1 Circadian locomotor rhythms of the cricket *Gryllus bimaculatus* under light/dark cycle (LD), constant dark (DD) (**a**) and constant light (LL) (**b**). Under LD, crickets show a nocturnal activity rhythm that persists for a period shorter (**a**) or longer (**b**) than 24 h under DD and LL, respectively. Under prolonged LL, the period becomes gradually shorter. The white and black bars above the actograms indicate light (white) and dark (black) conditions

Circadian rhythms are expressed in various behaviors, including locomotor (Tomioka and Chiba 1982b), stridulatory (Loher 1972), flight (Clopton 1984), feeding (Xu et al. 2008), oviposition (Loher 1979), egg hatching (Tomioka et al. 1991a), larval molting (Truman 1972; Fujishita and Ishizaki 1981), eclosion (Pittendrigh et al. 1958; Truman 1971), and mating activities (Sakai and Ishida 2001). Physiological functions also show circadian rhythms, including light sensitivity of compound eyes (Tomioka and Chiba 1982a; Wills et al. 1985), visual interneurons (Saifullah and Tomioka 2002; Uemura and Tomioka 2006), chemoreception in antennae (Saifullah and Page 2009), and cuticle formation (Wiedenmann et al. 1986; Ito et al. 2008). Figure 2.1a shows an example of the circadian rhythm of locomotor activity in the adult male cricket *Gryllus bimaculatus*. In the free-running state, the period is called the free-running period, and the phases corresponding to the original daytime and nighttime are called the subjective day and subjective night, respectively (Fig. 2.1a). The term circadian time (CT) is often used to determine phases in the free-running state. CT0 corresponds to the beginning of the subjective day, and CT12 corresponds to the beginning of the subjective night. Activity onsets are usually used to determine the phase and correspond to CT0 in diurnal insects and to CT12 in nocturnal insects. In this way, the CT of the rhythm is determined by dividing one circadian cycle from the activity onset to the next into 24 h.

The rhythm is essentially generated by an endogenous mechanism called the circadian clock that oscillates with a period of nearly 24 h, but under environmental cycles, it is modified by some direct responses to environmental factors, and the modification is called masking effects. The clock has flexibility to change its phase, period, waveform, or amplitude in response to daily or seasonal changes in environmental cycles. Insects possess two or more circadian clocks that compose

bilaterally symmetrical or hierarchical structures, which allow insects to cope with those day-to-day or seasonal changes in daily environmental cycles. This chapter outlines these general features of insect circadian rhythms.

2.2 Free-Running Period: Dependency on Environmental Conditions

Although the free-running period of the circadian rhythm is generally quite accurate and stable, it changes in response to environmental lighting and temperature conditions. Light usually has significant effects on the free-running period. In vertebrates, J. Aschoff established an empirical rule called Aschoff's rule for the light dependency of the free-running period: the free-running period is shorter in LL than in DD and becomes shorter with an increase in light intensity in diurnal animals, while the opposite is seen in nocturnal animals (Aschoff 1960; Pittendrigh 1960; Aschoff 1979). This is successfully applicable to most nocturnal insects: the free-running period is shorter in DD than in LL. For example, in the cricket *G. bimaculatus*, the free-running period under LL is longer than 24 h, which is significantly longer than that under DD (Fig. 2.1) (Tomioka and Chiba 1982b). Similar light-dependent changes in the free-running period have been known for several other nocturnal insect species, including the cockroaches *Rhyparobia* (*Leucophaea*) *maderae* and *Periplaneta americana* (Roberts 1960) and the beetle *Carabus problematicus* (Weber 1967), with some exceptions where the free-running period is shorter in LL, e.g., nocturnal pit-building activity in larvae of the ant-lion *Myrmeleon obscurus* (Youthed and Moran 1969). However, for diurnal insects, several *Nasonia* wasps are the only species that are thus far known to follow Aschoff's rule, showing a shorter free-running period in LL than in DD (Bertossa et al. 2013). Most other diurnal insects violate Aschoff's rule, with the free-running period being shorter under DD than LL. These include the dung beetle *Geotrupes sylvaticus* (Geisler 1961), the mosquito *Aedes aegypti* (Taylor and Jones 1969), the bean bug *Riptortus pedestris* (Numata and Matsui 1988), the blow fly *Calliphora vicina* (Hong and Saunders 1994), and the fruit fly *Drosophila melanogaster* (Konopka et al. 1989).

However, spontaneous changes in the free-running period sometimes occur under DD or LL. For example, some of the New Zealand weta *Hemideina thoracica* show locomotor rhythm free running with a period shorter than 24 h for the first several days in DD, but the period abruptly becomes longer, and in some cases, the period repeatedly becomes longer and shorter (Lewis 1994). Similar repetitive period changes were reported for the brow fly *Calliphora vicina* kept under DD (Kenny and Saunders 1991) and the onion fly *Delia antiqua* (Watari and Arai 1997). In the cricket *G. bimaculatus*, the period initially longer than 24 h after transfer to LL gradually becomes shorter and, in some cases, even shorter than 24 h, as shown under DD (Fig. 2.1b). The gradual period shortening caused by long-term exposure to LL is attributable to a single optic lobe clock, since the period change occurs after

unilateral ablation of the optic lobe. This seems not caused by reduced photoreceptor sensitivity because the expression level of the circadian photoreceptor gene (*opsin-long wavelength* (*opLW*)) is higher in crickets kept under LL for long term than in those kept under LD (Moriyama et al. 2022).

Under LL, the rhythm often disappears, resulting in behavioral arrhythmicity. It is debatable whether this LL-induced arrhythmicity is attributable to arrest of the clock at a specific phase called singularity, whether the clock maintains its oscillation but its control to overt rhythms is somehow disturbed or whether it is caused by desynchronization of multiple oscillators that control the overt rhythm. With careful experiments in *Drosophila pseudoobscura* eclosion rhythms, Winfree (1970) found that treatment of *Drosophil*a pupae with 50-sec dim blue light (10 μW/cm^2) at 6.8 h after LL/DD transition led the eclosion to be arrhythmic and suggested that the clock can be stopped by falling the oscillation to singularity.

2.3 Temperature Compensation

The free-running period of the circadian rhythm is well known to be rather stable against changes in temperature. Figure 2.2a shows an example of the temperature independence of the period in the cricket *G. bimaculatus*. This particular cricket was kept at 25 °C for the first 10 days and then transferred to 20 °C under constant darkness. The average free-running periods were 23.8 h and 22.9 h at 25 °C and 20 °C,

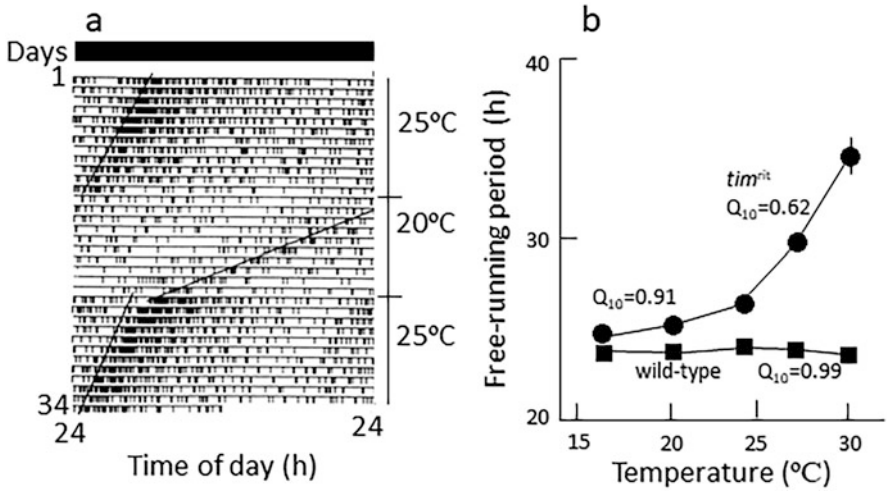

Fig. 2.2 Temperature compensation of free-running rhythm under DD. (**a**) Circadian locomotor rhythm of an adult male cricket *Gryllus bimaculatus* at 25 °C (days 1–12), 20 °C (days 13–22), and 25 °C (days 23–34). (**b**) Free-running periods of wild-type (Canton-S) and *tim*^{rit} mutant *Drosophila melanogaster* at various temperatures under DD. Redrawn from Ikeda and Tomioka (1993) and Matsumoto et al. (1999)

respectively (Ikeda and Tomioka 1993). The temperature coefficient Q_{10} in this species was 0.91, slightly smaller than 1.0. Similar values of Q_{10} have been reported for the fruit fly *D. melanogaster* (Fig. 2.2b) (Konopka et al. 1989; Matsumoto et al. 1999), the brow fly *C. vicina* (Saunders and Hong 2000), and the cockroach *R. maderae* (Pittendrigh and Caldarola 1973). Biochemical reactions occurring in biological systems, including insects, are temperature-dependent, and Q_{10} is usually 2.0–3.0. Therefore, there is a mechanism that maintains the free-running period to be constant irrespective of temperature, and the mechanism is called temperature compensation. The exact mechanism of temperature compensation remains to be elucidated. Although there are some possible hypotheses for the mechanism, it is apparently regulated by biochemical reactions because some mutations of clock-related genes, e.g., $timeless^{ritu}$ and $timeless^{blind}$, deeply affect temperature compensation (Fig. 2.2b) (Matsumoto et al. 1999; Singh et al. 2019).

2.4 Entrainment to Environmental Cycles

One of the most important functions of the circadian clock is to set daily events at an appropriate time of day. To pursue this entrainment, the clock must synchronize to daily environmental cycles. The clock uses some environmental cues, called zeitgebers, to achieve this. Insects use various zeitgebers, including light (Helfrich-Förster 2020), temperature (Tomioka and Yoshii 2006), food (Frisch and Aschoff 1987), and interindividual interactions (Levine et al. 2002). Among these, the most powerful zeitgeber is the light to dark cycle (LD).

When LD is shifted, the rhythm resynchronizes to the newly phased LD by gradual phase advances or delays. Figure 2.3a exemplifies the resynchronization or re-entrainment in the cricket *G. bimaculatus*. The process of re-enrainment requires several cycles, called transients, to restore the original phase relationship (Pittendrigh 1981a). The existence of transients is a sign of the endogenous nature of rhythm.

The resetting of the clock by light occurs in a circadian time-dependent manner. This trait is explored by experiments with a single light pulse exposure to free-running rhythms under DD. As shown in Fig. 2.3b, c, a light pulse given at early subjective night causes a delay shift of the rhythm, whereas it causes an advance shift when given at late subjective night. On subjective days, the pulse causes little phase shifts. The relationship between the CT at which the light pulse is given and the magnitude of the shifts thus caused is illustrated in the phase response curve (PRC) (Fig. 2.3d). The PRC for circadian rhythms has been obtained in a wide variety of insects, including the fruit flies *D. pseudoobscura* (Pittendrigh 1960) and *D. melanogaster* (Orr 1982), the brow flies *Lucilia cuprina* (Smith 1983) and *C. vicina* (Cymborowski et al. 1993), the cockroach *R. maderae* (Wiedenmann 1977), and the cricket *G. bimaculatus* (Okada et al. 1991). Although the shape of the PRC is basically shared among the tested species, the PRC is classified into two types, type 1 and type 0, based on the amplitude (Fig. 2.3e) (Winfree 1970). Type

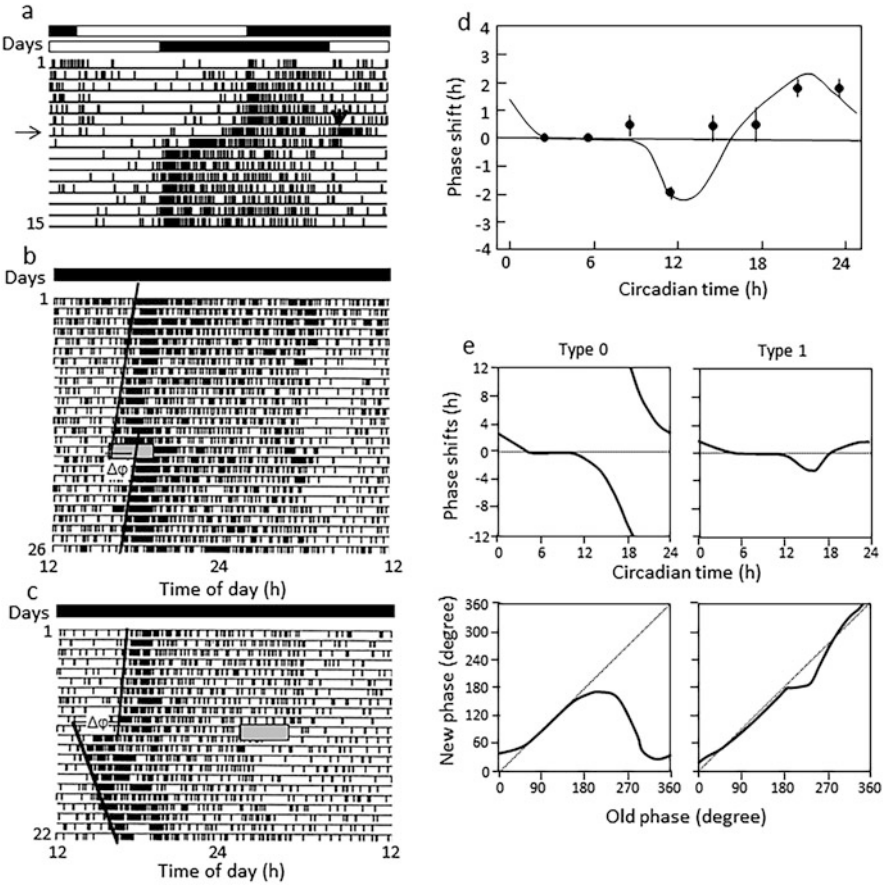

Fig. 2.3 Phase shifts of circadian rhythms. (**a**–**c**) Resynchronization to shifted LD (**a**), phase delay (**b**) or phase advance (**c**) caused by a 3-h light pulse (gray bars) in the adult male cricket *Gryllus bimaculatus*. In (**a**), an arrow indicates the day of phase shift, and a thick arrowhead indicates a positive masking effect. (**d**) Phase response curve obtained for locomotor rhythm by a 3-h light pulse in the adult male cricket *G. bimaculatus*. (**e**) Type 0 and type 1 PRCs and their PTCs. Redrawn from Okada et al. (1991) and Pittendrigh (1981b)

1 PRC has low amplitude, and if the time of activity onset is plotted with the phase before the pulse (old phase) on the horizontal axis and the phase after the pulse (new phase) on the vertical axis, the slope of the plot, called the phase transition curve (PTC), is approximately 1. In contrast, type 0 PRC has high amplitude, and the slope of the PTC is approximately 0. The amplitude of PRC depends on the light intensity and duration of light pulses. Thus, the type of PRC could be changed. For example, in the cockroach *Nauphoeta cinerea*, white light of 3-h duration causes type 1 PRC with low amplitude while that of 12-h duration type 0 PRC with high amplitude (Saunders and Thomson 1977).

2.5 Range of Entrainment

The rhythm can be entrained to light cycles with periods (Ts) longer or shorter than 24 h but close to 24 h. The PRC predicts not only the phase relationship between the rhythm and the light cycle in a steady-state entrainment but also the range of Ts that can entrain the rhythm. For example, when the cricket *G. bimaculatus* kept in DD is repetitively given a 3-h light pulse with a period of 26 h, then the cricket's rhythm could be predicted to synchronize to the cycle as the 3-h light pulse falls at CT 11. This is because a 3-h light pulse falling at CT11 is expected to cause a 2-h delay from the PRC (Fig. 2.3d) so that the period of the cricket's rhythm, which is close to 24 h, becomes 26 h. The limit of entrainment could also be predicted by the greatest delay and advance shift induced by a light pulse. In the cricket, with a 3-h light pulse, the maximal advance and delay are 2 h and 2.5 h, respectively (Fig. 2.3d) (Okada et al. 1991), so that the predicted range of entrainment is 22 h–26.5 h. The predicted range of entrainment matches well with the experimental results using LDs with L and D of the same duration in the cricket *G. bimaculatus*; the shorter limit is near $T = 21$ h, while the longer limit is close to 27 h (Tomioka 1993).

The rhythm is sometimes entrained to or free runs under LD cycles beyond the range of entrainment, e.g., shorter than 24 h, such as 12, 8, and 6 h. However, in such a shorter period, the rhythm rigidly maintains the entrained state or free-running state with a period of or close to 24 h (Fig. 2.4a, b). When entrained to the given LD, the clock reads the given LD cycles into a 24-h cycle. It is also known that the rhythm synchronizes to LD cycles with longer periods, e.g., 48 h. To such LDs, the rhythm entrains by repeating the 24-h rhythm twice or more in a given LD cycle.

2.6 Relative Coordination

When the power of the zeitgeber is weak, circadian rhythms are not entrained to the zeitgeber but only show a phase-dependent modulation of the free-running period. This state is called relative coordination (Aschoff 1981). Figure 2.4c shows an example of the relative coordination. In this particular case, the locomotor rhythm of the cricket *Gryllodes sigillatus* was recorded under constant darkness with a temperature cycle of 12 h 20 °C and 12 h 25 °C. The cricket cannot synchronize to the temperature cycle but shows a clear change in the free-running period, which is lengthened at 20 °C but shortened at 25 °C.

Relative coordination can also be observed when the rhythm is exposed to LD cycles outside the range of entrainment, as exemplified in Fig. 2.4d (Tomioka 1993). In this case, the cricket *G. bimaculatus* is exposed to LD 10.5:10.5 ($T = 21$ h). The cricket cannot be entrained to the given LD, free running with a relative coordination in which τ fluctuates as a function of the phase angle relationship with the light cycle. The period lengthens when the light onset falls during the subjective day, while it shortens if the light onset occurs during the subjective night.

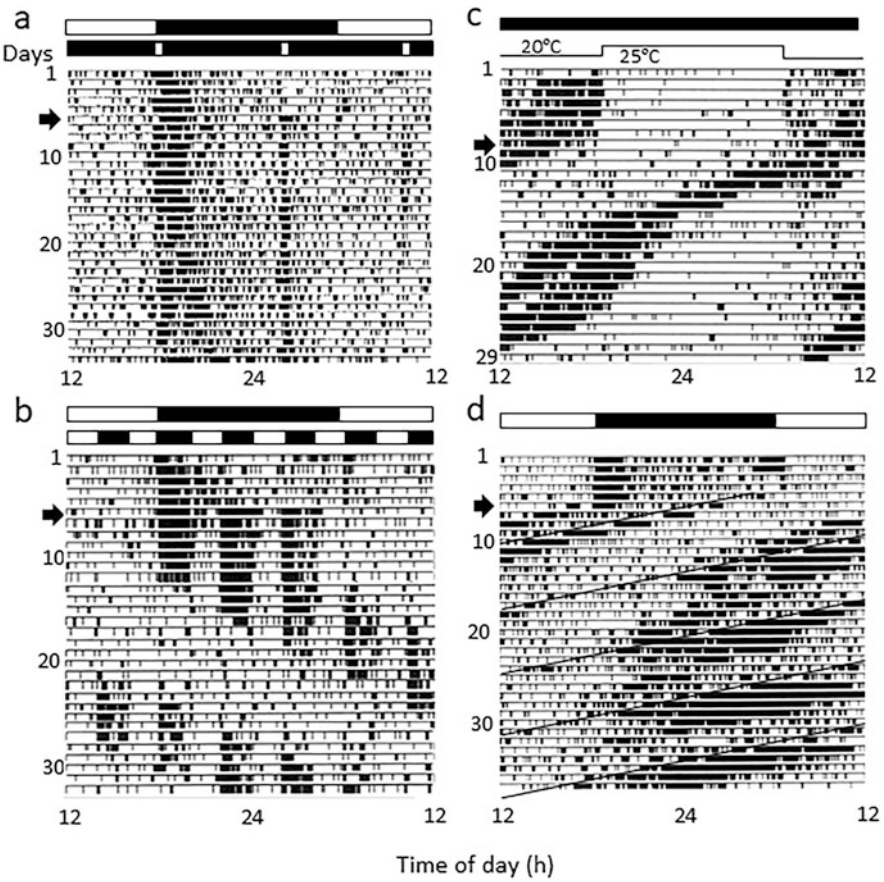

Fig. 2.4 Locomotor rhythms of adult male crickets *Gryllus bimaculatus* (**a**, **b**, **d**) and *Gryllodes sigillatus* (**c**) showing entrainment to LD1:7 (**a**), free-running under LD 2:2, and relative coordination under a 24-h temperature cycle (**c**) and under LD 10.5:10.5. (**d**) Arrows indicate the day when new lighting conditions or temperature cycles were started. Redrawn from Germ and Tomioka (1998) and Tomioka (1993)

2.7 Masking Effect of Light

Light has direct effects on behavior bypassing the clock. For example, lights on and off often modulate the activity rhythm in a time-dependent manner. There are two types of masking effects: positive and negative. Positive masking is an enhancement of activity by light, as shown for the cricket *G. bimaculatus* in Figs. 2.3a and 2.4a. In the cricket shown in Fig. 2.3a, intense locomotor activity occurred at lights on when the LD cycle was 6 h advanced, but this activity soon disappeared as the rhythm restored the original phase relationship with the shifted LD. Thus, positive masking often occurs in a phase-dependent manner, e.g., during late subjective night to early subjective day for nocturnal animals (Tomioka and Chiba 1987). Negative masking

is induced when light is given during the subjective night. When a short LD cycle is given, the locomotor rhythm often free runs, but the light phase strongly suppresses activity during subjective night so that the active phase is divided into pieces (Fig. 2.4b). To determine whether the activity observed under LD is caused by an endogenous clock or exogenous masking of light, the rhythm should be observed after transfer to DD, where endogenous components persist but exogenous masking disappears.

2.8 Multioscillator System and Internal Desynchronization of Rhythms

The daily temporal rhythms are often controlled by two or more circadian clocks. Splitting of the rhythm into two separately running components is strong evidence for the multioscillator nature of the circadian system. Splitting often occurs spontaneously under constant conditions or is artificially induced by blocking the light input to the clock on one side (Wiedenmann 1983; Tomioka et al. 1991b). Figure 2.5a exemplifies the spontaneous rhythm splitting in the cricket *G. bimaculatus* kept under LL. The splitting occurred through internal

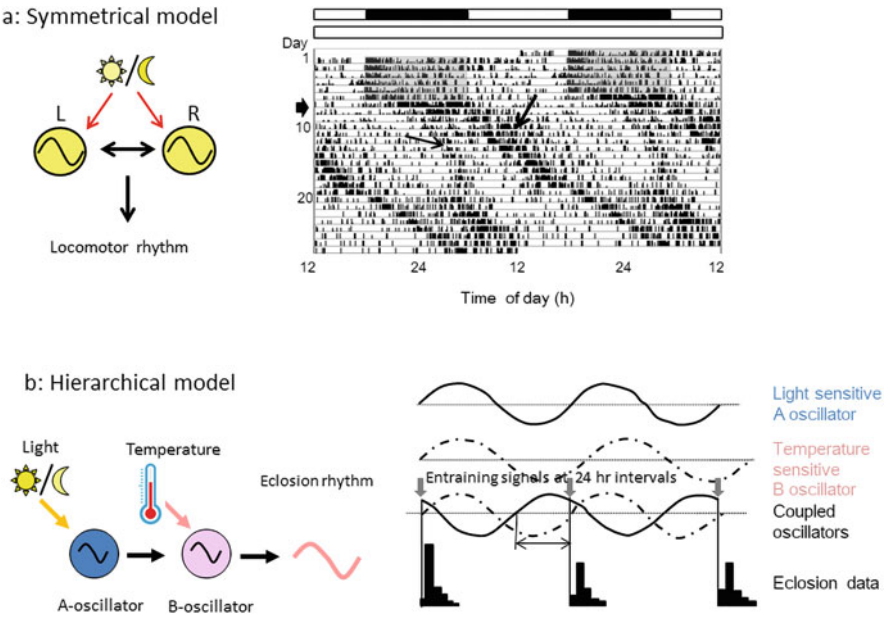

Fig. 2.5 Two-oscillator models. (**a**) Symmetrical two-oscillator model and splitting of locomotor rhythm caused by internal desynchronization of the two clocks in adult male cricket *Gryllus bimaculatus*. (**b**) Hierarchical two-oscillator model and controlled eclosion rhythm of *Drosophila pseudoobscura*. Redrawn from Moriyama et al. (2022) and Pittendrigh et al. (1958)

desynchronization of the two clocks. In crickets and cockroaches, the two clocks are located one in each optic lobe (Page 1982, 1983; Tomioka and Chiba 1984, 1992). The bilaterally paired nature of the clocks is revealed by surgical removal of the optic lobe in insects with split rhythms: unilateral lobe removal eliminates either of the two rhythms, often with significant changes in the free-running period of the remaining rhythm (Page 1978; Tomioka et al. 1991b; Lewis 1994). This period change suggests that the period of the system is determined by mutual interaction of the two clocks.

In addition to the bilaterally paired organization, there is a system with clocks that have a hierarchical relationship. The best-known clock system is that of the fruit fly *D. pseudoobscura*, which regulates eclosion rhythms. Adult emergence from pupal cases occurs in the early morning in *D. pseudoobscura*. With light pulse and temperature pulse experiments, the underlying regulatory mechanism was revealed to include a light-sensitive circadian oscillator (A oscillator) and a temperature-sensitive oscillator (B oscillator) (Pittendrigh 1981b). In this A-B two-oscillator model, the A oscillator is the master, entraining the B oscillator, which is the slave and determines the timing of eclosion (Fig. 2.5b). Light resets the A oscillator immediately but not the B oscillator, which gradually restores the original phase relationship with the A oscillator through the entraining effect from the A oscillator (Tackenberg et al. 2017). The resynchronizing process of the B oscillator appears as transients.

2.9 Aftereffects

The circadian rhythm is genetically programmed, and insects show the rhythm without experiencing any periodic environment. However, the rhythm is known to change its waveform to tune to a given photoperiod (Pittendrigh and Daan 1976). Figure 2.6 exemplifies the waveform modulation by entrainment to LDs of variable L-to-D ratios in the cricket *G. bimaculatus* (Koga et al. 2005). When experiencing longer light phases, the ratio of the active phase to the rest phase (α/ρ-ratio) was smaller, and the free-running period was shorter. The magnitude of changes in the α/ρ ratio was dependent on the number of cycles experienced. These phenomena are comparable to the aftereffects found in vertebrates: the white-footed mouse, *Peromyscus leucopus*, for example, the subjective night length and the free-running period were clearly dependent on the light cycle in which the animal had been exposed (Pittendrigh and Daan 1976). In vertebrates, these changes in the free-running period and circadian waveform are explained by photoperiod-dependent changes in the coupling state of two circadian oscillators, which regulate the lights-on (morning) peak and lights-off (evening) peak, respectively (Pittendrigh and Daan 1976). Additionally, in insects, there are some lines of evidence suggesting a similar role of oscillator coupling in controlling the circadian waveform (Wiedenmann and Loher 1984; Helfrich-Förster 2001; Koga et al. 2005). However, the waveform modulation could be attributable at least in part to a single optic lobe clock because

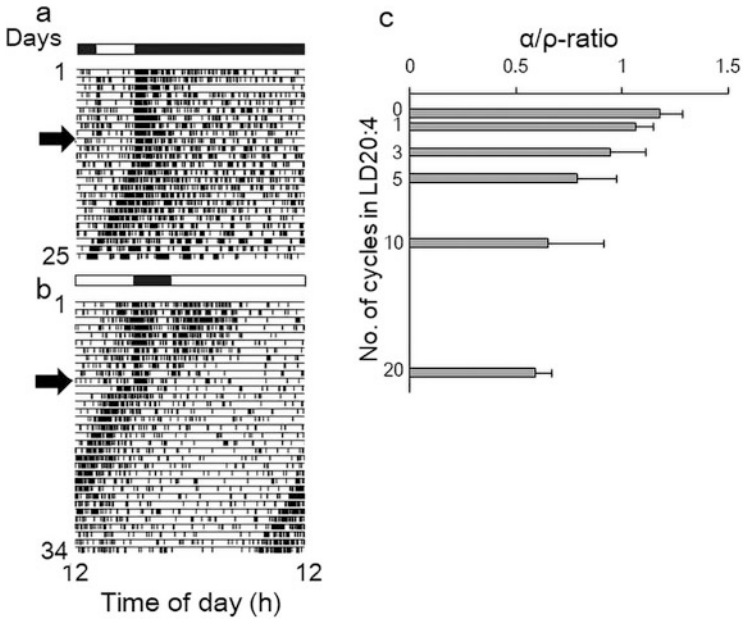

Fig. 2.6 History-dependent changes in locomotor activity rhythms in adult male crickets *Gryllus bimaculatus* after entrainment to (**a**) LD 4:20 and (**b**) LD 20:4. Arrows indicate the day of transfer from LD 4:20 (**a**) or LD 20:4 (**b**) to DD. (**c**) Effects of the number of experienced LD20:4 cycles on the α/ρ ratio. Redrawn from Koga et al. (2005)

the modulated waveform persisted in isolated and cultured optic lobes (Koga et al. 2005). More clear evidence for this was obtained in the cockroach *R. maderae*: experience of T cycles with a period longer or shorter than 24 h causes long-lasting changes in the period of optic lobe clocks, and the change was maintained even after the optic lobe was transplanted to another individual (Page 1982, 1983). A remaining important issue is how these history-dependent changes occur and are maintained. This question deserves to be solved at the cellular and molecular levels.

2.10 Future Perspectives

As we have seen in this chapter, circadian rhythms have properties unique to biological rhythms. These properties were previously explored behaviorally and physiologically but are now being studied at cellular and molecular levels. In particular, the oscillatory and photic entrainment mechanisms of the circadian clock are already largely understood, albeit for some insects, including *D. melanogaster*. These will be discussed in other chapters. However, there are still some unresolved issues, such as temperature compensation and aftereffects. Comprehensive analysis of genes by next-generation sequencers and regulation of

gene expression by RNAi and gene editing techniques are now widely used in studies on insect circadian rhythms. These innovations in circadian rhythm research are expected to help us to deepen our understanding of the various properties of clocks and their adaptive significance.

References

Aschoff J (1960) Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol 25:11–27. https://doi.org/10.1101/sqb.1960.025.01.004

Aschoff J (1979) Circadian rhythms: influences of internal and external factors on the period measured in constant conditions. Z Tierpsychol 49:225–249. https://doi.org/10.1111/j.1439-0310.1979.tb00290.x

Aschoff J (1981) Free-running and entrained circadian rhythms. In: Aschoff J (ed) Behavioral neurobiology, Biological rhythms, vol 4. Plenum Press, New York; London, pp 81–93

Bertossa RC, van Dijk J, Diao W, Saunders D, Beukeboom LW, Beersma DGM (2013) Circadian rhythms differ between sexes and closely related species of *Nasonia* wasps. PLoS One 8: e60167. https://doi.org/10.1371/journal.pone.0060167

Clopton JR (1984) Mosquito circadian flight rhythms: differential effects of constant light. Am J Phys 247:R960–R967. https://doi.org/10.1152/ajpregu.1984.247.6.R960

Cymborowski B, Gillanders SW, Hong SF, Saunders DS (1993) Phase shifts of the adult locomotor activity rhythm in *Calliphora vicina* induced by non-steroidal ecdysteroid agonist RH 5849. J Comp Physiol A 172:101–108. https://doi.org/10.1007/BF00214719

Frisch B, Aschoff J (1987) Circadian rhythms in honeybees: entrainment by feeding cycles. Physiol Entomol 12:41–49. https://doi.org/10.1111/j.1365-3032.1987.tb00722.x

Fujishita M, Ishizaki H (1981) Circadian clock and prothoracicotropic hormone secretion in relation to the larval-larval ecdysis rhythm of the saturniid *Samia cynthia ricini*. J Insect Physiol 27:121–128. https://doi.org/10.1016/0022-1910(81)90118-9

Geisler M (1961) Untersuchungen zur Tagesperiodik des Mistkäfers *Geotrupes silvaticus* Panz. Z Tierpsychol 18:389–420. https://doi.org/10.1111/j.1439-0310.1961.tb00233.x

Germ M, Tomioka K (1998) Circadian period modulation and masking effects induced by repetitive light pulses in locomotor rhythms of the cricket *Gryllus bimaculatus*. Zool Sci 15:309–316. https://doi.org/10.2108/zsj.15.309

Helfrich-Förster C (2001) The locomotor activity rhythm of *Drosophila melanogaster* is controlled by a dual oscillator system. J Insect Physiol 47:877–887. https://doi.org/10.1016/S0022-1910(01)00060-9

Helfrich-Förster C (2020) Light input pathways to the circadian clock of insects with an emphasis on the fruit fly *Drosophila melanogaster*. J Comp Physiol A 206:259–272. https://doi.org/10.1007/s00359-019-01379-5

Hong S-F, Saunders DS (1994) Effects of constant light on the rhythm of adult locomotor activity in the blow fly, *Calliphora vicina*. Physiol Entomol 19:319–324. https://doi.org/10.1111/j.1365-3032.1994.tb01058.x

Ikeda M, Tomioka K (1993) Temperature dependency of the circadian locomotor rhythm in the cricket *Gryllus bimaculatus*. Zool Sci 10:597–604. https://doi.org/10.34425/zs001120

Ito C, Goto SG, Shiga S, Tomioka K, Numata H (2008) Peripheral circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster*. Proc Natl Acad Sci 105:8446–8451. https://doi.org/10.1073/pnas.0800145105

Kenny NA, Saunders DS (1991) Adult locomotor rhythmicity as "hands" of the maternal photoperiodic clock regulating larval diapause in the blowfly, *Calliphora vicina*. J Biol Rhythm 6: 217–233. https://doi.org/10.1177/074873049100600303

Koga M, Ushirogawa H, Tomioka K (2005) Photoperiodic modulation of circadian rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 51:681–690. https://doi.org/10.1016/j.jinsphys.2005.01.006

Konopka RJ, Pittendrigh CS, Orr D (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. J Neurogenet 6:1–10. https://doi.org/10.1080/01677060701695391

Levine JD, Funes P, Dowse HB, Hall JC (2002) Resetting the circadian clock by social experience in *Drosophila melanogaster*. Science 298:2010–2012. https://doi.org/10.1126/science.1076008

Lewis RD (1994) Modelling the circadian system of the weta, *Hemideina thoracica* (Orthoptera: Stenopelmatidae). J R Soc N Z 24:395–421. https://doi.org/10.1080/03014223.1994.9517476

Loher W (1972) Circadian control of stridulation in the cricket *Teleogryllus commodus* Walker. J Comp Physiol 79:173–190. https://doi.org/10.1007/BF00697770

Loher W (1979) Circadian rhythmicity of locomotor behavior and oviposition in female *Teleogryllus commodus*. Behav Ecol Sociobiol 5:253–262. https://doi.org/10.1007/BF00293674

Matsumoto A, Tomioka K, Chiba Y, Tanimura T (1999) *tim*^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol 19:4343–4354. https://doi.org/10.1128/mcb.19.6.4343

Moriyama Y, Takeuchi K, Shinohara T, Miyagawa K, Matsuka M, Yoshii T et al (2022) *Timeless* plays an important role in compound eye-dependent photic entrainment of the circadian rhythm in the cricket *Gryllus bimaculatus*. Zool Sci 39:397–405. https://doi.org/10.2108/zs220011

Numata H, Matsui N (1988) Circadian rhythm of oviposition in the bean bug, *Riptortus clavatus* Thunberg (Heteroptera: Alydidae). Appl Entomol Zool 23:493–495. https://doi.org/10.1303/aez.23.493

Okada Y, Tomioka K, Chiba Y (1991) Circadian phase response curves for light in nymphal and adult crickets, *Gryllus bimaculatus*. J Insect Physiol 37:583–590. https://doi.org/10.1016/0022-1910(91)90035-X

Orr DP-Y (1982) Behavioral neurogenetic studies of a circadian clock in *Drosophila melanogaster*. Dissertation (PhD), California Institute of Technology, Pasadena. https://resolver.caltech.edu/CaltechTHESIS:10222019-143344602

Page TL (1978) Interactions between bilaterally paired components of the cockroach circadian system. J Comp Physiol 124:225–236. https://doi.org/10.1007/BF00657054

Page TL (1982) Transplantation of the cockroach circadian pacemaker. Science 216:73–75. https://doi.org/10.1126/science.216.4541.73

Page TL (1983) Effects of optic-tract regeneration on internal coupling in the circadian system of the cockroach. J Comp Physiol A 153:231–240. https://doi.org/10.1007/BF00612589

Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. Cold Spring Harb Symp Quant Biol 25:159–184. https://doi.org/10.1101/SQB.1960.025.01.015

Pittendrigh CS (1981a) Circadian systems: general perspective. In: Aschoff J (ed) Handbook of behavioral neurobiology, Biological rhythms, vol 4. Plenum Press, New York; London, pp 57–80

Pittendrigh CS (1981b) Circadian systems: entrainment. In: Aschoff J (ed) Handbook of behavioral neurobiology, Biological rhythms, vol 4. Plenum Press, New York; London, pp 95–124

Pittendrigh CS, Caldarola PC (1973) General homeostasis of the frequency of circadian oscillations. Proc Natl Acad Sci U S A 70:2697–2701. https://doi.org/10.1073/pnas.70.9.2697

Pittendrigh CS, Daan S (1976) A functional analysis of a circadian pacemaker in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. J Comp Physiol 106:333–355. https://doi.org/10.1007/BF01417860

Pittendrigh CS, Bruce VG, Kaus P (1958) On the significance of transients in daily rhythms. Proc Natl Acad Sci U S A 44:965–973. https://doi.org/10.1073/pnas.44.9.965

Roberts SKF (1960) Circadian activity in cockroaches. I. The free-running rhythm in steady-state. J Cell Comp Physiol 55:99–110. https://doi.org/10.1002/jcp.1030550112

Saifullah ASM, Tomioka K (2002) Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket, *Gryllus bimaculatus*. J Exp Biol 205:1305–1314. https://doi.org/10.1242/jeb.205.9.1305

Saifullah ASM, Page TL (2009) Circadian regulation of olfactory receptor neurons in the cockroach antenna. J Biol Rhythm 24:144–152. https://doi.org/10.1177/0748730408331166
Sakai T, Ishida N (2001) Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. Proc Natl Acad Sci 98:9221–9225. https://doi.org/10.1073/pnas.151443298
Saunders DS, Thomson EJ (1977) 'Strong' phase response curve for the circadian rhythm of locomotor activity in a cockroach (Nauphoeta cinerea). Nature 270:241–243. https://doi.org/10.1038/270241a0
Saunders DS, Hong S-F (2000) Effects of temperature and temperature-steps on circadian locomotor rhythmicity in the blow fly *Calliphora vicina*. J Insect Physiol 46:289–295. https://doi.org/10.1016/s0022-1910(99)00182-1
Singh S, Giesecke A, Damulewicz M, Fexova S, Mazzotta GM, Stanewsky R et al (2019) New *Drosophila* circadian clock mutants affecting temperature compensation induced by targeted mutagenesis of *timeless*. Front Physiol 10:1442. https://doi.org/10.3389/fphys.2019.01442
Smith PH (1983) Circadian control of spontaneous flight activity in the blowfly, *Lucilia cuprina*. Physiol Entomol 10:323–336. https://doi.org/10.1111/j.1365-3032.1983.tb00335.x
Tackenberg MC, Johnson CH, Page TL, Daan S (2017) Revealing oft-cited but unpublished papers of Colin Pittendrigh and coworkers. J Biol Rhythm 32:291–294. https://doi.org/10.1177/0748730417716685
Taylor B, Jones MDR (1969) The circadian rhythm of flight activity in the mosquito *Aedes aegypti* (L.): the phase-setting effects of light-on and light-off. J Exp Biol 51:59–70. https://doi.org/10.1242/jeb.51.1.59
Tomioka K (1993) Analysis of coupling between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Comp Physiol A 172:401–408. https://doi.org/10.1007/BF00213522
Tomioka K, Chiba Y (1982a) Persistence of circadian ERG rhythms in the cricket with optic tract severed. Naturwissenschaften 69:355–356. https://doi.org/10.1007/BF00396696
Tomioka K, Chiba Y (1982b) Post-embryonic development of circadian rhythm in the cricket, *Gryllus bimaculatus*. J Comp Physiol A 147:299–304. https://doi.org/10.1007/BF00609663
Tomioka K, Chiba Y (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the circadian locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zool Sci 1:375–382. https://doi.org/10.2108/zsj.1.375
Tomioka K, Chiba Y (1987) Entrainment of cricket circadian activity rhythm after 6-hour phase-shifts of light-dark cycle. Zool Sci 4:535–542. https://doi.org/10.2108/zsj.4.535
Tomioka K, Chiba Y (1992) Characterization of an optic lobe circadian pacemaker by in situ and in vitro recording of neuronal activity in the cricket *Gryllus bimaculatus*. J Comp Physiol A 171:1–7. https://doi.org/10.1007/BF00195955
Tomioka K, Yoshii T (2006) Entrainment of *Drosophila* circadian rhythms by temperature cycles. Sleep Biol Rhythms 4:240–247. https://doi.org/10.1111/j.1479-8425.2006.00227.x
Tomioka K, Wakatsuki T, Shimono K, Chiba Y (1991a) Circadian control of hatching in the cricket, *Gryllus bimaculatus*. J Insect Physiol 37:365–371. https://doi.org/10.1016/0022-1910(91)90087-G
Tomioka K, Yamada K, Yokoyama S, Chiba Y (1991b) Mutual interactions between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Comp Physiol A 169:291–298. https://doi.org/10.1007/BF00206993
Truman JW (1971) The role of the brain in the ecdysis rhythm of silkmoths: comparison with the photoperiodic termination of diapause. In: Menaker M (ed) Biochronometry. National Academy of Sciences, Washington, pp 483–504
Truman JW (1972) Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. J Exp Biol 57:805–820. https://doi.org/10.1242/jeb.57.3.805
Uemura H, Tomioka K (2006) Postembryonic changes in circadian photo-responsiveness rhythms of optic lobe interneurons in the cricket *Gryllus bimaculatus*. J Biol Rhythm 21:1–11. https://doi.org/10.1177/0748730406288716
Watari Y, Arai T (1997) Effects of photoperiod and aging on locomotor activity rhythms in the onion fly, *Delia antiqua*. J Insect Physiol 43:567–576. https://doi.org/10.1016/S0022-1910(97)00002-4

Weber F (1967) Die Periodenlange der circadianen Laufperiodizitat bei drei *Carabus*-Arten (Coleopteral, Ins.). Naturwissenschaften 54:122. https://doi.org/10.1007/BF00640591

Wiedenmann G (1977) Weak and strong phase shifting in the activity rhythm of *Leucophaea maderae* (Blaberidae) after light pulses of high intensity. Z Naturforsch 32c:464–465

Wiedenmann G (1983) Splitting in a circadian activity rhythm: the expression of bilaterally paired oscillators. J Comp Physiol 150:51–60. https://doi.org/10.1007/BF00605287

Wiedenmann G, Loher W (1984) Circadian control of singing in crickets: two different pacemakers for early-evening and before-dawn activity. J Insect Physiol 30:145–151. https://doi.org/10.1016/0022-1910(84)90118-5

Wiedenmann G, Lukat R, Weber F (1986) Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? J Insect Physiol 32:1019–1027. https://doi.org/10.1016/0022-1910(94)00092-U

Wills SA, Page TL, Colwell CS (1985) Circadian rhythms in the electroretinogram of the cockroach. J Biol Rhythm 1:25–37. https://doi.org/10.1177/074873048600100105

Winfree A (1970) The temporal morphology of a biological clock. In: Gerstenhaber M (ed) Lectures on mathematics in the life sciences. American Mathematical Society, Providence, pp 111–150

Xu K, Zheng X, Sehgal A (2008) Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. Cell Metab 8:289–300. https://doi.org/10.1016/j.cmet.2008.09.006

Youthed GJ, Moran VC (1969) The solar-day activity rhythm of Myrmeleontid larvae. J Insect Physiol 15:1103–1116. https://doi.org/10.1016/0022-1910(69)90147-4

Chapter 3
Neural and Molecular Mechanisms of Entrainment

Kenji Tomioka and Taishi Yoshii

Abstract Synchronization or entrainment to daily environmental cycles is one of the important properties of the circadian clock, which is required to set an appropriate timing of physiology or behavior. Insects use various entraining agents or zeitgebers for entrainment, including light, temperature, food, and social cues. The mechanisms underlying entrainment have been studied extensively at cellular and molecular levels. For light and temperature, molecular components for their perception and how they reset the clock molecular oscillatory mechanism have been the main topics of chronobiology. This chapter will focus on the mechanism of light and temperature entrainment. The entrainment to restricted feeding, which has been progressing in recent years, will also be discussed.

Keywords Circadian clock · Clock gene · Entrainment · Phase shift · Zeitgeber

3.1 Introduction

Adaptation to daily and seasonally changing environments is of utmost importance for insects to live on this planet. The daily cycle includes not only geophysical factors but also biological/ecological factors (Dunlap et al. 2004). To confront these daily cyclical factors, insects must set their behavioral and physiological events at an appropriate time of day. For example, crickets and cockroaches exhibit stable nocturnal activity with the onset of activity at lights off under light to dark cycles, and the time of day mosquitoes come to the stable to suck blood varies from species to species (Katô and Triumi 1950). The timing is determined by the circadian clock that synchronizes or entrains to environmental cycles. Insects use various geophysical and biological factors for their clock synchronization to daily cycles. These include light (Pittendrigh and Minis 1964), temperature (Zimmerman et al. 1968), food availability (Xu et al. 2011), and social cues such as individual interaction

K. Tomioka (✉) · T. Yoshii
Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan
e-mail: tomioka@cc.okayama-u.ac.jp

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(Bloch et al. 2013). Among them, light is the most powerful synchronizing agent or zeitgeber.

The mechanism for light entrainment has been extensively studied in various insects at physiological and molecular levels. Temperature is also known as an important zeitgeber, and the mechanism for temperature entrainment has recently been studied at cellular and molecular levels (Zimmerman et al. 1968; Glaser and Stanewsky 2005; Tomioka and Yoshii 2006; Sehadova et al. 2009). This chapter describes the behavioral, physiological, cellular, and molecular mechanisms for entrainment to light and temperature cycles. In addition, entrainment to restricted feeding is discussed. Social cues are also important entraining agents for social insects such as bees and ants (Bloch et al. 2013). Social entrainment is discussed in detail in Chap. 8.

3.2 Photic Entrainment and Circadian Photoreceptors

Among the zeitgebers or synchronizing agents that entrain the clock to environmental cycles, light is the most powerful (Saunders et al. 2002). Generally, light shifts the phase of circadian clocks in a phase-dependent manner: it delays the clock when given at early subjective night, whereas it advances at late subjective night (see Fig. 2.3) (Pittendrigh 1981). The transient cycles necessary for entrainment are dependent on light intensity and wavelength. In the cockroach *Periplaneta americana*, the most effective wavelength for phase shifts and ultimate entrainment is reportedly near 495 nm (Mote and Black 1981), suggesting the involvement of green-sensitive photoreceptors. Similar results were obtained in entrainment experiments in another cockroach species, *Blattella germanica* (Leppla et al. 1989). These results are quite consistent with our more recent results of RNA interference (RNAi) experiments in the cricket *Gryllus bimaculatus*, in which the green-light receptor, opsin-long wavelength (OpLW), is the circadian photoreceptor (Komada et al. 2015).

Photoreceptors necessary for photic entrainment were studied by occlusion with painting over the compound eyes and ocelli but later by surgical lesions or mutant screenings, which yielded more definitive results. Occlusion sometimes yielded inconsistent results. For example, occlusion of the compound eyes and ocelli in the cockroach *P. americana* resulted in arrhythmicity (Cloudsley-Thompson 1953), while in *Rhyparobia* (*Leucophaea*) *maderae* and *P. americana*, the same treatment reportedly caused free-running locomotor rhythms (Roberts 1960). In contrast, surgical lesions yielded clear results. Cutting the optic nerves that connect the compound eye and optic lobe clearly prevented the entrainment of activity rhythms in cockroaches and crickets (Nishiitsutsuji-Uwo and Pittendrigh 1968; Loher 1972; Tomioka and Chiba 1984) (Fig. 3.1a). In these experiments, ocelli were left intact so that ocelli were thought to have no important role in photic entrainment. However, they were shown to have some role in circadian control in singing rhythms of the cricket *Teleogryllus commodus* (Rence et al. 1988). In addition, the contribution of extraocular photoreceptors was later found in New Zealand weta *Hemideina*

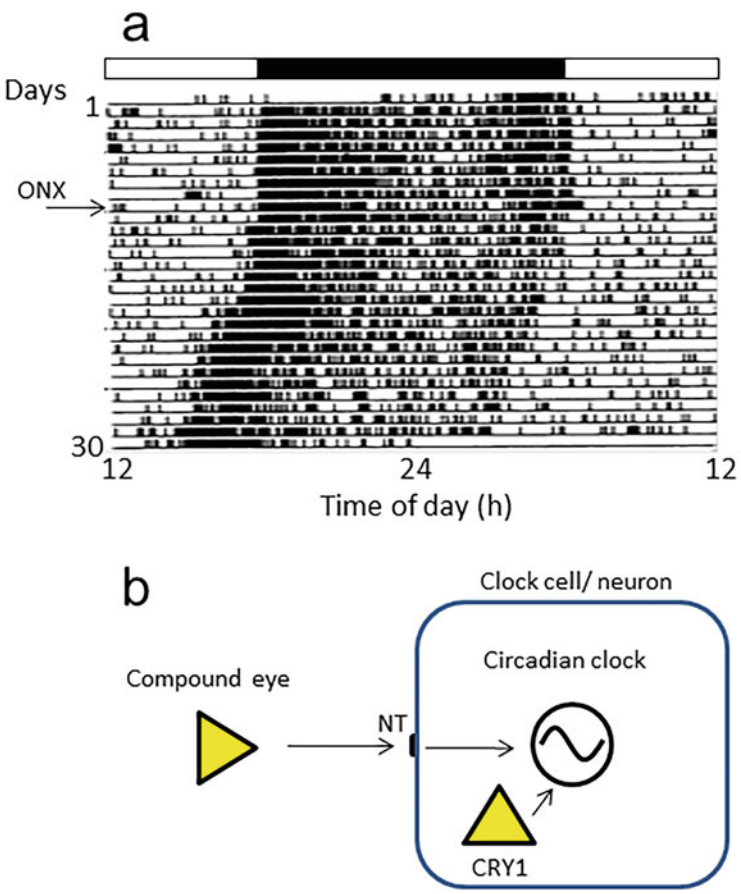

Fig. 3.1 Insect circadian photoreceptors. (**a**) Circadian locomotor activity rhythm of an adult male cricket, *Gryllus bimaculatus*, whose optic nerves were bilaterally cut on the day indicated by ONX (arrow). The cricket was kept under LD cycles, but after ONX, its rhythm free ran. (**b**) Two photic entrainment pathways in insects. CRY1 is an intracellular photoreceptor and directly acts on the clock machinery, whereas the light information perceived by the compound eye is conducted by the neural pathway and acts on the clock via neurotransmitters (NT)

thoracica and the cricket *Dianemobius nigrofasciatus* (Waddel et al. 1990; Shiga et al. 1999).

In *Drosophila melanogaster*, involvement of the extraocular photoreceptor in photic entrainment was clearly shown by mutant screening. Mutant flies carrying *sine oculis* with compound eyes genetically lost were shown to be entrained to light cycles (Helfrich and Englemann 1983). The photoreceptor molecule critical for photic entrainment was later found to be cryptochrome (CRY) by a gene screening experiment using *D. melanogaster* carrying a *per-luc* reporter (Emery et al. 1998; Stanewsky et al. 1998). The obtained *cry* mutant (*cryptochrome*^{baby}, *cry*^{b}) disrupted photic entrainment of the locomotor rhythm. Subsequent molecular studies revealed

that CRY is a member of the photolyase family and plays an important role in resetting the oscillatory loop by perceiving blue/UV light (Lin and Todo 2005; Fogle et al. 2011) and is expressed in cerebral clock neurons, compound eyes, and peripheral tissues (Ito et al. 2008; Yoshii et al. 2008) (Fig. 3.1b). However, the compound eye and ocelli also have a significant contribution to photic entrainment in *D. melanogaster* (Rieger et al. 2003), so the mutant of *cry* can still be entrained by light cycles, albeit to a much slower extent than that of wild-type flies (Kistenpfennig et al. 2012). Hofbauer-Buchner (HB) eyelets that are the adult remnant of Bolwig's organ, the larval visual organ (Yasuyama and Meinertzhagen 1999), also play a role in photic entrainment. HB eyelets directly innervate the accessory medulla and affect the neuronal activity of clock neurons (Muraro and Ceriani 2015; Schlichting et al. 2016). When lacking all the external visual organs and *cry*, flies are not able to synchronize to light-dark cycles (LD) at all (Helfrich-Förster et al. 2001).

D. melanogaster has only one *cry* gene in the genome, but many other insects have two *cry* genes, the so-called *cry1* and *cry2*. The *cry1* gene is a homolog of the *Drosophila cry*, whereas the *cry2* gene is similar to the mammalian *cry* genes. An important question to be answered is why and when CRY1 has become to be used as a circadian photoreceptor in some insects. It seems premature to answer this question, but there is some important information available on this issue. Based on phylogenetic analysis, Yuan et al. (2007) suggested that both insect *cry1* and *cry2* homologs existed at the base of metazoan radiation and that gene duplication procedures occurred at least two times to acquire the *cry2* cluster. *cry1* was produced by the first duplication, and *cry2* and vertebrate *cry*, both of which lack photoreceptivity, occurred by the second duplication. CRY1 is suggested to be a circadian photoreceptor in higher-order insects, including flies and butterflies (Yuan et al. 2007). However, it is apparently not a major circadian photoreceptor in lower-order insects, including cockroaches and crickets. Moreover, many insect orders, including one of the most primitive insects, the firebrat *Thermobia domestica*, lacks *cry1* but possesses *cry2* (Kotwica-Rolinska et al. 2022). These circumstances suggest that loss of *cry1* may have occurred separately depending on different insect orders. Thus, the role of *cry*s in entrainment mechanisms should be thoroughly examined in various insect orders to understand its commonality and specificity.

3.3 Molecular Mechanism of Photic Entrainment

3.3.1 Cry-Dependent Pathway

In the photic entrainment mechanism, the *cry*-dependent pathway is best understood in insects (Fig. 3.2). As described in detail in Chap. 4, the *D. melanogaster* clock is based on rhythmic expression of so-called clock genes. The major loop consists of *Clock* (*Clk*), *cycle* (*cyc*), *period* (*per*), and *timeless* (*tim*). The product proteins of *Clk* and *cyc* form a heterodimer CLK/CYC, which promotes transcription of *per* and *tim* during late day to early night, and PER and TIM proteins accumulate in the

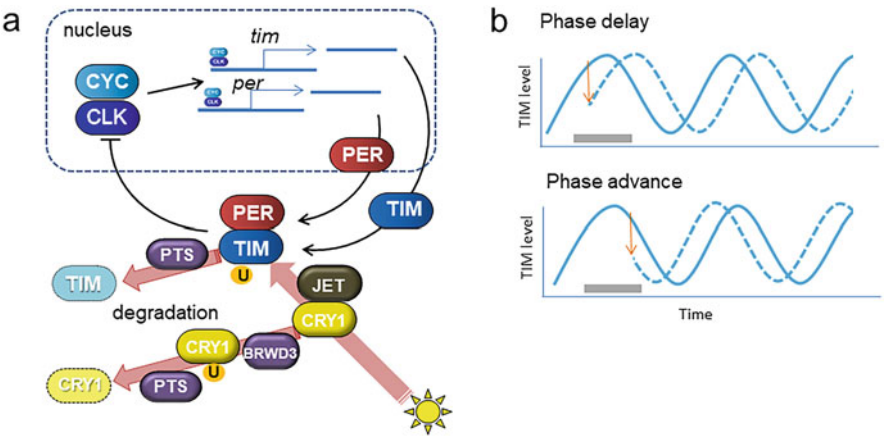

Fig. 3.2 Entrainment mechanism via the *cry* pathway in *Drosophila*. (**a**) Light-activated CRY1 works on TIM together with JET and ubiquitinates TIM. Ubiquitinated TIM leads to its degradation by proteasomes (PTS). CRY1 also leads to its degradation by PTS after ubiquitination by BRWD3. (**b**) Light-induced degradation of TIM results in phase delay in early subjective night (upper panel) but phase advance in late subjective night (lower panel). Gray bars indicate subjective night

cytoplasm during night. At late night, the PER/TIM heterodimer enters the nucleus to repress the transcriptional activity of CLK/CYC, reducing their own transcription and leading to a reduced level of their proteins, which corresponds to daytime. The reduction of PER/TIM releases CLK/CYC from the repression, and the clock oscillatory loop goes to the next round.

Molecular studies on CRY showed that CRY leads to degradation of TIM in a light-dependent manner (Fig. 3.2b). TIM degradation results in resetting of the molecular oscillation of the clock: at early night, the reduction in TIM delays the oscillation because the TIM level is necessary to increase to reach a level sufficient for repression of CLK/CYC. At late night, the reduction of TIM accelerates the oscillation to reach daytime earlier, advancing the clock's phase.

The CRY-dependent degradation of TIM requires another factor, JETLAG (JET) (Fig. 3.2a). JET is a member of the F-box and leucine-rich repeat protein (FBXL) family and a constituent of Cullin1-RING finger (CRL1) E3 ubiquitin ligases, recruiting substrates (Koh et al. 2006; Peschel et al. 2009). Light induces conformational changes in CRY, which enables CRY to bind to JET and TIM (Peschel et al. 2009; Ozturk et al. 2011; Zoltowski et al. 2011). The CRL1 E3 ligase ubiquitinates TIM, and ubiquitinated TIM is subsequently degraded by a proteasome-dependent mechanism (Koh et al. 2006; Peschel et al. 2009). In *D. melanogaster*, CRY is also degraded in a light-dependent manner (Fig. 3.2a). CRY degradation is also caused by another ubiquitin proteasome system, but the ubiquitination is caused by CRL4 E3 ligase, in which bromodomain and WD repeat domain containing 3, BRWD3, works as a component recruiting substrates (Ozturk et al. 2013).

3.3.2 *Compound Eye-Dependent Pathway*

Compound eyes are often used as a circadian photoreceptor necessary for photic entrainment of insect clocks. The neural pathway for compound eye-dependent entrainment has been studied in detail. As mentioned in Sect. 3.2, cutting the optic nerves prevents photic entrainment in crickets and cockroaches, which are solely dependent on the compound eye. In the cricket *G. bimaculatus*, partial destruction of the compound eye weakens entrainability, suggesting that the photic information received by photoreceptor cells in the compound eye is additively integrated in the entrainment pathway and supplied to the clock cells in the optic lobe (Tomioka et al. 1990).

Light information impacts the optic lobe clock cells through neurotransmitters and resets the clock's phase. The resetting mechanism has been unexplained for a long time but is now being rapidly elucidated in the cricket *G. bimaculatus* (Fig. 3.3). In this cricket, the clock molecular oscillatory mechanism consists of two major transcriptional/translational feedback loops, the *per*/*tim* loop and the *cry2* loop, which can oscillate independent of each other (Tokuoka et al. 2017). Both

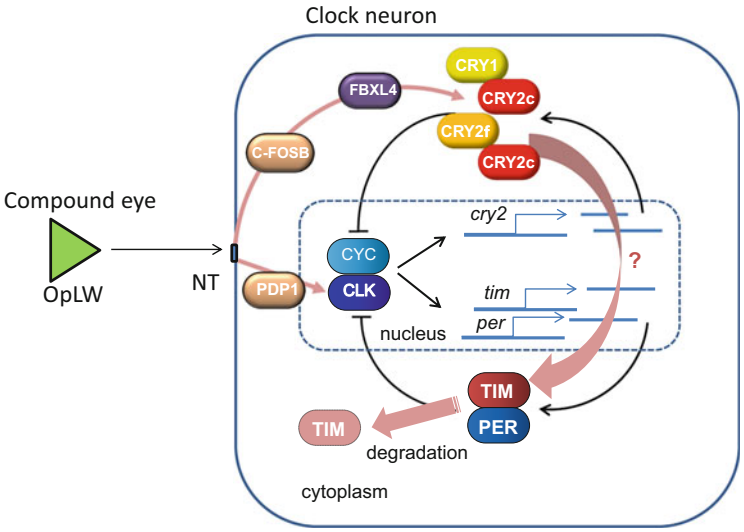

Fig. 3.3 Entrainment mechanism via the compound eye pathway in the cricket *Gryllus bimaculatus*. The cricket clock consists of the *per*/*tim*-loop and *cry2*-loop, which are coupled by sharing CLK/CYC as transcriptional activators (Tokuoka et al. 2017). In the *cry2*-loop, product proteins of *cry2* splicing variants and *cry1* form dimers and inhibit CLK/CYC. Light is perceived by green-sensitive opsin (opsin-long wavelength, OpLW). Light information acts on clock neurons in the optic lobe through neurotransmitters (NT), activating PDP1 or C-FOSB. *Pdp1* is upregulated only when the light off is delayed, leading to upregulation of *Clk*, which in turn upregulates *per* and *tim*, leading to prolonged subjective night, causing phase delay. C-FOSB induces FBXL4, which probably ubiquitinates CRYs and leads to degradation of TIM. Thereby, the *per*/*tim*-loop is reset, which subsequently resets the *cry*-loop

loops share the transcriptional activators CLK/CYC (Moriyama et al. 2012; Uryu et al. 2013; Tokuoka et al. 2017). The *per*/*tim* loop is similar to that of the *Drosophila* clock; *per* and *tim* are transactivated during late day to early night (Moriyama et al. 2008; Danbara et al. 2010). TIM also cycles in a similar time course to that in *D. melanogaster* (Moriyama et al. 2022). *cry2* oscillates in a similar time course to *per* and *tim*, but its product proteins form complexes between CRY2 variants and between CRY2 variants and CRY1, which negatively feedback to repress CLK/CYC transactivation (Tokuoka et al. 2017). In the photic entrainment pathway, light is perceived by a green light-sensitive photoreceptor, OpLW (Komada et al. 2015). The information resets the optic lobe circadian clock via two separate pathways: a *Pdp1* pathway and a *c-fos*/*cry* pathway. The former pathway is activated only when lights off is delayed (Kutaragi et al. 2016). Upregulation of *Pdp1* by delayed light off induces upregulation of *Clk*, which is followed by upregulation of *per* and *tim*, which prolongs the night phase to delay the clock (Kutaragi et al. 2016).

Once it switches to dark, the *Pdp1* pathway no longer functions, and the *c-fos*/*cry* pathway takes its place in resetting the clock (Kutaragi et al. 2018). In the *c-fos*/*cry* pathway, light induces *c-fosB*, which is a bZip transcription factor gene known to be upregulated by light exposure in mammalian circadian clocks (Kornhauser et al. 1990), in the optic lobe within 60 min of light exposure. Light-induced *c-fosB* mediates the induction of several *Fbxl* genes. RNAi-mediated gene silencing revealed that *Fbxl4* is involved in entrainment to light cycles by both advance and delay shifts (Takeuchi et al. 2023): RNAi of *Fbxl4* prevented or delayed entrainment to shifted light cycles. The photic entrainment is disrupted by double RNAi of *cry1* and *cry2* without any effect on light-dependent *c-fosB* induction, and RNAi of *c-fosB* strongly disrupted advance and delay shifts caused by light pulses given at late night and early subjective night, respectively (Kutaragi et al. 2018). These facts suggest that resetting of the clock by light in the dark starts with *c-fosB* induction and that *cry1* and *cry2* work downstream of *c-fosB* and are regulated by *Fbxl*.

We recently found that *tim* plays an essential role in photic entrainment via delay shifts (Moriyama et al. 2022). tim^{RNAi} disrupted reentrainment to delayed light cycles. Light pulse reduces TIM protein levels both at early and late night. TIM reduction at early night should be required for the delay shift of the clock. TIM reduction is probably coupled with the *cry2* oscillatory loop because *cry1*/*cry2* double knockdown by RNAi disrupted the normal entrainment to shifted light cycles in both advance and delay shifts (Kutaragi et al. 2018). This also suggests that TIM degradation is most likely downstream of CRYs. Interestingly, TIM degradation is shared by the compound eye-dependent pathway and the *cry*-dependent pathway, suggesting that TIM may have a common role in light entrainment of insect circadian clocks across species.

3.4 Temperature Entrainment

3.4.1 Role of Temperature in Phase Setting

Temperature is also an important entraining agent in insects. Temperature often cooperates with light cycles to set physiological or behavioral events to occur at an appropriate time of day. In the cricket *G. bimaculatus*, temperature is a weaker zeitgeber for circadian rhythm synchronization. Kannan et al. (2019) showed that *G. bimaculatus* entrains to temperature cycles of 25 °C and 30 °C under DD with a peak around the warm to cool transition and that when the temperature cycle was advanced by 6 h, the locomotor rhythm resynchronized to the shifted temperature cycle with long transient cycles. Temperature step-up and step-down by 5 °C shift the clock in the advance and delay directions, respectively, but no clear phase dependency was observed (Ikeda and Tomioka 1993). In addition to entrainment, temperature has substantial effects on activity rhythms in *G. bimaculatus*. Under 25 °C or higher ambient temperature levels, adult crickets show nocturnal activity, while they switch to show diurnal activity at 20 °C (Ikeda and Tomioka 1993).

In the fruit fly *Drosophila pseudoobscura*, temperature regulates the timing of adult eclosion (Pittendrigh 1960). Eclosion usually occurs in the morning, when the humidity is high, to avoid death from desiccation. This timing is set by two clocks (oscillators). One is the light-entrainable A oscillator, and the other is the temperature-sensitive B oscillator; usually, the A oscillator entrains the B oscillator through an internal mechanism (Pittendrigh 1981; Tackenberg et al. 2017). This A-B two-oscillator model is also applicable to locomotor rhythms in *D. melanogaster*, which shows bimodal activity with a morning and an evening peak (Fig. 3.4). The rhythm is driven by several groups of clock neurons, called small ventrally located lateral neurons (sLNv), fifth sLNv, large-LNv (lLNv), dorsally located LNs (LNd), three groups of dorsal neurons (DN1, DN2, DN3), and lateral posterior neurons (LPN) (Yoshii et al. 2005). Under lower-temperature levels, the morning peak occurs later, and the evening peak occurs earlier. Experiments simultaneously entraining the clock by light and temperature cycles revealed that the light-entrainable clock neurons, including sLNv, fifth sLNv, lLNv, and LNd, set the framework of the activity to occur within the daytime, whereas the temperature-entrainable clocks, i.e., DNs and LPNs, tune the onset of the evening peak according to temperature cycles (Miyasako et al. 2007) (Fig. 3.4). Therefore, temperature plays an important adaptive role in the circadian system.

3.4.2 Molecular Mechanism of Temperature Entrainment

In *D. melanogaster*, the mechanism of temperature entrainment has been studied at cellular and molecular levels (George and Stanewsky 2021). The cerebral clock neurons are thought to have no direct entrainability to temperature cycles because

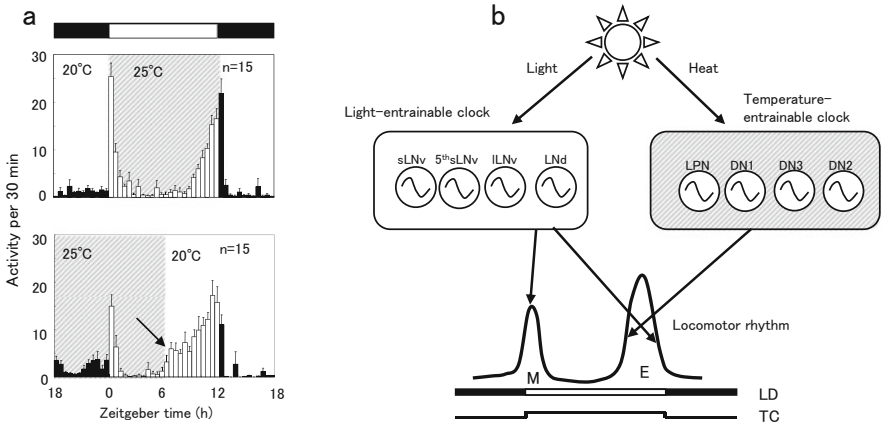

Fig. 3.4 *Drosophila* circadian locomotor rhythm is regulated by two groups of clocks, i.e., light-entrainable and temperature-entrainable clocks. (**a**) Average activity profiles of locomotor rhythm of adult male *Drosophila melanogaster* under a light-dark cycle (LD) (12 h light: 12 h darkness) and temperature cycle (TC) (12 h 20 °C: 12 h 25 °C). Gray shaded areas indicate 25 °C. White and black bars indicate light (while) and dark (black) phases. The white and black columns indicate activity in the light (white) and dark (black) phases, respectively. Error bars indicate SEM. When TC was advanced by 6 h, the onset of the evening peak advanced (arrow), but its offset stayed at lights off. (**b**) A cellular model of the *Drosophila* central clock system for light and temperature entrainment. Laterally located neurons (lLNv, sLNv, fifth sLNv, LNd) are all light-entrainable clocks, some of which determine the morning peak and the offset timing of the evening peak. Dorsally located neurons (DN1, DN2, and DN3) and lateral posterior neurons (LPNs) are temperature-entrainable clocks and regulate the onset timing of the evening peak. Redrawn from Miyasako et al. (2007)

those neurons in isolated brain kept in culture conditions cannot synchronize to temperature cycles. The temperature signal for entrainment is perceived by chordotonal organs, which are located at the joints between limb segments and antenna and are internally attached to the cuticle (Fig. 3.5). While the organs were originally thought to have functions as mechanoreceptors for stretching or vibration, they play an important role in temperature entrainment (Sehadova et al. 2009). The chordotonal organs express *no circadian temperature entrainment* (*nocte*) gene, encoding a large glutamine-rich protein, which is required for temperature entrainment (Glaser and Stanewsky 2005; Sehadova et al. 2009). NOCTE protein is thought to be required for the proper structural conformation and physiological function of the chordotonal organ, which is important for temperature entrainment. The temperature information perceived by the chordotonal organ is sent to the cerebral clock neurons. Among the clock neurons, the posterior DN1 (DN1ps) was recently demonstrated to monitor modest changes in environmental temperature that come not only from the chordotonal organs but also from the aristae of antennae (Yadlapalli et al. 2018).

The molecular mechanism for temperature entrainment is not yet fully understood, but some important information is available (Fig. 3.5b). Most likely, the temperature information is mediated by phospholipase C (PLC) because mutants

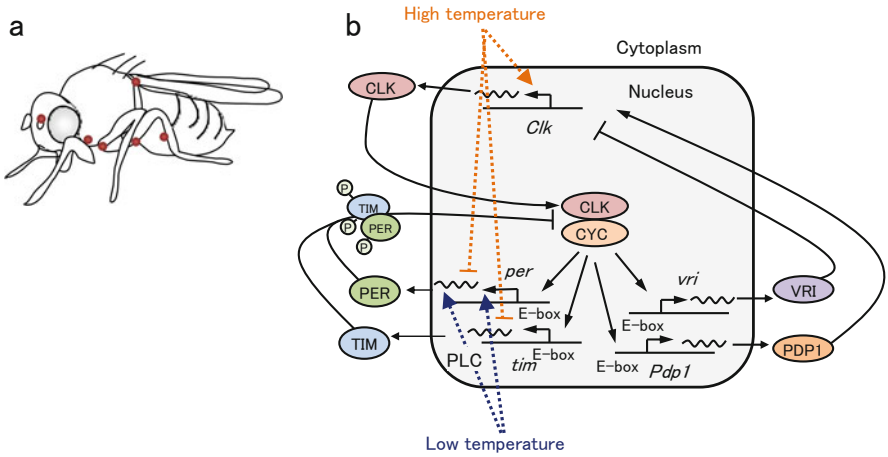

Fig. 3.5 Temperature entrainment mechanism in *Drosophila*. (**a**) Chordotonal organs that perceive ambient temperature. (**b**) A possible model for temperature entrainment. High temperature upregulates *Clock* (*Clk*) but downregulates *per* and *tim*, whereas low temperature upregulates *per* and stimulates its alternative splicing. The dotted arrow indicates upregulation, and the dotted lines with bar ends indicate suppression or downregulation. Redrawn with some modification from Tomioka and Yoshii (2006)

in *norpA* encoding PLC are not able to synchronize to temperature cycles (Glaser and Stanewsky 2005). Although molecular events downstream of PLC are less clear at present, changes in the expression of clock genes may be involved because PLC is suggested to be involved in 3′ splicing of *per* RNA (Collins et al. 2004). A similar temperature-dependent alternative splicing is also observed in the *tim* gene (Martin Anduaga et al. 2019). In fact, temperature has significant effects on the expression of clock genes in *Drosophila*.

Temperature entrainment of the molecular oscillation has been analyzed under constant light, in which the clock is stopped but the temperature cycle forces the clock to oscillate and entrain (Yoshii et al. 2005; Sehadova et al. 2009). Under continuous light conditions, light-activated CRY continuously leads to degradation of TIM, resulting in the arrest of the clock around circadian time (CT) 12. However, the temperature cycle induces molecular oscillation by stimulating clock gene expression. Temperature step-up and step-down have different effects on clock gene expression (Yoshii et al. 2007) (Fig. 3.5b). A temperature step-up from 20 °C to 30 °C stimulates *Clk* gene expression and reduction of *per* and *tim*. PER and TIM proteins are quickly degraded by exposure to a high temperature of 37 °C (Sidote et al. 1998), and TIM degradation is caused by the interaction between CRY and the PER/TIM complex (Fan et al. 2007). High-temperature-induced *Clk* upregulation is followed by upregulation of *per* and *tim* expression, whereas temperature step-down induces an increase in PER levels through upregulation of *per* mRNA (Yoshii et al. 2007). These facts may explain why temperature step-up induces phase advance and step-down phase delay in *D. pseudoobscura*

(Zimmerman et al. 1968). However, how temperature steps change the protein or mRNA levels of clock genes remains to be elucidated.

In other insects, knowledge of the molecular mechanisms of temperature entrainment is quite limited. Only fragmental information is available for molecular events underlying the temperature-induced phase shifts in the cricket *G. bimaculatus* (Kannan et al. 2019). QPCR analysis revealed that clock genes consisting of cricket's clock showed slightly earlier peaks under temperature cycles of 25 °C and 30 °C in DD compared with those under LD. When the temperature cycle was advanced by 6 h, they resynchronized to the shifted temperature cycle with a gradual phase advance. *per* and *tim* resynchronized more quickly than *cry2* and *cyc*. It is thus likely that clock genes play differential roles in resetting the clock in response to changes in environmental temperature cycles. However, further detailed analysis is necessary to clarify the temperature entrainment mechanism in *G. bimaculatus*.

3.5 Entrainment by Restricted Feeding

In mammals, daily scheduled time-restricted feeding induces food anticipatory behavioral rhythms: an increase in activity before feeding (Mistlberger 2011). Interestingly, similar food anticipatory rhythms can be observed even in mutants of canonical clock genes, suggesting the existence of distinct molecular mechanisms. A time-restricted feeding experiment has also been conducted in *D. melanogaster*, but activity rhythms were not clearly entrained by the feeding schedule (Oishi et al. 2004). From this result, one can conclude that the central brain clock is insensitive to feeding. However, it is well known that honey bees can memorize the time and place where food is available and forage at appropriate times, known as time-place learning (Renner 1960). This is a kind of anticipation and is associated with an increase in activity before food availability. The memory of timing can be transferred to another individual by transplanting a mushroom body (Martin et al. 1978). The same behavior has been observed in *D. melanogaster*, and clock genes are involved in time-place learning (Chouhan et al. 2015). At this point, however, it is an open question whether insect time-place learning is mediated by circadian entrainment.

Feeding is a potent zeitgeber for peripheral clocks in the fat body. Time-restricted feeding under DD conditions entrains rhythmic expression of clock and nonclock genes in the fat body in *Drosophila* (Xu et al. 2011) (Fig. 3.6). The fat body clock is not completely independent from the brain clock. Disruption of the brain clock attenuates free-running rhythms of gene expression in the fat body, which are mediated by a neuropeptide, neuropeptide F, produced in cerebral clock neurons (Erion et al. 2016). Additionally, misalignment of feeding and activity rhythms causes lower egg production (Xu et al. 2011). Therefore, it is suggested that the coordinated circadian rhythms between the fat body and central brain clocks are important for fitness.

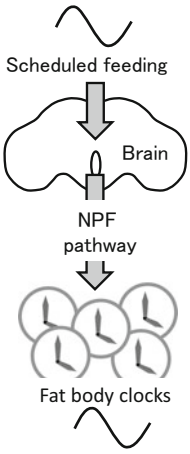

Fig. 3.6 Entrainment pathway for scheduled feeding in *Drosophila*. Zeitgeber information on time-restricted feeding is first transmitted to the brain and then transferred to fat bodies via the NPF signaling pathway. The entrained fat body clocks generate rhythmic gene expression

3.6 Future Perspectives

As we have reviewed in this chapter, the entrainment mechanisms of insect circadian clocks have been extensively studied at cellular and molecular levels, especially for light and temperature. In addition, the phase of the clock is also regulated by food availability and by social factors such as interactions among individuals. Insects live in diverse environments, and investigating which environmental factors they mainly use for entrainment of their clocks will help us understand the adaptive significance of the clocks and their zeitgebers. Zeitgebers may work together in complex ways to harmonize rhythms within the changing environmental cycle. The oscillatory mechanism of the clock is now being elucidated at the molecular level in various insects. Through investigating the entrainment mechanisms by these entraining agents, the commonalities and specificities of the mechanisms by which each agent acts on the oscillatory mechanism and controls the phase will be elucidated. Furthermore, in *Drosophila*, it has been shown that the zeitgebers used primarily vary from clock cell to clock cell. Thus, the mechanism of mutual phase control between these cells will be an important issue for the future.

References

Bloch G, Herzog ED, Levine JD, Schwartz WJ (2013) Socially synchronized circadian oscillators. Proc R Soc B 280:20130035. https://doi.org/10.1098/rspb.2013.0035

Chouhan NS, Wolf R, Helfrich-Förster C, Heisenberg M (2015) Flies remember the time of day. Curr Biol 25:1619–1624. https://doi.org/10.1016/j.cub.2015.04.032

Cloudsley-Thompson JL (1953) Studies on diurnal rhythms. III. Photoperiodism in the cockroach. Ann Mag Nat Hist 6:705–712. https://doi.org/10.1080/00222935308654473

Collins BH, Rosato E, Kyriacou CP (2004) Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. Proc Natl Acad Sci 101:1945–1950. https://doi.org/10.1073/pnas.0308240100

Danbara Y, Sakamoto T, Uryu O, Tomioka K (2010) RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 56: 1738–1745. https://doi.org/10.1016/j.jinsphys.2010.07.002

Dunlap JC, Loros J, DeCoursey PJ (2004) Chronobiology: biological timekeeping. Sinauer, Sunderland, MA

Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679. https://doi.org/10.1016/s0092-8674(00)81637-2

Erion R, King AN, Wu G, Hogenesch JB, Sehgal A (2016) Neural clocks and Neuropeptide F/Y regulate circadian gene expression in a peripheral metabolic tissue. elife 5:e13552. https://doi.org/10.7554/eLife.13552

Fan J-Y, Muskus MJ, Price JL (2007) Entrainment of the *Drosophila* circadian clock: more heat than light. Science 413:pe65. https://doi.org/10.1126/stke.4132007pe65

Fogle KJ, Parson KG, Dahm NA, Holmes TC (2011) CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. Science 331:1409–1413. https://doi.org/10.1126/science.1199702

George R, Stanewsky R (2021) Peripheral sensory organs contribute to temperature desynchronization of the circadian clock in *Drosophila melanogaster*. Front Physiol 12: 622545. https://doi.org/10.3389/fphys.2021.622545

Glaser FT, Stanewsky R (2005) Temperature synchronization of the *Drosophila* circadian clock. Curr Biol 15:1352–1363. https://doi.org/10.1016/j.cub.2005.06.056

Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:149–261. https://doi.org/10.1016/s0896-6273(01)00277-x

Helfrich C, Englemann W (1983) Circadian rhythm of the locomotor activity in *Drosophila melanogaster* and its mutants 'sine oculis' and 'small optic lobes'. Physiol Entomol 8:257–272. https://doi.org/10.1111/j.1365-3032.1983.tb00358.x

Ikeda M, Tomioka K (1993) Temperature dependency of the circadian locomotor rhythm in the cricket *Gryllus bimaculatus*. Zool Sci 10:597–604. https://doi.org/10.34425/zs001120

Ito C, Goto SG, Shiga S, Tomioka K, Numata H (2008) Peripheral circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster*. Proc Natl Acad Sci 105:8446–8451. https://doi.org/10.1073/pnas.0800145105

Kannan NN, Tomiyama Y, Nose M, Tokuoka A, Tomioka K (2019) Temperature entrainment of circadian locomotor and transcriptional rhythms in the cricket, *Gryllus bimaculatus*. Zool Sci 36:95–104. https://doi.org/10.2108/zs180148

Katô M, Triumi M (1950) Studies in the associative ecology of insects. I. Nocturnal succession of a mosquito association in the biting activity. Sci Rep Tohoku Univ Ser IV (Biol) 18:467–472

Kistenpfennig C, Hirsh J, Yoshii T, Helfrich-Förster C (2012) Phase-shifting the fruit fly clock without cryptochrome. J Biol Rhythm 27:117–125. https://doi.org/10.1177/0748730411434390

Koh K, Zheng X, Sehgal A (2006) JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. Science 312:1809–1812. https://doi.org/10.1126/science.1124951

Komada S, Kamae Y, Koyanagi M, Tatewaki K, Hassaneen E, Saifullah A et al (2015) Green-sensitive opsin is the photoreceptor for photic entrainment of an insect circadian clock. Zool Lett 1:11. https://doi.org/10.1186/s40851-015-0011-6

Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS (1990) Photic and circadian regulation of *c-fos* gene expression in hamster suprachiasmatic nucleus. Neuron 5:127–134. https://doi.org/10.1016/0896-6273(90)90303-w

Kotwica-Rolinska J, Chodáková L, Smýkal V, Damulewicz M, Provazník J, Wu BC-H et al (2022) Loss of timeless underlies an evolutionary transition within the circadian clock. Mol Biol Evol 39:msab346. https://doi.org/10.1093/molbev/msab346

Kutaragi Y, Miki T, Bando T, Tomioka K (2016) Transcriptional and non-transcriptional events are involved in photic entrainment of the circadian clock in the cricket *Gryllus bimaculatus*. Physiol Entomol 41:358–368. https://doi.org/10.1111/phen.12162

Kutaragi Y, Tokuoka A, Tomiyama Y, Nose M, Watanabe T, Bando T et al (2018) A novel photic entrainment mechanism for the circadian clock in an insect: involvement of *c-fos* and *cryptochrome*s. Zool Lett 4:26. https://doi.org/10.1186/s40851-018-0109-8

Leppla NC, Koehler PG, Agee HR (1989) Circadian rhythms of the German cockroach (Dictyoptera: Blattellidae): locomotion in response to different photoperiods and wavelengths of light. J Insect Physiol 35:63–66. https://doi.org/10.1016/0022-1910(89)90037-1

Lin C, Todo T (2005) The cryptochromes. Genome Biol 6:220. https://doi.org/10.1186/gb-2005-6-5-220

Loher W (1972) Circadian control of stridulation in the cricket *Teleogryllus commodus* Walker. J Comp Physiol 79:173–190. https://doi.org/10.1007/BF00697770

Martin Anduaga A, Evantal N, Patop IL, Bartok O, Weiss R, Kadener S (2019) Thermosensitive alternative splicing senses and mediates temperature adaptation in *Drosophila*. elife 8:e44642. https://doi.org/10.7554/eLife.44642

Martin U, Martin H, Lindauer M (1978) Transplantation of a time-signal in honey bees. J Comp Physiol 124:193–201. https://doi.org/10.1007/BF00657051

Mistlberger RE (2011) Neurobiology of food anticipatory circadian rhythms. Physiol Behav 104:535–545. https://doi.org/10.1016/j.physbeh.2011.04.015

Miyasako Y, Umezaki Y, Tomioka K (2007) Separate sets of cerebral clock neurons are responsible for light and temperature entrainment of *Drosophila* circadian locomotor rhythms. J Biol Rhythm 22:115–126. https://doi.org/10.1177/0748730407299344

Moriyama Y, Kamae Y, Uryu O, Tomioka K (2012) *Gb'Clock* is expressed in the optic lobe and required for the circadian clock in the cricket *Gryllus bimaculatus*. J Biol Rhythm 27:467–477. https://doi.org/10.1177/0748730412462207

Moriyama Y, Sakamoto T, Karpova SG, Matsumoto A, Noji S, Tomioka K (2008) RNA interference of the clock gene *period* disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. J Biol Rhythm 23:308–318. https://doi.org/10.1177/0748730408320486

Moriyama Y, Takeuchi K, Shinohara T, Miyagawa K, Matsuka M, Yoshii T et al (2022) *Timeless* plays an important role in compound eye-dependent photic entrainment of the circadian rhythm in the cricket *Gryllus bimaculatus*. Zool Sci 39:397–405. https://doi.org/10.2108/zs220011

Mote MI, Black KR (1981) Action spectrum and threshold sensitivity of entrainment of circadian running activity in the cockroach *Periplaneta americana*. Photochem Photobiol 34:257–265. https://doi.org/10.1111/j.1751-1097.1981.tb08995.x

Muraro NI, Ceriani MF (2015) Acetylcholine from visual circuits modulates the activity of arousal neurons in *Drosophila*. J Neurosci 35:16315–16327. https://doi.org/10.1523/jneurosci.1571-15.2015

Nishiitsutsuji-Uwo J, Pittendrigh CS (1968) Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. Z Vergl Physiol 58:1–13. https://doi.org/10.1007/BF00302433

Oishi K, Shiota M, Sakamoto K, Kasamatsu M, Ishida N (2004) Feeding is not a more potent Zeitgeber than the light-dark cycle in *Drosophila*. Neuroreport 15:739–743. https://doi.org/10.1097/00001756-200403220-00034

Ozturk N, Selby CP, Annayev Y, Zhong D, Sancar A (2011) Reaction mechanism of *Drosophila* cryptochrome. Proc Natl Acad Sci 108:516–521. https://doi.org/10.1073/pnas.1017093108

Ozturk N, VanVickle-Chavez SJ, Akileswaran L, Van Gelder RN, Sancar A (2013) Ramshackle (Brwd3) promotes light-induced ubiquitylation of *Drosophila* cryptochrome by DDB1-CUL4-ROC1 E3 ligase complex. Proc Natl Acad Sci 110:4980–4985. https://doi.org/10.1073/pnas.1303234110

Peschel N, Chen KF, Szabo G, Stanewsky R (2009) Light-dependent interactions between the *Drosophila* circadian clock factors Cryptochrome, Jetlag, and Timeless. Curr Biol 19:241–247. https://doi.org/10.1016/j.cub.2008.12.042

Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. Cold Spring Harb Symp Quant Biol 25:159–184. https://doi.org/10.1101/SQB.1960.025.01.015

Pittendrigh CS (1981) Circadian systems: entrainment. In: Aschoff J (ed) Handbook of behavioral neurobiology, Biological rhythms, vol 4. Plenum Press, New York; London, pp 95–124

Pittendrigh CS, Minis DH (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. Am Nat 98:261–294. https://doi.org/10.1086/282327

Rence BG, Lisy MT, Garves BR, Quilan BJ (1988) The role of ocelli in circadian singing rhythms of crickets. Physiol Entomol 13:201–212. https://doi.org/10.1111/j.1365-3032.1988.tb00924.x

Renner M (1960) The contribution of the honey bee to the study of time-sense and astronomical orientation. Cold Spring Harb Symp Quant Biol 25:361–367. https://doi.org/10.1101/sqb.1960.025.01.037

Rieger D, Stanewsky R, Helfrich-Förster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. J Biol Rhythm 18:377–391. https://doi.org/10.1177/0748730403256997

Roberts SKF (1960) Circadian activity in cockroaches. I. The free-running rhythm in steady-state. J Cell Comp Physiol 55:99–110. https://doi.org/10.1002/jcp.1030550112

Saunders DS, Steel CGH, Vafopoulou X, Lewis RD (2002) Insect clocks, 3rd edn. Elsevier, Amsterdam

Schlichting M, Menegazzi P, Lelito KR, Yao Z, Buhl E, Dalla Benetta E et al (2016) A neural network underlying circadian entrainment and photoperiodic adjustment of sleep and activity in *Drosophila*. J Neurosci 36:9084–9096. https://doi.org/10.1523/jneurosci.0992-16.2016

Sehadova H, Glaser F, Gentile C, Simoni A, Giesecke A, Albert J et al (2009) Temperature entrainment of *Drosophila*'s circadian clock involves the gene *nocte* and signaling from peripheral sensory tissues to the brain. Neuron 64:251–266. https://doi.org/10.1016/j.neuron.2009.08.026

Shiga S, Numata H, Yoshioka E (1999) Localization of the photoreceptor and pacemaker for the circadian activity rhythm in the band-legged ground cricket, *Dianemobius nigrofasciatus*. Zool Sci 16:193–201. https://doi.org/10.2108/zsj.16.193

Sidote D, Majercak J, Parikh V, Edery I (1998) Differential effects of light and heat on the *Drosophila* circadian clock proteins PER and TIM. Mol Cell Biol 18:2004–2013. https://doi.org/10.1128/MCB.18.4.2004

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA et al (1998) The *cryb* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692. https://doi.org/10.1016/S0092-8674(00)81638-4

Tackenberg MC, Johnson CH, Page TL, Daan S (2017) Revealing oft-cited but unpublished papers of Colin Pittendrigh and coworkers. J Biol Rhythm 32:291–294. https://doi.org/10.1177/0748730417716685

Takeuchi K, Matsuka M, Shinohara T, Hamada M, Tomiyama Y, Tomioka K (2023) *Fbxl4* regulates the photic entrainment of circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. Zool Sci 40:53–63. https://doi.org/10.2108/zs220047

Tokuoka A, Itoh TQ, Hori S, Uryu O, Danbara Y, Nose M et al (2017) *Cryptochrome* genes form an oscillatory loop independent of the *per/tim* loop in the circadian clockwork of the cricket *Gryllus bimaculatus*. Zool Lett 3:5. https://doi.org/10.1186/s40851-017-0066-7

Tomioka K, Chiba Y (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the circadian locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zool Sci 1:375–382. https://doi.org/10.2108/zsj.1.375

Tomioka K, Yoshii T (2006) Entrainment of *Drosophila* circadian rhythms by temperature cycles. Sleep Biol Rhythms 4:240–247. https://doi.org/10.1111/j.1479-8425.2006.00227.x

Tomioka K, Okada Y, Chiba Y (1990) Distribution of circadian photoreceptors in the compound eye of the cricket *Gryllus bimaculatus*. J Biol Rhythm 5:131–139. https://doi.org/10.1177/074873049000500403

Uryu O, Karpova SG, Tomioka K (2013) The clock gene *cycle* plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. J Insect Physiol 59:697–704. https://doi.org/10.1016/j.jinsphys.2013.04.011

Waddel B, Lewis RD, Engelmann W (1990) Localization of the circadian pacemakers of *Hemideina thoracica* (Orthoptera; Stenopelmatidae). J Biol Rhythm 5:131–139. https://doi.org/10.1177/074873049000500205

Xu K, DiAngelo JR, Hughes ME, Hogenesch JB, Sehgal A (2011) The circadian clock interacts with metabolic physiology to influence reproductive fitness. Cell Metab 13:639–654. https://doi.org/10.1016/j.cmet.2011.05.001

Yadlapalli S, Jiang C, Bahle A, Reddy P, Meyhofer E, Shafer OT (2018) Circadian clock neurons constantly monitor environmental temperature to set sleep timing. Nature 555:98–102. https://doi.org/10.1038/nature25740

Yasuyama K, Meinertzhagen IA (1999) Extraretinal photoreceptors at the compound eye's posterior margin in *Drosophila melanogaster*. J Comp Neurol 412:193–202. https://doi.org/10.1002/(sici)1096-9861(19990920)412:2

Yoshii T, Fujii K, Tomioka K (2007) Induction of *Drosophila* behavioral and molecular circadian rhythms by temperature steps in constant light. J Biol Rhythm 22:103–114. https://doi.org/10.1177/0748730406298176

Yoshii T, Todo T, Wülbeck C, Stanewsky R, Helfrich-Förster C (2008) Cryptochrome is present in the compound eyes and a subset of *Drosophila*'s clock neurons. J Comp Neurol 508:952–966. https://doi.org/10.1002/cne.21702

Yoshii T, Heshiki Y, Ibuki-Ishibashi T, Matsumoto A, Tanimura T, Tomioka K (2005) Temperature cycles drive *Drosophila* circadian oscillation in constant light that otherwise induces behavioural arrhythmicity. Eur J Neurosci 22:1176–1184. https://doi.org/10.1111/j.1460-9568.2005.04295.x

Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24:948–955. https://doi.org/10.1093/molbev/msm011

Zimmerman WF, Pittendrigh CS, Pavlidis T (1968) Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. J Insect Physiol 14:669–684. https://doi.org/10.1016/0022-1910(68)90226-6

Zoltowski BD, Vaidya AT, Top D, Widom J, Young MW, Crane BR (2011) Structure of full-length *Drosophila* cryptochrome. Nature 480:396–399. https://doi.org/10.1038/nature10618

Chapter 4
Molecular Mechanism of the Circadian Clock

David Doležel

Abstract Nearly all organisms possess a circadian clock, a genetically determined device that generates endogenous oscillations with a period of approximately 24 h. From a molecular perspective, the circadian clock relies on negative transcription-translation feedback loops. In insects, the molecular and genetic basis of the circadian clock machinery has been revealed by the remarkable genetic tools available to the fruit fly *Drosophila melanogaster*. However, the dawn of reverse genetics methods applicable to nonmodel species has led to recent significant advances in our understanding of the circadian clock beyond *Drosophila*. To illustrate the molecular mechanism behind the insect circadian clock, the first section focuses primarily on *Drosophila melanogaster* as the best established and most detailed insect model. Conserved components of the insect clocks are then identified at the genetic level, and lineage-specific idiosyncrasies and variations in setup are highlighted and further discussed. Functional evidence from non-*Drosophila* insects is reviewed, and the main descriptive data from molecular biology are presented in an evolutionary context and briefly summarized.

Keywords Cryptochrome · Evolution · Negative feedback · Oscillator · Period · Transcription

4.1 Introduction

Before we dive into the details and setup of molecular machinery driving the circadian clock in various insect species, the general background should be presented. The purpose of the biological clock ticking with a period of approximately 24 h lies on the time scale quite far from the time needed for typical biochemical processes, such as transcription, translation, protein phosphorylation,

D. Doležel (✉)
Biology Centre, Institute of Entomology, Czech Academy of Sciences, České Budějovice, Czech Republic
e-mail: david.dolezel@entu.cas.cz

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dephosphorylation, and degradation. Thus, a set of multiple interlocked loops, including some or even all of the above-listed regulatory processes, are found in the circadian clocks of various systems. It is unclear whether the circadian clock has evolved independently in kingdoms several times or if one ancestral clock has been heavily modified in different lineages of organisms during their evolution. At least, it is safe to say that we can find rather differently built clocks in cyanobacteria, plants, fungi, protozoa, and bilaterian animals (reviewed in Dunlap 1999). In cyanobacteria, the elegant phosphorylation cycle among just a few kinases can stably run even in vitro. Plant, fungal, and bilaterian clocks utilize a combination of interlocked feedback loops with transcription factors (also known as positive components) in the center that drive the expression of multiple genes, including the negative elements.

In Bilateria, two excellent models shaped our knowledge when research on one synergistically supported and motivated research on the other. First, *Drosophila* genetic tools lead to seminal discoveries of the *period* (*per*) and *timeless* (*tim*) genes. Then, new clock genes were identified in flies and mice, gradually building up the picture of the conserved animal clock setup with several specific feature characteristic of the fly and feature and component characteristic of the mouse clock. However, with the growing genomic sequencing, it became clear that the picture is not that simple and even some insect species remarkably differ from *Drosophila*. As functional genetic research further expanded, we can now see that insect clocks are more colorful than might have been expected at first. After all, with more than 400 million years of evolution and with millions of species known today, I would consider insect evolution as a remarkable collection of various solutions to the same or slightly different problems, a beautiful selection experiment for which the notes are not available. To illustrate the molecular mechanism behind the insect circadian clock, I will first focus mostly on the *Drosophila melanogaster* circadian clock as the best established and most detailed insect model (although, in some cases, the comparison to other insects will be provided immediately). Then, the conserved components of the insect clocks are identified, and lineage-specific idiosyncrasies are highlighted and further discussed.

4.2 Clock Setup in *Drosophila*

Unprecedented genetic tools predispose *D. melanogaster* to be a powerful model for gene discovery and elucidation of underlying molecular mechanisms. There are several excellent and detailed reviews on the *Drosophila* circadian clock and the mechanism involved in its regulation (Ozkaya and Rosato 2012; Hardin 2011; Peschel and Helfrich-Förster 2011; Stanewsky 2003; Hall 2003; Tataroglu and Emery 2015; Lim and Allada 2013a). A seminal screen by Ronald Konopka led to the identification of the *per* gene at the beginning of the 1970s (Konopka and Benzer 1971), whereas the gene was mapped by positional cloning more than a decade later (Zehring et al. 1984; Bargiello et al. 1984). The key feature of *per* regulation was a cyclical abundance of its transcript oscillating with a period of 24 h and, as

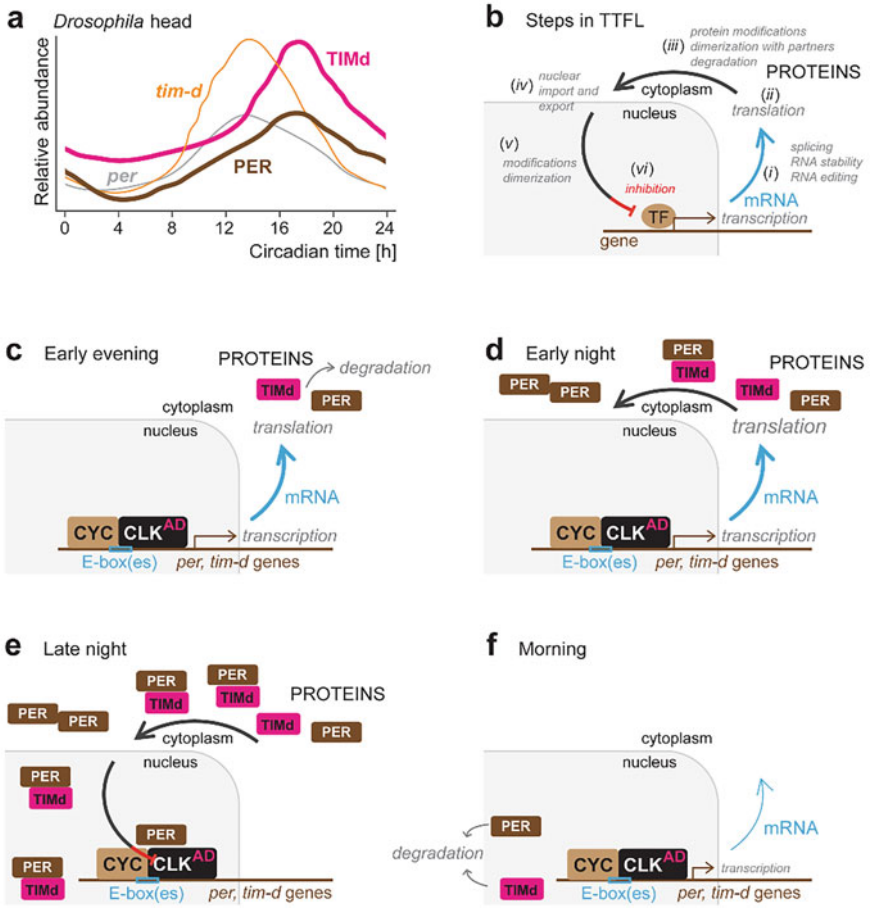

Fig. 4.1 *Drosophila*-negative transcription-translation feedback loop (TTFL). (**a**) Relative abundance of *per* and *tim-d* mRNAs compared to PER and TIMd (*Drosophila*-type TIM) proteins illustrates oscillation in abundance and a delay between the accumulation of mRNA and protein in whole head extracts (redrawn from Dunlap 1999). (**b**) Schematic depiction of TTFL with major steps and regulatory mechanisms, illustrating the activity and inhibition of a hypothetical *t*ranscription *f*actor (TF). (**c**) In the early evening, CLK-CYC drives transcription from *per* and *tim-d* genes. *A*ctivation *d*omain (AD) is located on CLK protein in *Drosophila*. The resulting mRNAs are translated, but the proteins are (mostly) degraded. (**d**) As the night progresses, PER and TIMd proteins are stabilized by heterodimerization, although PER-PER homodimers are also observed. (**e**) During the late night, PER and TIMd enter the cell nucleus, where PER molecules inhibit the transcription factor CLK. (**f**) As PER and TIMd are eventually degraded, the inhibition fades away, and a new round of transcription starts in the morning

discovered by Siwicki et al. (1988), the cyclical abundance of the PER protein, which peaked with a few-hour delay after the *per* mRNA reached its maximum. See Fig. 4.1a for an illustration of the expression pattern in the whole head extracts. The cyclical transcription implied a feedback loop (Hardin et al. 1990); however, a

simple transcription-translation feedback loop (TTFL) should produce oscillations on a scale of minutes. Thus, additional regulatory steps should be involved in TTFL participating in the circadian clock.

Figure 4.1b illustrates the general principle of the negative TTFL with major conceivable regulatory mechanisms adjusting the period of its oscillation. First, a transcription factor(s) drives the expression of mRNA coding for the negative element, which, before translation, needs to be processed, and its stability might be further regulated. To prevent degradation, the negative element protein(s) might be stabilized by modification steps such as phosphorylation, by interaction with partner protein(s) forming either the heterodimer or homodimer, and even by additional modifications (glycosylations, SUMOylation, etc.). The last step of the feedback is the interaction of the negative element with the transcription factor(s), preventing its activity. As a consequence, no mRNA coding for the negative element is transcribed. Transcription can only start once the nucleus-localized negative element protein is removed from the system. Thus, protein stability and subcellular localization are key regulatory steps of the whole process leading to 24-h oscillations.

4.2.1 *Period and* Drosophila-*Type Timeless*

In *Drosophila*, the first well-described TTFL comprises proteins PER and TIM, here referred to as *Drosophila*-type *tim-d* (TIMd for the protein) to distinguish it from its paralogous mammalian-type (*tim-m*, TIMm). Similar to *per*/PER expression, *tim-d* and TIMd oscillate in abundance in a 24-h cycle, when mRNA peaks circa 4 h before TIMd (Fig. 4.1a). Furthermore, the subcellular localization pattern of PER and TIMd changes over time, all of which led to the establishment of the following model. Let us focus on the most important clock neurons in the fly brain responsible for behavioral rhythmicity (the expression might differ in various cell types across the body; see Chap. 5). During the early evening (Fig. 4.1c), *per* and *tim-d* mRNAs are transcribed; however, the corresponding proteins are not detectable, as they are degraded. Then, during the early night, PER and TIMd are stabilized by dimerization, and both proteins are detected in the cytoplasm (Fig. 4.1d). Gradually, PER and TIMd accumulate, and then, during late night (Fig. 4.1e), both proteins are detected in the nucleus. At the same time, transcription of *per* and *tim-d* mRNAs stops because PER interacts with the transcription factor CLOCK (CLK). CLK, a basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) protein, forms a dimer with another bHLH-PAS protein CYCLE (CYC), and together they drive expression from the so-called E-box (cis-regulatory DNA elements with motif CACGTG). E-boxes are localized in the promoters of multiple genes, including *per* and *tim-d*, and are key for their cyclical expression. The inhibition of CLK-CYC continues as long as PER is present in the nucleus. After its depletion (Fig. 4.1f), the transcription of *per* and *tim-d* mRNAs resumes again.

The above-described model has been synthesized from data gathered on mutant and wild-type flies (Hardin et al. 1990; Siwicki et al. 1988; Zerr et al. 1990; Sehgal et al. 1994; Sehgal et al. 1995; Allada et al. 1998; Rutila et al. 1998; Glossop et al. 1999). The feedback mechanism was further reconstructed in *Drosophila* Schneider 2 (S2) cell cultures (Darlington et al. 1998), and the role of the E-box was addressed both in S2 cells and in flies using elegant in vivo luciferase reporters (Brandes et al. 1996; McDonald et al. 2001). The protein domains important for PER-TIMd interaction and nuclear localization were systematically explored in S2 cells (Saez and Young 1996; Saez et al. 2011), even though real-time monitoring in S2 cells using FRET (fluorescence resonance energy transfer) revealed that the spatial organization of the dimer differs in the cytoplasm and nucleus (Meyer et al. 2006). Furthermore, functional PER-PER homodimers exist in flies, and if their mutual dimerization is impaired, molecular and behavioral rhythmicity is severely disrupted (Landskron et al. 2009). In addition to nuclear localization signals (NLS), PER and TIMd also contain nuclear export signals (NES) (Ashmore et al. 2003; Hara et al. 2011; Jang et al. 2015; Singh et al. 2019; Cai et al. 2021). For details on TIMd and TIMm biology, see a thorough review by Cai and Chiu (2021).

A great deal of data was revealed thanks to a beautiful collection of genetic mutants created in *Drosophila*, which, after all, provide the most accurate understanding of the impact on the ultimate output, the fly's behavior. A fundamental feature of the circadian clock is temperature compensation, the phenomenon when the free-running period remains constant over a physiologically acceptable range of temperatures (the meaningful temperature range for recording fly locomotor activity is approximately 15–29°C). Some of the *per* and *tim-d* mutations result in a good temperature-compensated clock of either a long or short free-running period (Rothenfluh et al. 2000a, 2000b; Hamblen et al. 1998). However, per^{L} and multiple *tim-d* mutants produce a slower clock at high temperatures (Konopka et al. 1989; Matsumoto et al. 1999; Singh et al. 2019), which can be understood as a temperature-overcompensated clock, although one might argue that the mutated proteins are simply unstable at high temperatures. Interestingly, the mutated protein TIM^{SL} shortens the free-running period when expressed in the per^{L} mutant and completely restores the temperature compensation deficiency (Rutila et al. 1996). The opposite trend, temperature undercompensated clock, is produced by per^{SLIH}, per^{S}, and per^{T} mutations (Konopka et al. 1989; Konopka et al. 1994; Hamblen et al. 1998). The last three *per* mutants were particularly instrumental in elucidating how phosphorylation influences PER stability (Chiu et al. 2008; Chiu et al. 2011).

4.2.2 Posttranslational Modifications

The key part of the negative PER-TIMd TTFL is the delay between mRNA and protein peaks. The regulation of PER and TIMd stability is tightly connected with phosphorylation and dephosphorylation performed by several kinases and phosphatases. The phosphorylation of PER can also serve as a fascinating tale illustrating the

synergistic research on the fruit fly and mammalian clocks. In 1998, two seminal papers described DOUBLETIME, a casein kinase 1 (CK1) isoform[1] type, as a key clock component in *Drosophila* (Price et al. 1998; Kloss et al. 1998). In mammals, a homologous casein kinase 1ε (CK1ε) was identified as a component of the hamster clock when the *tau* mutation was mapped by positional cloning as an arginine-to-cysteine amino acid substitution at a highly conserved position within the kinase domain (Lowrey et al. 2000). Only a year later, Toh et al. (2001) described in humans the molecular basis for familial advanced sleep phase syndrome (FASPS) as a serine-to-glycine mutation within the CK1ε binding region of human PER2[2], which results in hypophosphorylated PER2.

The physical interaction between DBT kinase and PER is remarkably stable (Kloss et al. 2001). Interestingly and surprisingly, in vitro studies on DBT^{L} and DBT^{S} using nonphysiological substrates suggested that both mutant proteins have reduced kinase activity (Kivimaë et al. 2008; Venkatesan et al. 2019), even though the resulting behavioral phenotype of these mutants is the opposite (Price et al. 1998). The molecular mechanism behind this conundrum was first revealed for mammalian PER2 in the context of multi-kinase hierarchical activities. The current phosphoswitch model involves two competing phosphorylation sites on mouse PER2, the FASP site and the phosphodegron sites, which regulate PER2 stability in opposing ways (Zhou et al. 2015; Masuda et al. 2020). Importantly, different splicing isoforms of mammalian CK1δ, a paralog of mammalian CK1ε, have different priming phosphorylation capacities – that is, the ability to phosphorylate residues in a protein region where no other phosphorylation mark is attached. CK1δ1 and CK1ε are more active in priming phosphorylation of the FASP site, whereas CK1δ2 is more potent in priming the degron site (see the excellent review by Narasimamurthy and Virshup 2021).

In *D. melanogaster*, the intronless *dbt* gene encodes only one protein version; thus, the priming phosphorylation of PER must be regulated by a different mechanism. Similar to mammalian PER2, *Drosophila* PER contains multiple phosphoclusters (Chiu et al. 2008; Garbe et al. 2013; Kivimaë et al. 2008; Top et al. 2018). The phosphorylation of the N-terminal region (serine 47 and nearby amino acids) by DBT generates a high-affinity binding site for *supernumerary limbs* (SLIMB) (Chiu et al. 2008), an F-box protein targeting PER for ubiquitination and subsequent proteasomal degradation (Grima et al. 2002; Ko et al. 2002; see also Sect. 4.2.4). On the other hand, phosphorylation by NEMO/NLK kinase at the per-short domain, a region located in the center of PER protein (positions 585–600 in 1224-amino acid PER), stimulates additional phosphorylation of several nearby sites by DBT. This multisite phosphorylation of the central part of PER prevents N-terminal

[1] In this case, CK1 isoforms are coded by distinct genes, whereas the term isoform might also be used for variants originating from alternative splicing or alternative transcription start. See *Pdp1* and *Kay* genes in bZIP protein sections as examples of the latter.

[2] Circadian clock genes are often multiplicated in vertebrates, so there are three *per* genes, the so-called paralogs, encoding three proteins: PER1, PER2, and PER3.

phosphorylation, which is key for SLIMB binding, resulting in a time-delay phosphorylation circuit (Chiu et al. 2011). The complex phosphorylation pattern of PER seems to be one of the mechanisms behind the temperature compensation of the circadian clock in *Drosophila* (Joshi et al. 2022).

Several additional kinases were discovered as important components of the *Drosophila* clock affecting the negative TTFL with PER and TIMd. Some of them, such as NEMO (NMO), CK1α, and p38 phosphorylate PER (Chiu et al. 2011; Lam et al. 2018; Dusik et al. 2014), whereas CASEIN KINASE 2 (CK2) phosphorylates both PER and TIMd in *Drosophila* (Cai et al. 2021). SHAGGY (SGG), a kinase identified as a TIMd-phosphorylating enzyme, might also phosphorylate PER (Martinek et al. 2001; Top et al. 2016).

In addition to kinases, enzymes with the opposite role, phosphatases, participate in the regulatory loops of the circadian clock and counterbalance the impact of kinases on various TTFL components. PROTEIN PHOSPHATASE 2 (PP2), a multiunit enzyme, specifically dephosphorylates PER and regulates its abundance, PROTEIN PHOSPHATASE 1 (PP1) targets both PER and TIMd, and phosphatase of regenerating liver-1 (PRL1) selectively dephosphorylates TIMd in darkness (Sathyanarayanan et al. 2004; Fang et al. 2007; Kula-Eversole et al. 2021; Agrawal and Hardin 2016).

However, since many of the abovementioned kinases and phosphatases tend to recognize and (de)phosphorylate multiple substrates, their role in the clock has gradually become increasingly complex. For example, the transcription factor CLK is cyclically phosphorylated, with its minimum phosphorylation detected in the early night and its maximal phosphorylation in the morning (Lee et al. 1998). Its partner CYC is constitutively expressed (at least in *Drosophila* and related flies; see CLK-CYC idiosyncrasies in the lineage-specific description later in this chapter); thus, the cyclical phosphorylation of CLK seems to be the key rhythmic component of the circadian activator (Yu et al. 2006). The phase-specific hyperphosphorylation is DBT-dependent and leads to maximal repression of CLK activity. Remarkably, PP2, introduced in a previous paragraph as PER-regulating phosphatase, stabilizes CLK by counteracting the activity of DBT (Kim and Edery 2006). Furthermore, DBT-mediated degradation of CLK is further counterbalanced by CK2, which inhibits CLK degradation and reduces its activity (Szabo et al. 2013).

Not only is the phosphorylation state important to define the stability of the proteins and determine their interaction with partner proteins, but it might also specifically impact their subcellular localization, for example, the nuclear export of TIMd (Cai et al. 2021; Fang et al. 2007). An additional posttranslational modification, such as O-GlcNAcylation of PER, reduces PER interaction with CLK, a mechanism that might link the clock with the metabolic signals stemming from feeding activity (Li et al. 2019; Liu et al. 2021).

4.2.3 Entrainment by Light

The above-described machinery illustrates the molecular mechanism of the *Drosophila* clock "ticking" in constant darkness. However, the clock must be synchronized (entrained) with the external time of the surrounding environment. The most powerful cues are light and temperature. The major light-mediated synchronization involves the PER-TIMd feedback loop, although opsin-based receptors contribute as well (see Chap. 3). As was noted early with *tim-d* discovery and characterization, TIMd stabilizes PER, as there is no PER detected in the $tim\text{-}d^{01}$ mutant. At the same time, TIMd is degraded upon light illumination, which led to a model where entrainment is achieved via light-mediated degradation of TIMd, which is further relayed to PER depletion, resulting in either phase advance or phase delay of the clock (Hunter-Ensor et al. 1996; Myers et al. 1996; Zeng et al. 1996).

However, TIMd is not sensitive to light by itself; instead, the signal must be transduced from a photoreceptor, which then leads to tyrosine phosphorylation of TIMd and its subsequent degradation through a ubiquitin-proteasome mechanism (Naidoo et al. 1999). The actual photoreceptor of the light-input pathway turned out to be flavoprotein CRYPTOCHROME (Stanewsky et al. 1998; Emery et al. 1998), a member of a large protein family present in all kingdoms and including different types of photolyases (Mei and Dvornyk 2015; Xu et al. 2021). Here, we will refer to this protein as *Drosophila*-type (CRYd), although CRYd is found in the majority of Protostomia and even some basal Deuterostomia (Kotwica-Rolinska et al. 2022a). CRYd functions as a photoreceptor within clock neurons located deep in the fly brain (Emery et al. 2000a), and its mutation or complete depletion results in behavioral rhythmicity in constant light (Emery et al. 2000b; Dolezelova et al. 2007), a condition under which wild-type flies become completely arrhythmic. Simplified systems of cell cultures and yeast helped to shed light on the mechanism: S2 cell transfection experiments revealed that CRYd blocks TIMd+PER-dependent inhibition of CLK-mediated transcription under light but has no impact in darkness, and the yeast two-hybrid system identified a light-dependent interaction between CRYd and TIMd (Ceriani et al. 1999). Furthermore, Rosato et al. (2001) discovered CRYd interaction with PER. The most variable region of various CRY proteins lies in their C-terminus (C-tail), a key part necessary in *Drosophila* CRYd for regulating its interaction with TIMd upon light illumination. If the C-tail is removed from CRYd, either by a stop codon mutation or engineered in a synthetic construct, the resulting "C-tailless CRYd" is constitutively active and interacts with TIMd even in constant darkness, resulting in a long free-running period (Busza et al. 2004; Dissel et al. 2004).

The interaction between TIMd and CRYd is further affected by the N-terminal region in TIMd. The *s-tim-d* allele encodes a 1398 amino acid-long S-TIMd protein, whereas the *ls-tim-d* allele contains two alternative start codons, resulting in transcripts encoding 1398 (S-TIMd) and 1421 (L-TIMd) amino acid-long proteins, respectively (Rosato et al. 1997). The *ls-tim-d* allele originated in *D. melanogaster* in southern Italy circa 10,000 years ago and has spread in all directions (Tauber et al.

2007). L-TIMd shows a diminished interaction with CRYd (Sandrelli et al. 2007), *ls-tim-d* flies are significantly more rhythmic under continuous light than *s-tim-d* flies (Deppisch et al. 2022), and only *ls-tim-d* flies can synchronize to seminatural conditions with short "white nights" typical of high latitudes (Lamaze et al. 2022).

Constant light rhythmicity was observed in flies with mutated JETLAG (JET), an F-box protein with leucine-rich repeat (LRR) (Koh et al. 2006; Peschel et al. 2006). The jet^c mutation results in a single amino acid change in the LRR region, the part of the protein important for substrate binding. Yeast two-hybrid experiments identified an interaction between JET and CRYd and further revealed that this interaction is reduced between JET^c and CRYd. Interestingly, JET and TIMd do not bind each other in the yeast system, but their interaction was detected in S2 cells (one of several examples illustrating the limitations of yeast-based experiments for reconstructing the *Drosophila* system). The current (working) model assumes the light-dependent interaction between CRYd and JET, as well as the interaction of TIMd with JET, and both CRYd and TIMd are then degraded by the proteasome (Peschel et al. 2009).

TIMm, a protein also known as TIMEOUT or TIM2, was first identified in mice (Zylka et al. 1998) and subsequently in fruit flies (Benna et al. 2000). This protein is essential for development in both mammals and flies, which heavily complicates its functional reverse genetics research. Flies with only one *tim-m* functional allele possess reduced sensitivity to light (Benna et al. 2010). RNA-mediated silencing (RNAi) in crickets and linden bugs affects behavioral rhythmicity (Nose et al. 2017; Kotwica-Rolinska et al. 2022a), which, together with the involvement of *tim-m* in the neuronal activity rhythm of the suprachiasmatic nucleus in rats (Barnes et al. 2003) and the connection of human *tim-m* with familial advanced sleep phase syndrome (Kurien et al. 2019), suggests TIMm as a conceivable and conserved clock component. For a more detailed description of TIMd/m proteins in *Drosophila*, see the review by Cai and Chiu (2021). For a general overview of the light input into various circadian clocks, see the review by Johnsson et al. (2014).

4.2.4 Protein Degradation

The oscillation of the clock system, either in constant darkness or in a light/dark regime, depends largely on the well-regulated protein turnover. The SKP/CULLIN/F-box-containing complexes (SCF complexes) function as E3 ubiquitin ligases targeting proteins for 26S proteasomal degradation. SLIMB is an F-box/WD40-repeat protein participating in the CULLIN-1-based E3 ubiquitin ligase complex that binds phosphorylated residues in the N-terminal region of PER (Chiu et al. 2008), ubiquitinates PER, and thus stimulates its degradation. Flies with mutated *slimb* are arrhythmic, and the same phenotype is obtained when a dominant-negative form of SLIMB is expressed in clock cells (Ko et al. 2002; Grima et al. 2002). Furthermore, highly phosphorylated PER and TIMd are constitutively present in the constant darkness of *slimb* mutants. However, the cyclical oscillation of PER and TIMd abundance is maintained even in *slimb* mutants under the light/dark regime due to

the degradation pathway utilizing CRYd and JET proteins (see Sect. 4.2.3). Another ubiquitin ligase, the CULLIN-3 (CUL-3)-based complex, interacts with low-phosphorylated species of TIMd in the absence of PER. SLIMB, on the other hand, binds more phosphorylated TIMd, including PER-bound TIMd (Grima et al. 2012). CIRCADIAN TRIP (CTRIP) is an E3 ubiquitin ligase that seems to regulate the stability of both PER and CLK when *ctrip* downregulation results in a long free-running period, high CLK levels, and persistence of phosphorylated PER during the subjective day when PER is normally degraded (Lamaze et al. 2011).

A study aiming at the identification of cyclically ubiquitinated proteins, the cycling ubiquitylome, revealed a 2–2.5-fold oscillation in abundance for 52 proteins (15 % of all identified ubiquitinated proteins) (Szabo et al. 2018). A remarkable 29-fold oscillation was found for transcription of Megator (MTOR), a nuclear pore complex component. Additional cyclic ubiquitylation affects MTOR, which then feeds back to the pacemaker when it regulates the subcellular localization of the core clock proteins (Szabo et al. 2018).

4.2.5 bZIP Proteins PDP1, VRI, and KAY

The next feedback loop involves two basic leucine zipper (bZIP) transcription factors (Fig. 4.2a, b). First, *vrille* (*vri*) mRNA is cyclically transcribed by the CLK-CYC complex in a phase similar to *tim-d* expression (Blau and Young 1999; McDonald and Rosbash 2001). The second bZIP protein, PAR (proline and acidic rich) DOMAIN PROTEIN 1 (PDP1), particularly its isoform *epsilon* (PDP1ε), is cyclically translated from *Pdp1ε* mRNA, whose expression is also driven by the CLK-CYC complex from an upstream promoter in the *Pdpd1* gene. However, *Pdp1ε* peaks several hours after *vri* reached its maximum, and a similar delay was reported for the peaks of Pdp1ε and VRI proteins (Cyran et al. 2003). Even though PDP1ε and VRI remarkably differ in the organization of protein domains, both proteins contain a highly conserved DNA-binding domain. Indeed, PDP1ε and VRI bind identical cis-regulatory elements in DNA, the so-called V/P motif (also known as the D-box), but with the opposite impact (Fig. 4.2b). The early peaking VRI serves as a transcriptional repressor on the *Clk* promoter. A few hours later, PDP1ε reaches its maximum, replaces VRI, and activates *Clk* transcription. The resulting two-loop model with the negative (VRI) and positive (PDP1ε) components then explained the cyclical expression pattern of *Clk* mRNA, which runs in antiphase to the expression of *per*, *tim-d*, and *vri* mRNAs (Cyran et al. 2003). A third bZIP protein, specifically its α isoform produced from the upstream promoter in the *kayak* gene, further contributes to VRI/PDP1ε feedback. Downregulation of KAYAKα (KAYα), a homolog of mammalian FOS, in circadian pacemaker neurons prolongs period length. KAYα binds to VRI and thus inhibits its suppression of the *Clk* promoter. Surprisingly, KAYα also represses CLK activity. These opposite roles of KAYα in the two-loop model were interpreted as a mechanism bringing stability and precision to the system (Ling et al. 2012).

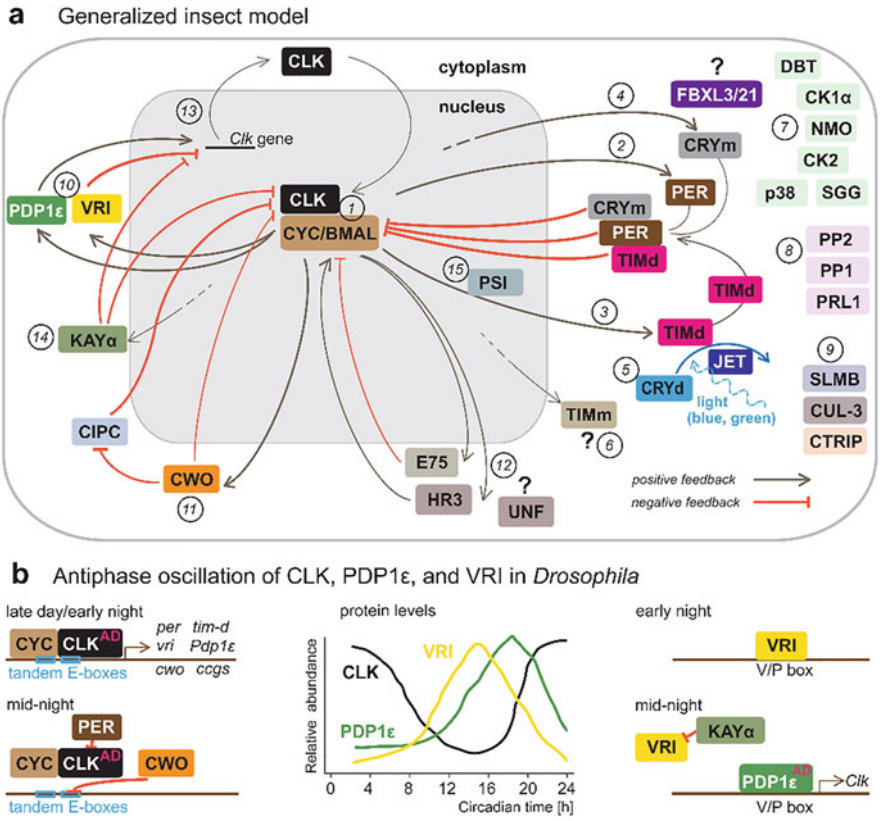

Fig. 4.2 (**a**) Major transcription-translation feedback loops (TTFL) identified in insects (note that some components might be missing in certain insect lineages). The end style and color indicate whether the loop activates the transcription factors CLK-CYC/BMAL (positive feedback) or inhibits their activity (negative feedback). Loops and key modulatory processes are numbered, and the same numbering is used in Fig. 4.3. For simplicity, promoters are not shown in panel **a**, and transcription with subsequent mRNA processing is depicted as arrows running from the nucleus to the cytoplasm. The core of system ① consists of bHLH PAS proteins CLK and CYC/BMAL that drive the expression of the majority of components, including PERIOD (PER) ②, *Drosophila*-type TIMELESS (TIMd) ③, mammalian-type CRYPTOCHROME (CRYm) ④, bZIP transcription factors VRILLE (VRI) and PAR DOMAIN PROTEIN 1 (PDP1) ⑩, bHLH protein CLOCKWORK ORANGE (CWO) ⑪, and nuclear receptors E75, HR3, and UNF ⑫. Additional components include *Drosophila*-type CRY (CRYd) ⑤, which interacts with TIMd upon light illumination and, with the involvement of F-box protein JETLAG (JET), mediates the major light entrainment of the system (see Chap. 3 for details on photic entrainment). Mammalian-type TIM (TIMm) ⑥ contributes to rhythmicity in some insects and is involved in light entrainment in *Drosophila*; however, the mechanisms behind both roles are unclear. Key regulatory steps involve posttranslational modification, such as phosphorylation by multiple kinases ⑦, dephosphorylation by phosphatases ⑧, and degradation machinery ⑨. In *Drosophila*, *Clk* is cyclically expressed ⑬, and splicing of *tim-d* mRNA is regulated by PSI ⑮ in a temperature-dependent manner. KAYAK ⑭, a bZIP protein related to mammalian FOS, contributes to rhythmicity in *Drosophila*. (**b**) Scheme illustrating the role of cis-regulatory elements in *Drosophila* promoters. "AD" in CLK and PDP1 proteins refers to the activation domain, *ccgs* stands for *clock-controled genes*. See the text for a detailed explanation

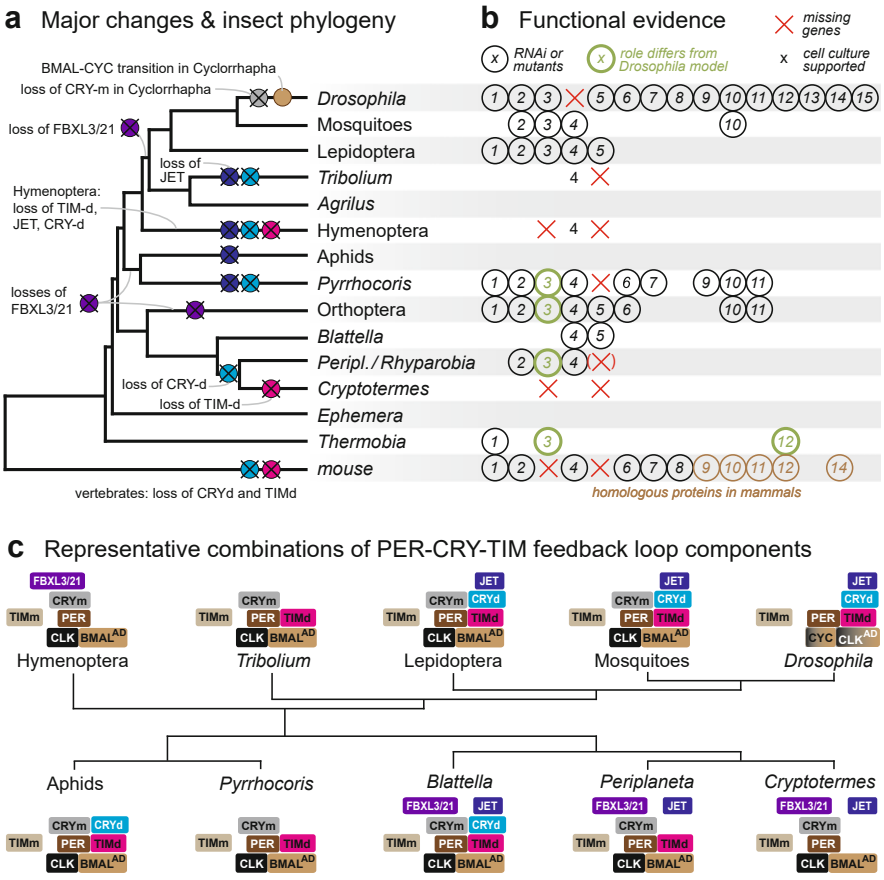

Fig. 4.3 Lineage-specific changes in the insect clock setup depicted from a genetic perspective. Colors correspond to Figs. 4.1 and 4.2. (**a**) A simplified phylogenetic tree with highlighted gene losses and the transition of BMAL to CYC. The genetic setup seems to be conserved in entire insect orders (Lepidoptera, Hymenoptera, Orthoptera) or lower taxonomic groups (aphids, mosquitoes), but in several cases, representative species are depicted. (**b**) Experimental evidence for the role of specific loops and components in particular insect lineages (numbers correspond to the scheme in Fig. 4.2a: CLK and CYC/BMAL ①; PER ②; TIMd ③; CRYm ④; CRYd ⑤; TIMm⑥; kinases ⑦; phosphatases ⑧; degradation machinery ⑨; bZIP transcription factors VRI and PDP1 ⑩; CWO ⑪; nuclear receptors E75, HR3, and UNF ⑫; cyclical expression of CLK ⑬; KAYAK ⑭; and splicing of *tim-d* mRNA regulated by PSI ⑮. If experiments were performed only in an artificial system of cell culture, the number is depicted without the circle. CRYd has been lost in *Periplaneta*, whereas its presence/absence is not fully clear in *Rhyparobia*; thus, X is depicted in parentheses. Deviation from the *Drosophila* model is highlighted by green color. As a reference, a mouse clock setup is depicted with homologous components shown in brown. (**c**) Depiction of major combinations of TTFL components in insects

4.2.6 Clockwork Orange (CWO)

Somewhat similar two-component feedback with opposite roles utilizes CWO, a protein belonging to the bHLH-ORANGE family related to mammalian DEC1 and DEC2, both of which are important for rhythmicity in mice (Honma et al. 2002). CWO was identified as a direct target of CLK (Kadener et al. 2007) and in parallel by genome-wide functional screening using an RNA interference (RNAi) system in flies (Matsumoto et al. 2007). CWO competes with the CLK-CYC dimer in binding to tandem E-boxes when the DNA-binding capacity is the highest for the CLK-CYC dimer, intermediate for CWO, and the weakest for the CLK-CYC-PER complex (Fig. 4.2b). Consequently, CWO binds E-boxes of core clock genes (*per*, *tim-d*, *vri*, *Pdp1*) in antiphase to CLK-CYC (Zhou et al. 2016). A recent study by Rivas et al. (2021) uncovered CLOCK INTERACTING PROTEIN CIRCADIAN (CIPC), an ortholog of mouse CIPC, as an additional component of the loop or more precisely a subloop within the CWO feedback (Fig. 4.2a). *Drosophila* CIPC decreases CLK-CYC-mediated transcription; however, the degree of repression is variable: the strongest for *per*, intermediate for *tim-d*, and minimal for *vri*. Flies with *Cipc* silenced by RNAi and *Cipc* null mutant flies produce a short free-running period. At the same time, the expression of *Cipc* is suppressed by CWO. Therefore, in addition to displacing CLK-CYC from tandem E-boxes and suppressing CLK-CYC transcription, CWO also indirectly activates CLK-CYC by removing CIPC repression (Rivas et al. 2021).

4.2.7 Nuclear Receptors E75, HR3, and UNF

The mammalian clock contains a feedback loop with RORα and REV-ERBα, two orphan nuclear receptors cyclically expressed by CLK-BMAL1 (*b*rain and *m*uscle *a*RNT-like *1*, a mammalian homolog of *Drosophila* CYC). Both RORα and REV-ERBα recognize and compete for the same DNA motif RORE, which is localized in the promoter of mammalian *Bmal1* and *Clk* genes. RORα serves as a transcription activator, whereas REV-ERBα suppresses transcription. The role of homologous nuclear receptors was addressed by RNAi in the basal insect *Thermobia domestica* when Kamae et al. (2014) identified E75 (homolog of REV-ERBα) and HR3 (homolog of RORα) as clock components. Both E75 and HR3 are cyclically expressed, and silencing either of them by RNAi influenced the phase of *tim-d*, *cyc*, and *Clk* expression in *Thermobia*.

Jaumouille et al. (2015) systematically analyzed all 18 nuclear receptor genes in *Drosophila* by RNAi in clock neurons. While E75 was identified as an important component of the fruit fly clock, silencing HR3 did not influence the rhythmicity. Interestingly, the silencing of another nuclear receptor, DHR51 (unfulfilled, UNF), was significant for the free-running period. S2 cell and *Drosophila* in vivo

experiments then confirmed that E75 together with UNF coregulate CLK-CYC-mediated transcription of *per* when they bind to *per* regulatory sequences.

4.2.8 *Regulation of the* Drosophila *Clock at the RNA Level*

Circadian rhythmicity is further regulated at the level of posttranscriptional RNA processing, which includes alternative splicing, polyadenylation, mRNA stability, and regulation by microRNAs (for a detailed review, see Lim and Allada 2013a). The first of them, alternative splicing, was first documented for the *per* gene, where the last exon is retained at high temperatures, while splicing is enhanced at low temperatures. Interestingly, this exon is positioned in the 3' untranslated region (3' UTR) of mRNA, and its splicing leads to an advanced phase in *per* mRNA accumulation, which in turn results in advanced evening activity at low temperatures (Majercak et al. 1999).

Alternative splicing was also reported for *tim-d*, where two cold-specific isoforms are upregulated at low temperatures. At high temperatures, another isoform, *tim-d-medium*, is produced. This isoform is characterized by retention of the intron between exons 13 and 14, resulting in a premature stop codon and an unstable, and probably nonfunctional, TIMd-medium protein (Martin Anduaga et al. 2019). Thermosensitive splicing of this *tim-d* intron requires the alternative splicing regulator *P*-element *s*omatic *i*nhibitor (PSI) (Foley et al. 2019) and additional spliceosome factors (Shakhmantsir et al. 2019).

The length of the poly adenine (A) tail heavily influences mRNA stability. The deadenylase POP2 specifically shortens the *tim-d* mRNA poly(A) tail, thus destabilizing the transcript and leading to lower TIMd levels. Interestingly, POP2 activity is inhibited by PER, the partner of TIMd (Grima et al. 2019).

However, another level in RNA metabolism, its translation, is subject to circadian clock regulation. ATAXIN-2 (ATX2), an RNA-binding protein, is necessary for PER accumulation in clock neurons. ATX2 is crucial for the functions of *twenty-four* (TYF), a key activator of PER translation, which is associated with a 5'-cap-binding complex (Lim et al. 2011). TYF and ATX2 interact with polyadenylate-binding protein (PABP) (Lim and Allada 2013b; Zhang et al. 2013b).

4.2.9 *Network Properties of the Clock*

Although the clock mechanism was first accepted as a cell-autonomous oscillatory system, circadian rhythmicity also requires intercellular communication and is, therefore, a result of the circadian network. The first evidence was indicated by the discovery of a neuropeptide *pigment-dispersing factor* (*pdf*) mutant (Renn et al. 1999). Then, specific neurons regulating the morning and evening activity were identified and genetically manipulated (Grima et al. 2004; Stoleru et al. 2004).

The study by Dissel et al. (2014) further emphasized the role of particular groups of different clock cells and their mutual communication. The interaction among different neuronal oscillators (distinct groups of neurons), each characterized by a specific neuropeptide and coupled to other oscillator groups (neurons) in the network, drives the rhythmic activity of flies (Yao and Shafer 2014). The importance of clock groups was shown in calcium imaging experiments when the Ca^{2+} rhythms displayed by a particular group of cells corresponded to the morning or evening locomotor activity peaks (Liang et al. 2016), and the key role of neuropeptide signaling in these intracellular communications was further supported by Ca^{2+} imaging (Liang et al. 2017).

4.2.10 Temperature and the Clock

Temperature is an important and variable factor in the environment that interferes with the functioning of the circadian clock. The clock is only useful for the organism if it keeps ticking at a constant (or near-constant) speed within a physiologically acceptable range of temperatures. This temperature independence, known as temperature compensation, is particularly important for poikilotherms, which are organisms without regulation of body temperature, such as insects. On the other hand, chemical reactions are strongly influenced by temperature (Arrhenius 1889). An explanation of this logical "conflict" could be a combination of reactions, where some reactions have opposite effects on the free-running period than others (Hastings and Sweeney 1957). Thus, the period-extending reactions would, for instance, prolong the free-running period even more at high temperatures. At the same time, this lengthening would be compensated by other reactions that more strongly shorten the free-running period at high temperatures. These opposing reactions could impact the stability of some negative or positive components of the feedback loops and might influence their subcellular localization (nuclear export and import), their interaction with partners, their activity, or perhaps a combination of (all) the abovementioned mechanisms. We cannot exclude the possibility that temperature compensation may be partially affected at the level of interneuronal communication. In this case, temperature compensation would also be a network property of the clock.

Although the molecular mechanism behind temperature compensation remains largely elusive, genetic and biochemical data suggest that phosphorylation of distinct regions of PER protein could be consistent with the model proposed above, even though the details of the mechanism will most likely differ between mammals and *Drosophila* (see Sect. 4.2.2.). Components of the negative feedback loop are important for temperature compensation, as illustrated by mutations specifically interfering with PER-PER homodimerization in *Drosophila* (Landskron et al. 2009) and a subset of TIMd mutations (Matsumoto et al. 1999; Singh et al. 2019; Rutila et al. 1996; see Sect. 4.2.1.).

In addition to single amino acid mutant variants isolated in genetic screens, even certain *Drosophila per* alleles occurring abundantly in nature show altered temperature compensation. *Drosophila* PER contains a series of threonine-glycine (Thr-Gly) repeats that are polymorphic in length. The most common variants consist of 14, 17, 20, or 23 Thr-Gly pairs, and their frequencies in naturally occurring European populations display a clear latitudinal cline (Costa et al. 1992). Furthermore, a detailed analysis involving transgenic flies in which the Thr-Gly repeats and flanking regions were modified revealed the role of this PER in temperature compensation (Sawyer et al. 1997).

Although the free-running period remains constant (compensated) in a certain temperature range, the daily distribution of activity is often affected by temperature. Furthermore, circadian clock genes impact daily activity patterns under light-dark regimes. In *D. melanogaster*, morning and evening activity peaks are separated by midday siesta. At high temperatures, the morning peak is earlier, and the evening peak is delayed, whereas, at low temperatures, the trend is reversed. The temporal regulation of the morning and evening peaks includes alternative splicing of *per* mRNA in its 3' UTR and complex alternative splicing of *tim-d* transcripts (see Sect. 4.2.8; Majercak et al. 1999; Martin Anduaga et al. 2019; Foley et al. 2019).

4.3 Lineage-Specific Variations in the Clock Setup

The remarkable progress in genomics and transcriptomics during the last decade resolved the phylogeny of insects with reasonable precision and dated the separation of all major insect lineages (Misof et al. 2014; Johnson et al. 2018; Kawahara et al. 2019; McKenna et al. 2019; Wipfler et al. 2019). At the same time, these phylogenomics-oriented studies produced a remarkable wealth of data in which clock genes can be identified. In the case of nonmodel insects, the transcriptomes are particularly valuable, as the complete coding sequences are often retrieved and gene paralogs can be reliably defined. Thus, the presence of different circadian clock genes in the insect phylogeny could be usefully mapped and important gene changes and losses identified. Pinpointing gene loss is a nontrivial endeavor. If the gene in question is well conserved across insects (let us say at the level of a protein it encodes) yet is absent in some species, we may claim that we are not able to identify it in a genome of certain insects[3]. However, genome assemblies of nonmodel insects are often fragmented; moreover, some genomes are remarkably large. For example, the genome of the migratory locust is more than twice the size of a human genome (Wang et al. 2014). Therefore, the absence of a gene in an individual species must be interpreted with extreme caution. Nevertheless, if the genomes and transcriptomes of

[3] The automatic gene annotation is prone to artifacts, and therefore careful phylogenetic analysis is often needed to assign a gene/protein to a particular type (especially when multiple paralogs exist, such as in the case of cryptochromes).

all species within a specific monophyletic group (i.e., a group of organisms with a common ancestor) do not contain a particular gene, and if the quality of these genomes/transcriptomes is reasonably good, then the gene loss becomes the most parsimonious explanation.

This part of the chapter aspires to compare the circadian clock across insects using *D. melanogaster* as the reference model. Here, it is important to keep in mind that the depth of our knowledge remarkably varies among different insect groups. For some, only genomic/transcriptomic evidence is available, whereas, in others, the circadian clock was functionally studied by gene silencing using RNAi and, in some species, stable genetic mutations were introduced. Another set of insights includes temporal expression analyses and immunohistochemistry, valuable data that might support mechanistic explanation, even though functional reverse genetic evidence is not available.

We will start with the closest relatives of *D. melanogaster* and gradually focus on the major changes in the circadian clock setup. The key TTFL loops and modification enzymes, such as kinases and phosphatases, depicted in a generalized insect clock model (Fig. 4.2a) are first approached from a phylogenetic perspective in Fig. 4.3, where panel "a" defines major gene losses and changes during insect evolution, whereas panel "b" summarizes the experimental evidence for each loop (using the numbers in circles) in a particular lineage. The description of the circadian clock at the RNA and protein levels is summarized separately in Table 4.1.

The *D. melanogaster* clock setup seems to be, at least according to available descriptive data, conserved in cyclorrhaphan flies, including the housefly *Musca domestica* (Codd et al. 2007; Bazalova and Dolezel 2017), the olive fly (Bertolini et al. 2018), and the Medfly *Ceratitis capitata* (Kotwica-Rolinska et al. 2022a). The *tim-d* mutant of *Chymomyza costata*, a drosophilid fly living in temperate regions, not only confirmed the role of *tim-d* in its molecular oscillator (Kobelkova et al. 2010) but also supported the involvement of the circadian machinery in seasonality (Pavelka et al. 2003; Stehlik et al. 2008).

4.3.1 *Unique Features of the* Drosophila *(Cyclorrhaphan) Clock*

Two or perhaps three major and probably connected features are unique to *Drosophila* and related fly species. First, *Drosophila* CLK contains a transactivation domain at its C-terminus, and a similar pattern is found in cyclorrhaphan Diptera (including *Musca*, the olive fly, and the medfly). Perhaps connected to that, *Clk* mRNA is cyclically expressed in these species, whereas the *cyc* level is constant (Cyran et al. 2003; Codd et al. 2007; Bertolini et al. 2018). BMAL1, the mammalian homolog of *Drosophila* CYC, is characterized by cyclical *bmal1* expression in mice, and the transactivation domain, the so-called the BMAL1 C-terminal region

Table 4.1 Overview of descriptive analyses characterizing circadian clock factors

	CLK BMAL*	PER	TIMd	CRYm	CRYd	TIMm	KIN.	PHOS.	SLMB	PDP1 VRI	CWO	E75 HR3**	PDF
Drosophila	*mRNA* IHC, G4	*mRNA* IHC, G4	*mRNA* IHC, G4	X	*mRNA* IHC, G4	*mRNA*	*mRNA* IHC	*mRNA* IHC	*(mRNA)*	*mRNA* IHC	*mRNA* IHC	*(mRNA)*	*mRNA* IHC, G4
Mosquitoes	*mRNA*	*mRNA* IHC	*mRNA*	*mRNA*	*mRNA*					*mRNA*			IHC
Lepidoptera	IHC	*mRNA* IHC	*mRNA* IHC	*mRNA*	*mRNA* IHC		*mRNA* IHC						IHC
Coleoptera***		IHC			(X)								
Hymenoptera	*mRNA*	*mRNA* IHC	X	*mRNA*	X	*mRNA*							IHC
Aphids	*mRNA*	*mRNA* IHC	*mRNA*	*mRNA*	*mRNA* IHC					*mRNA*			X
Heteroptera***	*mRNA*	*mRNA* IHC	*mRNA* IHC	*mRNA* (IHC)	(X)					*mRNA*			IHC
Orthoptera	*mRNA*	*mRNA* IHC	*mRNA*	*mRNA*	*mRNA* IHC	*mRNA*	IHC			*mRNA*	*mRNA*	*mRNA*	IHC
Blattella		IHC											IHC
Peripl./Rhyparobia		*mRNA*	*mRNA*	*mRNA*	X								IHC
Termites***			(X)		X								IHC
Ephemeroptera		IHC											
Thermobia	*mRNA*	IHC	*mRNA*									*mRNA*	

The table summarizes the description at the level of mRNA (either by in situ hybridization or by addressing the expression level by RNase protection assay, quantitative real-time polymerase reaction, microarray analysis, or GAL4 transgenic reporters, indicated as G4) and protein (IHC, immunohistochemistry) in representative insect groups. This summary is a synthesis of publications cited in the mechanistic descriptions of this chapter and is further supported by studies on *Drosophila* (Houl et al. 2006; Richier et al. 2008), mosquitoes (Baik et al. 2020; Rund et al. 2011), Lepidoptera (Iwai et al. 2008; Sauman et al. 2005; Sehadova et al. 2004; Kobelkova et al. 2015; Zavodska et al. 2012; Zhu et al. 2008), Coleoptera (Frisch et al. 1996), Hymenoptera (Fuchikawa et al. 2017; Beer et al. 2018), aphids (Barbera et al. 2017; Barbera et al. 2022; Colizzi et al. 2021), Heteroptera (Dolezel et al. 2008; Ikeno et al. 2008; Kotwica-Rolinska et al. 2017; Stroppa and Garcia 2019; Vafopoulou and Steel 2012; Koide et al. 2021; Kotwica-Rolinska et al. 2022b), Orthoptera mostly represented by crickets (Sehadova et al. 2003; Shao et al. 2006), *Blattella* (Wen and Lee 2008), *Periplaneta* and *Rhyparobia* (Gestrich et al. 2018), termites (Zavodska et al. 2008), Ephemeroptera (Zavodska et al. 2003a), and *Thermobia* (Zavodska et al. 2003b)

*CYC in *Drosophila*. **In *Drosophila*, UNF participates in this negative feedback loop ***Some circadian clock genes were lost only in a subset of certain orders (*cry-d* in some Coleoptera and Heteroptera, *tim-d* in the majority of termites), and the loss of CRYd is unclear for *Rhyparobia*. For details on clock setups, see Kotwica-Rolinska et al. 2022a)

(BCTR), lies in the C-terminus[4]. The same protein architecture was identified in the BMAL of the silk moth (Chang et al. 2003) and even sand flies (Meireles-Filho et al. 2006), indicating the unique and relatively recent rearrangement of the positive components in the *Drosophila* clock (Fig. 4.3a, c). The role of CLK-BMAL as positive regulators of the circadian machinery was experimentally confirmed by RNAi in Heteroptera, Orthoptera, and *Thermobia* (Kotwica-Rolinska et al. 2022a; Uryu et al. 2013; Moriyama et al. 2012; Kamae et al. 2014) (Fig. 4.3b). In

[4]Some authors use the term CYC in all insect species, whereas others distinguish between BMAL (activation domain is present) and CYC (activation domain has been lost). See also Fig. 4.3c.

Lepidoptera, stable genetic mutants were created for both CLK and BMAL (Markert et al. 2016; Zhang et al. 2017) in addition to the reconstruction of the feedback in cell culture (Chang et al. 2003). Furthermore, the *bmal* transcript is cyclically expressed in numerous (and phylogenetically distant) insects, including sand flies (Meireles-Filho et al. 2006), crickets (Uryu et al. 2013), and *Thermobia* (Kamae et al. 2014).

The second unique feature of the *Drosophila* setup is the absence of mammalian-type CRYPTOCHROME (CRYm), also known as CRY2, CRYII, or mCRY (Kotwica-Rolinska et al. 2022a). In mammals, two paralogous mammalian-type CRYs are key components of the negative TTFL (Kume et al. 1999; van der Horst et al. 1999). A similar transcriptional repressive function was confirmed in cell cultures for CRYm from mosquitoes, Lepidoptera, Coleoptera, and Hymenoptera (Yuan et al. 2007). Functionally, CRYm was identified as a clock component by RNAi in mosquitoes, Heteroptera, crickets, and cockroaches (Meuti et al. 2015; Ikeno et al. 2011a; Tokuoka et al. 2017; Bazalova et al. 2016; Werckenthin et al. 2020). Stable genetic mutants were created in the linden bug *Pyrrhocoris apterus* and the monarch butterfly *Danaus plexippus* (Kotwica-Rolinska et al. 2022a; Zhang et al. 2017). The latter model was instrumental in explaining that monarch CRYm regulates circadian repression of BMAL through the activation domain (AD) via two independent mechanisms (Zhang et al. 2017). The perfect correlation of CRYm loss with the absence of AD in BMAL/CYC of certain dipteran insects implies that once CRYm was lost, probably in an ancestor of Cyclorrhapha, repression of AD on BMAL was no longer used. The activation domain is localized on CLK in insects that have lost CRYm (Thakkar et al. 2022).

The third specificity of *Drosophila* is the involvement of the nuclear receptor UNF (Jaumouille et al. 2015), whereas basal insect *Thermobia* utilizes HR3, a homolog of the well-established mammalian component RORα (Kamae et al. 2014) (see Sect. 4.2.7 also). It is tempting to speculate that UNF recruitment to the circadian machinery might be connected to the shift of the activation domain from BMAL to CLK because the oscillating expression of *Clk* and noncyclical expression of *cyc*, characteristic only for cyclorrhaphan flies, imply evolutionary changes in the promoters of both genes. It will be interesting to see if HR3 and/or UNF participate in the circadian clock of other insect groups.

4.3.2 Losses of CRYd and TIMd

The mammalian clock setup is characterized by the absence of CRYd and TIMd. Therefore, it was a remarkable surprise to see a comparable situation in Hymenoptera when the genome of the honey bee *Apis mellifera* was analyzed (Rubin et al. 2006). A recent detailed inspection confirmed that TIMd and CRYd were lost in all Hymenoptera. Furthermore, TIMd has been lost in the animal kingdom at least three times, in Hymenoptera, vertebrates, and the majority of termites, and these three losses always correlate with the loss of CRYd (Kotwica-Rolinska et al. 2022a; Fig. 4.3a).

However, in several lineages, CRYd has disappeared and TIMd is still present. We can see a particularly nice gradient of gene losses in cockroaches and termites (which are from a phylogenetic point of view a subset of cockroaches): the basal cockroaches (such as *Blattella germanica*) possess the complete toolkit, *Periplaneta* lost CRYd, and termites also lost TIMd (except for just one basal termite species *Porotermes*, where a portion of *tim-d* transcript was found). It will be extremely interesting to see how the light entrainment pathway functions in these three types of clocks detected in cockroaches/termites.

In Orthoptera, a sister group to cockroaches, the role of both *cryptochrome* types was tested by RNAi applied to the cricket *Gryllus bimaculatus*, leading to a new model with a CRYm-CRYd oscillatory loop independent of PER-TIMd (Tokuoka et al. 2017). Furthermore, light input seems to require c-FOS, a bZIP protein participating in the mammalian entrainment pathway (Kornhauser et al. 1990). In the proposed cricket model, the light signal is perceived by the compound eyes, from which the information is transmitted to the clock neurons to stimulate *c-fos* mRNA expression, which is finally relayed on the CRYm-CRYd loop that feeds back on CLK-CYC/BMAL (Kutaragi et al. 2018).

Interestingly, RNAi silencing of *Gryllus tim-d* did not result in behavioral arrhythmicity; instead, the free-running period was significantly shorter (Danbara et al. 2010). A similar short free-running period was observed after *tim-d* silencing in the firebrat *T. domestica* (Kamae and Tomioka 2012). These findings contrast with the fundamental role of *tim-d* in the circadian clock of *Drosophila* but also in Lepidoptera (Nartey et al. 2021). The role of *tim-d* was rigorously addressed in the linden bug *P. apterus* (Heteroptera), an insect that does not have *cry-d* and instead possesses *cry-m* (Bajgar et al. 2013). Genetically created null mutant in *tim-d* showed a free-running period of their locomotor activity shortened by more than 1 h, but the rhythmicity was robust. These *tim-d* phenotypes provided a possible explanation for evolutionary changes in the clock setup within animals in general and insects in particular (Kotwica-Rolinska et al. 2022a): “The dispensability of TIMd in *P. apterus* suggests a scenario of transition between clock architectures relying on distinct components of their negative feedback loops. The proposed model implies that the clock would be functional in each step of the transition from the ancestral state to the PER & CRYm system known today in vertebrates. A similar clock gene combination in Hymenoptera (PER & CRYm) indicates a convergent evolution of the circadian system, although functional evidence from this insect group is not yet available. The circadian clock observed in *P. apterus* could then correspond to an early clock setup that facilitates *tim-d* loss without a complete collapse of circadian cycling. However, the timing and causality of the proposed events might have been lineage-specific, where either the loss of cry-d triggered the transition of TIMd to its modulatory role, or alternatively, the loss of JET or change in TIMd properties compromised its interaction with CRYd, which in turn was subsequently lost.”

4.3.3 JET and FBXL3/21 Proteins

JET, a protein essential for the interaction between CRYd and TIMd in *Drosophila*, which results in their degradation, is found in numerous protostomian lineages but is also independently lost at least six times in insects (Kotwica-Rolinska et al. 2022a). Thus, various combinations of JET with TIMd and CRYd exist in insects (Fig. 4.3c): JET, TIMd, and CRYd (Diptera, Lepidoptera, some Coleoptera, *Blatella*); JET, TIMd, and no CRYd (*Periplaneta*); and JET, no TIMd, and no CRYd (*Cryptotermes*). Similarly, JET was lost, but TIMd and CRYd are present in aphids (Cortés et al. 2010). In *Pyrrhocoris*, JET was lost and only TIMd is present, whereas all these three genes were lost in Hymenoptera.

In mammals, CRYm interacts with FBXL3 and FBXL21, two closely related paralogs also known as *overtime* and *after-hours*, respectively (Godinho et al. 2007; Siepka et al. 2007; Hirano et al. 2013). In protostomes and basal deuterostomes, only the ancestral protein FBXL3 is found. Similar to JET, FBXL3 has been lost multiple times in insects and is present in four lineages: Ephemeroptera, Blattodea (cockroaches + termites), Thysanoptera, and Hymenoptera (Kotwica-Rolinska et al. 2022a). Thus, FBXL3 is not necessary for CRYm function in some systems, such as the circadian clock of Lepidoptera or *Pyrrhocoris*. Nonetheless, it will be very interesting to see whether FBXL3 participates in CRYm regulation in Hymenoptera, a group of insects with a clock setup remarkably similar to that of mammals.

4.3.4 Conserved Components of Insect Clocks

PER, an iconic circadian clock protein found in all insects, seems to participate in the rhythmicity in all tested species, albeit to a different extent. It is absolutely necessary for *Drosophila*, but in *Pyrrhocoris*, approximately one-third of genetic mutants are still robustly rhythmic (Kotwica-Rolinska et al. 2022a). Similarly, RNAi silencing of *per* in the Madeira cockroach resulted in only a partial phenotype (Werckenthin et al. 2020). It is possible that in the abovementioned (and many other) insect species, CRYm serves as the most important negative element, whereas the PER contribution to the negative feedback is smaller than that in *Drosophila*. However, depletion of PER in the silk moth *Bombyx mori* leads to arrhythmicity (Ikeda et al. 2019), even though the lepidopteran clock relies on CRYm (Zhang et al. 2017).

The role of bZIP proteins VRI and PDP1 was tested in *Pyrrhocoris*, where *vri* silencing nonsignificantly shortened the free-running period, while *Pdp1* knockdown resulted in arrhythmicity (Kotwica-Rolinska et al. 2022a). In crickets, however, cosilencing of *cyc* was necessary to obtain *vri*- and *Pdp1*-dependent changes in the free-running period (Narasaki-Funo et al. 2020). In the northern house mosquito *Culex pipiens*, both *vri* and *Pdp1* were tested for their role in diapause, and their cyclical expression was confirmed (Chang and Meuti 2020).

CWO, a bHLH protein whose depletion slows down the clock in *Drosophila*, was silenced with a comparable 1-h extension of periodicity in *Pyrrhocoris* (Kotwica-Rolinska et al. 2022a) and crickets (Tomiyama et al. 2020).

The role of components working at the protein level, i.e., kinases and F-box proteins, was minimally tested outside *Drosophila*. Silencing *dbt* robustly extended the free-running period in *Pyrrhocoris*, and a less extreme yet remarkable phenotype was observed after *slibm* knockdown, whereas *nmo* knockdown marginally sped up the clock (Kotwica-Rolinska et al. 2022a).

4.3.5 Descriptive Studies

The majority of the mechanisms described in this chapter were elaborated in *D. melanogaster* and further challenged and expanded in several insect models. Although an occasional spontaneous mutation or a variant is mapped in a nonmodel organism, these examples are relatively rare for chronobiology outside of *D. melanogaster* (Pavelka et al. 2003; Kozak et al. 2019). Therefore, reverse genetic tools have been key for the analysis of nonmodel organisms. One of the most powerful tools is RNAi, which, if spread in the organism systemically, can be a cost-effective and fast approach for chronobiological studies. However, the target transcript might only be partially reduced; thus, the data interpretation becomes, at least in some specific cases, nontrivial. Therefore, stable genetic modifications are more attractive from an interpretation point of view, but these techniques are also quite laborious and time demanding. Nevertheless, some insect models have become accessible to stable modification and are successfully used in chronobiology (Markert et al. 2016; Zhang et al. 2017; Kotwica-Rolinska et al. 2019; Ikeda et al. 2019).

In some groups, however, no reverse genetics data are available. However, many of these insect species are extremely interesting for their biology and even for their role as agricultural pests or for their ability to transmit pathogens. Thus, even descriptive evidence might provide important and valuable hints about the molecular mechanism behind their circadian clocks. Therefore, Table 4.1 briefly summarizes the evidence obtained at the RNA and protein levels across selected insect groups. For the anatomy of the insect circadian clock, see Chap. 5.

4.4 Conclusion and Future Perspective

The genetic architecture of the above-described circadian clocks observed in various insect lineages invites us to speculate why and how this diversity has originated. Insects are a large group with more than 400 million years of evolution (Misof et al. 2014). Some lineages, such as aphids, underwent noteworthy gene expansion, resulting in >35 thousand genes, which is remarkably more than the gene count in

D. melanogaster (~13.5 thousand) or even in the body louse *Pediculus humanus* (~11 thousand) (Thomas et al. 2020).

A possible mechanistic explanation for the various clock setups might be provided by mammalian research when Pett et al. (2018) showed that the importance of individual feedback loops differs in a tissue-specific manner. In mammals, this mechanistic flexibility may account for organ-specific differences in clock gene expression and allow for the hierarchical organization of the mammalian clock. A somewhat similar tissue-specific clock architecture exists in *D. melanogaster*, where CRYd serves as a light photoreceptor in clock neurons (Emery et al. 2000a), whereas CRYd is a CLK/CYC repressor in the periphery (Collins et al. 2006). Indeed, the role in the peripheral clock allowed the identification of *cry-d*^{b} mutation in a luciferase reporter-based screen (Stanewsky et al. 1998).

In insects, we can assume that similar flexibility in clock setup allows for gene loss, which has some impact on rhythmicity, albeit not detrimental. Indeed, this is the case for the *tim-d* gene in *P. apterus* and probably other insect species, where TIMd participates only as a modulator of the free-running period (Kotwica-Rolinska et al. 2022a; Danbara et al. 2010). Thus, modifications of a certain loop may, for example, impact the light entrainment capacities of the system, whereas the clock remains either fully functional or with only a mild impact on its properties. Whether the combination of these changes is beneficial or disadvantageous for the particular organism depends on the life strategy in a specific environment. For example, organisms living in high geographical latitudes face multiple challenges, including extreme photoperiods (day-to-night ratio). Under such conditions, the less light-sensitive clock might be an advantage.

Another conceivable selection pressure might be the role of circadian clock genes in different time-measuring systems than the circadian clock itself. The possible role of (at least a subset of) clock genes was suggested in photoperiodic timing (Ikeno et al. 2010, 2011b, 2013; Kotwica-Rolinska et al. 2017, 2022b; Sakamoto et al. 2009) but not for the tidal and lunar rhythms (Takekata et al. 2014; Zhang et al. 2013a). Therefore, components recruited by a different time-measuring system (s) might be, in addition to their role in the circadian clock, under selection pressure connected to the properties of the noncircadian system.

Taken together, during the last two decades, we could see remarkable progress in understanding the circadian clock mechanism at the molecular level. Research on *D. melanogaster* has been dominating insect molecular chronobiology for decades, and the fruit fly will always be an excellent model with unparalleled tools and opportunities. However, emerging reverse genetic tools available to nonmodel organisms will facilitate research on genetically interesting clock setups and chronobiological, ecological, and physiological phenomena unavailable, or weakly expressed, in *D. melanogaster*.

References

Agrawal P, Hardin PE (2016) An RNAi screen to identify protein phosphatases that function within the *Drosophila* circadian clock. G3 6:4227–4238. https://doi.org/10.1534/g3.116.035345

Allada R, White NE, So WV, Hall J, Rosbash M (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93:791–804. https://doi.org/10.1016/S0092-8674(00)81440-3

Arrhenius S (1889) Über die reaktionsgeschwindigkeit bei der inversion von Rohrzucker durch Saeuren. Z Phys Chem 4:226–248. https://doi.org/10.1515/zpch-1889-0416

Ashmore LJ, Sathyanarayanan S, Silvestre DW, Emerson MM, Schotland P, Sehgal A (2003) Novel insights into the regulation of the timeless protein. J Neurosci 23:7810–7819. https://doi.org/10.1523/jneurosci.23-21-07810.2003

Baik LS, Nave C, Au DD, Guda T, Chevez JA, Ray A et al (2020) Circadian regulation of light-evoked attraction and avoidance behaviors in daytime- versus nighttime-biting mosquitoes. Curr Biol 30:3252–3259. https://doi.org/10.1016/j.cub.2020.06.010

Bajgar A, Jindra M, Dolezel D (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc Natl Acad Sci USA 110:4416–4421. https://doi.org/10.1073/pnas.1217060110

Barbera M, Collantes-Alegre JM, Martinez-Torres D (2017) Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrthosiphon pisum*. Insect Biochem Mol Biol 83:54–67. https://doi.org/10.1016/j.ibmb.2017.02.006

Barbera M, Collantes-Alegre JM, Martinez-Torres D (2022) Mapping and quantification of cryptochrome expression in the brain of the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 31:159–169. https://doi.org/10.1111/imb.12747

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioral rhythms by gene transfer in *Drosophila*. Nature 312:752–754. https://doi.org/10.1038/312752a0

Barnes JW, Tischkau SA, Barnes JA, Mitchell JW, Burgoon PW, Hickok JR et al (2003) Requirement of mammalian *timeless* for circadian rhythmicity. Science 302:439–442. https://doi.org/10.1126/science.1086593

Bazalova O, Dolezel D (2017) Daily activity of the housefly, *Musca domestica*, is influenced by temperature independent of 3′ UTR *period* gene splicing. G3 7:2637–2649. https://doi.org/10.1534/g3.117.042374

Bazalova O, Kvicalova M, Valkova T, Slaby P, Bartos P, Netusil R et al (2016) Cryptochrome 2 mediates directional magnetoreception in cockroaches. Proc Natl Acad Sci USA 113:1660–1665. https://doi.org/10.1073/pnas.1518622113

Beer K, Kolbe E, Kahana NB, Yayon N, Weiss R, Menegazzi P et al (2018) Pigment-dispersing factor-expressing neurons convey circadian information in the honey bee brain. Open Biol 8:170224. https://doi.org/10.1098/rsob.170224

Benna C, Bonaccorsi S, Wülbeck C, Helfrich-Förster C, Gatti M, Kyriacou CP et al (2010) *Drosophila timeless2* is required for chromosome stability and circadian photoreception. Curr Biol 20:346–352. https://doi.org/10.1016/j.cub.2009.12.048

Benna C, Scannapieco P, Piccin A, Sandrelli F, Zordan F, Rosato E et al (2000) A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. Curr Biol 10:R512–R513. https://doi.org/10.1016/s0960-9822(00)00594-7

Bertolini E, Kistenpfennig C, Menegazzi P, Keller A, Koukidou M, Helfrich-Förster C (2018) The characterization of the circadian clock in the olive fly *Bactrocera oleae* (Diptera:Tephritidae) reveals a *Drosophila*-like organization. Sci Rep 8:816. https://doi.org/10.1038/s41598-018-19255-8

Blau J, Young MW (1999) Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell 99:661–671. https://doi.org/10.1016/S0092-8674(00)81554-8

Brandes C, Plautz JD, Stanewsky R, Jamison CF, Straume M, Wood KV et al (1996) Novel features of *Drosophila period* transcription revealed by real-time luciferase reporting. Neuron 16:687–692. https://doi.org/10.1016/S0896-6273(00)80088-4

Busza A, Emery-Le M, Rosbash M, Emery P (2004) Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception. Science 304:1503–1506. https://doi.org/10.1126/science.1096973

Cai YD, Chiu JC (2021) Timeless in animal circadian clocks and beyond. FEBS J 289:6559. https://doi.org/10.1111/febs.16253

Cai YD, Xue Y, Truong CC, Del Carmen-Li J, Ochoa C, Vanselow JT et al (2021) CK2 inhibits TIMELESS nuclear export and modulates CLOCK transcriptional activity to regulate circadian rhythms. Curr Biol 31(502-514):e507. https://doi.org/10.1016/j.cub.2020.10.061

Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ et al (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285:553–556. https://doi.org/10.1126/science.285.5427.553

Chang DC, McWatters HG, Williams JA (2003) Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*. J Biol Chem 278:38149–38158. https://doi.org/10.1074/jbc.M306937200

Chang V, Meuti ME (2020) Circadian transcription factors differentially regulate features of the adult overwintering diapause in the Northern house mosquito, *Culex pipiens*. Insect Biochem Mol Biol 121:103365. https://doi.org/10.1016/j.ibmb.2020.103365

Chiu JC, Ko HW, Edery I (2011) NEMO/NLK phosphorylates PERIOD to initiate a time delay phosphorylation circuit that sets circadian clock speed. Cell 145:357–370. https://doi.org/10.1016/j.cell.2011.04.002

Chiu JC, Vanselow JT, Kramer A, Edery I (2008) The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. Genes Dev 22:1758–1772. https://doi.org/10.1101/gad.1682708

Codd V, Dolezel D, Stehlik J, Piccin A, Garner KJ, Racey SN et al (2007) Circadian rhythm gene regulation in the housefly *Musca domestica*. Genetics 177:1539–1751. https://doi.org/10.1534/genetics.107.079160

Colizzi FS, Beer K, Cuti P, Deppisch P, Martinez Torres D, Yoshii T et al (2021) Antibodies against the clock proteins period and cryptochrome reveal the neuronal organization of the circadian clock in the pea aphid. Front Physiol 12:705048. https://doi.org/10.3389/fphys.2021.705048

Collins B, Mazzoni EO, Stanewsky R, Blau J (2006) *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor. Curr Biol 16:441–449. https://doi.org/10.1016/j.cub.2006.01.034

Cortés T, Ortiz-Rivas B, Martínez-Torres D (2010) Identification and characterization of circadian clock genes in the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 19:123–139. https://doi.org/10.1111/j.1365-2583.2009.00931.x

Costa R, Peixoto AA, Barbujani G, Kyriacou CP (1992) A latitudinal cline in a *Drosophila* clock gene. Proc Biol Sci 250:43–49. https://doi.org/10.1098/rspb.1992.0128

Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NRJ, Hardin PE et al (2003) *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112:329–341. https://doi.org/10.1016/S0092-8674(03)00074-6

Danbara Y, Sakamoto T, Uryu O, Tomioka K (2010) RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 56:1738–1745. https://doi.org/10.1016/j.jinsphys.2010.07.002

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TDL et al (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science 280:1599–1603. https://doi.org/10.1126/science.280.5369.1599

Deppisch P, Prutscher JM, Pegoraro M, Tauber E, Wegener C, Helfrich-Forster C (2022) Adaptation of *Drosophila melanogaster* to long photoperiods of high-latitude summers is facilitated by the *ls-timeless* allele. J Biol Rhythms 37:185–201. https://doi.org/10.1177/07487304221082448

Dissel S, Codd V, Fedic R, Garner KJ, Costa R, Kyriacou CP et al (2004) A constitutively active cryptochrome in *Drosophila melanogaster*. Nat Neurosci 7:834–840. https://doi.org/10.1038/nn1285

Dissel S, Hansen CN, Ozkaya O, Hemsley M, Kyriacou CP, Rosato E (2014) The logic of circadian organization in *Drosophila*. Curr Biol 24:2257–2266. https://doi.org/10.1016/j.cub.2014.08.023

Dolezel D, Zdechovanova L, Sauman I, Hodkova M (2008) Endocrine-dependent expression of circadian clock genes in insects. Cell Mol Life Sci 65:964–969. https://doi.org/10.1007/s00018-008-7506-7

Dolezelova E, Dolezel D, Hall JC (2007) Rhythm defects caused by newly engineered null mutations in *Drosophila*'s *cryptochrome* gene. Genetics 177:329–345. https://doi.org/10.1534/genetics.107.076513

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290. https://doi.org/10.1016/s0092-8674(00)80566-8

Dusik V, Senthilan PR, Mentzel B, Hartlieb H, Wulbeck C, Yoshii T et al (2014) The MAP kinase p38 is part of *Drosophila melanogaster's* circadian clock. PLoS Genet 10:e1004565. https://doi.org/10.1371/journal.pgen.1004565

Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679. https://doi.org/10.1016/S0092-8674(00)81637-2

Emery P, Stanewsky R, Hall JC, Rosbash M (2000b) *Drosophila* cryptochromes - a unique circadian-rhythm photoreceptor. Nature 404:456–457. https://doi.org/10.1038/35006558

Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, Rosbash M (2000a) *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron 26:493–504. https://doi.org/10.1016/S0896-6273(00)81181-2

Fang Y, Sathyanarayanan S, Sehgal A (2007) Post-translational regulation of the *Drosophila* circadian clock requires protein phosphatase 1 (PP1). Genes Dev 21:1506–1518. https://doi.org/10.1101/gad.1541607

Foley LE, Ling J, Joshi R, Evantal N, Kadener S, Emery P (2019) *Drosophila* PSI controls circadian period and the phase of circadian behavior under temperature cycle via *tim* splicing. eLife 8:e50063. https://doi.org/10.7554/eLife.50063

Frisch B, Fleissner G, Fleissner G, Brandes C, Hall JC (1996) Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock gene product: Labeling of cells with possible importance for the beetle's circadian rhythms. Cell Tissue Res 286:411–429. https://doi.org/10.1007/s004410050711

Fuchikawa T, Beer K, Linke-Winnebeck C, Ben-David R, Kotowoy A, Tsang VWK et al (2017) Neuronal circadian clock protein oscillations are similar in behaviourally rhythmic forager honeybees and in arrhythmic nurses. Open Biol 7:170047. https://doi.org/10.1098/rsob.170047

Garbe DS, Fang Y, Zheng XZ, Sowcik M, Anjum R, Gygi SP et al (2013) Cooperative interaction between phosphorylation sites on PERIOD maintains circadian period in *Drosophila*. PLoS Genetics 9:e1003749. https://doi.org/10.1371/journal.pgen.1003749

Gestrich J, Giese M, Shen W, Zhang Y, Voss A, Popov C et al (2018) Sensitivity to Pigment-Dispersing Factor (PDF) is cell-type specific among PDF-expressing circadian clock neurons in the Madeira cockroach. J Biol Rhythms 33:35–51. https://doi.org/10.1177/0748730417739471

Glossop NR, Lyons LC, Hardin PE (1999) Interlocked feedback loops within the *Drosophila* circadian oscillator. Science 286:766–768. https://doi.org/10.1126/science.286.5440.766

Godinho SI, Maywood ES, Shaw L, Tucci V, Barnard AR, Busino L et al (2007) The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. Science 316:897–900. https://doi.org/10.1126/science.1141138

Grima B, Chelot E, Xia R, Rouyer F (2004) Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature 431:869–873. https://doi.org/10.1038/nature02935

Grima B, Dognon A, Lamouroux A, Chelot E, Rouyer F (2012) CULLIN-3 controls TIMELESS oscillations in the *Drosophila* circadian clock. PLoS Biol 10:e1001367. https://doi.org/10.1371/journal.pbio.1001367

Grima B, Lamouroux A, Chelot E, Papin C, Limbourg-Bouchon B, Rouyer F (2002) The F-box protein slimb controls the levels of clock proteins period and timeless. Nature 420:178–182. https://doi.org/10.1038/nature01122

Grima B, Papin C, Martin B, Chelot E, Ponien P, Jacquet E et al (2019) PERIOD-controlled deadenylation of the *timeless* transcript in the *Drosophila* circadian clock. Proc Natl Acad Sci U S A 116:5721–5726. https://doi.org/10.1073/pnas.1814418116

Hall JC (2003) Genetics and molecular biology of rhythms in *Drosophila* and other insects. Adv Genet 48:1–280. https://doi.org/10.1016/s0065-2660(03)48000-0

Hamblen MJ, White NE, Emery PTJ, Kaiser K, Hall JC (1998) Molecular and behavioral analysis of four *period* mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics 149:165–178. https://doi.org/10.1093/genetics/149.1.165

Hara T, Koh K, Combs DJ, Sehgal A (2011) Post-translational regulation and nuclear entry of TIMELESS and PERIOD are affected in new *timeless* mutant. J Neurosci 31:9982–9990. https://doi.org/10.1523/JNEUROSCI.0993-11.2011

Hardin PE (2011) Molecular genetic analysis of circadian timekeeping in *Drosophila*. Adv Genet 48(74):141–173. https://doi.org/10.1016/B978-0-12-387690-4.00005-2

Hardin PE, Hall JC, Rosbash M (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature 343:536–540. https://doi.org/10.1038/343536a0

Hastings JW, Sweeney BM (1957) On the mechanism of temperature independence in a biological clock. Proc Natl Acad Sci U S A 43:804–811. https://doi.org/10.1073/pnas.43.9.804

Hirano A, Yumimoto K, Tsunematsu R, Matsumoto M, Oyama M, Kozuka-Hata H et al (2013) FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. Cell 152:1106–1118. https://doi.org/10.1016/j.cell.2013.01.054

Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M et al (2002) *Dec1* and *Dec2* are regulators of the mammalian molecular clock. Nature 419:841–844. https://doi.org/10.1038/nature01123

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M et al (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627–630. https://doi.org/10.1038/19323

Houl JH, Yu W, Dudek SM, Hardin PE (2006) *Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. J Biol Rhythms 21:93–103. https://doi.org/10.1177/0748730405283697

Hunter-Ensor M, Ousley A, Sehgal A (1996) Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. Cell 84:677–685. https://doi.org/10.1016/s0092-8674(00)81046-6

Ikeda K, Daimon T, Sezutsu H, Udaka H, Numata H (2019) Involvement of the clock gene *period* in the circadian rhythm of the silkmoth *Bombyx mori*. J Biol Rhythms 34:283–292. https://doi.org/10.1177/0748730419841185

Ikeno T, Ishikawa K, Numata H, Goto SG (2013) Circadian clock gene *Clock* is involved in the photoperiodic response of the bean bug *Riptortus pedestris*. Physiol Entomol 38:157–162. https://doi.org/10.1111/phen.12013

Ikeno T, Katagiri C, Numata H, Goto SG (2011a) Causal involvement of mammalian-type *cryptochrome* in the circadian cuticle deposition rhythm in the bean bug *Riptortus pedestris*. Insect Mol Biol 20:409–415. https://doi.org/10.1111/j.1365-2583.2011.01075.x

Ikeno T, Numata H, Goto SG (2008) Molecular characterization of the circadian clock genes in the bean bug, *Riptortus pedestris*, and their expression patterns under long- and short-day conditions. Gene 419:56–61. https://doi.org/10.1016/j.gene.2008.05.002

Ikeno T, Numata H, Goto SG (2011b) Photoperiodic response requires mammalian-type *cryptochrome* in the bean bug *Riptortus pedestris*. Biochem Biophys Res Commun 410:394–397. https://doi.org/10.1016/j.bbrc.2011.05.142

Ikeno T, Tanaka SI, Numata H, Goto SG (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol 8:116. https://doi.org/10.1186/1741-7007-8-116

Iwai S, Trang LTD, Sehadova H, Takeda M (2008) Expression analyses of *casein kinase 2α* and *casein kinase 2β* in the silkmoth, *Bombyx mori*. Comp Biochem Physiol B 149:38–46. https://doi.org/10.1016/j.cbpb.2007.08.004

Jang AR, Moravcevic K, Saez L, Young MW, Sehgal A (2015) *Drosophila* TIM binds importin α1, and acts as an adapter to transport PER to the nucleus. PLoS Genet 11:e1005205. https://doi.org/10.1371/journal.pgen.1004974

Jaumouille E, Machado Almeida P, Stahli P, Koch R, Nagoshi E (2015) Transcriptional regulation via nuclear receptor crosstalk required for the *Drosophila* circadian clock. Curr Biol 25:1502–1508. https://doi.org/10.1016/j.cub.2015.04.017

Johnson KP, Dietrich CH, Friedrich F, Beutel RG, Wipfler B, Peters RS et al (2018) Phylogenomics and the evolution of hemipteroid insects. Proc Natl Acad Sci U S A 115:12775–12780. https://doi.org/10.1073/pnas.1815820115

Johnsson A, Helfrich-Förster C, Engelmann W (2014) How light resets circadian clocks. In: Björn L (ed) Photobiology. Springer, New York, pp 243–297. https://doi.org/10.1007/978-1-4939-1468-5_18

Joshi R, Cai YD, Xia Y, Chiu J, Emery P (2022) PERIOD phosphoclusters control temperature compensation of the *Drosophila* circadian clock. Front Physiol 13:888262. https://doi.org/10.1101/2021.12.23.474078

Kadener S, Stoleru D, McDonald M et al (2007) *Clockwork Orange* is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. Genes Dev 21:1675–1686. https://doi.org/10.1101/Gad.1552607

Kamae Y, Tomioka K (2012) *timeless* is an essential component of the circadian clock in a primitive insect, the firebrat *Thermobia domestica*. J Biol Rhythms 27:126–134. https://doi.org/10.1177/0748730411435997

Kamae Y, Uryu O, Miki T, Tomioka K (2014) The nuclear receptor genes HR3 and E75 are required for the circadian rhythm in a primitive insect. PLoS One 9:e114899. https://doi.org/10.1371/journal.pone.0114899

Kawahara AY, Plotkin D, Espeland M, Meusemann K, Toussaint EFA, Donath A et al (2019) Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. Proc Natl Acad Sci U S A 116:22657–22663. https://doi.org/10.1073/pnas.1907847116

Kim EY, Edery I (2006) Balance between DBT/CKIε kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. Proc Natl Acad Sci U S A 103:6178–6183. https://doi.org/10.1073/pnas.0511215103

Kivimaë S, Saez L, Young MW (2008) Activating PER repressor through a DBT-directed phosphorylation switch. PLoS Biol 6:1570–1583. https://doi.org/10.1371/journal.pbio.0060183

Kloss B, Price JL, Saez L (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell 94:97–107. https://doi.org/10.1016/S0092-8674(00)81225-8

Kloss B, Rothenfluh A, Young MW, Saez L (2001) Phosphorylation of PERIOD is influenced by cycling physical associations of DOUBLE-TIME, PERIOD, and TIMELES in the *Drosophila* clock. Neuron 30:699–706. https://doi.org/10.1016/S0896-6273(01)00320-8

Ko HW, Jiang J, Edery I (2002) A role for Slimb in the degradation of *Drosophila* PERIOD protein phosphorylated by DOUBLETIME. Nature 420:673–678. https://doi.org/10.1038/Nature01272

Kobelkova A, Bajgar A, Dolezel D (2010) Functional molecular analysis of a circadian clock gene *timeless* promoter from the drosophilid fly *Chymomyza costata*. J Biol Rhythms 25:399–409. https://doi.org/10.1177/0748730410385283

Kobelkova A, Zavodska R, Sauman I, Bazalova O, Dolezel D (2015) Expression of clock genes *period* and *timeless* in the central nervous system of the Mediterranean flour moth, *Ephestia kuehniella*. J Biol Rhythms 30:104–116. https://doi.org/10.1177/0748730414568430

Koh K, Zheng X, Sehgal A (2006) JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. Science 312:1809–1812. https://doi.org/10.1126/science.1124951

Koide R, Xi J, Hamanaka Y, Shiga S (2021) Mapping PERIOD-immunoreactive cells with neurons relevant to photoperiodic response in the bean bug *Riptortus pedestris*. Cell Tissue Res 385: 571–583. https://doi.org/10.1007/s00441-021-03451-6

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Nat Acad Sci U S A 68:2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Konopka RJ, Hamblencoyle MJ, Jamison CF, Hall JC (1994) An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. J Biol Rhythms 9:189–216. https://doi.org/10.1177/074873049400900303

Konopka RJ, Pittendrigh C, Orr D (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. J Neurogenet 6:1–10. https://doi.org/10.3109/01677068909107096

Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS (1990) Photic and circadian regulation of *c-fos* gene expression in hamster suprachiasmatic nucleus. Neuron 5:127–134. https://doi.org/10.1016/0896-6273(90)90303-W

Kotwica-Rolinska J, Chodáková L, Chvalova D, Pauchova L, Provaznik J, Hejnikova M et al (2019) CRISPR/Cas9 genome editing introduction and optimization in the non-model insect *Pyrrhocoris apterus*. Front Physiol 10:891. https://doi.org/10.3389/fphys.2019.00891

Kotwica-Rolinska J, Chodáková L, Smykal V, Damulewicz M, Provaznik J, Wu BC et al (2022a) Loss of *timeless* underlies an evolutionary transition within the circadian clock. Mol Biol Evol 39:msab346. https://doi.org/10.1093/molbev/msab346

Kotwica-Rolinska J, Damulewicz M, Chodáková L, Krištofová L, Dolezel D (2022b) Pigment Dispersing Factor is a circadian clock output and regulates photoperiodic response in the linden bug, *Pyrrhocoris apterus*. Front Physiol 13:884909. https://doi.org/10.3389/fphys.2022.884909

Kotwica-Rolinska J, Pivarciova L, Vaneckova DD (2017) The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage. Physiol Entomol 42:266–273. https://doi.org/10.1111/phen.12197

Kozak GM, Wadsworth CB, Kahne SC, Bogdanowicz SM, Harrison RG, Coates BS et al (2019) Genomic basis of circannual rhythm in the European corn borer moth. Curr Biol 29:3501–3509. https://doi.org/10.1016/j.cub.2019.08.053

Kula-Eversole E, Lee DH, Samba I, Yildirim E, Levine DC, Hong HK et al (2021) Phosphatase of regenerating Liver-1 selectively times circadian behavior in darkness via function in PDF neurons and dephosphorylation of TIMELESS. Curr Biol 31:138–149. https://doi.org/10.1016/j.cub.2020.10.013

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin XW et al (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98:193–205. https://doi.org/10.1016/S0092-8674(00)81014-4

Kurien P, Hsu PK, Leon J, Wu D, McMahon T, Shi G et al (2019) TIMELESS mutation alters phase responsiveness and causes advanced sleep phase. Proc Natl Acad Sci U S A 116:12045–12053. https://doi.org/10.1073/pnas.1819110116

Kutaragi Y, Tokuoka A, Tomiyama Y, Nose M, Watanabe T, Bando T et al (2018) A novel photic entrainment mechanism for the circadian clock in an insect: involvement of *c-fos* and *cryptochromes*. Zool Lett 4:26. https://doi.org/10.1186/s40851-018-0109-8

Lam VH, Li YH, Liu X, Murphy KA, Diehl JS, Kwok RS et al (2018) CK1α collaborates with DOUBLETIME to regulate PERIOD function in the *Drosophila* circadian clock. J Neurosci 38: 10631–10643. https://doi.org/10.1523/JNEUROSCI.0871-18.2018

Lamaze A, Chen C, Leleux S, Xu M, George R, Stanewsky R (2022) A natural *timeless* polymorphism allowing circadian clock synchronization in "white nights". Nat Commun 13:1724. https://doi.org/10.1038/s41467-022-29293-6

Lamaze A, Lamouroux A, Vias C, Hung HC, Weber F, Rouyer F (2011) The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in *Drosophila*. EMBO Rep 12:549–557. https://doi.org/10.1038/embor.2011.64

Landskron J, Chen KF, Wolf E, Stanewsky R (2009) A role for the PERIOD:PERIOD homodimer in the *Drosophila* circadian clock. PLoS Biol 7:820–835. https://doi.org/10.1371/journal.pbio.1000003

Lee C, Bae K, Edery I (1998) The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. Neuron 21:857–867. https://doi.org/10.1016/S0896-6273(00)80601-7

Li YH, Liu X, Vanselow JT, Zheng H, Schlosser A, Chiu JC (2019) O-GlcNAcylation of PERIOD regulates its interaction with CLOCK and timing of circadian transcriptional repression. PLoS Genet 15:1007953. https://doi.org/10.1371/journal.pgen.1007953

Liang X, Holy TE, Taghert PH (2016) Synchronous *Drosophila* circadian pacemakers display nonsynchronous Ca^{2+} rhythms *in vivo*. Science 351:976–981. https://doi.org/10.1126/science.aad3997

Liang X, Holy TE, Taghert PH (2017) A series of suppressive signals within the *Drosophila* circadian neural circuit generates sequential daily outputs. Neuron 94:1173–1189. https://doi.org/10.1016/j.neuron.2017.05.007

Lim C, Allada R (2013a) Emerging roles for post-transcriptional regulation in circadian clocks. Nature Neurosci 16:1544–1550. https://doi.org/10.1038/nn.3543

Lim C, Allada R (2013b) ATAXIN-2 activates PERIOD translation to sustain circadian rhythms in *Drosophila*. Science 340:875–879. https://doi.org/10.1126/science.1234785

Lim C, Lee J, Choi C, Kilman VL, Kim J, Park SM et al (2011) The novel gene twenty-four defines a critical translational step in the *Drosophila* clock. Nature 470:399–403. https://doi.org/10.1038/nature09728

Ling J, Dubruille R, Emery P (2012) KAYAK-α modulates circadian transcriptional feedback loops in *Drosophila* pacemaker neurons. J Neurosci 32:16959–16970. https://doi.org/10.1523/JNEUROSCI.1888-12.2012

Liu X, Blazenovic I, Contreras AJ, Pham TM, Tabuloc CA, Li YH et al (2021) Hexosamine biosynthetic pathway and O-GlcNAc-processing enzymes regulate daily rhythms in protein O-GlcNAcylation. Nat Commun 12:4173. https://doi.org/10.1038/s41467-021-24301-7

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR et al (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science 288:483–491. https://doi.org/10.1126/science.288.5465.483

Majercak J, Sidote D, Hardin PE, Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron 24:219–230. https://doi.org/10.1016/S0896-6273(00)80834-X

Markert MJ, Zhang Y, Enuameh MS, Reppert SM, Wolfe SA, Merlin C (2016) Genomic access to monarch migration using TALEN and CRISPR/Cas9-mediated targeted mutagenesis. G3 6:905–915. https://doi.org/10.1534/g3.116.027029

Martin Anduaga A, Evantal N, Patop IL, Bartok O, Weiss R, Kadener S (2019) Thermosensitive alternative splicing senses and mediates temperature adaptation in *Drosophila*. eLife 8:e44642. https://doi.org/10.7554/eLife.44642

Martinek S, Inonog S, Manoukian AS, Young MW (2001) A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. Cell 105:769–779. https://doi.org/10.1016/S0092-8674(01)00383-X

Masuda S, Narasimamurthy R, Yoshitane H, Kim JK, Fukada Y, Virshup DM (2020) Mutation of a PER2 phosphodegron perturbs the circadian phosphoswitch. Proc Nat Acad Sci U S A 117:10888–10896. https://doi.org/10.1073/pnas.2000266117

Matsumoto A, Tomioka K, Chiba Y, Tanimura T (1999) *tim*^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol 19:4343-4354. https://doi.org/10.1128/MCB.19.6.4343

Matsumoto A, Ukai-Tadenuma M, Yamada RG, Houl J, Uno KD, Kasukawa T et al (2007) A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the *Drosophila* circadian clock. Genes Dev 21:1687–1700. https://doi.org/10.1101/Gad.1552207

McDonald MJ, Rosbash M (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell 107:567–578

McDonald MJ, Rosbash M, Emery P (2001) Wild-type circadian rhythmicity is dependent on closely spaced E boxes in the *Drosophila timeless* promoter. Mol Cell Biol 21:1207–1217. https://doi.org/10.1128/Mcb.21.4.1207-1217.2001

McKenna DD, Shin S, Ahrens D, Balke M, Beza-Beza C, Clarke DJ et al (2019) The evolution and genomic basis of beetle diversity. Proc Natl Acad Sci U S A 116:24729–24737. https://doi.org/10.1073/pnas.1909655116

Mei Q, Dvornyk V (2015) Evolutionary history of the photolyase/cryptochrome superfamily in eukaryotes. PLoS One 10:e0135940. https://doi.org/10.1038/s41598-021-89345-7

Meireles-Filho AC, Amoretty PR, Souza NA, Kyriacou CP, Peixoto AA (2006) Rhythmic expression of the cycle gene in a hematophagous insect vector. BMC Mol Biol 7:38. https://doi.org/10.1186/1471-2199-7-38

Meuti ME, Stone M, Ikeno T, Denlinger DL (2015) Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, *Culex pipiens*. J Exp Biol 218:412–422. https://doi.org/10.1242/jeb.113233

Meyer P, Saez L, Young MW (2006) PER-TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. Science 311:226–229. https://doi.org/10.1126/science.1118126

Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C et al (2014) Phylogenomics resolves the timing and pattern of insect evolution. Science 346:763–767. https://doi.org/10.1126/science.1257570

Moriyama Y, Kamae Y, Uryu O, Tomioka K (2012) *Gb'clock* is expressed in the optic lobe and is required for the circadian clock in the cricket *Gryllus bimaculatus*. J Biol Rhythms 27:467–477. https://doi.org/10.1177/0748730412462207

Myers MP, WagerSmith K, Rothenfluh-Hilfiker A, Young MW (1996) Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science 271:1736–1740. https://doi.org/10.1126/science.271.5256.1736

Naidoo N, Song W, Hunter-Ensor M, Sehgal A (1999) A role for the proteasome in the light response of the timeless clock protein. Science 285:1737–1741. https://doi.org/10.1126/science.285.5434.1737

Narasaki-Funo Y, Tomiyama Y, Nose M, Bando T, Tomioka K (2020) Functional analysis of *Pdp1* and *vrille* in the circadian system of a cricket. J Insect Physiol 127:104156. https://doi.org/10.1016/j.jinsphys.2020.104156

Narasimamurthy R, Virshup DM (2021) The phosphorylation switch that regulates ticking of the circadian clock. Mol Cell 81:1133–1146. https://doi.org/10.1016/j.molcel.2021.01.006

Nartey MA, Sun X, Qin S, Hou CX, Li MW (2021) CRISPR/Cas9-based knockout reveals that the clock gene *timeless* is indispensable for regulating circadian behavioral rhythms in *Bombyx mori*. Insect Sci 28:1414–1425. https://doi.org/10.1111/1744-7917.12864

Nose M, Tokuoka A, Bando T, Tomioka K (2017) *timeless2* plays an important role in reproduction and circadian rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 105:9. https://doi.org/10.1016/j.jinsphys.2017.12.007

Ozkaya O, Rosato E (2012) The circadian clock of the fly: a neurogenetics journey through time. Adv Genet 77:79–123. https://doi.org/10.1016/B978-0-12-387687-4.00004-0

Pavelka J, Shimada K, Kostal V (2003) TIMELESS: a link between fly's circadian and photoperiodic clocks? Eur J Entomol 100:255–265. https://doi.org/10.14411/eje.2003.041

Peschel N, Chen KF, Szabo G, Stanewsky R (2009) Light-dependent interactions between the *Drosophila* circadian clock factors cryptochrome, jetlag, and timeless. Curr Biol 19:241–247. https://doi.org/10.1016/j.cub.2008.12.042

Peschel N, Helfrich-Förster C (2011) Setting the clock - by nature: circadian rhythm in the fruitfly *Drosophila melanogaster*. FEBS Lett 585:1435–1442. https://doi.org/10.1016/j.febslet.2011.02.028

Peschel N, Veleri S, Stanewsky R (2006) *Veela* defines a molecular link between cryptochrome and timeless in the light-input pathway to *Drosophila's* circadian clock. Proc Nat Acad Sci U S A 103:17313–17318. https://doi.org/10.1073/pnas.0606675103

Pett JP, Kondoff M, Bordyugov G, Kramer A, Herzel H et al (2018) Co-existing feedback loops generate tissue-specific circadian rhythms. Life Sci Alliance 1:e201800078. https://doi.org/10.26508/lsa.201800078

Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW (1998) *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell 94:83–95. https://doi.org/10.1016/S0092-8674(00)81224-6

Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802. https://doi.org/10.1016/S0092-8674(00)81676-1

Richier B, Michard-Vanhee C, Lamouroux A, Papin C, Rouyer F (2008) The clockwork orange *Drosophila* protein functions as both an activator and a repressor of clock gene expression. J Biol Rhythms 23:103–116. https://doi.org/10.1177/0748730407313817

Rivas GBS, Zhou J, Merlin C, Hardin PE (2021) CLOCKWORK ORANGE promotes CLOCK-CYCLE activation via the putative *Drosophila* ortholog of CLOCK INTERACTING PROTEIN CIRCADIAN. Curr Biol 31:4207–4218.e4. https://doi.org/10.1016/j.cub.2021.07.017

Rosato E, Codd V, Mazzotta G, Piccin A, Zordan M, Costa R et al (2001) Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. Curr Biol 11:909–917. https://doi.org/10.1016/s0960-9822(01)00259-7

Rosato E, Trevisan A, Sandrelli F, Zordan M, Kyriacou CP, Costa R (1997) Conceptual translation of *timeless* reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res 25:455–458. https://doi.org/10.1093/nar/25.3.455

Rothenfluh A, Abodeely M, Price JL, Young MW (2000b) Isolation and analysis of six *timeless* alleles that cause short- or long-period circadian rhythms in *Drosophila*. Genetics 156:665–675. https://doi.org/10.1093/genetics/156.2.665

Rothenfluh A, Young MW, Saez L (2000a) A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. Neuron 26:505–514. https://doi.org/10.1016/s0896-6273(00)81182-4

Rubin EB, Shemesh Y, Cohen M, Elgavish S, Robertson HM, Bloch G (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. Genome Res 16:1352–1365. https://doi.org/10.1101/gr.5094806

Rund SS, Hou TY, Ward SM, Collins FH, Duffield GE (2011) Genome-wide profiling of diel and circadian gene expression in the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci U S A 108:E421–E430. https://doi.org/10.1073/pnas.1100584108

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 93:805–814. https://doi.org/10.1016/S0092-8674(00)81441-5

Rutila JE, Zeng H, Le M, Curtin KD, Hall JC, Rosbash M (1996) The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron 17:921–929. https://doi.org/10.1016/s0896-6273(00)80223-8

Saez L, Derasmo M, Meyer P, Stieglitz J, Young MW (2011) A key temporal delay in the circadian cycle of *Drosophila* is mediated by a nuclear localization signal in the Timeless protein. Genetics 188:591–U166. https://doi.org/10.1534/genetics.111.127225

Saez L, Young MW (1996) Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless. Neuron 17:911–920. https://doi.org/10.1016/S0896-6273(00)80222-6

Sakamoto T, Uryu O, Tomioka K (2009) The clock gene *period* plays an essential role in photoperiodic control of nymphal development in the cricket *Modicogryllus siamensis*. J Biol Rhythms 24:379–390. https://doi.org/10.1177/0748730409341523

Sandrelli F, Tauber E, Pegoraro M, Mazzotta G, Cisotto P, Landskron J et al (2007) A molecular basis for natural selection at the *timeless* locus in *Drosophila melanogaster*. Science 316:1898–1900. https://doi.org/10.1126/science.1138426

Sathyanarayanan S, Zheng X, Xiao R, Sehgal A (2004) Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. Cell 116:603–615. https://doi.org/10.1016/s0092-8674(04)00128-x

Sauman I, Briscoe AD, Zhu HS, Shi DD, Froy O, Stalleicken J et al (2005) Connecting the navigational clock to sun compass input in monarch butterfly brain. Neuron 46:457–467. https://doi.org/10.1016/j.neuron.2005.03.014

Sawyer LA, Hennessy JM, Peixoto AA, Rosato E, Parkinson H, Costa R et al (1997) Natural variation in a *Drosophila* clock gene and temperature compensation. Science 278:2117–2120. https://doi.org/10.1534/genetics.106.058792

Sehadova H, Markova EP, Sehnal FS, Takeda M (2004) Distribution of circadian clock-related proteins in the cephalic nervous system of the silkworm, *Bombyx mori*. J Biol Rhythms 19:466–482. https://doi.org/10.1177/0748730404269153

Sehadova H, Sauman I, Sehnal F (2003) Immunocytochemical distribution of pigment-dispersing hormone in the cephalic ganglia of polyneopteran insects. Cell Tissue Res 312:113–125. https://doi.org/10.1007/s00441-003-0705-5

Sehgal A, Price JL, Man B, Young MW (1994) Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant *timeless*. Science 263:1603–1606. https://doi.org/10.1126/science.8128246

Sehgal A, Rothenfluhhilfiker A, Hunterensor M, Chen YF, Myers MP, Young MW (1995) Rhythmic expression of *timeless* – a basis for promoting circadian cycles in *period* gene autoregulation. Science 270:808–810. https://doi.org/10.1126/science.270.5237.808

Shakhmantsir I, Nayak S, Grant GR, Sehgal A (2019) Spliceosome factors target *timeless* (*tim*) mRNA to control clock protein accumulation and circadian behavior in *Drosophila*. eLife 7: e39821. https://doi.org/10.7554/eLife.39821

Shao QM, Sehadova H, Ichihara N, Sehnal FS, Takeda M (2006) Immunoreactivities to three circadian clock proteins in two ground crickets suggest interspecific diversity of the circadian clock structure. J Biol Rhythms 21:118–131. https://doi.org/10.1177/0748730405283660

Siepka SM, Yoo SH, Park J, Song WM, Kumar V, Hu YN et al (2007) Circadian mutant overtime reveals F-box protein FBXL3 regulation of *cryptochrome* and *period* gene expression. Cell 129: 1011–1023. https://doi.org/10.1016/j.cell.2007.04.030

Singh S, Giesecke A, Damulewicz M, Fexova S, Mazzotta GM, Stanewsky R et al (2019) New *Drosophila* circadian clock mutants affecting temperature compensation induced by targeted mutagenesis of *timeless*. Front Physiol 10:1442. https://doi.org/10.3389/fphys.2019.01442

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC (1988) Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron 1:141–150. https://doi.org/10.1016/0896-6273(88)90198-5

Stanewsky R (2003) Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J Neurobiol 54:111–147. https://doi.org/10.1002/Neu.10164

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA et al (1998) The cry^b mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692. https://doi.org/10.1016/S0092-8674(00)81638-4

Stehlik J, Zavodska R, Shimada K, Sauman I, Kostal V (2008) Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*. J Biol Rhythms 23:129–139. https://doi.org/10.1177/0748730407313364

Stoleru D, Peng Y, Agosto J, Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. Nature 431:862–868. https://doi.org/10.1038/nature02926

Stroppa MM, Garcia BA (2019) Clock gene *timeless* in the chagas disease vector *Triatoma infestans* (Hemiptera: Reduviidae). Am J Trop Med Hyg 101:1369–1372. https://doi.org/10.4269/ajtmh.19-0169

Szabo A, Papin C, Cornu D, Chelot E, Lipinszki Z, Udvardy A et al (2018) Ubiquitylation dynamics of the clock cell proteome and TIMELESS during a circadian cycle. Cell Rep 23:2273–2282. https://doi.org/10.1016/j.celrep.2018.04.064

Szabo A, Papin C, Zorn D, Ponien P, Weber F, Raabe T et al (2013) The CK2 kinase stabilizes CLOCK and represses its activity in the *Drosophila* circadian oscillator. PloS Biol 11:e1001645. https://doi.org/10.1371/journal.pbio.1001645

Takekata H, Numata H, Shiga S, Goto SG (2014) Silencing the circadian clock gene *Clock* using RNAi reveals dissociation of the circatidal clock from the circadian clock in the mangrove cricket. J Insect Physiol 68:16–22. https://doi.org/10.1016/j.jinsphys.2014.06.012

Tataroglu O, Emery P (2015) The molecular ticks of the *Drosophila* circadian clock. Curr Opin Insect Sci 7:51–57. https://doi.org/10.1016/j.cois.2015.01.002

Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C et al (2007) Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science 316:1895–1898. https://doi.org/10.1126/science.1138412

Thakkar N, Giesecke A, Bazalova O, Martinek J, Smykal V, Stanewsky R et al (2022) Evolution of casein kinase 1 and functional analysis of new *doubletime* mutants in *Drosophila*. Front Physiol 13:1062632. https://doi.org/10.3389/fphys.2022.10626

Thomas GWC, Dohmen E, Hughes DST, Murali SC, Poelchau M, Glastad K et al (2020) Gene content evolution in the arthropods. Genome Biol 21:15. https://doi.org/10.1186/s13059-019-1925-7

Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM et al (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science 291:1040–1043. https://doi.org/10.1126/science.1057499

Tokuoka A, Itoh TQ, Hori S, Uryu O, Danbara Y, Nose M et al (2017) cryptochrome genes form an oscillatory loop independent of the *per/tim* loop in the circadian clockwork of the cricket *Gryllus bimaculatus*. Zoological Lett 3:5. https://doi.org/10.1186/s40851-017-0066-7

Tomiyama Y, Shinohara T, Matsuka M, Bando T, Mito T, Tomioka K (2020) The role of *clockwork orange* in the circadian clock of the cricket *Gryllus bimaculatus*. Zoological Lett 6:12. https://doi.org/10.1186/s40851-020-00166-4

Top D, Harms E, Syed S, Adams EL, Saez L (2016) GSK-3 and CK2 kinases converge on Timeless to regulate the master clock. Cell Rep 16:357–367. https://doi.org/10.1016/j.celrep.2016.06.005

Top D, O'Neil JL, Merz GE, Dusad K, Crane BR, Young MW (2018) CK1/Doubletime activity delays transcription activation in the circadian clock. eLife 7:e32679. https://doi.org/10.7554/eLife.32679

Uryu O, Karpova SG, Tomioka K (2013) The clock gene cycle plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. J Insect Physiol 59:697–704. https://doi.org/10.1016/j.jinsphys.2013.04.011

Vafopoulou X, Steel CG (2012) Metamorphosis of a clock: remodeling of the circadian timing system in the brain of *Rhodnius prolixus* (Hemiptera) during larval-adult development. J Comp Neurol 520:1146–1164. https://doi.org/10.1002/cne.22743

Venkatesan A, Fan JY, Bouyain S, Price JL (2019) The circadian tau mutation in casein kinase 1 is part of a larger domain that can be mutated to shorten circadian period. Int J Mol Sci 20:4. https://doi.org/10.3390/ijms20040813

Wang X, Fang X, Yang P, Jiang X, Jiang F, Zhao D et al (2014) The locust genome provides insight into swarm formation and long-distance flight. Nat Commun 5:2957. https://doi.org/10.1038/ncomms3957

Wen CJ, Lee HJ (2008) Mapping the cellular network of the circadian clock in two cockroach species. Arch Insect Biochem Physiol 68:215–231. https://doi.org/10.1002/arch.20236

Werckenthin A, Huber J, Arnold T, Koziarek S, Plath MJA, Plath JA et al (2020) Neither *per*, nor *tim1*, nor *cry2* alone are essential components of the molecular circadian clockwork in the Madeira cockroach. PLoS One 15:e0235930. https://doi.org/10.1371/journal.pone.0235930

Wipfler B, Letsch H, Frandsen PB, Kapli P, Mayer C, Bartel D et al (2019) Evolutionary history of Polyneoptera and its implications for our understanding of early winged insects. Proc Natl Acad Sci U S A 116:3024–3029. https://doi.org/10.1073/pnas.1817794116

Xu L, Chen S, Wen B, Shi H, Chi C, Liu C et al (2021) Identification of a novel class of photolyases as possible ancestors of their family. Mol Biol Evol 38:4505–4519. https://doi.org/10.1093/molbev/msab191

Yao Z, Shafer OT (2014) The *Drosophila* circadian clock is a variably coupled network of multiple peptidergic units. Science 343:1516–1520. https://doi.org/10.1126/science.1251285

Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. Genes Dev 20:723–733. https://doi.org/10.1101/gad.1404406

Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24:948–955. https://doi.org/10.1093/molbev/msm011

Zavodska R, Fexova S, von Wowern G, Han GB, Dolezel D, Sauman I (2012) Is the sex communication of two pyralid moths, *Plodia interpunctella* and *Ephestia kuehniella*, under circadian clock regulation? J Biol Rhythms 27:206–216. https://doi.org/10.1177/0748730412440689

Zavodska R, Sauman I, Sehnal F (2003a) Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. J Biol Rhythms 18:106–122. https://doi.org/10.1177/0748730403251711

Zavodska R, Sauman I, Sehnal F (2003b) The cycling and distribution of PER-like antigen in relation to neurons recognized by the antisera to PTTH and EH in *Thermobia domestica*. Insect Biochem Mol Biol 33:1227–1238. https://doi.org/10.1016/j.ibmb.2003.06.009

Zavodska R, Wen CJ, Hrdy I, Sauman I, Lee HJ, Sehnal F (2008) Distribution of corazonin and pigment-dispersing factor in the cephalic ganglia of termites. Arthropod Struct Dev 37:273–286. https://doi.org/10.1016/j.asd.2008.01.005

Zehring WA, Wheeler DA, Reddy P, Konopka RJ, Kyriacou CP, Rosbash M et al (1984) P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. Cell 39:369–376. https://doi.org/10.1016/0092-8674(84)90015-1

Zeng HK, Qian ZW, Myers MP, Rosbash M (1996) A light-entrainment mechanism for the *Drosophila* circadian clock. Nature 380:129–135. https://doi.org/10.1038/380129a0

Zerr DM, Hall JC, Rosbash M, Siwicki KK (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. J Neurosci 10:2749–2762. https://doi.org/10.1523/JNEUROSCI.10-08-02749.1990

Zhang L, Hastings MH, Green EW, Tauber E, Sladek M, Webster SG et al (2013a) Dissociation of circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. Curr Biol 23:1863–1873. https://doi.org/10.1016/j.cub.2013.08.038

Zhang Y, Ling J, Yuan C, Dubruille R, Emery P (2013b) A role for *Drosophila* ATX2 in activation of PER translation and circadian behavior. Science 340:879–882. https://doi.org/10.1126/science.1234746

Zhang Y, Markert MJ, Groves SC, Hardin PE, Merlin C (2017) Vertebrate-like CRYPTOCHROME 2 from monarch regulates circadian transcription via independent repression of CLOCK and BMAL1 activity. Proc Natl Acad Sci U S A 114:E7516–E7525. https://doi.org/10.1073/pnas.1702014114

Zhou J, Yu W, Hardin PE (2016) CLOCKWORK ORANGE enhances PERIOD mediated rhythms in transcriptional repression by antagonizing E-box binding by CLOCK-CYCLE. PLoS Genet 12:e1006430. https://doi.org/10.1371/journal.pgen.1006430

Zhou M, Kim JK, Eng GW, Forger DB, Virshup DM (2015) A Period2 phosphoswitch regulates and temperature compensates circadian period. Mol Cell 60:77–88. https://doi.org/10.1016/j.molcel.2015.08.022

Zhu HS, Sauman I, Yuan Q, Casselman A, Emery-Le M, Emery P et al (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biol 6:138–155. https://doi.org/10.1371/journal.pbio.0060004

Zylka MJ, Shearman LP, Levine JD, Jin XW, Weaver DR, Reppert SM (1998) Molecular analysis of mammalian *timeless*. Neuron 21:1115–1122. https://doi.org/10.1016/S0896-6273(00)80628-5

Chapter 5
Neurocircuitry of Circadian Clocks

Taishi Yoshii and Ayumi Fukuda

Abstract Classical studies using several insect species have demonstrated that the principal circadian clock cells that generate circadian oscillations and control behavioral rhythms are located in a specific brain region. The discovery of a clock gene, *period* (*per*), in *Drosophila melanogaster* further facilitated the identification of specific cells by labeling gene expression. Since most of the *per*-expressing brain neurons display circadian molecular oscillations in the levels of *per* mRNA and its protein expression, they have conventionally been defined as "circadian clock neurons." In *Drosophila*, approximately 150 neurons (out of 200,000 brain neurons) have been identified as clock neurons. However, elucidating the role of clock neurons, even with the *Drosophila* model, has been a major challenge. In 1995, it was discovered that 16 clock neurons expressed a neuropeptide, pigment-dispersing factor (PDF), the most important neurotransmitter for the insect circadian clock. This was where *Drosophila* genetics and neuroscience met in chronobiology, leading to a significant development in the functional analysis of clock neurons in *Drosophila* and the identification of clock neurons in nonmodel insect species. This chapter will summarize the latest findings of the clock neuron network in *Drosophila* and other insect species.

Keywords Clock network · Clock neuron · Coupling · Neurotransmitter · PDF · PERIOD

5.1 Introduction

The most commonly observed circadian rhythms are shown in behavior, e.g., sleep-wake cycles or nocturnal/diurnal activity. Today, we know that cells containing circadian clocks are widespread throughout the body (Ito and Tomioka 2016; Chap. 6). Needless to say, however, the biggest question in the past was where in

T. Yoshii (✉) · A. Fukuda
Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan
e-mail: yoshii@okayama-u.ac.jp

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the animal's body the clock was located. During the late 1960s and 1970s, several pioneering studies were conducted on insects to answer this question. The first study was performed by Nishiitsutuji-Uwo and Pittendrigh (1968), who surgically lesioned parts of the brain and measured locomotor activity rhythms in the cockroach *Rhyparobia* (*Leucophaea maderae*). The cockroaches in which a brain region called the optic lobe was bilaterally lesioned displayed arrhythmic locomotor activity. Later, the same conclusion was drawn from studies of other insects, such as beetles and crickets (Loher 1972; Fleissner 1982; Tomioka and Chiba 1984). Two significant findings have been made to reinforce the hypothesis that the optic lobe is the locus of the principal circadian pacemaker: (1) Transplantation of the optic lobes into the optic lobeless brain restored the activity rhythm of the donor cockroach in *R. maderae* (Page 1982). 2) The isolated optic lobes displayed circadian rhythms in neural activity in a self-sustained manner in the cricket *Gryllus bimaculatus* and *R. maderae* (Tomioka and Chiba 1986; Colwell and Page 1990; Tomioka and Chiba 1992). Thus, the optic lobes of these insects contain pacemakers that control the activity rhythm.

The optic lobes mainly consist of three neuropils, namely, the lamina, medulla, and lobula, which process visual information from the compound eyes and send the processed information to the midbrain. The anatomical relationship between the light input pathway and the circadian pacemaker is plausible, given the importance of light entrainment. In mammals, light information is conveyed directly from the eye via the retinohypothalamic tract to entrain the mammalian pacemaker located in the suprachiasmatic nucleus (SCN) (Panda et al. 2002; Ruby et al. 2002). This analogy between insects and mammals suggests that the origin of the pacemaker center should be tightly linked to photoreception.

5.2 Small Ventral Lateral Neurons in *Drosophila*

Classical lesion experiments have beautifully revealed the brain region important for rhythm generation. However, this method is not suitable for identifying the locus of the pacemaker at the cellular level. The discovery of the *period* (*per*) gene in the fruit fly *Drosophila melanogaster*, a model organism in genetics, overcame this difficulty by using modern cell labeling techniques, which enabled the identification of the cells expressing the *per* gene in the brain. In situ hybridization and immunohistochemistry, which label mRNA and protein expression, respectively, have revealed that *per* is expressed in cells distributed in a wide range of brain regions (Liu et al. 1988; Siwicki et al. 1988). Therefore, the difficulty in identifying pacemaker cells persisted.

Since then, developments in fluorescent immunohistochemistry, confocal laser microscopy, and transgenic fly lines, such as the GAL4-UAS system, have contributed significantly to determining the precise anatomical location of *per*-expressing brain neurons (Kaneko and Hall 2000; Helfrich-Förster 2003). Today, we know that *per* is expressed in approximately 150 brain neurons classified into nine groups

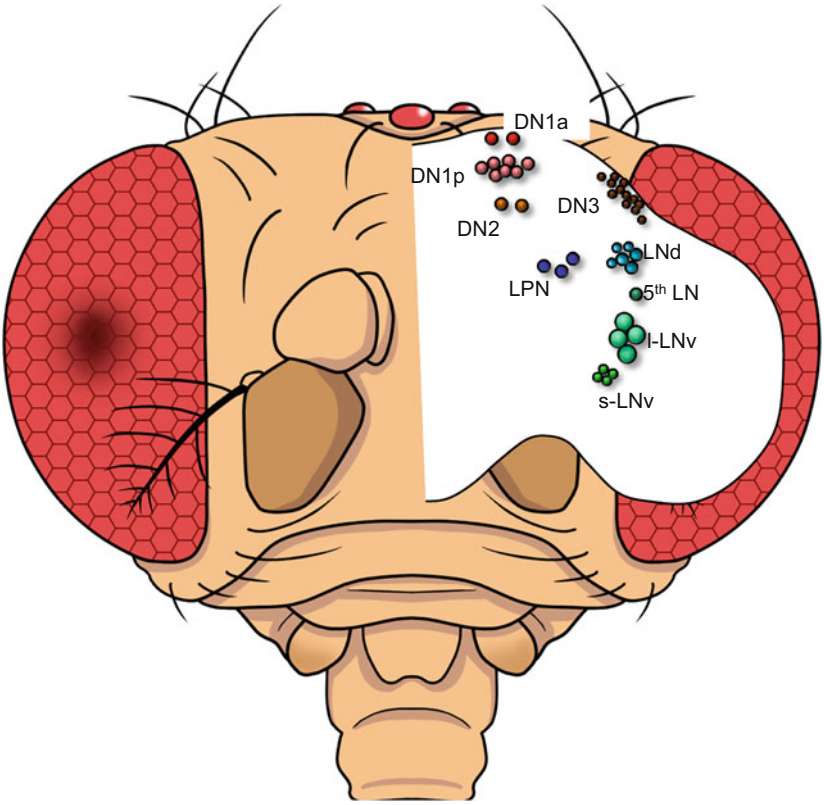

Fig. 5.1 The brain of *Drosophila melanogaster* and the distribution of clock neurons. The *Drosophila* central clock consists of approximately 150 neurons in the brain. The clock neurons are divided mainly into nine groups based on their localization and size of cell bodies: small ventral lateral neuron (s-LNv), large ventral lateral neuron (l-LNv), fifth lateral neuron (fifth LN), dorsal lateral neuron (LNd), lateral posterior neuron (LPN), anterior dorsal neuron 1 (DN1a), posterior dorsal neuron 1 (DN1p), dorsal neuron 2 (DN2), and dorsal neuron 3 (DN3)

(Fig. 5.1). First, *per*-expressing neurons are divided mainly into the lateral and dorsal neuron groups. Lateral neurons are located between the optic lobe and midbrain and are further subdivided into small ventral lateral neuron (s-LNv), large ventral lateral neuron (l-LNv), fifth lateral neuron (fifth LN, also known as fifth s-LNv), dorsal lateral neuron (LNd), and lateral posterior neuron (LPN) groups. Dorsal neurons are located in the rim of dorsal brain regions and are further subdivided into anterior dorsal neuron 1 (DN1a), posterior dorsal neuron 1 (DN1p), dorsal neuron 2 (DN2), and dorsal neuron 3 (DN3) groups.

The *disconnected* (*disco*) gene, which encodes a C_2H_2-type zinc-finger transcription factor, plays a role in nervous system development. *disco* mutants lack many optic lobe neurons, including *per*-expressing lateral neurons, and are behaviorally arrhythmic (Dushay et al. 1989; Zerr et al. 1990; Hardin et al. 1992). Since the DN

groups are intact in *disco* mutants, researchers assume that lateral neurons are the central pacemaker neurons that control activity rhythms such as locomotion and eclosion. Within the lateral neuron groups, s-LNv and l-LNv neurons express a neuropeptide, pigment-dispersing factor (PDF) (Helfrich-Förster 1995). Only a few *disco* mutants retain some of the s-LNv neurons, and they are behaviorally rhythmic (Helfrich-Förster 1998), which further suggests that s-LNv neurons are the essential pacemakers.

The discovery of the *Pdf* mutant has also significantly advanced the functional analysis of s-LNv neurons. Wild-type *Drosophila* shows bimodal locomotor activity rhythms with two peaks in the morning and evening in light-dark cycles (LD) and the rhythms free run with a period of approximately 24 h in constant darkness (DD) (Konopka and Benzer 1971; Hamblen-Coyle et al. 1992). *Pdf* mutants display weak free-running activity rhythms with a period of approximately 22 h in DD for the first few days, and then the rhythms damp out (Renn et al. 1999). These phenotypes are attributed to the loss of PDF in s-LNv neurons, since *Pdf* knockdown only in s-LNv neurons (but not in l-LNv neurons) reproduces the weak activity rhythm of *Pdf* mutants (Shafer and Taghert 2009). This s-LNv master pacemaker hypothesis is further supported by the fact that *per* expression only in s-LNv neurons is sufficient for generating free-running activity rhythms in DD (Grima et al. 2004). Taken together, s-LNv neurons are the most influential clock neurons, and the PDF signaling output from s-LNv neurons conveys important circadian timing information to downstream neurons.

5.3 Outputs from the *Drosophila* Clock Neurons

Fourteen neurotransmitters, including PDF, have been identified in *Drosophila* cerebral clock neurons. Although studies on their functional roles are still in progress, those reported are listed in Table 5.1. Among them, nine neurotransmitters have been reported to be involved in intercellular communication between clock neurons (Fig. 5.2). Here, we summarize what we know about circadian outputs from clock neurons.

5.3.1 Pigment-Dispersing Factor

PDF was found to be the first circadian neurotransmitter (Helfrich-Förster 1995). In 2005, three independent groups identified the PDF receptor (PDFR) gene (Hyun et al. 2005; Mertens et al. 2005; Lear et al. 2005). Interestingly, PDFR is expressed in many clock neurons, including PDF-positive s-LNv neurons, PDF-negative LNd, and other DN groups (Im and Taghert 2010). The function of PDF/PDFR signaling is to synchronize PDF-positive and PDFR-positive clock neurons to adjust the phase of molecular oscillations and Ca^{2+} rhythms in the clocks (Peng et al. 2003; Lin et al.

Table 5.1 Circadian neurotransmitters

Neurotransmitter	Clock neuron group	Effect
PDF	s-LNv, l-LNv	Free-running rhythm Morning activity Evening activity
NPF	LNd, l-LNv	Evening activity Sleep Gene expression in the fat body
sNPF	s-LNv, LNd	Morning activity Emergence rhythm
ITP	5th LN, LNd	Free-running rhythm Sleep
DH31	DN1p, LPN	Sleep Temperature preference rhythm Free-running rhythm
CCHa1	DN1a	Morning activity Evening activity Activity level Sleep
AstA	LPN	Sleep Feeding
AstC	LNd, DN1p, DN3, LPN	Evening activity Oogenesis rhythm
CNMa	DN1p	Sleep
Trissin	LNd	Unknown
IPNa	DN1a	Unknown
Glutamate	DN1a, DN1p, DN3 Fifth LN?, LNd?	Free-running rhythm
Acetylcholine	5th LN, LNd	Free-running rhythm
Glycine	s-LNv?, l-LNv?	Free-running rhythm

2004; Shafer et al. 2008; Yoshii et al. 2009; Liang et al. 2016, 2017; Fig. 5.2a). The mammalian counterpart for PDF/PDFR signaling is vasoactive intestinal polypeptide (VIP)/VIP receptor signaling, which also functions to couple clock neurons in the SCN (Mieda 2020; Ono et al. 2021). Both PDFR and VIP receptors belong to a class II G-protein-coupled receptor family (Mertens et al. 2005), implying an evolutionarily conserved neural mechanism in a wide range of animal species.

PDF is expressed only in s-LNv and l-LNv neurons (Helfrich-Förster 1995). s-LNv and l-LNv neurons are morphologically different, although both groups are located very close to each other in the lateral brain (Helfrich-Förster 1997). s-LNv neurons have smaller cell bodies and send projections into the dorsal brain, where fifth LN, LNd, and DN1 neurons also send their projections, presumably to communicate with each other. l-LNv neurons have larger cell bodies and send the projections in two directions: one goes to the optic lobe with complex arborizations and the other to the contralateral hemisphere. Both s-LNv and l-LNv neurons also send fibers into the accessory medulla (Helfrich-Förster et al. 2007). The role of PDF

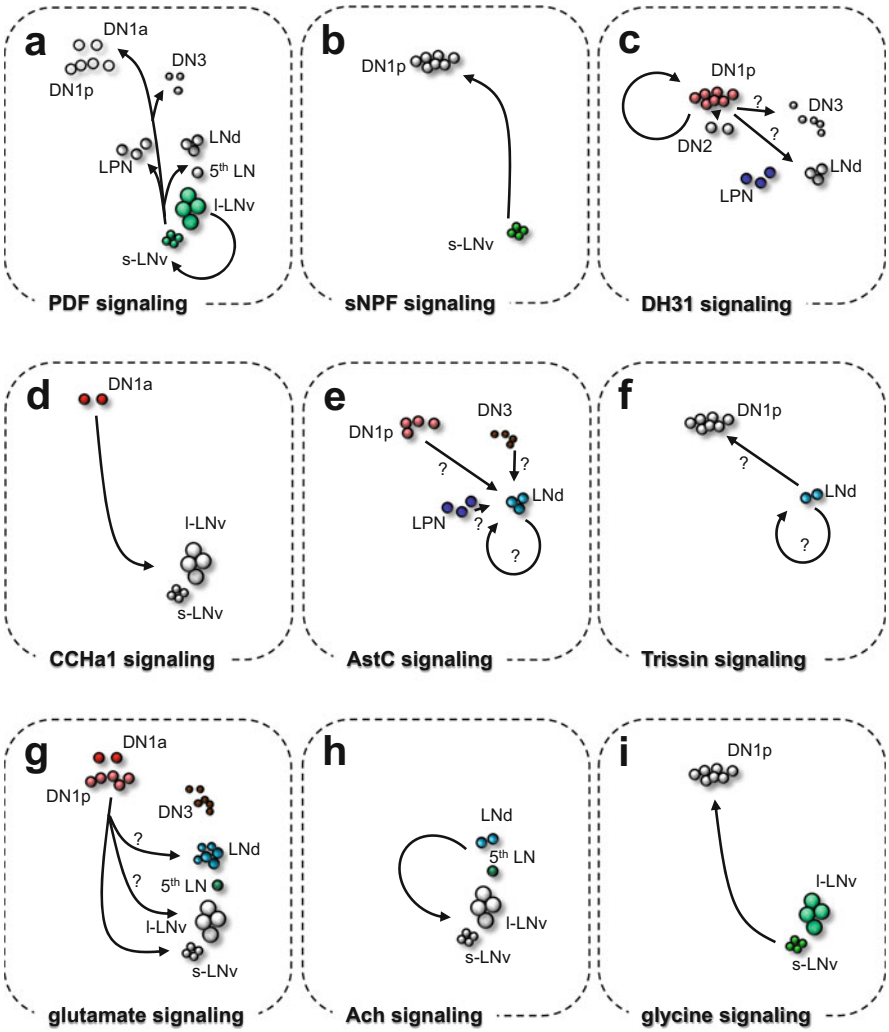

Fig. 5.2 Intercellular communication pathways of the *Drosophila* circadian clock network. The colored clock neurons contain neurotransmitters indicated in each panel and transmit signals to other (or own) clock neurons (arrows). The colors correspond to those used in Fig. 5.1. Since the distributions of the receptors for some ligands are not identified, some correspondences between output and input neurons are uncertain

signaling from s-LNv neurons is to synchronize s-LNv neurons and other PDF receptor-positive clock neurons, such as fifth LN, LNd, and DN1 neurons, which is supposed to be important for the maintenance of free-running rhythms in DD (Peng et al. 2003; Shafer et al. 2008; Yoshii et al. 2009). The projection pattern of l-LNv neurons implies that l-LNv neurons send a signal into the visual processing neurons in the optic lobe and into the contralateral brain for bilateral clock

synchronization (Helfrich-Förster et al. 2007). The l-LNv neurons indeed receive a signal from contralateral l-LNv neurons to change membrane potential but via gap junctions, not via PDF (Cao and Nitabach 2008).

It has been inferred that l-LNv neurons are less important in free-running conditions. This is because clock protein oscillations are dampened in l-LNv neurons under DD (Yang and Sehgal 2001) and *Pdf* knockdown in l-LNv neurons does not affect free-running rhythms in DD (Shafer and Taghert 2009). Pdf^{01} mutants display diminished morning activity and phase-advanced evening activity in LD. The rescue of PDF expression in l-LNv neurons restores the wild-type evening activity (Cusumano et al. 2009; Schlichting et al. 2016; Menegazzi et al. 2017; Schlichting et al. 2019b), suggesting that the role of PDF in l-LNv neurons is to set the phase of evening activity in LD. In contrast, PDF signaling from s-LNv neurons is essential for morning activity (Shafer and Taghert 2009). *pdfr* mutants also display phase-advanced evening activity (Hyun et al. 2005). The rescue of *pdfr* expression in DN1p or LNd and fifth LN neurons under the *pdfr* mutant background is sufficient for the wild-type morning and evening activity (Lear et al. 2009; Zhang et al. 2010a; Schlichting et al. 2016). According to the latest study by Schlichting et al. (2016), l-LNv neurons send PDF signaling to s-LNv neurons (PDF receptor-positive), and then s-LNv neurons pass it on to DN1p, LNd, and fifth LN neurons, which in turn generate wild-type evening activity. Since PDF is the first neuropeptide for the *Drosophila* circadian clock, it has been extensively studied. The functional analysis of PDF led directly to the analysis of s-LNv and l-LNv neurons, revealing the complexity of the circadian neural network.

5.3.2 Neuropeptides

To date, 11 neuropeptides expressed in clock neurons have been identified in *Drosophila*. Johard et al. (2009) found that three neuropeptides, neuropeptide F (NPF), short-neuropeptide F (sNPF), and ion transport peptide (ITP), were expressed in subsets of clock neurons. *Drosophila* shows sleeplike behavior that is typically characterized as periods of quiescence lasting longer than 5 min (Hendricks et al. 2000; Shaw et al. 2000). NPF-positive clock neurons modulate evening activity and sleep behavior (Hermann et al. 2012; Chung et al. 2017). In addition, NPF signaling from LNd neurons entrains rhythmic gene expression in fat bodies, which are comparable organs to the mammalian liver (Chung et al. 2017).

sNPF and NPF are similar in name, but they are encoded by different genes. Immunohistochemistry using antibodies against the sNPF precursor revealed that it is expressed in s-LNv neurons and two of six LNd neurons (Johard et al. 2009). sNPF signaling from s-LNv neurons negatively affects the Ca^{2+} level in DN1 neurons, which is correlated with the morning activity peak (Liang et al. 2017; Fig. 5.2b). sNPF also mediates circadian signaling from s-LNv neurons to prothoracicotropic hormone-expressing neurons, which control circadian emergence rhythms (Selcho et al. 2017).

One LNd and fifth LN neurons express the ITP neuropeptide (Johard et al. 2009). The ITP-positive LNd neuron is one of the three NPF-positive LNd neurons. Knockdown of *itp* expression in clock neurons reduces the evening activity peak in LD and lengthens the free-running period in DD (Hermann-Luibl et al. 2014). Furthermore, simultaneous knockdown of *itp* and *Pdf* increases the level of night activity in LD and makes arrhythmic. Taken together, ITP plays a role in the output of clock neurons, especially in relation to PDF signaling.

The neuropeptide diuretic hormone 31 (DH31) is expressed in DN1p and LPN neurons (Kunst et al. 2014; Reinhard et al. 2022). A loss-of-function allele of the *DH31* gene increases sleep late at night but shows normal circadian rhythms. However, DH31 does contribute to circadian activity rhythms. The double mutants of *Pdf* and *DH31* are nearly arrhythmic in DD (Goda et al. 2019), suggesting that DH31 plays a role in maintaining rhythms in the absence of PDF. DH31 receptor (DH31R) is expressed in DN1p neurons, and thus DH31 signaling from DN1p neurons may feedback on themselves (Goda et al. 2018; Fig. 5.2c). Flies change their preferred temperature over the course of a day, showing a so-called temperature preference rhythm with a peak in the evening (Kaneko et al. 2012). The DH31-PDFR signaling pathway in DN2 neurons plays a role in the temperature preference rhythm (Goda et al. 2016).

The neuropeptide CCHamide1 (CCHa1) is expressed in DN1a neurons (Fujiwara et al. 2018). The CCHa1 receptor (CCHa1R) is expressed in LNv neurons (Abruzzi et al. 2017; Fujiwara et al. 2018). Since PDFR is expressed in DN1a neurons and s-LNv neurons send projections to the dorsal brain in close proximity to DN1a neurons, DN1a and s-LNv neurons are reciprocally coupled via CCHa1 and PDF signaling (Fig. 5.2a, d). Mutant flies of *CCHa1* display diminished morning activity, reduced total activity, and enhanced sleep amount (Fujiwara et al. 2018). The mammalian homolog of CCHa1R is the receptor of gastrin-releasing peptide, which plays a role in the clock neuron network (Mieda 2020; Ono et al. 2021). Thus, similar to PDFR, the receptor (but not the ligand) is well conserved across animal species.

Allatostatin A (AstA) is a neuropeptide expressed in LPN neurons (Ni et al. 2019). Activation of LPN neurons promotes sleep and reduces feeding, and at least the sleep phenotype is partly mediated by AstA signaling (Chen et al. 2016; Ni et al. 2019; Reinhard et al. 2022). Since LPN neurons receive PDF signaling, LPN neurons are downstream of LNv neurons, and AstA signaling from LPN neurons may mediate coupling between LNv clock neurons and sleep-promoting neurons.

Allatostatin C (AstC) neuropeptide expression in clock neurons was first discovered by RNA-sequencing analysis (Abruzzi et al. 2017). Immunohistochemistry using an anti-AstC antibody further revealed that AstC is expressed in four to six DN1p, a subset of LNd, a subset of DN3, and LPN neurons (Díaz et al. 2019; Zhang et al. 2021a; Reinhard et al. 2022; Meiselman et al. 2022). Knockdown of *AstC* mRNA in clock neurons results in a phase-delayed evening activity, which is mediated by AstC-R2 (one of two AstC receptors) expressed in LNd neurons (Díaz et al. 2019). AstC is also involved in circadian rhythms in the progression of oogenesis in mated females (Allemand 1976). AstC signaling from DN1p neurons

outputs to the pars intercerebralis (PI) region, through which circadian oogenesis rhythms are generated (Zhang et al. 2021a, 2021b). Thus, AstC signaling is used in two directions. One is a signal to LNd neurons to communicate between clock neurons (Fig. 5.2e), and the other is an output of temporal information to downstream cells.

Transcriptome analyses in clock neurons reveal that two novel neuropeptides, CNMamide (CNMa) and Trissin, are expressed in DN1p and LNd neurons, respectively (Abruzzi et al. 2017; Ma et al. 2021). DN1p neurons input temperature information and modulate sleep in a temperature-dependent manner (Yadlapalli et al. 2018). CNMa signaling mediates the interaction between DN1p and PI neurons to control temperature-dependent sleep (Jin et al. 2021). In contrast, the function of Trissin has not yet been reported. Transcriptome analysis by Abruzzi et al. (2017) revealed that the receptor of Trissin is expressed in LNd and DN1 neurons, suggesting that Trissin mediates LNd-LNd and LNd-DN1 couplings (Fig. 5.2f).

IPNamide was discovered as the second circadian neuropeptide after PDF (Shafer et al. 2006). IPNamide is expressed in DN1a neurons, but its function has not been reported. IPNamide is encoded by the *neuropeptide-like precursor 1* gene (*Nplp1*), which encodes three other peptides, MTYamide, APK, and VQQ (Baggerman et al. 2002). Thus, it is difficult to analyze the function of IPNamide alone.

5.3.3 Glutamate, Acetylcholine, and Glycine

Vesicular glutamate transporter (VGlut) is expressed in DN1a, some DN1p, and DN3 neurons, suggesting that these clock neurons use glutamate as a neurotransmitter (Hamasaka et al. 2007). Glutamate signaling is mediated by two main receptors, a glutamate-gated chloride channel, GluCl, and a metabotropic G-protein-coupled receptor, mGluRA. The receptors are expressed in s-LNv, l-LNv, and LNd neurons (Hamasaka et al. 2007; Collins et al. 2012; Guo et al. 2016). Glutamate signaling from DN1p neurons synchronizes DN1p and LNv neurons (Collins et al. 2012; Guo et al. 2016; Fig. 5.2g). Since s-LNv neurons send PDF signaling to DN1p neurons (Shafer et al. 2008), there is reciprocal coupling between s-LNv and DN1p neurons. This coupling is essential for the robustness of molecular oscillations and normal activity rhythms (Hamasaka et al. 2007; Collins et al. 2014). *VGlut* may also be expressed in LNd and/or fifth LN neurons, as RNA interference for *VGlut* in LNd and fifth LN neurons influences activity rhythms (Duhart et al. 2020; Fig. 5.2g).

Johard et al. (2009) also reported that acetylcholine (Ach) is used as the circadian neurotransmitter, as choline acetyltransferase (ChAT) is expressed in two of six LNd and fifth LN neurons. ChAT is coexpressed with sNPF in the two LNd neurons, which is different from the three NPF-positive LNd neurons. Thus, six LNd neurons are divided into two sNPF- and ChAT-coexpressing neurons, two NPF-positive neurons, one ITP- and NPF-coexpressing neuron, and one with an unknown neurotransmitter. Cholinergic signaling, which is an excitatory input, from LNd neurons targets s-LNv and l-LNv neurons because its receptor is expressed (McCarthy et al.

2011; Lelito and Shafer 2012; Fig. 5.2h). Knockdowns of *ChAT* or *vesicular acetylcholine transporter* (*vAchT*) in LNd neurons do not change the speed of free-running activity rhythms in DD but reduce the robustness of the rhythms (Duhart et al. 2020).

The other fast neurotransmitter used in the *Drosophila* clock is glycine (Frenkel et al. 2017). Knockdown of the glycine transporter *dGlyT* in LNv (s-LNv and l-LNv) neurons results in lengthening of the free-running period in DD. Glycine applications on the cultured brain inhibit the neural activity of DN1p neurons. These results suggest that glycine is used in LNv neurons as a neurotransmitter and that its receptor is expressed in DN1p neurons. Since s-LNv neurons are responsible for the speed of the free-running period, one can assume that glycine mediates the neurotransmission from s-LNv neurons to DN1p neurons (Fig. 5.2i).

5.3.4 Gap Junctions

Most studies of the neural network of the *Drosophila* circadian clock are concerned with chemical synapses. Perhaps this is because genetic screening targeting ligands or their receptors is an advantage of *Drosophila* research. However, insect clock neurons are known to couple at electrical synapses as well (Schneider and Stengl 2006; Li et al. 2018). In the case of *Drosophila*, the electrical synapse is composed of gap junctions by eight innexin proteins (innexins 1–8). Knockdown of *Innexin1* and *Innexin2* expression in clock neurons results in longer free-running periods than control strains in DD (Ramakrishnan and Sheeba 2021). Additionally, knockdown of *Innexin2* expression leads to a phase shift of PER oscillation and reduces PDF expression in the morning. These results suggest that gap junction-mediated signaling between clock neurons is important for maintaining circadian molecular oscillations.

5.3.5 Output Modes

Clarifying when and how circadian neurotransmitters are released is still challenging, although some recent advances may have developed experimental methods to approach this long-lasting problem (Leopold et al. 2019; Ding et al. 2019). PDF immunostaining reveals that the PDF level in the terminals of the s-LNv projections cycles with a peak in the morning (Park et al. 2000). Similar observations have been made on ITP, CCHa1, DH31, AstA, and AstC neuropeptides, but the phases of their rhythms are different (Hermann-Luibl et al. 2014; Fujiwara et al. 2018; Díaz et al. 2019; Reinhard et al. 2022). The cycling of the neuropeptide contents may not reflect their synaptic release directly, but it implies that they are released in a circadian manner. A study using a fluorescent sensor for visualizing neuropeptide release has revealed that s-LNv neurons release neuropeptides in the morning with a slight delay

from the peak of PDF level at their axonal terminals (Klose et al. 2021). Thus, it is very likely that other circadian neuropeptides are also rhythmically released, by which timing information is transmitted to postsynaptic downstream neurons. However, the importance of rhythmic PDF release to activity rhythms is still controversial (Kula et al. 2006; Prakash et al. 2017).

The cyclic chemical transmissions can be complexly organized by the circadian structure remodeling of clock neurons. Fernández et al. (2008) found that the axonal terminals of s-LNv neurons change morphology, higher complexity during the day and lower complexity during the night, and the daily morphological changes are regulated by the molecular clock. Through rhythmic structure remodeling, s-LNv neurons change synaptic partners throughout the day (Gorostiza et al. 2014). A recent study proposed that s-LNv circadian remodeling is important for integrating light and temperature inputs (Fernandez et al. 2020). Similar morphological changes have been reported in fifth LN, LNd, and DN1a neurons (Duhart et al. 2020; Song et al. 2021). Thus, the circadian remodeling of axonal terminals may be a general property of clock neurons.

5.4 Functional Differentiation of Individual Clock Neuron Groups in *Drosophila*

5.4.1 Morning and Evening Oscillators in the Drosophila *Circadian Clock*

Drosophila shows two distinct activity peaks in the morning and evening (Helfrich-Förster 2000), and the two activity peaks are controlled by two oscillators with different properties (Yoshii et al. 2012). Grima et al. (2004) and Stoleru et al. (2004) proposed that the two oscillators are separate groups of clock neurons: the morning oscillator (M oscillator) corresponds to s-LNv neurons and the evening oscillator (E oscillator) to LNd neurons. Later, the fifth LN neuron was identified (Rieger et al. 2006) and considered the evening oscillator. The M and E oscillators have different response modes to light. An exposure of dim light, which is equivalent to a light intensity of a quarter moon, in the night phase results in a phase advance of the M peak and a phase delay of the E peak (Bachleitner et al. 2007). This result fits well with the classical two-oscillator model proposed from studies performed in rodents (Pittendrigh and Daan 1976). In this model, the M oscillator accelerates, and the E oscillator decelerates the speed of oscillations upon light exposure. By changing the speed of oscillation depending on light exposure, both oscillators can flexibly adapt to different photoperiods, by which the circadian clock enables the measurement of day length to predict the coming season.

The *Drosophila* two-oscillator model has certainly inspired the functional analysis of clock neurons. Many studies support this model, but on the other hand, some studies note that it is oversimplified. For example, per^{0} mutants with a *per* rescue

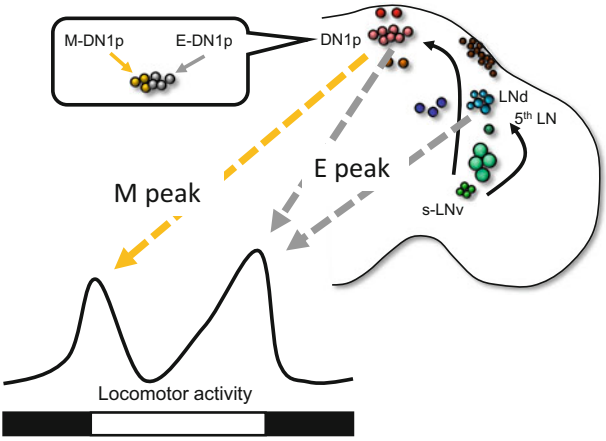

Fig. 5.3 Current model for generating morning (M) and evening (E) activity peaks in *Drosophila*. *Drosophila* shows two activity peaks in the morning and evening under LD. In principle, s-LNv neurons and fifth LN and LNd neurons are designated as the M oscillator and E oscillator, respectively. DN1p neurons contain the M (M-DN1p) and E oscillators (E-DN1p) and mediate the output from s-LNv neurons

expression only in a subset of DN1p neurons display relatively normal morning and evening activity (Zhang et al. 2010b). Additionally, flies without s-LNv neurons still display a clear morning activity peak (Sheeba et al. 2010). per^0 mutants with a *per* rescue only in M or E oscillator do not completely restore typical morning and evening activity under various photoperiods (Menegazzi et al. 2020). On the other hand, CRISPR-mediated *per* or *tim* gene knockout only in M oscillator causes loss of the morning activity peak (Delventhal et al. 2019). These seemingly contradictory results are because it does not take into account the complex neural network between clock neurons (Jaumouillé et al. 2021). Since LNv and DN1 neurons interact intricately with each other through multiple neurotransmitters, it may be challenging to analyze functions only by manipulating specific clock neuron groups (Yao and Shafer 2014; Yao et al. 2016). Figure 5.3 shows a current M-E two-oscillator model that highlights the importance of the DN1p group for generating both M and E peaks (Chatterjee et al. 2018). In this model, two types of DN1p neurons (M-DN1p and E-DN1p neurons) control the M and E peaks. M-DN1p neurons receive a signal from the M oscillator (s-LNv neurons) and control M peak. E-DN1p neurons and E oscillators (fifth LN and LNd neurons) control the E peak, but they are concurrently influenced by the M oscillator.

5.4.2 s-LNv Neurons as the Master Clock?

s-LNv neurons have been considered to be the master pacemaker clock. This hypothesis is based on the following facts: (1) the stability of the free-running

rhythm in DD is significantly weakened by the loss of PDF or PDF-positive LNv neurons, and (2) the free-running rhythm is restored by *per* rescue only in s-LNv neurons (Helfrich-Förster 1998; Renn et al. 1999; Grima et al. 2004; Cusumano et al. 2009). However, several lines of evidence point to different ideas. For example, per^{0} mutant flies with a *per* rescue only in the E oscillator (fifth LN and LNd neurons) can display a free-running rhythm under constant dim light conditions (Rieger et al. 2009). Disruption of the molecular clock only in s-LNv neurons is insufficient to render flies arrhythmic in DD (Delventhal et al. 2019; Schlichting et al. 2019a, 2019b; Jaumouillé et al. 2021). Furthermore, silencing of neural activity in M or E oscillators, even with normal molecular oscillations, also renders fly arrhythmic (Bulthuis et al. 2019), which suggests that the disconnection of intercellular communication between clock neurons causes the loss of the free-running rhythm. Thus, s-LNv neurons remain essential for self-sustained DD rhythms, but the importance of other clock neurons has been overlooked.

5.4.3 DN1p Neurons as Circadian Output Centers

DN1p neurons are composed of heterogeneous neurons. Six of 15 DN1p neurons express CRY and PDFR (Yoshii et al. 2008; Im and Taghert 2010), and they use AstC, DH31, glutamate, and CNMa as neurotransmitters (Kunst et al. 2014; Chatterjee et al. 2018; Ma et al. 2021). CRY-positive DN1p neurons are involved in sexual interactions (Fujii and Amrein 2010; Hanafusa et al. 2013), feeding (Barber et al. 2016), sleep regulation (Guo et al. 2016, 2017; Lamaze et al. 2018), memory extinction (Zhang et al. 2021b), activity rhythms (Nettnin et al. 2021), and reproductive rhythms (Zhang et al. 2021a). These reports suggest that CRY-positive DN1p neurons may be the output center of the circadian clock, which transmits timing information to downstream neurons to generate various behavioral rhythms. This may be why DN1p neurons have many different neurotransmitters.

5.5 Downstream Neurons of the *Drosophila* Circadian Clock

PI neurons have been considered the circadian output region for many years (Nishiitsutsuji-Uwo et al. 1967; Cymborowski 1973; Sokolove and Loher 1975; Takekata et al. 2018). In *Drosophila*, there are three distinct populations of PI neurons that express three different peptides: diuretic hormone 44 (DH44), SIFamide (SIFa), and *Drosophila* insulin-like peptide (dilp2). DN1p and LNd neurons directly or indirectly contact PI neurons (Cavanaugh et al. 2014; Barber et al. 2016, 2021). DH44-positive and SIFa-positive PI neurons mediate the output pathways to control circadian activity rhythms, whereas SIFa-positive and

dilp2-positive PI neurons control feeding rhythms and metabolism (Cavanaugh et al. 2014; Barber et al. 2016, 2021; Dreyer et al. 2019). Some DN1p neurons also contact tubercular-bulbar neurons that, in turn, connect ellipsoid body ring neurons (Guo et al. 2018; Lamaze et al. 2018), which include those that promote sleep and those involved in the output of activity rhythms (Liang et al. 2019).

DN1p neurons are not the only ones coupled to output pathways. As mentioned above, s-LNv neurons communicate with PTTH neurons via sNPF signaling (Selcho et al. 2017). PDF (and sNFP) signaling from s-LNv neurons plays roles in reproductive dormancy mediated by dilp2-positive PI neurons (Nagy et al. 2019) and memory mediated by the mushroom body (Flyer-Adams et al. 2020; Inami et al. 2021). The other output pathway from s-LNv neurons is the neuropeptide leucokinin (LK)-positive neurons (Cavey et al. 2016). Both *Lk* and *Lk receptor* mutants reduce the power of activity rhythms in DD. All the output pathways mentioned above have been analyzed morphologically, physiologically, and behaviorally. Recent electron microscopic data further revealed entire postsynaptic neurons of all clock neurons (Scheffer et al. 2020), showing that the output of the circadian clock spreads across a wide range of brain neurons.

5.6 Clock Neuron Networks in Other Insect Species

In insect species other than *Drosophila melanogaster*, immunostaining against PDF has provided the most reliable results for identifying putative clock neurons. This is because PDF antibodies are specific to many species due to the high conservation of PDF peptide sequences (Meelkop et al. 2011). Similar to all insect species studied, PDF cells reside in the lateral protocerebrum, and they extend neuronal processes toward the central brain and optic lobes (Helfrich-Förster 2005). In the cockroach *R. maderae*, ectopic transplantation of the accessory medulla, including PDF neurons, can restore activity rhythms in optic lobeless arrhythmic cockroaches, strongly suggesting the importance of PDF neurons (Reischig and Stengl 2003). Injections of synthetic PDF peptides into the brain phase shift activity rhythms in the cockroach (Petri and Stengl 1997) and cricket (Singaravel et al. 2003). The knockdown of *Pdf* mRNA expression by RNA interference or the knockout of the *Pdf* gene by CRISPR/Cas9 results in arrhythmicity or a short free-running period in the German cockroach (Lee et al. 2009), cricket (Hassaneen et al. 2011), and bug (Kotwica-Rolinska et al. 2022). Furthermore, circadian rhythms at the PDF level and the structural changes of the projections from PDF neurons have also been detected (Abdelsalam et al. 2008; Wei and Stengl 2011). Putting all these results together, it is likely that, as in *Drosophila*, PDF and PDF neurons are essential for circadian activity rhythms in insects. However, things are not so simple.

While the identification of PER-expressing cells in the brain has been attempted in several insect species, the locations of the PER-expressing cells are often different from those of *Drosophila* (Table 5.2; Helfrich-Förster 2005; Beer and Helfrich-Förster 2020). PER is not colocalized in PDF neurons in some insect species (Frisch

Table 5.2 List of studies investigating insect clock neurons

Reference	Species	Labeling method
Frisch et al. (1996)	Coleoptera (*Pachymorpha sexguttata*)	PER, PDF immunostaining
Sauman and Reppert (1996)	Lepidoptera (*Antheraea pernyi*)	PER immunostaining *per* in situ hybridization
Wise et al. (2002)	Lepidoptera (*Manduca sexta*)	PER immunostaining *per* in situ hybridization
Lupien et al. (2003)	Orthoptera (*Teleogryllus commodus*) Orthoptera (*Teleogryllus oceanicus*)	PER, PDF immunostaining
Bloch et al. (2003)	Hymenoptera (*Apis mellifera*)	PER, PDF immunostaining
Závodská et al. (2003a)	Thysanura (*Thermobia domestica*)	PER immunostaining
Závodská et al. (2003b)	Archaeognatha (*Lepismachilis y-signata*) Odonata (*Ischnura elegans*) Ephemeroptera (*Siphlonurus armatus*) Plecoptera (*Perla burmeisteriana*) Orthoptera (*Locusta migratoria*) Orthoptera (*Schistocerca gregaria*) Hemiptera (*Aquarius paludum*) Hemiptera (*Notonecta glauca*) Hymenoptera (*Apis mellifera*) Coleoptera (*Pachnoda marginata*) Diptera (*Neobellaria bullata*) Diptera (*Phormia regina*) Trichoptera (*Hydropsyche contubernalis*)	PER immunostaining
Sehadová et al. (2004)	Lepidoptera (*Bombyx mori*)	PER, CRY, CYC, DBT immunostaining
Sauman et al. (2005)	Lepidoptera (*Danaus plexippus*)	PER, CRY, TIM immunostaining *per*, *cry* in situ hybridization
Závodská et al. (2005)	Blattodea (*Periplaneta americana*)	PER immunostaining
Shao et al. (2006)	Orthoptera (*Dianemobius nigrofasciatus*) Orthoptera (*Allonemobius allardi*)	PER, CRY, DBT immunostaining
Shao et al. (2008a)	Orthoptera (*Dianemobius nigrofasciatus*)	CLK, CYC immunostaining
Shao et al. (2008b)	Orthoptera (*Allonemobius allardi*)	CLK, CYC immunostaining
Zhu et al. (2008)	Lepidoptera (*Danaus plexippus*)	TIM, CRY1, CRY2 immunostaining *cry2* in situ hybridization
Shiga and Numata (2009)	Diptera (*Protophormia terraenovae*)	PER, PDF immunostaining
Wen and Lee (2008)		PER, PDF immunostaining

(continued)

Table 5.2 (continued)

Reference	Species	Labeling method
	Blattodea (*Blattella germanica*) Blattodea (*Blattella bisignata*)	
Vafopoulou et al. (2010)	Hemiptera (*Rhodnius prolixus*)	PER, TIM, PDF immunostaining
Mohamed et al. (2014)	Lepidoptera (*Antheraea pernyi*)	PER, CLK, CYC immunostaining
Kobelková et al. (2015)	Lepidoptera (*Ephestia kuehniella*)	PER immunostaining *per*, *tim* in situ hybridization
Barberà et al. (2017)	Hemiptera (*Acyrthosiphon pisum*)	*per*, *tim* in situ hybridization
Fuchikawa et al. (2017)	Hymenoptera (*Apis mellifera*)	PER, PDF immunostaining
Beer et al. (2018)	Hymenoptera (*Apis mellifera*)	PER, PDF immunostaining
Kay et al. (2018)	Hymenoptera (*Camponotus floridanus*)	PER, PDF immunostaining
Kutaragi et al. (2018)	Orthoptera (*Gryllus bimaculatus*)	*per*, *cry2* in situ hybridization
Colizzi et al. (2021)	Hemiptera (*Acyrthosiphon pisum*)	PER, CRY1, PDF immunostaining
Koide et al. (2021)	Hemiptera (*Riptortus pedestris*)	PER, PDF immunostaining
Barberà et al. (2022)	Hemiptera (*Acyrthosiphon pisum*)	*cry1*, *cry2* in situ hybridization

et al. 1996; Sauman and Reppert 1996; Závodská et al. 2003b; Koide et al. 2021). Furthermore, in the cricket, the surgical lesion of the outer medulla and lamina neuropils results in arrhythmicity even though the accessory medulla, including PDF neurons, is still intact (Okamoto et al. 2001). Therefore, we have to be cautious in simply concluding PDF neurons as clock neurons. It is quite possible that there is a diversity of clock neuron networks, along with the diversity of insect species. Even within the genus *Drosophila*, there are some variations in PDF/clock protein expression patterns (Hermann et al. 2013; Menegazzi et al. 2017). In contrast, several studies have shown clock neuron networks similar to those of *Drosophila* in the blowfly *Protophormia terraenovae* (Shiga and Numata 2009), the honeybee *Apis mellifera* (Fuchikawa et al. 2017; Beer et al. 2018), the ant *Camponotus floridanus* (Kay et al. 2018; Fig. 5.4a), and the aphid *Acyrthosiphon pisum* (Barberà et al. 2017; Colizzi et al. 2021; Fig. 5.4b). In addition, subsets of PER-positive neurons in the blowfly, ant, and honeybee also exhibit PDF, although this is not true in the aphid because it seems that the *Pdf* gene is lost in the aphid genome (Huybrechts et al. 2010).

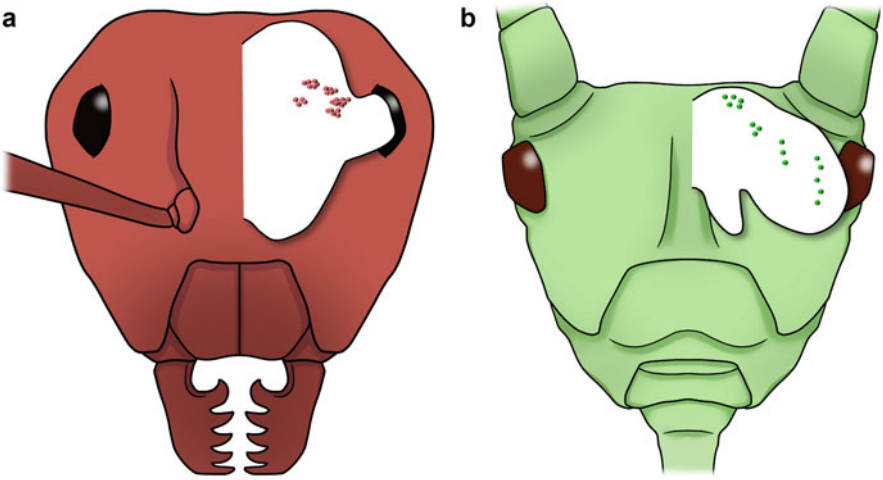

Fig. 5.4 The brains of the ant *Camponotus floridanus* (**a**) and aphid *Acyrthosiphon pisum* (**b**) and their clock neurons. In both insects, clock neurons form clusters similar to *Drosophila* lateral and dorsal neurons (Fig. 5.1). The ant clock neurons consist of approximately 200 neurons, whereas the aphid clock neurons consist of approximately 40 neurons

5.7 Bilateral Coupling Between Two Optic Lobe Clocks

Drosophila is not always used as a model of insect chronobiology. The coupling between two clocks residing in the left and right brain is such a subject of study. Even in advanced *Drosophila* genetics, it is not possible to manipulate one side of the body asymmetrically. The tiny brain of *Drosophila* also makes it difficult to manipulate the brain surgically. In contrast, robust insect species with larger brains, such as crickets and cockroaches, are good models.

Page and his colleagues demonstrated that excision of one optic lobe (either right or left) in the cockroach *R. maderae* did not affect the ability to generate free-running activity rhythms, but their periods were longer than those of intact animals (Page et al. 1977; Page 1978). They proposed that two clock components that reside in the left and right optic lobes were mutually coupled and each clock worked to shorten the period of the other clock. The cricket clocks are more intriguing because the coupling between the two optic lobe clocks seems weaker than that of cockroaches. If the optic nerve is unilaterally disconnected from the optic lobe, this optic lobe should be blind and free-run as if it is in DD, while the contralateral optic lobe should be entrained by light cycles unless the two optic lobes exchange the light information. In this situation, crickets display two rhythms simultaneously, a phenomenon called "splitting" (Wiedenmann 1983; Tomioka et al. 1991; Tomioka 1993). The two rhythms do not run completely independently; the free-running period is modulated by the coupling of two optic lobe clocks (Tomioka et al. 1991; Tomioka 1993). Figure 5.5 shows a model of the bilateral optic lobe clocks that interact with

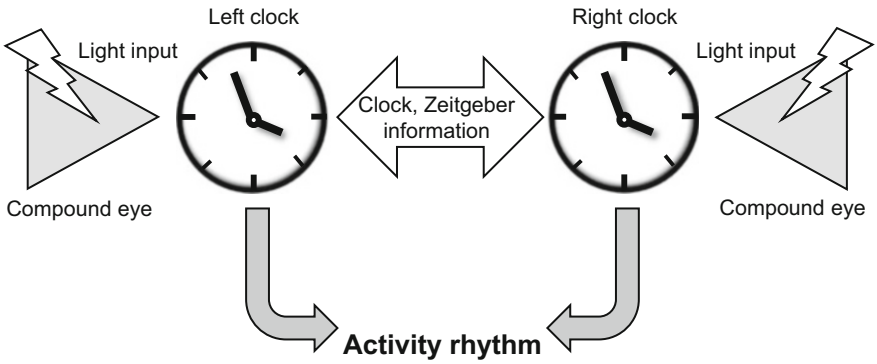

Fig. 5.5 A model of two clocks located in bilateral optic lobes in the cockroach and cricket. The left and right clocks independently receive light information from the compound eyes on each side. Although the two clocks can separately drive activity rhythms, they mutually interact to exchange time and zeitgeber information, enabling the generation of a coherent activity rhythm

each other to exchange zeitgeber and time information. PDF and serotonin are used in this bilateral coupling pathway (Saifullah and Tomioka 2002, 2003). In particular, PDF neurons form commissures projecting in the contralateral optic lobe (Helfrich-Förster 1997; Reischig et al. 2004), which is suitable for the coupling pathway, and its morphology is conserved across many insect species. It should also be mentioned that there are many more interneurons that bridge two sides of the optic lobes and possibly mediate the coupling (Yukizane and Tomioka 1995; Reischig and Stengl 2002).

A series of studies on the bilateral coupling of two optic lobe clocks have left the detailed mechanisms unknown (Page et al. 1977; Page 1978; Wiedenmann 1983; Tomioka et al. 1991; Tomioka 1993). However, these studies provide key points into insect circadian networks: (1) coupling may be needed for exchanging zeitgeber and time information, and (2) the strength of coupling may vary among species.

5.8 Conclusion Remarks

There is still not enough data to summarize the whole picture of insect clock networks. It would be important to try immunostainings with specific antibodies in many more insect species. Even for species that have already been studied previously, it would be significant to perform the latest fluorescent immunostaining with a confocal microscope and newly generated antibodies. In addition, neurotransmitters other than PDF have not yet been focused on nonmodel insects. In *Drosophila*, the first immunostaining against PER was performed in the late 1980s (Siwicki et al. 1988), but the presently known classification of clock neurons is based on studies conducted approximately in the year 2000. Surprisingly, more detailed and precise classification is still an ongoing subject (Schubert et al. 2018; Reinhard et al. 2022).

Thus, the study of clock neuron networks will continue to be an active area of insect chronobiology.

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References

Abdelsalam S, Uemura H, Umezaki Y, Saifullah ASM, Shimohigashi M, Tomioka K (2008) Characterization of PDF-immunoreactive neurons in the optic lobe and cerebral lobe of the cricket, *Gryllus bimaculatus*. J Insect Physiol 54:1205–1212. https://doi.org/10.1016/j.jinsphys.2008.05.001

Abruzzi KC, Zadina A, Luo W, Wiyanto E, Rahman R, Guo F et al (2017) RNA-seq analysis of *Drosophila* clock and non-clock neurons reveals neuron-specific cycling and novel candidate neuropeptides. PLoS Genet 13:e1006613. https://doi.org/10.1371/journal.pgen.1006613

Allemand R (1976) Influence de modifications des conditions lumineuses sur les rythmes circadiens de vitellogenese et d'ovulation chez *Drosophila melanogaster*. J Insect Physiol 22:1075–1080. https://doi.org/10.1016/0022-1910(76)90116-5

Bachleitner W, Kempinger L, Wülbeck C, Rieger D, Helfrich-Förster C (2007) Moonlight shifts the endogenous clock of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 104:3538–3543. https://doi.org/10.1073/pnas.0606870104

Baggerman G, Cerstiaens A, De Loof A, Schoofs L (2002) Peptidomics of the larval *Drosophila melanogaster* central nervous system. J Biol Chem 277:40368–40374. https://doi.org/10.1074/jbc.M206257200

Barber AF, Erion R, Holmes TC, Sehgal A (2016) Circadian and feeding cues integrate to drive rhythms of physiology in *Drosophila* insulin-producing cells. Genes Dev 30:2596–2606. https://doi.org/10.1101/gad.288258.116

Barber AF, Fong SY, Kolesnik A, Fetchko M, Sehgal A (2021) *Drosophila* clock cells use multiple mechanisms to transmit time-of-day signals in the brain. Proc Natl Acad Sci U S A 118:e2019826118. https://doi.org/10.1073/pnas.2019826118

Barberà M, Collantes-Alegre JM, Martínez-Torres D (2017) Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrthosiphon pisum*. Insect Biochem Mol Biol 83:54–67. https://doi.org/10.1016/j.ibmb.2017.02.006

Barberà M, Collantes-Alegre JM, Martínez-Torres D (2022) Mapping and quantification of cryptochrome expression in the brain of the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 31:159–169. https://doi.org/10.1111/imb.12747

Beer K, Helfrich-Förster C (2020) Model and non-model insects in chronobiology. Front Behav Neurosci 14:601676. https://doi.org/10.3389/fnbeh.2020.601676

Beer K, Kolbe E, Kahana NB, Yayon N, Weiss R, Menegazzi P et al (2018) Pigment-dispersing factor-expressing neurons convey circadian information in the honey bee brain. Open Biol 8:170224. https://doi.org/10.1098/rsob.170224

Bloch G, Solomon SM, Robinson GE, Fahrbach SE (2003) Patterns of PERIOD and pigment-dispersing hormone immunoreactivity in the brain of the European honeybee (*Apis mellifera*): age- and time-related plasticity. J Comp Neurol 464:269–284. https://doi.org/10.1002/cne.10778

Bulthuis N, Spontak KR, Kleeman B, Cavanaugh DJ (2019) Neuronal activity in non-LNv clock cells is required to produce free-running rest:activity rhythms in *Drosophila*. J Biol Rhythm 34:249–271. https://doi.org/10.1177/0748730419841468

Cao G, Nitabach MN (2008) Circadian control of membrane excitability in *Drosophila melanogaster* lateral ventral clock neurons. J Neurosci 28:6493–6501. https://doi.org/10.1523/JNEUROSCI.1503-08.2008

Cavanaugh DJ, Geratowski JD, Wooltorton JRA, Spaethling JM, Hector CE, Zheng X et al (2014) Identification of a circadian output circuit for rest:activity rhythms in *Drosophila*. Cell 157:689–701. https://doi.org/10.1016/j.cell.2014.02.024

Cavey M, Collins B, Bertet C, Blau J (2016) Circadian rhythms in neuronal activity propagate through output circuits. Nat Neurosci 19:587–595. https://doi.org/10.1038/nn.4263

Chatterjee A, Lamaze A, De J, Mena W, Chélot E, Martin B et al (2018) Reconfiguration of a multi-oscillator network by light in the *Drosophila* circadian clock. Curr Biol 28:2007–2017.e4. https://doi.org/10.1016/j.cub.2018.04.064

Chen J, Reiher W, Hermann-Luibl C, Sellami A, Cognigni P, Kondo S et al (2016) Allatostatin A signalling in *Drosophila* regulates feeding and sleep and is modulated by PDF. PLoS Genet 12:e1006346. https://doi.org/10.1371/journal.pgen.1006346

Chung BY, Ro J, Hutter SA, Miller KM, Guduguntla LS, Kondo S et al (2017) *Drosophila* neuropeptide F signaling independently regulates feeding and sleep-wake behavior. Cell Rep 19:2441–2450. https://doi.org/10.1016/j.celrep.2017.05.085

Colizzi FS, Beer K, Cuti P, Deppisch P, Martínez Torres D, Yoshii T et al (2021) Antibodies against the clock proteins Period and Cryptochrome reveal the neuronal organization of the circadian clock in the pea aphid. Front Physiol 12:705048. https://doi.org/10.3389/fphys.2021.705048

Collins B, Kane EA, Reeves DC, Akabas MH, Blau J (2012) Balance of activity between LN(v)s and glutamatergic dorsal clock neurons promotes robust circadian rhythms in *Drosophila*. Neuron 74:706–718. https://doi.org/10.1016/j.neuron.2012.02.034

Collins B, Kaplan HS, Cavey M, Lelito KR, Bahle AH, Zhu Z et al (2014) Differentially timed extracellular signals synchronize pacemaker neuron clocks. PLoS Biol 12:e1001959. https://doi.org/10.1371/journal.pbio.1001959

Colwell CS, Page TL (1990) A circadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. J Comp Physiol A 166:643–649. https://doi.org/10.1007/BF00240014

Cusumano P, Klarsfeld A, Chelot E, Picot M, Richier B, Rouyer F (2009) PDF-modulated visual inputs and cryptochrome define diurnal behavior in *Drosophila*. Nat Neurosci 12:1431–1437. https://doi.org/10.1038/nn.2429

Cymborowski B (1973) Control of the circadian rhythm of locomotor activity in the house cricket. J Insect Physiol 19:1423–1440. https://doi.org/10.1016/0022-1910(73)90173-X

Delventhal R, O'Connor RM, Pantalia MM, Ulgherait M, Kim HX, Basturk MK et al (2019) Dissection of central clock function in *Drosophila* through cell-specific CRISPR-mediated clock gene disruption. elife 8:e48308. https://doi.org/10.7554/eLife.48308

Díaz MM, Schlichting M, Abruzzi KC, Long X, Rosbash M (2019) Allatostatin-C/AstC-R2 is a novel pathway to modulate the circadian activity pattern in *Drosophila*. Curr Biol 29:13–22.e3. https://doi.org/10.1016/j.cub.2018.11.005

Ding K, Han Y, Seid TW, Buser C, Karigo T, Zhang S et al (2019) Imaging neuropeptide release at synapses with a genetically engineered reporter. elife 8:e46421. https://doi.org/10.7554/eLife.46421

Dreyer AP, Martin MM, Fulgham CV, Jabr DA, Bai L, Beshel J et al (2019) A circadian output center controlling feeding:fasting rhythms in *Drosophila*. PLoS Genet 15:e1008478. https://doi.org/10.1371/journal.pgen.1008478

Duhart JM, Herrero A, de la Cruz G, Ispizua JI, Pírez N, Ceriani MF (2020) Circadian structural plasticity drives remodeling of E cell output. Curr Biol 30:5040–5048.e5. https://doi.org/10.1016/j.cub.2020.09.057

Dushay MS, Rosbash M, Hall JC (1989) The *disconnected* visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. J Biol Rhythm 4:1–27. https://doi.org/10.1177/074873048900400101

Fernández MP, Berni J, Ceriani MF (2008) Circadian remodeling of neuronal circuits involved in rhythmic behavior. PLoS Biol 6:e69. https://doi.org/10.1371/journal.pbio.0060069

Fernandez MP, Pettibone HL, Bogart JT, Roell CJ, Davey CE, Pranevicius A et al (2020) Sites of circadian clock Neuron plasticity mediate sensory integration and entrainment. Curr Biol 30: 2225–2237.e5. https://doi.org/10.1016/j.cub.2020.04.025

Fleissner G (1982) Isolation of an insect circadian clock. J Comp Physiol 149:311–316. https://doi.org/10.1007/BF00619146

Flyer-Adams JG, Rivera-Rodriguez EJ, Yu J, Mardovin JD, Reed ML, Griffith LC (2020) Regulation of olfactory associative memory by the circadian clock output signal Pigment-Dispersing Factor (PDF). J Neurosci 40:9066–9077. https://doi.org/10.1523/JNEUROSCI.0782-20.2020

Frenkel L, Muraro NI, Beltrán González AN, Marcora MS, Bernabó G, Hermann-Luibl C et al (2017) Organization of circadian behavior relies on glycinergic transmission. Cell Rep 19:72–85. https://doi.org/10.1016/j.celrep.2017.03.034

Frisch B, Fleissner GG, Brandes C, Hall JC (1996) Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock-gene product: labeling of cells with possible importance for the beetle's circadian rhythms. Cell Tissue Res 286:411–429. https://doi.org/10.1007/s004410050711

Fuchikawa T, Beer K, Linke-Winnebeck C, Ben-David R, Kotowoy A, Tsang VWK et al (2017) Neuronal circadian clock protein oscillations are similar in behaviourally rhythmic forager honeybees and in arrhythmic nurses. Open Biol 7:170047. https://doi.org/10.1098/rsob.170047

Fujii S, Amrein H (2010) Ventral lateral and DN1 clock neurons mediate distinct properties of male sex drive rhythm in *Drosophila*. Proc Natl Acad Sci U S A 107:10590–10595. https://doi.org/10.1073/pnas.0912457107

Fujiwara Y, Hermann-Luibl C, Katsura M, Sekiguchi M, Ida T, Helfrich-Förster C et al (2018) The CCHamide1 neuropeptide expressed in the anterior dorsal neuron 1 conveys a circadian signal to the ventral lateral neurons in *Drosophila melanogaster*. Front Physiol 9:1276. https://doi.org/10.3389/fphys.2018.01276

Goda T, Doi M, Umezaki Y, Murai I, Shimatani H, Chu ML et al (2018) Calcitonin receptors are ancient modulators for rhythms of preferential temperature in insects and body temperature in mammals. Genes Dev 32:140–155. https://doi.org/10.1101/gad.307884.117

Goda T, Tang X, Umezaki Y, Chu ML, Kunst M, Nitabach MN et al (2016) *Drosophila* DH31 neuropeptide and PDF receptor regulate night-onset temperature preference. J Neurosci 36: 11739–11754. https://doi.org/10.1523/JNEUROSCI.0964-16.2016

Goda T, Umezaki Y, Alwattari F, Seo HW, Hamada FN (2019) Neuropeptides PDF and DH31 hierarchically regulate free-running rhythmicity in *Drosophila* circadian locomotor activity. Sci Rep 9:838. https://doi.org/10.1038/s41598-018-37107-3

Gorostiza EA, Depetris-Chauvin A, Frenkel L, Pírez N, Ceriani MF (2014) Circadian pacemaker neurons change synaptic contacts across the day. Curr Biol 24:2161–2167. https://doi.org/10.1016/j.cub.2014.07.063

Grima B, Chelot E, Xia R, Rouyer F (2004) Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature 431:869–873. https://doi.org/10.1038/nature02935

Guo F, Chen X, Rosbash M (2017) Temporal calcium profiling of specific circadian neurons in freely moving flies. Proc Natl Acad Sci U S A 114:E8780–E8787. https://doi.org/10.1073/pnas.1706608114

Guo F, Holla M, Díaz MM, Rosbash M (2018) A circadian output circuit controls sleep-wake arousal in *Drosophila*. Neuron 100:624–635.e4. https://doi.org/10.1016/j.neuron.2018.09.002

Guo F, Yu J, Jung HJ, Abruzzi KC, Luo W, Griffith LC et al (2016) Circadian neuron feedback controls the *Drosophila* sleep-activity profile. Nature 536:292–297. https://doi.org/10.1038/nature19097

Hamasaka Y, Rieger D, Parmentier ML, Grau Y, Helfrich-Förster C, Nässel DR (2007) Glutamate and its metabotropic receptor in *Drosophila* clock neuron circuits. J Comp Neurol 505:32–45. https://doi.org/10.1002/cne.21471

Hamblen-Coyle MJ, Wheeler DA, Rutila JE, Rosbash M, Hall JC (1992) Behavior of period-altered circadian rhythm mutants of *Drosophila* in light: dark cycles (Diptera: Drosophilidae). J Insect Behav 5:417–446. https://doi.org/10.1007/BF01058189

Hanafusa S, Kawaguchi T, Umezaki Y, Tomioka K, Yoshii T (2013) Sexual interactions influence the molecular oscillations in DN1 pacemaker neurons in *Drosophila melanogaster*. PLoS One 8:e84495. https://doi.org/10.1371/journal.pone.0084495

Hardin PE, Hall JC, Rosbash M (1992) Behavioral and molecular analyses suggest that circadian output is disrupted by *disconnected* mutants in *D. melanogaster*. EMBO J 11:1–6. https://doi.org/10.1002/j.1460-2075.1992.tb05020.x

Hassaneen E, El-Din Sallam A, Abo-Ghalia A, Moriyama Y, Karpova SG, Abdelsalam S et al (2011) Pigment-dispersing factor affects nocturnal activity rhythms, photic entrainment, and the free-running period of the circadian clock in the cricket *Gryllus bimaculatus*. J Biol Rhythm 26: 3–13. https://doi.org/10.1177/0748730410388746

Helfrich-Förster C (1995) The *period* clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 92:612–616. https://doi.org/10.1073/pnas.92.2.612

Helfrich-Förster C (1997) Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster*. J Comp Neurol 380:335–354. https://doi.org/10.1002/(sici)1096-9861(19970414)380:3<335::aid-cne4>3.0.co;2-3

Helfrich-Förster C (1998) Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of *disconnected* mutants. J Comp Physiol A 182:435–453. https://doi.org/10.1007/s003590050192

Helfrich-Förster C (2000) Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster*—sex-specific differences suggest a different quality of activity. J Biol Rhythm 15:135–154. https://doi.org/10.1177/074873040001500208

Helfrich-Förster C (2003) The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster*. Microsc Res Tech 62:94–102. https://doi.org/10.1002/jemt.10357

Helfrich-Förster C (2005) Organization of endogenous clocks in insects. Biochem Soc Trans 33: 957–961. https://doi.org/10.1042/BST20050957

Helfrich-Förster C, Shafer OT, Wülbeck C, Grieshaber E, Rieger D, Taghert P (2007) Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. J Comp Neurol 500:47–70. https://doi.org/10.1002/cne.21146

Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A et al (2000) Rest in *Drosophila* is a sleep-like state. Neuron 25:129–138. https://doi.org/10.1016/s0896-6273(00)80877-6

Hermann C, Saccon R, Senthilan PR, Domnik L, Dircksen H, Yoshii T et al (2013) The circadian clock network in the brain of different *Drosophila* species. J Comp Neurol 521:367–388. https://doi.org/10.1002/cne.23178

Hermann C, Yoshii T, Dusik V, Helfrich-Förster C (2012) Neuropeptide F immunoreactive clock neurons modify evening locomotor activity and free-running period in *Drosophila melanogaster*. J Comp Neurol 520:970–987. https://doi.org/10.1002/cne.22742

Hermann-Luibl C, Yoshii T, Senthilan PR, Dircksen H, Helfrich-Förster C (2014) The ion transport peptide is a new functional clock neuropeptide in the fruit fly *Drosophila melanogaster*. J Neurosci 34:9522–9536. https://doi.org/10.1523/JNEUROSCI.0111-14.2014

Huybrechts J, Bonhomme J, Minoli S, Prunier-Leterme N, Dombrovsky A, Abdel-Latief M et al (2010) Neuropeptide and neurohormone precursors in the pea aphid, *Acyrthosiphon pisum*. Insect Mol Biol 19:87–95. https://doi.org/10.1111/j.1365-2583.2009.00951.x

Hyun S, Lee Y, Hong ST, Bang S, Paik D, Kang J et al (2005) *Drosophila* GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron 48:267–278. https://doi.org/10.1016/j.neuron.2005.08.025

Im SH, Taghert PH (2010) PDF receptor expression reveals direct interactions between circadian oscillators in *Drosophila*. J Comp Neurol 518:1925–1945. https://doi.org/10.1002/cne.22311

Inami S, Sato T, Kurata Y, Suzuki Y, Kitamoto T, Sakai T (2021) Consolidation and maintenance of long-term memory involve dual functions of the developmental regulator Apterous in clock neurons and mushroom bodies in the *Drosophila* brain. PLoS Biol 19:e3001459. https://doi.org/10.1371/journal.pbio.3001459

Ito C, Tomioka K (2016) Heterogeneity of the peripheral circadian systems in *Drosophila melanogaster*: a review. Front Physiol 7:8. https://doi.org/10.3389/fphy.2016.00008

Jaumouillé E, Koch R, Nagoshi E (2021) Uncovering the roles of clocks and neural transmission in the resilience of *Drosophila* circadian network. Front Physiol 12:663339. https://doi.org/10.3389/fphys.2021.663339

Jin X, Tian Y, Zhang ZC, Gu P, Liu C, Han J (2021) A subset of DN1p neurons integrates thermosensory inputs to promote wakefulness via CNMa signaling. Curr Biol 31:2075–2087.e6. https://doi.org/10.1016/j.cub.2021.02.048

Johard HA, Yoishii T, Dircksen H, Cusumano P, Rouyer F, Helfrich-Förster C et al (2009) Peptidergic clock neurons in *Drosophila*: ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. J Comp Neurol 516:59–73. https://doi.org/10.1002/cne.22099

Kaneko H, Head LM, Ling J, Tang X, Liu Y, Hardin PE et al (2012) Circadian rhythm of temperature preference and its neural control in *Drosophila*. Curr Biol 22:1851–1857. https://doi.org/10.1016/j.cub.2012.08.006

Kaneko M, Hall JC (2000) Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. J Comp Neurol 422:66–94. https://doi.org/10.1002/(sici)1096-9861(20000619)422:1<66::aid-cne5>3.0.co;2-2

Kay J, Menegazzi P, Mildner S, Roces F, Helfrich-Förster C (2018) The circadian clock of the ant *Camponotus floridanus* is localized in dorsal and lateral neurons of the brain. J Biol Rhythm 33:255–271. https://doi.org/10.1177/0748730418764738

Klose MK, Bruchez MP, Deitcher DL, Levitan ES (2021) Temporally and spatially partitioned neuropeptide release from individual clock neurons. Proc Natl Acad Sci U S A 118:e2101818118. https://doi.org/10.1073/pnas.2101818118

Kobelková A, Závodská R, Sauman I, Bazalová O, Dolezel D (2015) Expression of clock genes *period* and *timeless* in the central nervous system of the Mediterranean flour moth, *Ephestia kuehniella*. J Biol Rhythm 30:104–116. https://doi.org/10.1177/0748730414568430

Koide R, Xi J, Hamanaka Y, Shiga S (2021) Mapping PERIOD-immunoreactive cells with neurons relevant to photoperiodic response in the bean bug *Riptortus pedestris*. Cell Tissue Res 385:571–583. https://doi.org/10.1007/s00441-021-03451-6

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 68:2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Kotwica-Rolinska J, Damulewicz M, Chodakova L, Kristofova L, Dolezel D (2022) Pigment dispersing factor is a circadian clock output and regulates photoperiodic response in the linden bug, *Pyrrhocoris apterus*. Front Physiol 13:884909. https://doi.org/10.3389/fphys.2022.884909

Kula E, Levitan ES, Pyza E, Rosbash M (2006) PDF cycling in the dorsal protocerebrum of the *Drosophila* brain is not necessary for circadian clock function. J Biol Rhythm 21:104–117. https://doi.org/10.1177/0748730405285715

Kunst M, Hughes ME, Raccuglia D, Felix M, Li M, Barnett G et al (2014) Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in *Drosophila*. Curr Biol 24:2652–2664. https://doi.org/10.1016/j.cub.2014.09.077

Kutaragi Y, Tokuoka A, Tomiyama Y, Nose M, Watanabe T, Bando T et al (2018) A novel photic entrainment mechanism for the circadian clock in an insect: involvement of *c-fos* and *cryptochromes*. Zool Lett 4:26. https://doi.org/10.1186/s40851-018-0109-8

Lamaze A, Krätschmer P, Chen KF, Lowe S, Jepson JEC (2018) A wake-promoting circadian output circuit in *Drosophila*. Curr Biol 28:3098–3105.e3. https://doi.org/10.1016/j.cub.2018.07.024

Lear BC, Merrill CE, Lin JM, Schroeder A, Zhang L, Allada R (2005) A G protein-coupled receptor, *groom-of-PDF*, is required for PDF neuron action in circadian behavior. Neuron 48: 221–227. https://doi.org/10.1016/j.neuron.2005.09.008

Lear BC, Zhang L, Allada R (2009) The neuropeptide PDF acts directly on evening pacemaker neurons to regulate multiple features of circadian behavior. PLoS Biol 7:e1000154. https://doi.org/10.1371/journal.pbio.1000154

Lee CM, Su MT, Lee HJ (2009) Pigment dispersing factor: an output regulator of the circadian clock in the German cockroach. J Biol Rhythm 24:35–43. https://doi.org/10.1177/0748730408327909

Lelito KR, Shafer OT (2012) Reciprocal cholinergic and GABAergic modulation of the small ventrolateral pacemaker neurons of *Drosophila*'s circadian clock neuron network. J Neurophysiol 107:2096–2108. https://doi.org/10.1152/jn.00931.2011

Leopold AV, Shcherbakova DM, Verkhusha VV (2019) Fluorescent biosensors for neurotransmission and neuromodulation: engineering and applications. Front Cell Neurosci 13:474. https://doi.org/10.3389/fncel.2019.00474

Li MT, Cao LH, Xiao N, Tang M, Deng B, Yang T et al (2018) Hub-organized parallel circuits of central circadian pacemaker neurons for visual photoentrainment in *Drosophila*. Nat Commun 9:4247. https://doi.org/10.1038/s41467-018-06506-5

Liang X, Ho MCW, Zhang Y, Li Y, Wu MN, Holy TE et al (2019) Morning and evening circadian pacemakers independently drive premotor centers via a specific dopamine relay. Neuron 102: 843–857.e4. https://doi.org/10.1016/j.neuron.2019.03.028

Liang X, Holy TE, Taghert PH (2016) Synchronous *Drosophila* circadian pacemakers display nonsynchronous Ca^{2+} rhythms in vivo. Science 351:976–981. https://doi.org/10.1126/science.aad3997

Liang X, Holy TE, Taghert PH (2017) A series of suppressive signals within the *Drosophila* circadian neural circuit generates sequential daily outputs. Neuron 94:1173–1189.e4. https://doi.org/10.1016/j.neuron.2017.05.007

Lin Y, Stormo GD, Taghert PH (2004) The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. J Neurosci 24:7951–7957. https://doi.org/10.1523/JNEUROSCI.2370-04.2004

Liu X, Lorenz L, Yu QN, Hall JC, Rosbash M (1988) Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. Genes Dev 2:228–238. https://doi.org/10.1101/gad.2.2.228

Loher W (1972) Circadian control of stridulation in the cricket *Teleogryllus commodus* Walker. J Comp Physiol 79:173–190. https://doi.org/10.1007/BF00697770

Lupien M, Marshall S, Leser W, Pollack GS, Honegger HW (2003) Antibodies against the PER protein of *Drosophila* label neurons in the optic lobe, central brain, and thoracic ganglia of the crickets *Teleogryllus commodus* and *Teleogryllus oceanicus*. Cell Tissue Res 312:377–391. https://doi.org/10.1007/s00441-003-0720-6

Ma D, Przybylski D, Abruzzi KC, Schlichting M, Li Q, Long X et al (2021) A transcriptomic taxonomy of *Drosophila* circadian neurons around the clock. elife 10:e63056. https://doi.org/10.7554/eLife.63056

McCarthy EV, Wu Y, Decarvalho T, Brandt C, Cao G, Nitabach MN (2011) Synchronized bilateral synaptic inputs to *Drosophila melanogaster* neuropeptidergic rest/arousal neurons. J Neurosci 31:8181–8193. https://doi.org/10.1523/JNEUROSCI.2017-10.2011

Meelkop E, Temmerman L, Schoofs L, Janssen T (2011) Signalling through pigment dispersing hormone-like peptides in invertebrates. Prog Neurobiol 93:125–147. https://doi.org/10.1016/j.pneurobio.2010.10.004

Meiselman MR, Alpert MH, Cui X, Shea J, Gregg I, Gallio M et al (2022) Recovery from cold-induced reproductive dormancy is regulated by temperature-dependent AstC signaling. Curr Biol 32:1362–1375. https://doi.org/10.1016/j.cub.2022.01.061

Menegazzi P, Beer K, Grebler V, Schlichting M, Schubert FK, Helfrich-Förster C (2020) A functional clock within the main morning and evening neurons of *D. melanogaster* is not

sufficient for wild-type locomotor activity under changing day length. Front Physiol 11:229. https://doi.org/10.3389/fphys.2020.00229

Menegazzi P, Dalla Benetta E, Beauchamp M, Schlichting M, Steffan-Dewenter I, Helfrich-Förster C (2017) Adaptation of circadian neuronal network to photoperiod in high-latitude European Drosophilids. Curr Biol 27:833–839. https://doi.org/10.1016/j.cub.2017.01.036

Mertens I, Vandingenen A, Johnson EC, Shafer OT, Li W, Trigg JS et al (2005) PDF receptor signaling in *Drosophila* contributes to both circadian and geotactic behaviors. Neuron 48:213–219. https://doi.org/10.1016/j.neuron.2005.09.009

Mieda M (2020) The central circadian clock of the suprachiasmatic nucleus as an ensemble of multiple oscillatory neurons. Neurosci Res 156:24–31. https://doi.org/10.1016/j.neures.2019.08.003

Mohamed AA, Wang Q, Bembenek J, Ichihara N, Hiragaki S, Suzuki T et al (2014) *N-acetyltransferase* (*nat*) is a critical conjunct of photoperiodism between the circadian system and endocrine axis in *Antheraea pernyi*. PLoS One 9:e92680. https://doi.org/10.1371/journal.pone.0092680

Nagy D, Cusumano P, Andreatta G, Anduaga AM, Hermann-Luibl C, Reinhard N et al (2019) Peptidergic signaling from clock neurons regulates reproductive dormancy in *Drosophila melanogaster*. PLoS Genet 15:e1008158. https://doi.org/10.1371/journal.pgen.1008158

Nettnin EA, Sallese TR, Nasseri A, Saurabh S, Cavanaugh DJ (2021) Dorsal clock neurons in *Drosophila* sculpt locomotor outputs but are dispensable for circadian activity rhythms. iScience 24:103001. https://doi.org/10.1016/j.isci.2021.103001

Ni JD, Gurav AS, Liu W, Ogunmowo TH, Hackbart H, Elsheikh A et al (2019) Differential regulation of the *Drosophila* sleep homeostat by circadian and arousal inputs. elife 8:e40487. https://doi.org/10.7554/eLife.40487

Nishiitsutsuji-Uwo J, Petropulos SF, Pittendrigh CS (1967) Central nervous system control of circadian rhythmicity in the cockroach. I. Role of the pars intercerebralis. Biol Bull 133:679–696. https://doi.org/10.2307/1539928

Nishiitsutsuji-Uwo J, Pittendrigh C (1968) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? Z Vergl Physiol 58:14–46. https://doi.org/10.1007/BF00302434

Okamoto A, Mori H, Tomioka K (2001) The role of the optic lobe in circadian locomotor rhythm generation in the cricket, *Gryllus bimaculatus*, with special reference to PDH-immunoreactive neurons. J Insect Physiol 47:889–895. https://doi.org/10.1016/s0022-1910(01)00061-0

Ono D, Honma KI, Honma S (2021) Roles of neuropeptides, VIP and AVP, in the mammalian central circadian clock. Front Neurosci 15:650154. https://doi.org/10.3389/fnins.2021.650154

Page TL (1978) Interactions between bilaterally paired components of the cockroach circadian system. J Comp Physiol 124:225–236. https://doi.org/10.1007/BF00657054

Page TL (1982) Transplantation of the cockroach circadian pacemaker. Science 216:73–75. https://doi.org/10.1126/science.216.4541.73

Page TL, Caldarola PC, Pittendrigh CS (1977) Mutual entrainment of bilaterally distributed circadian pacemaker. Proc Natl Acad Sci U S A 74:1277–1281. https://doi.org/10.1073/pnas.74.3.1277

Panda S, Sato TK, Castrucci AM, Rollag MD, DeGrip WJ, Hogenesch JB et al (2002) Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. Science 298:2213–2216. https://doi.org/10.1126/science.1076848

Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, Hall JC (2000) Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. Proc Natl Acad Sci U S A 97:3608–3613. https://doi.org/10.1073/pnas.070036197

Peng Y, Stoleru D, Levine JD, Hall JC, Rosbash M (2003) *Drosophila* free-running rhythms require intercellular communication. PLoS Biol 1:32–40. https://doi.org/10.1371/journal.pbio.0000013

Petri B, Stengl M (1997) Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. J Neurosci 17:4087–4093. https://doi.org/10.1523/JNEUROSCI.17-11-04087.1997

Pittendrigh CS, Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. J Comp Physiol A 106:333–355. https://doi.org/10.1007/BF01417860

Prakash P, Nambiar A, Sheeba V (2017) Oscillating PDF in termini of circadian pacemaker neurons and synchronous molecular clocks in downstream neurons are not sufficient for sustenance of activity rhythms in constant darkness. PLoS One 12:e0175073. https://doi.org/10.1371/journal.pone.0175073

Ramakrishnan A, Sheeba V (2021) Gap junction protein Innexin2 modulates the period of free-running rhythms in *Drosophila melanogaster*. iScience 24:103011. https://doi.org/10.1016/j.isci.2021.103011

Reinhard N, Bertolini E, Saito A, Sekiguchi M, Yoshii T, Rieger D et al (2022) The lateral posterior clock neurons of *Drosophila melanogaster* express three neuropeptides and have multiple connections within the circadian clock network and beyond. J Comp Neurol 530:1507–1529. https://doi.org/10.1002/cne.25294

Reischig T, Petri B, Stengl M (2004) Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. Cell Tissue Res 318:553–564. https://doi.org/10.1007/s00441-004-0927-1

Reischig T, Stengl M (2002) Optic lobe commissures in a three-dimensional brain model of the cockroach *Leucophaea maderae*: a search for the circadian coupling pathways. J Comp Neurol 443:388–400. https://doi.org/10.1002/cne.10133

Reischig T, Stengl M (2003) Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). J Exp Biol 206:1877–1886. https://doi.org/10.1242/jeb.00373

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802. https://doi.org/10.1016/s0092-8674(00)81676-1

Rieger D, Shafer OT, Tomioka K, Helfrich-Förster C (2006) Functional analysis of circadian pacemaker neurons in *Drosophila melanogaster*. J Neurosci 26:2531–2543. https://doi.org/10.1523/JNEUROSCI.1234-05.2006

Rieger D, Wülbeck C, Rouyer F, Helfrich-Förster C (2009) *Period* gene expression in four neurons is sufficient for rhythmic activity of *Drosophila melanogaster* under dim light conditions. J Biol Rhythm 24:271–282. https://doi.org/10.1177/0748730409338508

Ruby NF, Brennan TJ, Xie X, Cao V, Franken P, Heller HC et al (2002) Role of melanopsin in circadian responses to light. Science 298:2211–2213. https://doi.org/10.1126/science.1076701

Saifullah ASM, Tomioka K (2002) Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Exp Biol 205:1305–1314. https://doi.org/10.1242/jeb.205.9.1305

Saifullah ASM, Tomioka K (2003) Pigment-dispersing factor sets the night state of the medulla bilateral neurons in the optic lobe of the cricket, *Gryllus bimaculatus*. J Insect Physiol 49:231–239. https://doi.org/10.1016/s0022-1910(02)00270-6

Sauman I, Briscoe AD, Zhu H, Shi D, Froy O, Stalleicken J et al (2005) Connecting the navigational clock to sun compass input in monarch butterfly brain. Neuron 46:457–467. https://doi.org/10.1016/j.neuron.2005.03.014

Sauman I, Reppert SM (1996) Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of Period protein regulation. Neuron 17:889–900. https://doi.org/10.1016/s0896-6273(00)80220-2

Scheffer LK, Xu CS, Januszewski M, Lu Z, Takemura SY, Hayworth KJ et al (2020) A connectome and analysis of the adult *Drosophila* central brain. elife 9:e57443. https://doi.org/10.7554/eLife.57443

Schlichting M, Díaz MM, Xin J, Rosbash M (2019a) Neuron-specific knockouts indicate the importance of network communication to *Drosophila* rhythmicity. elife 8:e48301. https://doi.org/10.7554/eLife.48301

Schlichting M, Menegazzi P, Lelito KR, Yao Z, Buhl E, Dalla Benetta E et al (2016) A neural network underlying circadian entrainment and photoperiodic adjustment of sleep and activity in *Drosophila*. J Neurosci 36:9084–9096. https://doi.org/10.1523/JNEUROSCI.0992-16.2016

Schlichting M, Weidner P, Diaz M, Menegazzi P, Dalla Benetta E, Helfrich-Förster C et al (2019b) Light-mediated circuit switching in the *Drosophila* neuronal clock network. Curr Biol 29:3266–3276.e3. https://doi.org/10.1016/j.cub.2019.08.033

Schneider NL, Stengl M (2006) Gap junctions between accessory medulla neurons appear to synchronize circadian clock cells of the cockroach *Leucophaea maderae*. J Neurophysiol 95:1996–2002. https://doi.org/10.1152/jn.00835.2005

Schubert FK, Hagedorn N, Yoshii T, Helfrich-Förster C, Rieger D (2018) Neuroanatomical details of the lateral neurons of *Drosophila melanogaster* support their functional role in the circadian system. J Comp Neurol 526:1209–1231. https://doi.org/10.1002/cne.24406

Sehadová H, Markova EP, Sehnal F, Takeda M (2004) Distribution of circadian clock-related proteins in the cephalic nervous system of the silkworm, *Bombyx mori*. J Biol Rhythm 19:466–482. https://doi.org/10.1177/0748730404269153

Selcho M, Millán C, Palacios-Muñoz A, Ruf F, Ubillo L, Chen J et al (2017) Central and peripheral clocks are coupled by a neuropeptide pathway in *Drosophila*. Nat Commun 8:15563. https://doi.org/10.1038/ncomms15563

Shafer OT, Helfrich-Förster C, Renn SC, Taghert PH (2006) Reevaluation of *Drosophila melanogaster*'s neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol 498:180–193. https://doi.org/10.1002/cne.21021

Shafer OT, Kim DJ, Dunbar-Yaffe R, Nikolaev VO, Lohse MJ, Taghert PH (2008) Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of *Drosophila* revealed by real-time cyclic AMP imaging. Neuron 58:223–237. https://doi.org/10.1016/j.neuron.2008.02.018

Shafer OT, Taghert PH (2009) RNA-interference knockdown of *Drosophila pigment dispersing factor* in neuronal subsets: the anatomical basis of a neuropeptide's circadian functions. PLoS One 4:e8298. https://doi.org/10.1371/journal.pone.0008298

Shao QM, Bembenek J, Trang le TD, Hiragaki S, Takeda M (2008a) Molecular structure, expression patterns, and localization of the circadian transcription modulator CYCLE in the cricket, *Dianemobius nigrofasciatus*. J Insect Physiol 54:403–413. https://doi.org/10.1016/j.jinsphys.2007.10.013

Shao QM, Hiragaki S, Takeda M (2008b) Co-localization and unique distributions of two clock proteins CYCLE and CLOCK in the cephalic ganglia of the ground cricket, *Allonemobius allardi*. Cell Tissue Res 331:435–446. https://doi.org/10.1007/s00441-007-0534-Z

Shao QM, Sehadová H, Ichihara N, Sehnal F, Takeda M (2006) Immunoreactivities to three circadian clock proteins in two ground crickets suggest interspecific diversity of the circadian clock structure. J Biol Rhythm 21:118–131. https://doi.org/10.1177/0748730405283660

Shaw PJ, Cirelli C, Greenspan RJ, Tononi G (2000) Correlates of sleep and waking in *Drosophila melanogaster*. Science 287:1834–1837. https://doi.org/10.1126/science.287.5459.1834

Sheeba V, Fogle KJ, Holmes TC (2010) Persistence of morning anticipation behavior and high amplitude morning startle response following functional loss of small ventral lateral neurons in *Drosophila*. PLoS One 5:e11628. https://doi.org/10.1371/journal.pone.0011628

Shiga S, Numata H (2009) Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, *Protophormia terraenovae*. J Exp Biol 212:867–877. https://doi.org/10.1242/jeb.027003

Singaravel M, Fujisawa Y, Hisada M, Saifullah AS, Tomioka K (2003) Phase shifts of the circadian locomotor rhythm induced by pigment-dispersing factor in the cricket *Gryllus bimaculatus*. Zool Sci 20:1347–1354. https://doi.org/10.2108/zsj.20.1347

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC (1988) Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron 1:141–150. https://doi.org/10.1016/0896-6273(88)90198-5

Sokolove PG, Loher W (1975) Rôle of eyes, optic lobes, and pars intercerebralis in locomotory and stridulatory circadian rhythms of *Teleogryllus commodus*. J Insect Physiol 21:785–799. https://doi.org/10.1016/0022-1910(75)90009-8

Song BJ, Sharp SJ, Rogulja D (2021) Daily rewiring of a neural circuit generates a predictive model of environmental light. Sci Adv 7:eabe4284. https://doi.org/10.1126/sciadv.abe4284

Stoleru D, Peng Y, Agosto J, Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. Nature 431:862–868. https://doi.org/10.1038/nature02926

Takekata H, Numata H, Shiga S (2018) Effects of pars intercerebralis removal on circatidal rhythm in the mangrove cricket, *Apteronemobius asahinai*. J Comp Physiol A 204:801–810. https://doi.org/10.1007/s00359-018-1281-1

Tomioka K (1993) Analysis of coupling between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Comp Physiol A 172:401–408. https://doi.org/10.1007/BF00213522

Tomioka K, Chiba Y (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the circadian locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zool Sci 1:385–394. https://doi.org/10.34425/zs000039

Tomioka K, Chiba Y (1986) Circadian rhythm in the neurally isolated lamina-medulla-complex of the cricket, *Gryllus bimaculatus*. J Insect Physiol 32:747–755. https://doi.org/10.1016/0022-1910(86)90077-6

Tomioka K, Chiba Y (1992) Characterization of optic lobe circadian pacemaker by in situ and in vitro recording of neuronal activity in the cricket *Gryllus bimaculatus*. J Comp Physiol A 171:1–7. https://doi.org/10.1007/BF00195955

Tomioka K, Yamada K, Yokoyama S, Chiba Y (1991) Mutual interactions between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Comp Physiol A 169:291–298. https://doi.org/10.1007/BF00206993

Vafopoulou X, Terry KL, Steel CG (2010) The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (hemiptera). J Comp Neurol 518:1264–1282. https://doi.org/10.1002/cne.22274

Wei H, Stengl M (2011) Light affects the branching pattern of peptidergic circadian pacemaker neurons in the brain of the cockroach *Leucophaea maderae*. J Biol Rhythm 26:507–517. https://doi.org/10.1177/0748730411419968

Wen CJ, Lee HJ (2008) Mapping the cellular network of the circadian clock in two cockroach species. Arch Insect Biochem Physiol 68:215–231. https://doi.org/10.1002/arch.20236

Wiedenmann G (1983) Splitting in a circadian activity rhythm: the expression of bilaterally paired oscillators. J Comp Physiol 150:51–60. https://doi.org/10.1007/BF00605287

Wise S, Davis NT, Tyndale E, Noveral J, Folwell MG, Bedian V et al (2002) Neuroanatomical studies of period gene expression in the hawkmoth, *Manduca sexta*. J Comp Neurol 447:366–380. https://doi.org/10.1002/cne.10242

Yadlapalli S, Jiang C, Bahle A, Reddy P, Meyhofer E, Shafer OT (2018) Circadian clock neurons constantly monitor environmental temperature to set sleep timing. Nature 555:98–102. https://doi.org/10.1038/nature25740

Yang Z, Sehgal A (2001) Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. Neuron 29:453–467. https://doi.org/10.1016/s0896-6273(01)00218-5

Yao Z, Bennett AJ, Clem JL, Shafer OT (2016) The *Drosophila* clock neuron network features diverse coupling modes and requires network-wide coherence for robust circadian rhythms. Cell Rep 17:2873–2881. https://doi.org/10.1016/j.celrep.2016.11.053

Yao Z, Shafer OT (2014) The *Drosophila* circadian clock is a variably coupled network of multiple peptidergic units. Science 343:1516–1520. https://doi.org/10.1126/science.1251285

Yoshii T, Rieger D, Helfrich-Förster C (2012) Two clocks in the brain: an update of the morning and evening oscillator model in *Drosophila*. Prog Brain Res 199:59–82. https://doi.org/10.1016/B978-0-444-59427-3.00027-7

Yoshii T, Todo T, Wülbeck C, Stanewsky R, Helfrich-Förster C (2008) Cryptochrome is present in the compound eyes and a subset of *Drosophila*'s clock neurons. J Comp Neurol 508:952–966. https://doi.org/10.1002/cne.21702

Yoshii T, Wülbeck C, Sehadova H, Veleri S, Bichler D, Stanewsky R et al (2009) The neuropeptide pigment-dispersing factor adjusts period and phase of *Drosophila*'s clock. J Neurosci 29:2597–2610. https://doi.org/10.1523/JNEUROSCI.5439-08.2009

Yukizane M, Tomioka K (1995) Neural pathways involved in mutual interactions between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Comp Physiol A 176:601–610. https://doi.org/10.1007/BF01021580

Závodská R, Sauman I, Sehnal F (2003a) The cycling and distribution of PER-like antigen in relation to neurons recognized by the antisera to PTTH and EH in *Thermobia domestica*. Insect Biochem Mol Biol 33:1227–1238. https://doi.org/10.1016/j.ibmb.2003.06.009

Závodská R, Šauman I, Sehnal F (2003b) Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. J Biol Rhythm 18:106–122. https://doi.org/10.1177/0748730403251711

Závodská R, Sehadová H, Sauman I, Sehnal F (2005) Light-dependent PER-like proteins in the cephalic ganglia of an apterygote and a pterygote insect species. Histochem Cell Biol 123:407–418. https://doi.org/10.1007/s00418-004-0728-3

Zerr DM, Hall JC, Rosbash M, Siwicki KK (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. J Neurosci 10:2749–2762. https://doi.org/10.1523/JNEUROSCI.10-08-02749.1990

Zhang C, Daubnerova I, Jang YH, Kondo S, Žitňan D, Kim YJ (2021a) The neuropeptide allatostatin C from clock-associated DN1p neurons generates the circadian rhythm for oogenesis. Proc Natl Acad Sci U S A 118:e2016878118. https://doi.org/10.1073/pnas.2016878118

Zhang L, Chung BY, Lear BC, Kilman VL, Liu Y, Mahesh G et al (2010a) DN1(p) circadian neurons coordinate acute light and PDF inputs to produce robust daily behavior in *Drosophila*. Curr Biol 20:591–599. https://doi.org/10.1016/j.cub.2010.02.056

Zhang Y, Liu Y, Bilodeau-Wentworth D, Hardin PE, Emery P (2010b) Light and temperature control the contribution of specific DN1 neurons to *Drosophila* circadian behavior. Curr Biol 20:600–605. https://doi.org/10.1016/j.cub.2010.02.044

Zhang Y, Zhou Y, Zhang X, Wang L, Zhong Y (2021b) Clock neurons gate memory extinction in *Drosophila*. Curr Biol 31:1337–1343.e4. https://doi.org/10.1016/j.cub.2021.01.008

Zhu H, Sauman I, Yuan Q, Casselman A, Emery-Le M, Emery P et al (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biol 6:e4. https://doi.org/10.1371/journal.pbio.0060004

Chapter 6
Peripheral Circadian Clock

Chihiro Ito

Abstract Insects exhibit circadian rhythms in a variety of behavioral and physiological processes. These rhythms are controlled not only by the central clock located in the brain but also by peripheral clocks ubiquitously distributed in peripheral tissues or organs. Peripheral clocks temporally control the local physiology in peripheral tissues and organs, some of which affect their behavior and life span. The central and peripheral clocks share the molecules for oscillation and entrainment. However, some peripheral clocks exhibit different oscillatory mechanisms. The characteristics of peripheral clocks vary between species and among individuals. This chapter describes the localization and features of peripheral clocks and the relationship between the central and peripheral clocks in several insects.

Keywords Autonomous oscillation · Local physiology · Master clock · Peripheral clock · Slave oscillator

6.1 Introduction

Circadian rhythms are observed in various behavioral and physiological processes. These rhythms are controlled by circadian clocks, allowing organisms on the Earth to anticipate periodic environmental changes and prepare their physiological states for changes. Circadian clocks are distributed ubiquitously throughout the body and reside not only in the central nervous system but also in peripheral tissues or organs (e.g., Tomioka et al. 2012 for review). With no clear definition yet, it is accepted in general that the circadian clocks residing in the central nervous system are the central clocks, whereas those in peripheral tissues or organs are peripheral clocks. The central clock governs periodical behaviors such as locomotor activity and sleep-wake cycles, while the peripheral clocks regulate temporal physiology in each tissue

C. Ito (✉)
Department of Biochemistry, Faculty of Pharmacy, Osaka Medical and Pharmaceutical University, Takatsuki, Osaka, Japan
e-mail: chihiro.ito@ompu.ac.jp

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and organ. In insects, peripheral clocks have been shown to exist in the compound eyes, antennae, proboscis, intestinal organs, reproductive organs, excretory and osmoregulatory organs, and endocrine organs. Furthermore, peripheral clocks have also been found in tissues or cells, including fat body, oenocytes, and epidermal cells, among others (Fig. 6.1, Table 6.1).

Methods for characterizing clocks and their locations have drastically changed since the discovery of clock genes (Konopka and Benzer 1971; Bargiello et al. 1984; Reddy et al. 1984; Zehring et al. 1984). Previously, the features or the presence of peripheral clocks was demonstrated in several insects using traditional physiological techniques such as surgery, tissue or organ culture, decapitation, ligation, and transplantation. After the discovery of clock genes in the fruit fly *Drosophila melanogaster*, the approach for identifying peripheral clocks shifted to using molecular biological techniques. Therefore, recent studies on peripheral clocks have focused on *D. melanogaster* armed with advantageous molecular and genetic tools. In particular, because targeted disruption or ablation of the central or peripheral clocks to clarify the relationship between the central and peripheral clocks is difficult in other insects, most studies have been conducted in *D. melanogaster*.

This chapter provides an overview of the rhythms produced by peripheral clocks and their regulatory mechanisms in insects. Additionally, its possible physiological role or effects on behavior or life span and the relationship between the central and peripheral clocks are discussed.

6.2 General Features of Peripheral Circadian Clocks

Circadian rhythm is defined by the following three features: (1) The rhythm persists or free runs for approximately 24 h, even under constant conditions where no daily periodic information is input from the environment. (2) The rhythm is entrained by daily environmental cycles. (3) The free-running period is temperature-compensated. In addition, another recently introduced criterion for the rhythm is its dysfunction when clock genes are disrupted. These features can also be applied to circadian rhythms that are controlled by peripheral clocks. However, not all peripheral circadian clocks meet these definitions. For example, some peripheral clocks often dampen their oscillations under constant conditions or lack entrainment ability to environmental cycles or temperature compensation of free-running periods (Plautz et al. 1997; Wiedenmann et al. 1986; Versteven et al. 2020). These peripheral clocks often require input from the central clocks to maintain their oscillation and/or to entrain environmental cycles. The degree of dependency on the central clocks varies among tissues and organs (Ito et al. 2008; Ito and Tomioka 2016).

The core of the circadian clock is a molecular oscillator composed of multiple clock genes and proteins that comprise interlocked transcriptional-translational feedback loops (e.g., Hardin 2011). The central and peripheral clocks consist of similar, but not identical, molecules (Ito and Tomioka 2016). In *D. melanogaster*, CRYPTOCHROME (CRY), a blue light-absorbing pigment, serves as a

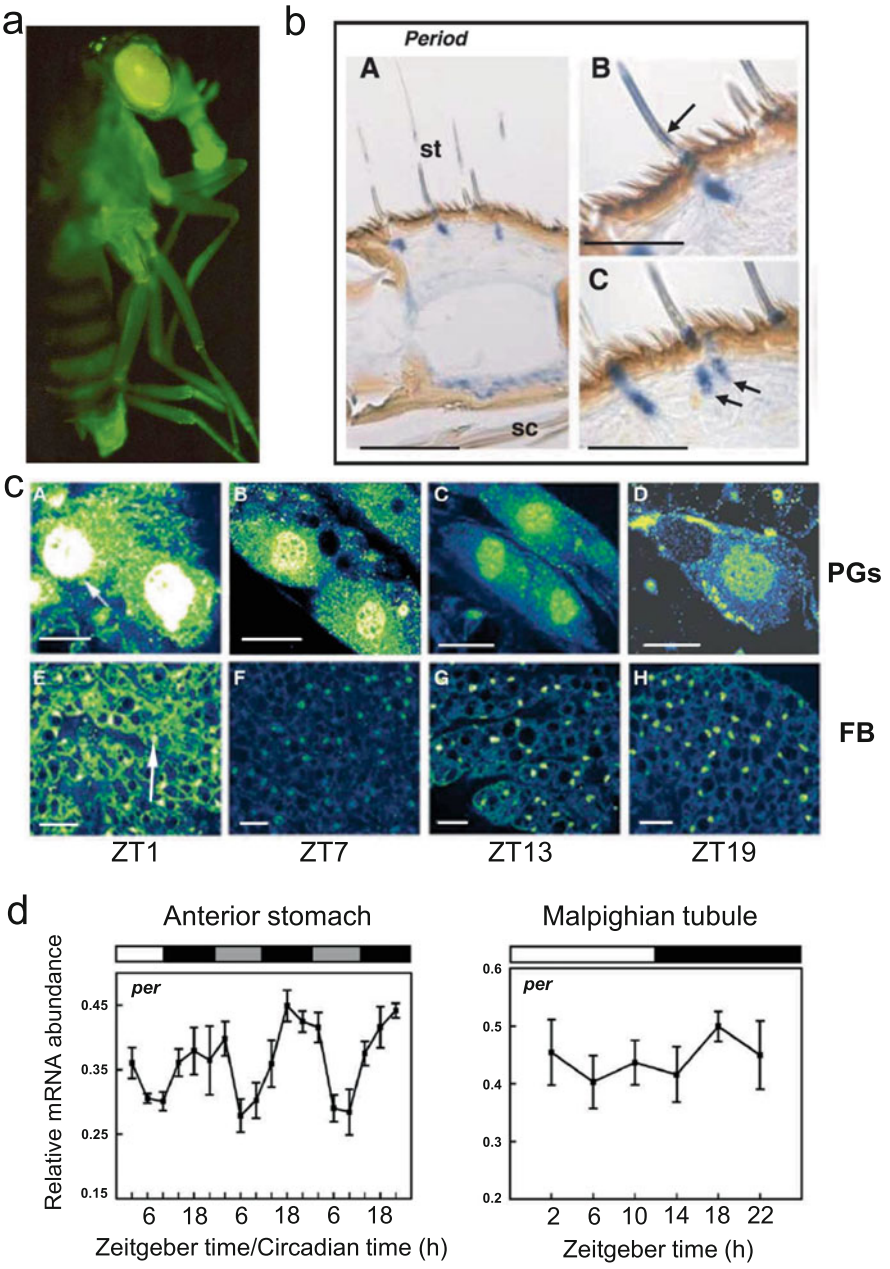

Fig. 6.1 The ubiquitous expression of clock genes and proteins was confirmed by various methods in many tissues and organs of insects. (**a**) *per*-driven green fluorescent protein (GFP) expression can be seen throughout the fly body in *Drosophila melanogaster*. From Plautz et al. (1997) with permission from AAAS. (**b**) Expression of *per* (A–C) on longitudinal sections of male antennae in *Mamestra brassicae*, as determined by in situ hybridization. (A) *per* expression on the sensilla side of the antennae with labeled cells located at the base of olfactory sensilla trichodea (st); sc, scales. Bars: 50 µm. (B and C) Sensilla trichodea at higher magnification are shown. (B) Labeling

photoreceptor for photic entrainment in the central clock but acts as a component of the molecular oscillator. In several moth species, translocation of PER into the nucleus does not occur and remains in the cytoplasm of the central clock neurons (Collins et al. 2006; Ivanchenko et al. 2001; Krishnan et al. 2001). However, PER is detectable in the nucleus of several peripheral tissues, such as the antennae and male reproductive system (Sauman and Reppert 1996; Gvakharia et al. 2000; Iwai et al. 2006; Schuckel et al. 2007; Kotwica et al. 2009). It is interesting to understand why these differences in the molecular mechanisms between the central and peripheral clocks have evolved. The functional significance of these differences should be clarified in future studies.

6.3 Various Peripheral Clocks

6.3.1 Compound Eyes

In the visual system, circadian rhythms are observed at various levels and include the expression of clock genes, morphological changes, and sensitivities in several insects. The visual system of insects is composed of the retina and three optic neuropils: the lamina, medulla, and lobula (van der Kooi et al. 2021). The electrical activity of the retina in response to a light stimulus is measured using an electroretinogram (ERG). The ERG amplitude of the compound eye shows a circadian change that reflects a rhythmic change in retinal sensitivity to light in some insects, such as flies, crickets, and cockroaches (Chen et al. 1992, 1999; Tomioka and Chiba 1982; Wills et al. 1985). In crickets and cockroaches, the ERG rhythm peaks during the night, when insects are in the active phase (Tomioka and Chiba 1982; Wills et al. 1985). The ERG rhythm has been shown to persist even when the optic lobe, which

Fig. 6.1 (continued) inside a sensillum (arrow). (C) Two labeled somata at the base of a sensillum trichodeum (arrows). Bars: 20 μm in (B, C). From Merlin et al. (2006) with permission from John Wiley and Sons. (**c**) Daily cycling in vivo in abundance and nuclear localization of PER immunofluorescence in *Rhodnius prolixus*. Green/yellow/white indicates PER immunofluorescence. Tissue samples were dissected at four time points during day 12 after a blood meal: 1 h after lights off, 7 h after lights off, 1 h after lights on, and 7 h after lights on. A–D show prothoracic gland (PG) cells; E–H show fat body (FB) cells. Note the cycling of nuclear PER with peaks at early photophase. Arrows in A and E show fluorescent nuclei with PER accumulation. Bars: 10 μm. From Vafopoulou and Steel (2014) under CC-BY 3.0 (https://creativecommons.org/licenses/by/3.0/). (**d**) Abundance of *per* transcripts over time in the anterior stomach and Malpighian tubule of the cricket *Gryllus bimaculatus*. A robust rhythm was observed in the anterior stomach under LD cycles and DD but no rhythm existed in Malpighian tubules under LD cycles. The abundance of *per* was measured by reverse transcriptase quantitative RT-qPCR using total RNA extracted from the tissues of adult crickets collected at 4 h intervals, starting at 2 h after lights on (ZT2). The values shown are relative to the amount of *rpl18a* mRNA. Vertical bars indicate SEM. Black, white, and gray bars indicate night/subjective night, day, and subjective day, respectively. From Uryu and Tomioka (2010) with permission from Elsevier

Table 6.1 Example of insect peripheral tissues or organs that express clock genes or proteins

Species	Tissue or organs	Clock genes or proteins	Method	Reference
Drosophila melanogaster	Esophagus, proventriculus, hindgut, Malpighian tubules, fat body, rectum, spermatheca,	PER, TIM	IHC	Giebultowicz et al. (2001)
	Epidermal cells	PER	IHC	Ito et al. (2008)
	Prothoracic gland, proboscis, wing, legs	*per*	*per-luciferase, per-GFP*	Emery et al. (1997), Plautz et al. (1997)
Gryllus bimaculatus	Terminal abdominal ganglion, anterior stomach, midgut, testes, Malpighian tubules	*per*	RT-qPCR	Uryu and Tomioka (2010)
Schistocerca gregaria	Testes, accessory glands	*per*, *tim*	RT-qPCR	Tobback et al. (2012)
Bombyx mori	Testes, ovary	*per*, *tim*	Northern blot	Iwai et al. (2006)
	Head, flight muscle, testes, antenna		RT-qPCR	
Manduca sexta	Antenna	PER	IHC	Schuckel et al. (2007)
Danaus plexippus	Antenna	*per*, *tim*, *cry1*, *cry2*	RT-qPCR	Merlin et al. (2009)
Helicoverpa armigera	Compound eye	*per*, *tim*, *cry1* *cry2*	RT-qPCR	Yan et al. (2014, 2019)
Cydia pomonella	Testes	PER, *per*	RNase protection assay, in situ hybridization, IHC	Gvakharia et al. (2000)
Spodoptera littoralis	Midgut, fat body	*Per*	RT-qPCR	Suszczynska et al. (2017)
	Antenna	*Per*, *cry1*, *cry2*	RT-qPCR	Merlin et al. (2007)
Pyrrhocoris apterus	Gut	*Cyc*, *Clk*, *Pdp1*, *cry*	RT-qPCR	Bajgar et al. (2013)
		CRY2	IHC	
Rhodnius prolixus	Prothoracic grand, fat body, salivary gland	PER	IHC	Vafopoulou and Steel (2014)

The expression of clock genes or proteins has been detected by immunohistochemistry (IHC), in situ hybridization, Northern blot, RT-qPCR, RNase protection assay, or reporter assay

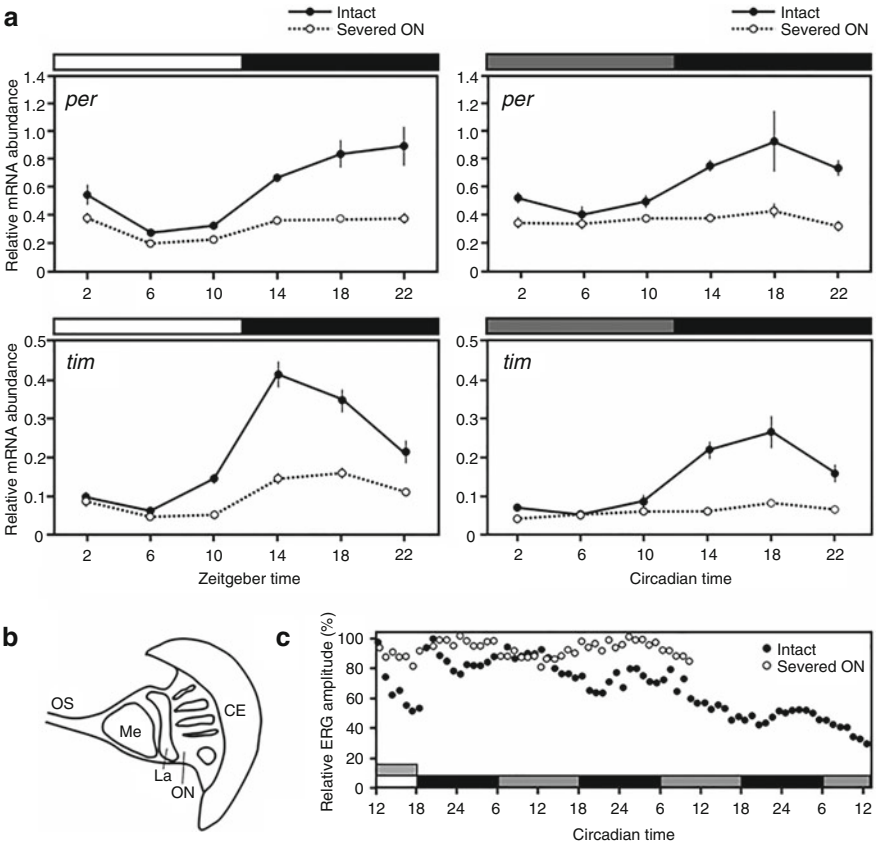

Fig. 6.2 Circadian rhythms in the compound eye in the cricket *Gryllus bimaculatus*. (**a**) Daily expression profiles of clock genes, *per*, and *tim*, in the compound eye of the cricket *Gryllus bimaculatus* under light-dark cycles and constant darkness. Closed and open circles indicate intact eyes and eyes with severed optic nerves (ON), respectively. Error bars indicate SEM. White, black, and gray bars above the panels indicate the light phase (white), dark phase (black), and subjective dark phase, respectively. (**b**) indicates the anatomical structure of the compound eye and a part of the optic lobe. *CE* compound eye, *ON* optic nerve, *La* lamina, *Me* medulla, *OS* optic stalk. (**c**) The ERG rhythm in an intact compound eye and a compound eye with severed ON in *Gryllus bimaculatus*. Redrawn from Ohguro et al. (2021)

includes the pacemaker controlling locomotor activity rhythm in the crickets and cockroaches, is separated from the brain (Tomioka and Chiba 1982; Wills et al. 1985). These findings suggest that the ERG rhythm is controlled by a clock within the optic lobe-compound eye complex. Recently, Ohguro et al. (2021) showed that the expression of the clock genes *per*, *tim*, *cry2*, and *cyc* was rhythmic in the compound eye under light-dark (LD) cycles and constant darkness (DD) in the cricket *Gryllus bimaculatus* (Fig. 6.2). The severance of optic nerve damage weakened the expression of these clock genes. However, more than half of a compound eye with severed optic nerves showed a significant circadian ERG rhythm, although

the amplitude was lowered (Fig. 6.2). This study clarified that the compound eye of the cricket houses an autonomous circadian oscillator that controls the ERG rhythm and its oscillation is affected by the central clock in the optic lobe.

In *G. bimaculatus*, *opsin* genes are rhythmically expressed in the compound eye (Komada et al. 2015), suggesting that they may also contribute to circadian sensitivity in the compound eyes. In the compound eyes of the cotton bollworm, a nocturnal moth *Helicoverpa armigera*, the transcription levels of three *opsin* genes encoding opsins with a peak absorbance at ultraviolet (UV, 300–400 nm), blue (400–500 nm), and long wavelengths (LW, 500–600 nm), respectively, exhibited a daily rhythm with a different pattern (Yan et al. 2014). Under LD cycles, transcript levels of UV- and blue-sensitive *opsin* peaked after lights on, that is, early in the morning, and decreased to the trough during the light phase, whereas transcript levels of LW-sensitive *opsin* tended to decrease during the day and increase at night. The rhythmic expression of *opsin* genes persisted for 1 day under DD but dampened 2 days after transfer to DD or constant light (LL) (Yan et al. 2014), suggesting that the expression of *opsin* genes is under the control of the circadian clock, which attenuates under constant conditions. The authors speculated the biological significance of daily changes in opsin mRNA levels from the behavioral patterns of nocturnal moths. The light of short wavelengths like UV and blue light is strong during the day. Increased transcript levels of UV- and blue-sensitive *opsin*s, therefore, are beneficial to the night-active moth because they can recognize light of a short wavelength to avoid UV damage and restrict their activity to the night. The increase in transcript levels of LW-sensitive *opsin* at night may be useful in dim-light environments. In *H. armigera*, the cyclic expression of core clock genes *cry1*, *cry2*, *per*, and *tim* was also observed under LD cycles and DD in the compound eyes, suggesting that the peripheral circadian clock resides in the eyes and might contribute to the rhythmic expression of *opsin* genes (Yan et al. 2014, 2019). Whether the peripheral clocks in the compound eyes are responsible for the rhythmic expression of opsins should be addressed in future studies.

Rhythmic changes in sensitivity to light are also based on morphological changes in the compound eyes. In *G. bimaculatus*, the rhabdomere size is larger at night than during the day, and this diurnal morphological change might lead to an ERG rhythm (Sakura et al. 2003). Circadian changes in neuronal morphology have been observed in flies, including the housefly *Musca domestica*, blowfly *Calliphora vicina*, and fruit fly *D. melanogaster* (Pyza and Meinertzhagen 1993, 1995, 1999; Pyza and Cymborowski 2001). For example, in *D. melanogaster*, in the cross-sectional area of L1 and L2 monopolar cells, first-order interneurons in the lamina increase in size with two peaks in the morning and evening (Pyza and Meinertzhagen 1999). The dendrite length of L2 exhibits circadian changes, being the longest in the morning and evening (Górska-Andrzejak et al. 2005). This rhythm is abolished in the per^0 mutant, indicating the involvement of core clock genes in the rhythmic regulation of neuronal morphology. Such circadian plasticity in the fly's visual system is modulated by the pigment dispersing factor (PDF) and ion transport peptide (ITP) produced by the fifth sLNv, a clock neuron located in the brain that projects to the lamina (Damulewicz and Pyza 2011; Damulewicz et al. 2013, 2015). The expression

of clock genes and clock-controlled genes, such as *Atpα*, *nrv2* (encoding α and β subunits of Na^+/K^+-ATPase, respectively, a major Na^+ pump in the cells), *brp* (encoding Bruchpilot, presynaptic scaffolding protein), and *Pdfr* (encoding PDF receptor), was confirmed in the retina and lamina (Damulewicz et al. 2013, 2015). These clock-controlled genes and their protein products seem to play a crucial role in circadian rhythms in the lamina and contribute to morphological changes in the eye (Pyza et al. 2004; Górska-Andrzejak et al. 2009). Thus, sensitivity to light is precisely modulated by peripheral and central clocks through several levels of gene expression and morphological changes because light information contributes to not only visually guided behavior but also the photic entrainment of the circadian clock.

Phototransduction consumes a large amount of oxygen and generates high levels of reactive oxygen species in the retina (Chartier et al. 2012). Damulewicz et al. (2017) showed that the mRNA levels of *heme oxygenase*, an enzyme that catalyzes the breakdown of heme to produce biliverdin, iron, and carbon monoxide and serves as a cytoprotective and anti-apoptotic agent (Loboda et al. 2008), exhibit circadian changes in the *Drosophila* retina. Diurnal expression of *heme oxygenase* is abolished in the per^0 mutant. Interestingly, heme oxygenase also regulates the circadian clock and protects photoreceptors against DNA damage-induced degeneration. The authors suggested that the peripheral clock in the retina probably regulates the circadian expression of *heme oxygenase* but the central clock may also play a role (Damulewicz et al. 2015). In any case, the peripheral clocks in the compound eye control various local physiologies.

Recently, the peripheral clocks in the compound eyes have been shown to affect the regulation of sleep patterns (Damulewicz et al. 2020). Furthermore, in flies, disruption of the peripheral clock in the eye triggered arrhythmic locomotor activity or shortened the free-running period of the locomotor activity rhythm. Blocking neurotransmission from the retina photoreceptors and disruption of the clock in *glass*-expressing cells decreased the amplitude of PER cycling in sLNv and lLNv, the essential pacemakers of the central clock in the brain, most likely by changing light transmission to clock neurons (Fig. 6.3) (Damulewicz et al. 2020). Thus, the peripheral clocks in the compound eye regulate daily changes in the visual system and, in turn, alter behavior.

6.3.2 Antenna and Proboscis

Several studies have shown that the antennae harbor circadian clocks that regulate daily rhythmic changes in the olfactory sense as well as sun-compass orientation. Olfaction is crucial for insects to detect various odors, including sex pheromones for mating and food-related chemicals. Insects usually have a pair of major olfactory sensory organs, that is, the antennae and maxillary palps. They are covered by sensilla. Each sensillum contains multiple olfactory receptor neurons (ORNs) (Singh et al. 2019). Olfactory information is conveyed directly from ORNs to the

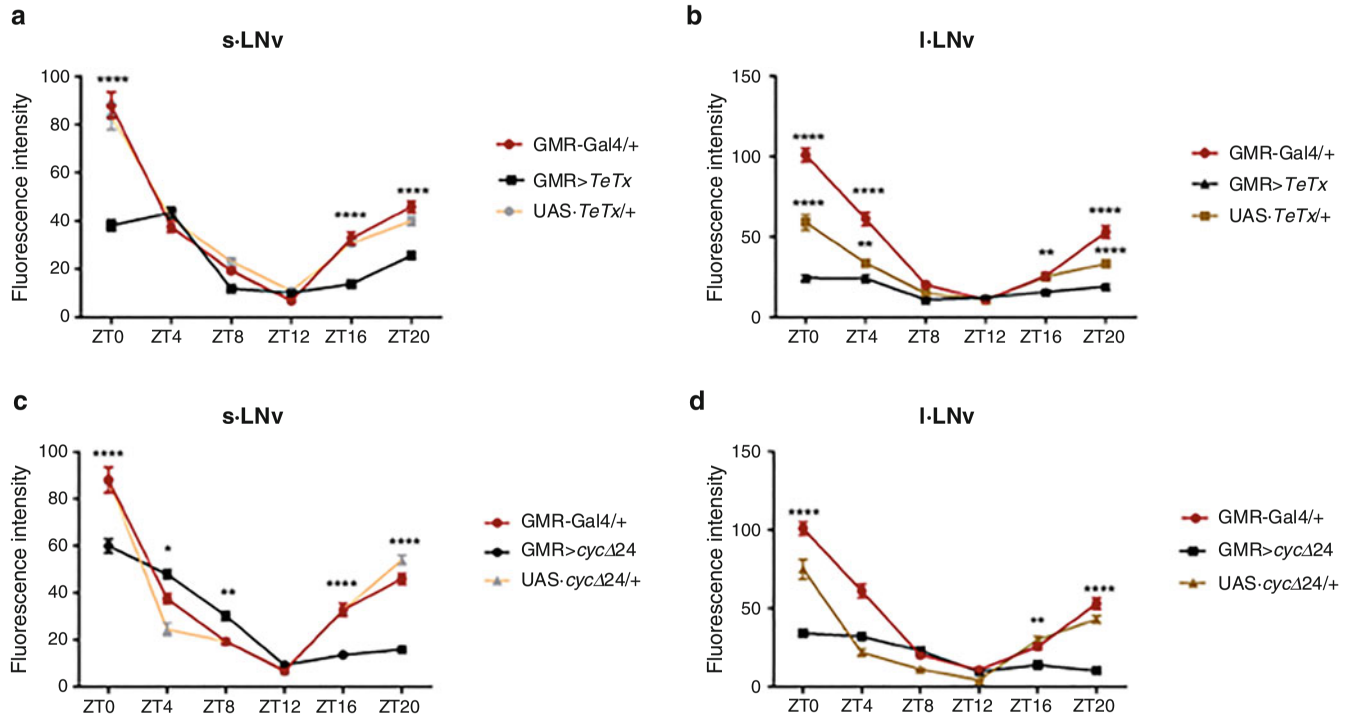

Fig. 6.3 Peripheral clocks located in the photoreceptors affect PER expression in the central clock neurons in *Drosophila melanogaster*. The immunofluorescence signal intensity for PER was measured in the central clock neurons, LNvs (sLNvs and lLNvs). When neurotransmission from retinal photoreceptors was blocked using GMR > *TeTx* (**a**, **b**), the amplitude of PER expression rhythm was significantly reduced compared with control flies carrying GMR-Ga4/+ or UAS-*TeTx*/+. A similar reduction was also observed when the clock of the photoreceptor was disrupted using GMR > *cycΔ24* (**c**, **d**) compared with control flies carrying GMR-Gal4/+ or UAS-*cycΔ24*/+. Asterisks (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$) show statistically significant differences between experimental flies and controls at specific time points. From Damulewicz et al. (2020) under CC-BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

antennal lobe, the first major information processing center in the deutocerebrum, and then transferred to higher brain centers (Singh et al. 2019). In several insects, the olfactory system is under circadian control.

An electroantennogram (EAG) is a record of the odor-induced electrical activity in an antenna. In various insects, the EAG response exhibits a circadian rhythm. In *D. melanogaster*, rhythmic *per* expression was observed in isolated antennae, which persisted under DD and entrained to LD cycles (Plautz et al. 1997). Analysis of EAG in response to ethyl acetate, a food-related odor, revealed that in *Drosophila*, the olfactory response exhibited a circadian rhythm with a peak in the middle of the night. The EAG rhythms were abolished in per^0 and tim^0 mutants, the null mutants for *per* and *tim*, respectively, showing that clock genes driving the central clock are also important for ticking in the peripheral clock. Olfactory rhythms are also abolished in *per* 7.2:2 transgenic flies, in which *per* expression is restricted to only the lateral neurons (LNvs; the central clock cells) in the brain (Krishnan et al. 1999). Tanoue et al. (2004) showed that olfactory sensory neurons are not only necessary but also sufficient for olfactory rhythms. Targeted ablation of LNvs using apoptosis-promoting factors did not affect the EAG rhythms. However, targeted disruption of the circadian clock by a dominant negative form of CLK or CYC in antennal neurons abolished the EAG rhythm. Targeted rescue of olfactory sensory neurons in cyc^{01} flies by expressing wild-type CYC recovered normal EAG rhythm. These findings suggest that the peripheral clock in olfactory sensory neurons operates autonomously and independently from the central clock. Moreover, they suggest that odorant receptors (ORs) and/or OR-dependent processes are under clock control. The mRNA levels of *G-protein-coupled receptor kinase 2* (*Gprk 2*), a member of the family of serine/threonine kinases modulating G-protein-coupled receptors, and its protein expression were shown to be regulated by the circadian clock in the antenna (Tanoue et al. 2008). Circadian clock-dependent rhythms in GPRK2 levels control the rhythmic accumulation of odorant receptors in the dendrites of olfactory sensory neurons. The levels of GPRK2 determined the amplitude of EAG responses to ethyl acetate in basiconic sensillae, suggesting that clock-dependent rhythms in GPRK2 contribute to rhythm generation in EAG responses (Tanoue et al. 2008). Coherence of the activity rhythm was observed when the flies were housed in a group. For coherence of the activity rhythm, the olfactory system is necessary, suggesting that the antennal peripheral clock may play an important role in circadian behavior (Levine et al. 2002).

In the cockroach *Rhyparobia* (*Leucophaea*) *maderae*, the EAG amplitude in response to food-related odors exhibits a circadian rhythm with a peak early in the morning. In addition, the spike frequency of ORNs exhibits circadian rhythms with peak activity early in the day. Ablation of the optic lobes renders a loss of amplitude in the EAG rhythm, indicating that the rhythm is controlled by the central clock in the optic lobe (Page and Koelling 2003). In contrast, the rhythm in the spike frequency of ORNs persisted even after the ablation of the optic lobes (Saifullah and Page 2009). These findings indicate that a circadian clock outside the optic lobes, probably located in the individual ORN, could control ORN responses. The circadian clock in the optic lobe likely synchronizes the phase of each ORN clock.

Females of *R. maderae* exhibited a circadian rhythm of olfactory response to male sex pheromone-related odors, with a peak in the early subjective day and a trough near dusk (Rymer et al. 2007). The phase of the EAG rhythm was essentially identical to that obtained using food-related odors (Page and Koelling 2003). Mating behavior also showed a circadian rhythm, but the mating was consistent with a trough when the EAG response peaked (Rymer et al. 2007). Thus, it is not clear whether the EAG rhythm contributes to the behavioral response rhythm in cockroaches.

Pheromonal communication is extensively regulated by circadian clocks in moths (reviewed in Levi-Zada and Byers 2021). The rhythmic expression of clock genes in the antenna of moths, such as *Manduca sexta*, *Mamestra brassicae*, *Bombyx mori*, and *Spodoptera littoralis*, has been shown using various methods (Iwai et al. 2006; Merlin et al. 2006, 2007; Schuckel et al. 2007). These studies suggest that the peripheral clock resides in the antenna of moths. The EAG response to sex pheromones exhibited circadian rhythms in *S. littoralis* and *M. sexta*, implying the possibility of the presence of a peripheral clock in the antenna in the moths (Hoballah et al. 2005; Merlin et al. 2007; Fenske et al. 2018), although the output physiological rhythm is unclear. Whether the antenna of the moths harbors peripheral clocks should be addressed in future studies.

In the monarch butterfly *Danaus plexippus*, the antennal circadian clock is involved in navigation during long-distance migration. The butterflies migrate from the northern part of North America to Mexico during the fall. During migration, they use the sun compass for navigation and the circadian clock to compensate for the positional changes of the sun in the sky. Merlin et al. (2009) showed that covering the bilateral antennae of the butterflies with black paint or bilateral antennal removal eliminated the correct orientation against the sun, suggesting that the antennal peripheral clock plays an important role in the circadian compensation of the sun compass required for migration. A single functional antenna was sufficient for sun compass orientation; however, antennae with conflicting timing by painting it black disrupted sun compass orientation (Guerra et al. 2012). These results suggest that clock outputs from each antenna are processed and integrated into the circadian compensated sun compass circuit in the brain, which is not yet fully understood (Guerra et al. 2012). Further investigations are needed to elucidate the neuronal circuit of the circadian compensated sun compass.

In *D. melanogaster*, the proboscis contains a circadian clock related to circadian control of the gustatory system. *Drosophila* senses taste via gustatory receptors (GRs) expressed in gustatory receptor neurons (GRNs) on the proboscis, leg, wing margins, and ovipositors. The expression of clock genes was detected in the vicinity of proboscis GRs using *per*-driven green fluorescent protein, and its oscillation was observed in individually cultured proboscis under LD cycles and DD (Plautz et al. 1997). The amplitude of the rhythm was damped under DD but recovered after retransfer to LD cycles, suggesting that the photosensitive circadian clock is localized in the proboscis (Plautz et al. 1997). The electrophysiological responses (spike amplitude, frequency, and duration) of GRNs to food stimuli exhibit a circadian rhythm (Chatterjee et al. 2010). GRNs are most sensitive to tastants in the morning

when the flies are in the active phase and feeding activity is maximal, suggesting that the peripheral clock in the GRNs plays an important role in food detection (Chatterjee et al. 2010). When food-related chemicals contact chemoreceptors, flies extend their proboscis to attempt feeding. This behavior is called the proboscis extension reflex (PrER) response. The PrER responses are under circadian control, and the peripheral clocks in the GRNs are sufficient and necessary to drive the PrER rhythm. The GRN clocks generate the circadian rhythm of *Gprk 2* expression rhythm to peak at night, indicating that a similar mechanism for ORNs is functional in GRNs as well. When the GRN clocks were disrupted, feeding and activity increased, as in a starvation state (Chatterjee et al. 2010).

6.3.3 Digestive Organs, Excretory Organs, and Fat Body

The digestive system of insects consists of a long enclosed tube called the alimentary canal, which is divided into three regions: the foregut, midgut, and hindgut. The foregut consists of the esophagus, crop, and proventriculus, whereas the hindgut consists of the ileum and rectum (Chapman 2012). Malpighian tubules (MTs) are elongated and are normally located in the posterior part of the alimentary canal. The fat body is a unique organ in insects and is distributed throughout the body, preferentially underneath the integument and surrounding the gut and reproductive organs. The fat body plays multiple roles in storage, metabolism, and detoxification (Arrese and Soulages 2010; Chapman 2012).

In several insects, the expression of clock genes and proteins has been detected in digestive and excretory systems and the fat body. For example, clock genes *cyc*, *Clk*, *Pdp1*, and *cry2* are expressed in the gut of *Pyrrhocoris apterus* (Bajgar et al. 2013). Expression of *per* and *tim* was detected in the anterior stomach, midgut, and MTs; however, no circadian expression was observed in the MTs of *G. bimaculatus* (Uryu and Tomioka 2010). PER and TIM were detected in the esophagus, proventriculus, hindgut, MTs, rectum, and fat body of *D. melanogaster* (Giebultowicz et al. 2001).

Many insects show rhythmic feeding (Suszczynska et al. 2017). It is natural for the activity of enzymes related to digestion and detoxification to change with feeding timing. The caterpillar of nocturnal *Spodoptera* spp. *S exigua*, *S. littoralis*, and *S. litura* reared on an artificial diet under LD cycles showed rhythmic feeding behavior peaking at night. In the latter two species, this rhythmic feeding persisted under DD conditions, suggesting that feeding behavior is regulated by a circadian clock in the larvae of both species (Suszczynska et al. 2017; Zhang et al. 2021). To investigate whether the peripheral clock is involved in rhythmic feeding, the expression of several clock genes in the midgut and fat body of *S. littoralis* larvae was assessed. Circadian clock genes were expressed in both the midgut and fat body. However, the expression of the main circadian clock component, *per*, was arrhythmic in the midgut and rhythmic in the fat body. In the midgut, daily expression of genes encoding digestion-related enzymes was observed, and digestive enzyme activities were rhythmic under LD cycles, implying that enzyme activity could be

associated with rhythmic feeding (Suszczynska et al. 2017). In addition, the expression of genes encoding metabolic enzymes was rhythmic in the fat body under LD cycles (Suszczynska et al. 2017). Transcriptomic analyses of *S. litura* larval midguts and fat body under LD cycles showed daily rhythmicity in the transcriptional levels of genes for digestion (e.g., amylase) and detoxification (e.g., CYP450s), with a peak during the daytime when larvae were most inactive without feeding, and their digestion was at its highest levels. Thus, in *S. litura* larvae, night feeding and daytime digestion/detoxification processes are separated by the circadian clock system and may play a protective role (Zhang et al. 2021).

D. melanogaster displays a circadian rhythm during feeding, with a peak in the early morning (Xu et al. 2008). Targeted disruption of clock oscillations in the fat body abolished feeding rhythm—the levels of energy storage decreased, but overall food consumption increased. Microarray analysis showed that the expression of approximately 60% (81/137) of transcripts was regulated by the peripheral clock in the fat body in *D. melanogaster*, indicating that the expression of the remaining 40% of transcripts was controlled by external factors such as light and hormonal signals from the central clock in the brain. The signaling of neuropeptide F (NPF) and insulin-like peptide from the brain drives the rhythmic expression of metabolic genes in the fat body (Barber et al. 2016; Erion et al. 2016; Xu et al. 2011). Dietary restriction (DR) is known to boost fat metabolism and increase life span. DR increased the amplitude of transcripts of the core clock genes and clock protein levels in the brain and peripheral tissues (Katewa et al. 2016). Circadian regulation is critical for the DR-dependent increase in life span (Katewa et al. 2016).

MTs are the excretory and osmoregulatory systems of insects that excrete nitrogenous waste products (Chapman 2012). MTs also function in detoxification and metabolism (Chapman 2012). In *D. melanogaster* MTs, the transcript and protein levels of *per* and *tim* cycle in a circadian manner. Molecular oscillations persist in the MTs of decapitated flies and in those cultured in vitro (Giebultowicz and Hege 1997; Hege et al. 1997; Giebultowicz et al. 2000) and are entrained to LD cycles even in vitro (Giebultowicz et al. 2000). When MTs were transplanted to a host fly that had been kept under LD cycles with antiphase, the transplanted MTs maintained their own phase (Giebultowicz et al. 2000) (Fig. 6.4). These results suggest that MTs house an autonomous circadian oscillator that is independent of the central clock (Giebultowicz et al. 2000). However, the circadian output physiology of MTs in *Drosophila* remains unclear.

6.3.4 Epidermis: Cuticle Deposition

In several insects, cuticle deposition is controlled by the circadian clock. The cuticle, a tough membrane secreted by the epidermal cell layer, is composed of cuticular proteins and chitin, a linear polymer mainly composed of N-acetylglucosamine (Neville 1975; Chapman 2012). Multiple chitin chains made from chitin molecules assemble to form chitin fibrils that are wrapped by cuticular proteins (chitin-protein

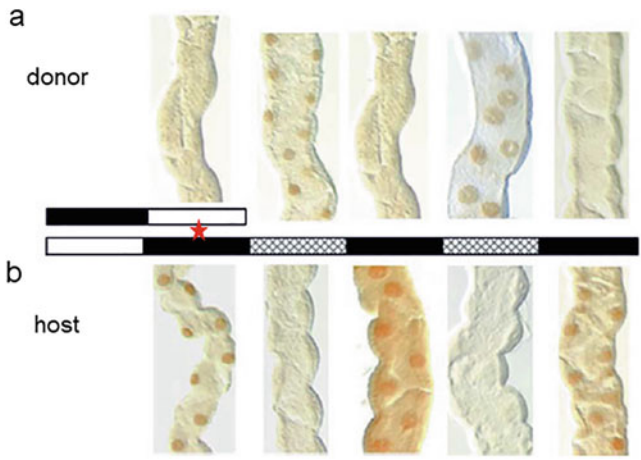

Fig. 6.4 Expression of TIM protein in the *Drosophila melanogaster* Malpighian tubules (MTs) transplanted from flies reared in LD cycles into flies reared in reversed LD. Transplantation was conducted at a red star, and the host's and transplanted donor's MTs were collected every 12 h for 2 days. Left most panels in a and b show representative examples of TIM staining in MTs in donor and host flies, respectively, before the operation, and the following photographs show stainings of transplanted donor MTs (**a**) and host MTs (**b**) collected at subjective day (shaded bars) and subjective night (black bars) after transplantation. White and black bars indicate day and night, respectively. Note that the transplanted MTs maintained the rhythm with their original phase. From Giebultowicz et al. (2000) with permission from Elsevier

fibrils). These chitin-protein fibrils form a cuticle layer with a sheetlike structure (Fig. 6.5). The insect exoskeleton consists of three cuticle layers: epicuticle, exocuticle, and endocuticle. After ecdysis, the endocuticle, closest to the epidermal cell layer, grows due to the accumulation of alternating lamellar layers of helicoidally oriented fibrils and non-lamellar layers of unidirectionally oriented fibrils of chitin fibers in different orientations (Neville 1975) (Fig. 6.5). This process is called cuticle deposition or cuticle growth. Many insects exhibit rhythmic cuticle deposition (Neville 1983). Cuticle deposition occurs in a circadian manner in some insects, such as cockroaches, locusts, and bean bugs, and is called cuticle deposition rhythm (Lukat 1978; Neville 1983; Weber 1995; Ikeno et al. 2010). Two layers with different chitin orientations in the endocuticle can be distinguished as alternating dark-bright bands under a Nomarski differential interference contrast microscope or polarization microscope because of different light transmission properties after the removal of cuticle protein (Neville 1983).

The cuticle deposition rhythms are investigated by observing alternating bright and dark layers and have been intensively studied in locusts, cockroaches, and fruit flies. The cuticle deposition rhythm was shown to be controlled by the peripheral oscillator in the epidermis of locusts and cockroaches in vitro culture experiments (Neville 1967; Lukat 1978; Lukat et al. 1989; Weber 1985, 1995). Although the rhythm of cuticle formation is controlled by the circadian clock, the underlying circadian system seems imperfect. In the desert locust *Schistocerca gregaria*,

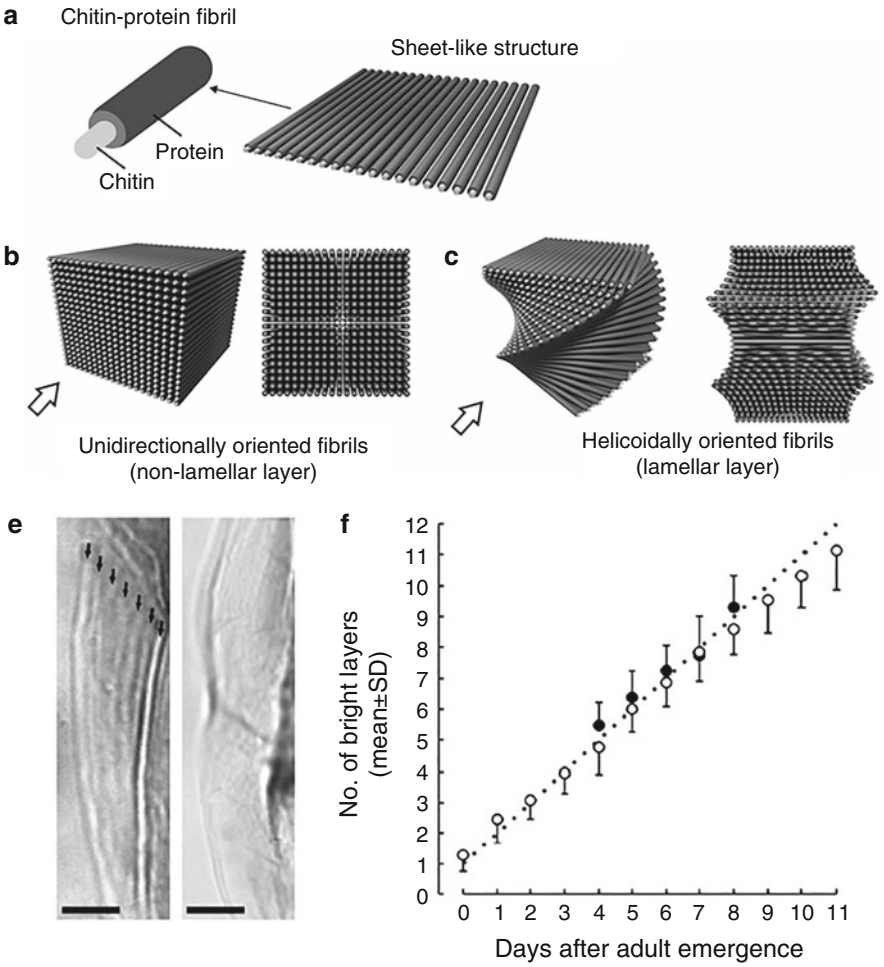

Fig. 6.5 The general structure of insect exoskeleton and the cuticle deposition rhythm in *Drosophila melanogaster*. (**a**) Chitin-protein fibrils form a cuticle layer of a sheet-like structure. (**b**) Non-lamellar layer is composed of unidirectionally oriented fibrils. (**c**) Lamellar layer is composed of helicoidally oriented fibrils. In the left drawing from the arrow, the difference in chitin orientation between the non-lamellar (**b**) and lamellar layer (**c**) is obvious (right). (**d**) Sagittal view of the endocuticle in the third furca of *D. melanogaster* on day 6. Alternating bright and dark layers are observed in wild-type flies kept under LD cycles (left), but not in the per^{01} mutant kept under DD right). Arrows show the seven bright layers. (Scale bars: 10 μm.) (e) The daily increase of growth layers in the endocuticle of wild-type flies under LD cycles (open circles) and DD (filled circles). The dotted line shows the hypothetical values assuming that the bright layer increases at the rate of one per day ($N = 30$–43). From Ito et al. (2008) (Copyright 2008 National Academy of Sciences, USA)

rhythmic cuticle deposition, which can be observed in the hind tibia, persisted under LD cycles and DD for approximately 2 weeks, and the free-running period was temperature-compensated (Neville 1965a, b). These findings suggest that the circadian clock controls the cuticle deposition rhythm. However, alternating cuticle layers are formed in direct response to environmental cycles, such as LD 6:6 and LD 24:24, which are far from 24 h (Neville 1963). This indicates that the direct response to LD cycles is more strongly reflected than the circadian rhythm. In the cockroach *Blaberus craniifer*, the rhythm of cuticle formation is regulated by a circadian clock but does not entrain to LD cycles (Wiedenmann et al. 1986). This indicates that the clock controlling the cuticle deposition rhythm in *B. craniifer* may lack an input system for light information from the environment. The cuticle deposition rhythm is thought to be important for the formation of alternating layers of chitin in different arrangements and for increasing the physical strength of the body wall (Neville 1967, 1975). If the clocks in the epidermal cells involved in cuticle deposition are synchronized to form the same type of cuticle layer, the cuticle deposition rhythm need not be entrained into the environmental cycles. In other words, clocks that control the rhythm of cuticle formation do not necessarily require a perfect clock mechanism with an input system from the environment.

The molecular mechanisms of the cuticle deposition rhythm, including oscillation and entrainment, have been well studied in *Drosophila*. In *D. melanogaster*, daily growth layers were observed in the phragma and the second and third furcae in the thorax (Johnston and Ellison 1982) (Fig. 6.5). They are the apodemata, that is, the internal cuticular processes of the body wall on which the muscles are inserted. In vitro culture experiments and immunohistochemistry for PER demonstrated that the cuticle deposition rhythm was produced by a peripheral circadian clock residing in epidermal cells (Ito et al. 2008). In several clock gene mutant lines, alternating bright and dark cuticle layers were no longer produced, but a uniform cuticle layer was formed (Ito et al. 2008) (Fig. 6.5). This reinforces that the circadian clock in the brain and the epidermal cell clock use the same set of clock genes to oscillate. These results suggest that the epidermal cell clocks may be involved in changing the orientation of chitin fibers to generate two types of cuticular layers (Ito et al. 2008). In *Locusta migratoria*, the helicoidal chitin arrangement changed to a unidirectional chitin arrangement when chitin deacetylase was knocked down (Yu et al. 2016). Although it has been speculated that several factors are involved in controlling chitin arrangement, whether chitin deacetylase is under circadian control should be examined in future studies.

The cuticle deposition rhythm in the furca of *D. melanogaster* was entrained to LD cycles even when the thorax was cultured, suggesting that the photic entrainment system is independent of the central clock and resides in the thorax. The entrainment ability of LD cycles was lost in cry^b mutants, which have defective CRY, and entrainability was rescued by the overexpression of *cry* throughout the body. The cuticle deposition rhythm was still observed in cry^b mutants, in which many other peripheral circadian clocks are arrhythmic (Stanewsky et al. 1998; Ivanchenko et al. 2001; Krishnan et al. 2001; Collins et al. 2006). Thus, only CRY is responsible for light reception and entrainment of the cuticle deposition rhythm in *Drosophila.* The

cuticle deposition rhythm is also entrained to temperature cycles both in vivo and in vitro (Ito et al. 2011). Interestingly, LD cycles and temperature cycles with the same period in which the thermophase coincided with the photophase synergistically entrained the cuticle deposition rhythm, that is, the variation in the number of cuticle layers among individuals was quite small (Ito et al. 2011). These results suggest that the number of cuticle growth layers may be useful for estimating age in wild populations.

6.3.5 Male Reproductive Organ: Sperm Release

It has been shown that many male moths exhibit a circadian rhythm in sperm release and related physiological processes. Rhythm is regulated by the circadian clock residing in the male reproductive system (Giebultowicz et al. 1989; Bebas et al. 2001). During spermatogenesis in lepidopteran insects, both nucleated eupyrene (fertile) and apyrene spermatozoa (infertile) are produced in testicular follicles. Both types of spermatozoa develop as clones. After elongation and differentiation, spermatids develop into eupyrene or apyrene sperm bundles that are enclosed by somatic cyst cells (Friedländer et al. 2005). Under LD cycles, some mature sperm bundles accumulated in the testis are released into the vas deferens once a day within a few hours before the dark period. Sperm bundles migrate from the testis into the upper vasa deferentia (UVD) through the terminal epithelium, which lies between the testicular follicles and the vas deferens (VD). Subsequently, cyst cells degenerate, and their remnants are phagocytosed by barrier cells (Giebultowicz and Hege 1997). Sperm bundles remain in the VD and are coated with glycoproteins that are rhythmically secreted from the VD epithelium (Bebas et al. 2002b). Sperm bundles are then transferred into the seminal vesicles (SV) within a few hours after lights on because of an increase in myogenic muscle contractions of the VD wall (Giebultowicz et al. 1996). The rhythmic expression of vacuolar ATPase alters the pH of the UVD lumen (Bebas et al. 2002a). Daily batches of released sperm accumulate in the duplex, which is a storage organ from which bundles are retrieved during mating. The rhythm of sperm release was first reported in the flour moth, *Ephestia kuehniella* (Riemann et al. 1974). This rhythm has now been confirmed in several species, including the cotton leafworm *S. littoralis* (Bebas et al. 2001), the spongy moth *Lymantria dispar* (Giebultowicz et al. 1988), and the codling moth *Cydia pomonella* (Giebultowicz and Brooks 1998). The rhythm of sperm release was shown to persist when testis-VD complexes of *L. disper* were cultured in LD cycles or DD (Giebultowicz et al. 1989). The phase of this rhythm is reset by shifting the LD cycles in vitro (Giebultowicz et al. 1989). Thus, the testis-VD complex contains a light-entrainable circadian oscillator that regulates the sperm release rhythm. Such a peripheral oscillator has also been confirmed in *S. littoralis* as well (Bebas et al. 2001). Under LL, the rhythm of sperm release and related physiological rhythms are disrupted both in vivo and in vitro in *S. littoralis* (Bebas et al. 2001). Under LL, the amount of sperm moving from the testes was reduced, and the

dispersal of spermatozoa from sperm bundles was impaired. The sperm of males kept under LL conditions did not have fertilizing ability (Giebultowicz et al. 1990; Bębas and Cymborowski 1999). These findings suggest that the rhythm of sperm release is crucial for normal reproduction in the moths.

The importance of rhythmic sperm release has also been shown in the desert locust *S. gregaria* and the fruit fly *D. melanogaster* (Beaver et al. 2002; Tobback et al. 2012). Expression analyses have revealed rhythmic expression of *per* mRNA and PER protein at the testis level in *C. pomonella*, *B. mori*, and *S. littoralis* (Gvakharia et al. 2000; Iwai et al. 2006; Kotwica et al. 2009). Additionally, in *S. littoralis*, an in vitro *per* RNA interference caused a delay in sperm release, indicating that a molecular oscillator plays an essential role in regulating rhythmic sperm release in *S. littoralis* (Kotwica et al. 2009).

6.3.6 Prothoracic Gland

Molting and eclosion behaviors are strictly regulated by the circadian clock system in many insects (Steel and Vafopoulou 2002). The prothoracic gland (PG) is an important endocrine organ that synthesizes and immediately releases ecdysteroids that regulate growth, molting, and metamorphosis. Ecdysteroid synthesis is mainly triggered by prothoracicotropic hormone (PTTH), a cerebral neuropeptide that surges before molting (Chapman 2012).

The circadian clock system resides in the PG in several insects. The first series of studies were performed in the moth *Samia cynthia ricini* without measuring hormonal levels. With ligation between the head and thorax, PTTH release is involved in the timing of the gut purge, which occurs at the end of the final larval instar to evacuate the gut before pupation (Fujishita and Ishizaki 1982). Mizoguchi and Ishizaki (1982) subsequently showed that the gut purge is regulated by the circadian clock with photic entrainment ability in the PG using localized illumination and transplantation of the PG.

The involvement of the circadian rhythm in the production and secretion of ecdysteroids has been shown in the blood-sucking bug *Rhodnius prolixus* by direct measurement of hormone titers in the hemolymph using radioimmunoassay (Ampleford and Steel 1985; Vafopoulou and Steel 1991). PTTH was rhythmically released under circadian control throughout most of the larval-adult development (Vafopoulou and Steel 1996a, b). When a single PG was cultured in vitro, rhythmic ecdysteroid synthesis (and secretion) and its persistence under DD conditions were observed (Vafopoulou and Steel 1998), suggesting that PG has an endogenous circadian clock and that rhythmic PTTH release is not required for rhythmic steroid synthesis. When the bugs were kept under LL conditions, the circadian rhythm of PTTH release and ecdysteroid synthesis was abolished. Rhythmic PTTH release and ecdysteroid synthesis were immediately restored after transfer to DD (Vafopoulou and Steel 2001). To examine the possible involvement of PTTH in rhythm induction in PGs, both hormonal rhythms were measured in bugs paralyzed by tetrodotoxin,

Table 6.2 Hierarchical relationship between the brain clock and the PG clock in *Drosophila melanogaster*

	Free-running period		
Flies	Brain clock	PG clock	Eclosion rhythm
tim>dbt^{L}	Long	Long	Long
tim>dbt^{L}*phm-gal80*	Long	Normal	Long
phm>dbt^{L}	Normal	Long	Normal
tim>w	Normal	Normal	Normal
tim>w+phm-gal80	Normal	Normal	Normal
phm>w	Normal	Normal	Normal
tim>dbt^{S}	Short	Short	Short
tim > dbt^{S} *+ phm-gal80*	Short	Normal	Short
Phm >dbt^{S}	Normal	Short	Normal

Eclosion rhythm is regulated by the central clock in the brain and peripheral clocks in the PG. Phenotypes of the pattern of the eclosion rhythm are simplistically shown when all clocks or only the brain or PG clocks have been sped up or slowed down. Eclosion rhythm finally follows the free-running period of the brain clock. From Selcho et al. (2017)

which eliminates action potentials in peripheral nerves. In paralyzed bugs, no PTTH release was observed, while ecdysteroid synthesis occurred but with a phase different from that in normal bugs (Vafopoulou and Steel 2001). These results suggest that both brain and PG oscillators are likely photosensitive and rhythmically released PTTH regulates the phase of rhythmic ecdysteroid synthesis.

In *D. melanogaster*, the timing of adult emergence from the pupae, termed eclosion, is controlled by a circadian system that consists of two hierarchically organized oscillators located in the LNvs and PG, respectively. The targeted disruption of the clock either in LNvs or PG using *tim* overexpression, which disrupts circadian oscillation, rendered the eclosion arrhythmic (Myers et al. 2003). The eclosion rhythm and molecular oscillation of the TIM in the PG were diminished when LNvs were ablated. The results suggest that both LNv and PG clocks are necessary for the eclosion rhythm and that the PG clock is a slave oscillator driven by the master LNv clock. To further dissect this relationship, Morioka et al. (2012) observed the clock gene transcript rhythm and posttranscriptional rhythm in PG in vitro and found that the PER oscillation of the PG clock receives light information from the central clock, but TIM oscillation does not. Interestingly, under DD conditions, TIM maintained its oscillation, while PER could not in PG, although both molecular oscillations were robust under LD cycles. Control from the central nervous system may contribute to maintaining the robust coordinated oscillations of the PER and TIM, which are otherwise dissociated from each other. Thus, the oscillator in *Drosophila* PG is largely governed by the central clock. Disrupting the clock either in the brain or PG by overexpressing a dominant negative form of CYC impaired the eclosion rhythm, suggesting that both the brain and PG are necessary for eclosion rhythms (Selcho et al. 2017). Manipulating the speed of the clock by expressing different *double-time* alleles only in the brain was sufficient to change the period of the eclosion rhythm, whereas the speeding up or slowing down

of the PG clock did not alter the eclosion rhythm (Table 6.2). These findings indicate the dominant role of the central pacemaker over the peripheral clock in the PG (Selcho et al. 2017). The central pacemaker neurons, sLNvs, transmit timing information to the PG via PTTH-producing neurons. sLNvs communicate with PTTH neurons via the short neuropeptide F (sNPF). PTTH neurons projecting to the PG secrete PTTH and activate ecdysone synthesis in the PG to control the eclosion rhythm. Knockdown of *ptth* in PTTH neurons or the PTTH receptor *torso* in the PG leads to arrhythmic emergence (Selcho et al. 2017). Thus, the circadian system with hierarchically coupled oscillators in the brain and PG drives the eclosion rhythms.

6.3.7 *Oenocyte*

Oenocytes are large secretory cells present on the inner surface of the abdomen in insects. Oenocytes synthesize long-chain fatty acids to produce hydrocarbons and pheromones (Huang et al. 2022). Rhythmic expression of core clock genes was observed in oenocytes under LD and DD conditions in *D. melanogaster* (Krupp et al. 2008). Cyclic expression was abolished in the per^{0} mutant, lacking *per* in DD. Cyclic expression was never restored in the *per*7.2:2 transgenic flies, which express PER only in the subset of the central clock neurons and not in the peripheral tissues. These results suggest that oenocytes contain a *per*-dependent peripheral clock. The oenocyte circadian clock is involved in the rhythmic expression of cuticular pheromones, as limiting *per* expression only to the central pacemaker using a transgenic *per*7.2:2 strain or disrupting the molecular oscillation in oenocytes by overexpressing a dominant negative form of CYC abolishes this rhythm. The oenocyte clock regulates the rhythmic synthesis and release of cuticular monosaturated hydrocarbons through the circadian expression of *desaturase1* (*desat1*), a gene required for the production of male cuticular sex pheromones.

The phase of the expression of clock genes in oenocytes was modulated by PDF signaling, and it was altered when PDF signaling was disrupted in pdf^{01} and $pdfr^{5304}$ mutants lacking PDF and PDF receptors, respectively. In these mutants, however, clock gene expression was robustly rhythmic, as in wild-type flies, and the phase relationship among clock genes remained normal (Krupp et al. 2013). These results suggest that the peripheral oscillator in oenocytes is a slave oscillator that can maintain its oscillation independent of the central master oscillator, but its phase is modulated by the central master clock.

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6.4 Summary and Perspective

The discovery of peripheral clocks had preceded in the insects than in other animals. Initially, the central clocks were studied more extensively than the peripheral clocks to understand the basis of the molecular mechanism for oscillation and entrainment. Some groups, however, have been working intensively on peripheral clocks in insects. Over the last two decades, our understanding of the peripheral circadian system and its relevant output physiology has advanced substantially. In parallel with understanding the features of peripheral clocks, including the mechanisms for oscillation and entrainment, knowledge of the relationship between the central and peripheral clocks has increased. As described in this chapter, there are several types of peripheral clocks (Ito and Tomioka 2016). These are (1) self-sustained and are not affected by the central oscillator (e.g., Giebultowicz et al. 2000); (2) autonomous, but their phase is modified by the central oscillator (e.g., Erion et al. 2016; Krupp et al. 2008, 2013; Saifullah and Page 2009), (3) not self-sustained and are driven by the central clock (e.g., Myers et al. 2003; Uryu and Tomioka 2010); or (4) affected by the central clocks and, in turn, feed their output back to the central clocks (e.g., Damulewicz et al. 2020). Why these circadian systems operate in the body is an interesting question. The adaptive significance and evolution of the types of peripheral clocks should be addressed.

Interestingly, peripheral clocks have an impact on a wide variety of physiologies and, in turn, behaviors and life spans, as described above. Due to technical limitations, most studies that uncover the detailed features of peripheral clocks and their relevance to central clocks have been conducted mainly in *D. melanogaster*.

Therefore, in insects other than *Drosophila*, the function of some peripheral clocks, especially their output physiology, remains to be elucidated. A survey of tissue-specific clock-controlled transcriptomes would help clarify this issue.

References

Ampleford EJ, Steel CGH (1985) Circadian control of a daily rhythm in hemolymph ecdysteroid titer in the insect *Rhodnius prolixus* (Hemiptera). Gen Comp Endocrinol 59:453–459. https://doi.org/10.1016/0016-6480(85)90404-6

Arrese EL, Soulages JL (2010) Insect fat body: energy, metabolism, and regulation. Annu Rev Entomol 55:207–225. https://doi.org/10.1146/annurev-ento-112408-085356

Bajgar A, Jindra M, Dolezel D (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc Nat Acad Sci USA 110:4416–4421. https://doi.org/10.1073/pnas.1217060110

Barber AF, Erion R, Holmes TC, Sehgal A (2016) Circadian and feeding cues integrate to drive rhythms of physiology in *Drosophila* insulin-producing cells. Genes Dev 30:2596–2606. https://doi.org/10.1101/gad.288258.116

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. Nature 312:752–754. https://doi.org/10.1038/312752a0

Beaver LM, Gvakharia BO, Vollintine TS, Hege DM, Stanewsky R, Giebultowicz JM (2002) Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 99:2134–2139. https://doi.org/10.1073/pnas.032426699

Bębas P, Cymborowski B (1999) Effect of constant light on male sterility in the cotton leafworm *Spodoptera littoralis*. Physiol Entomol 24:165–170. https://doi.org/10.1046/j.1365-3032.1999.00127.x

Bebas P, Cymborowski B, Giebultowicz JM (2001) Circadian rhythm of sperm release in males of the cotton leafworm, *Spodoptera littoralis*: in vivo and in vitro studies. J Insect Physiol 47:859–866. https://doi.org/10.1016/S0022-1910(01)00058-0

Bebas P, Cymborowski B, Giebultowicz JM (2002a) Circadian rhythm of acidification in insect vas deferens regulated by rhythmic expression of vacuolar h(+)-ATPase. J Exp Biol 205:37–44. https://doi.org/10.1242/jeb.205.1.37

Bebas P, Maksimiuk E, Gvakharia B, Cymborowski B, Giebultowicz JM (2002b) Circadian rhythm of glycoprotein secretion in the vas deferens of the moth, *Spodoptera littoralis*. BMC Physiol 2:15. https://doi.org/10.1186/1472-6793-2-15

Chapman RF (2012) The insects: structure and function, 5th edn. Cambridge University Press, Cambridge

Chartier FJ-M, Hardy ÉJ-L, Laprise P (2012) Crumbs limits oxidase-dependent signaling to maintain epithelial integrity and prevent photoreceptor cell death. J Cell Biol 198:991–998. https://doi.org/10.1083/jcb.201203083

Chatterjee A, Tanoue S, Houl JH, Hardin PE (2010) Regulation of gustatory physiology and appetitive behavior by the *Drosophil*a circadian clock. Curr Biol 20:300–309. https://doi.org/10.1016/j.cub.2009.12.055

Chen B, Meinertzhagen IA, Shaw SR (1999) Circadian rhythms in light-evoked responses of the fly's compound eye, and the effects of neuromodulators 5-HT and the peptide PDF. J Comp Physiol A 185:393–404. https://doi.org/10.1007/s003590050400

Chen DM, Christianson JS, Sapp RJ, Stark WS (1992) Visual receptor cycle in normal and *period* mutant *Drosophila*: microspectrophotometry, electrophysiology, and ultrastructural morphometry. Vis Neurosci 9:125–135. https://doi.org/10.1017/s0952523800009585

Collins B, Mazzoni EO, Stanewsky R, Blau J (2006) *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor. Curr Biol 16:441–449. https://doi.org/10.1016/j.cub.2006.01.034

Damulewicz M, Ispizua JI, Ceriani MF, Pyza EM (2020) Communication among photoreceptors and the central clock affects sleep profile. Front Physiol 11:993. https://doi.org/10.3389/fphys.2020.00993

Damulewicz M, Loboda A, Bukowska-Strakova K, Jozkowicz A, Dulak J, Pyza E (2015) Clock and clock-controlled genes are differently expressed in the retina, lamina and in selected cells of the visual system of *Drosophila melanogaster*. Front Cell Neurosci 9:353. https://doi.org/10.3389/fncel.2015.00353

Damulewicz M, Loboda A, Jozkowicz A, Dulak J, Pyza E (2017) Interactions between the circadian clock and heme oxygenase in the retina of *Drosophila melanogaster*. Mol Neurobiol 54:4953–4962. https://doi.org/10.1007/s12035-016-0026-9

Damulewicz M, Pyza E (2011) The clock input to the first optic neuropil of *Drosophila melanogaster* expressing neuronal circadian plasticity. PLoS One 6:e21258. https://doi.org/10.1371/journal.pone.0021258

Damulewicz M, Rosato E, Pyza E (2013) Circadian regulation of the Na+/K+-ATPase alpha subunit in the visual system is mediated by the pacemaker and by retina photoreceptors in *Drosophila melanogaster*. PLoS One 8:e73690. https://doi.org/10.1371/journal.pone.0073690

Emery IF, Noveral JM, Jamison CF, Siwicki KK (1997) Rhythms of *Drosophila period* gene expression in culture. Proc Natl Acad Sci U S A 94:4092–4096. https://doi.org/10.1073/pnas.94.8.4092

Erion R, King AN, Wu G, Hogenesch JB, Sehgal A (2016) Neural clocks and neuropeptide F/Y regulate circadian gene expression in a peripheral metabolic tissue. eLife 5:e13552. https://doi.org/10.7554/eLife.13552

Fenske MP, Nguyen LP, Horn EK, Riffell JA, Imaizumi T (2018) Circadian clocks of both plants and pollinators influence flower seeking behavior of the pollinator hawkmoth *Manduca sexta*. Sci Rep 8:2842. https://doi.org/10.1038/s41598-018-21251-x

Friedländer M, Seth RK, Reynolds SE (2005) Eupyrene and apyrene sperm: dichotomous spermatogenesis in Lepidoptera. Adv Insect Physiol 32:206–308. https://doi.org/10.1016/S0065-2806(05)32003-0

Fujishita M, Ishizaki H (1982) Temporal organization of endocrine events in relation to the circadian clock during larval-pupal development in *Samia cynthia ricini*. J Insect Physiol 28: 77–84. https://doi.org/10.1016/0022-1910(82)90025-7

Górska-Andrzejak J, Keller A, Raabe T, Kilianek L, Pyza E (2005) Structural daily rhythms in GFP-labelled neurons in the visual system of *Drosophila melanogaster*. Photochem Photobiol Sci 4:721–726. https://doi.org/10.1039/b417023g

Górska-Andrzejak J, Salvaterra PM, Meinertzhagen IA, Krzeptowski W, Görlich A, Pyza E (2009) Cyclical expression of Na+/K+-ATPase in the visual system of *Drosophila melanogaster*. J Insect Physiol 55:459–468. https://doi.org/10.1016/j.jinsphys.2009.02.003

Giebultowicz JM, Bell RA, Imberski RB (1988) Circadian rhythm of sperm movement in the male reproductive tract of the gypsy moth, *Lymantria dispar*. J Insect Physiol 34:527–532. https://doi.org/10.1016/0022-1910(88)90194-1

Giebultowicz JM, Blackburn MB, Thomas-Laemont PA, Weyda F, Raina AK (1996) Daily rhythm in myogenic contractions of vas deferens associated with sperm release cycle in a moth. J Comp Physiol A 178:629–636. https://doi.org/10.1007/BF00227376

Giebultowicz JM, Brooks NL (1998) The circadian rhythm of sperm release in the codling moth, *Cydia pomonella*. Entomol Exp Appl 88:229–234. https://doi.org/10.1046/j.1570-7458.1998.00367.x

Giebultowicz JM, Hege DM (1997) Circadian clock in malpighian tubules. Nature 386:664. https://doi.org/10.1038/386664a0

Giebultowicz JM, Ivanchenko M, Vollintine T (2001) Organization of the insect circadian system: spatial and developmental expression of clock genes in peripheral tissues of *Drosophila melanogaster*. In: Denlinger DL, Giebultowicz JM, Saunders DS (eds) Insect timing: circadian rhythmicity to seasonality. Elsevier, Amsterdam, pp 31–42. https://doi.org/10.1016/B978-044450608-5/50035-0

Giebultowicz JM, Ridgway RL, Imberski RB (1990) Physiological basis for sterilizing effects of constant light in *Lymantria dispar*. Physiol Entomol 15:149–156. https://doi.org/10.1111/j.1365-3032.1990.tb00502.x

Giebultowicz JM, Riemann JG, Raina AK, Ridgway RL (1989) Circadian system controlling release of sperm in the insect testes. Science 245:1098–1100. https://doi.org/10.1126/science.245.4922.1098

Giebultowicz JM, Stanewsky R, Hall JC, Hege DM (2000) Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. Curr Biol 10:107–110. https://doi.org/10.1016/s0960-9822(00)00299-2

Guerra PA, Merlin C, Gegear RJ, Reppert SM (2012) Discordant timing between antennae disrupts sun compass orientation in migratory monarch butterflies. Nat Commun 3:958. https://doi.org/10.1038/ncomms1965

Gvakharia BO, Kilgore JA, Bebas P, Giebultowicz JM (2000) Temporal and spatial expression of the *period* gene in the reproductive system of the codling moth. J Biol Rhythm 15:4–12. https://doi.org/10.1177/074873040001500102

Hardin PE (2011) Molecular genetic analysis of circadian timekeeping in *Drosophila*. Adv Genet 74:141–173. https://doi.org/10.1016/b978-0-12-387690-4.00005-2

Hege DM, Stanewsky R, Hall JC, Giebultowicz JM (1997) Rhythmic expression of a per-reporter in the malpighian tubules of decapitated *Drosophila*: evidence for a brain-independent circadian clock. J Biol Rhythm 12:300–308. https://doi.org/10.1177/074873049701200402

Hoballah ME, Stuurman J, Turlings TC, Guerin PM, Connétable S, Kuhlemeier C (2005) The composition and timing of flower odour emission by wild petunia axillaris coincide with the

antennal perception and nocturnal activity of the pollinator *Manduca sexta*. Planta 222:141–150. https://doi.org/10.1007/s00425-005-1506-8

Huang K, Liu Y, Perrimon N (2022) Roles of insect oenocytes in physiology and their relevance to human metabolic diseases. Front Insect Sci 2:859847. https://doi.org/10.3389/finsc.2022.859847

Ikeno T, Tanaka SI, Numata H, Goto SG (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol 8:116. https://doi.org/10.1186/1741-7007-8-116

Ito C, Goto SG, Shiga S, Tomioka K, Numata H (2008) Peripheral circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster*. Proc Natl Acad Sci U S A 105:8446–8451. https://doi.org/10.1073/pnas.080014510

Ito C, Goto SG, Tomioka K, Numata H (2011) Temperature entrainment of the circadian cuticle deposition rhythm in *Drosophila melanogaster*. J Biol Rhythm 26:14–23. https://doi.org/10.1177/0748730410391640

Ito C, Tomioka K (2016) Heterogeneity of the peripheral circadian systems in *Drosophila melanogaster*: a review. Front Physiol 7:8. https://doi.org/10.3389/fphys.2016.00008

Ivanchenko M, Stanewsky R, Giebultowicz JM (2001) Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central clocks. J Biol Rhythm 16:205–215. https://doi.org/10.1177/074873040101600303

Iwai S, Fukui Y, Fujiwara Y, Takeda M (2006) Structure and expressions of two circadian clock genes, *period* and *timeless* in the commercial silkmoth, *Bombyx mori*. J Insect Physiol 52:625–637. https://doi.org/10.1016/j.jinsphys.2006.03.001

Johnston JS, Ellison JR (1982) Exact age determination in laboratory and field-caught *Drosophila*. J Insect Physiol 28:773–779. https://doi.org/10.1016/0022-1910(82)90138-X

Katewa SD, Akagi K, Bose N, Rakshit K, Camarella T, Zheng X et al (2016) Peripheral circadian clocks mediate dietary restriction-dependent changes in lifespan and fat metabolism in *Drosophila*. Cell Metab 23:143–154. https://doi.org/10.1016/j.cmet.2015.10.014

Komada S, Kamae Y, Koyanagi M, Tatewaki K, Hassaneen E, Saifullah ASM et al (2015) Green-sensitive opsin is the photoreceptor for photic entrainment of an insect circadian clock. Zool Lett 1:11. https://doi.org/10.1186/s40851-015-0011-6

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 68:2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Kotwica J, Bebas P, Gvakharia BO, Giebultowicz JM (2009) RNA interference of the *period* gene affects the rhythm of sperm release in moths. J Biol Rhythm 24:25–34. https://doi.org/10.1177/0748730408329109

Krishnan B, Dryer SE, Hardin PE (1999) Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature 400:375–378. https://doi.org/10.1038/22566

Krishnan B, Levine JD, Lynch MK, Dowse HB, Funes P, Hall JC, Hardin PE, Dryer SE (2001) A new role for cryptochrome in a *Drosophila* circadian oscillator. Nature 411:313–317. https://doi.org/10.1038/35077094

Krupp JJ, Billeter JC, Wong A, Choi C, Nitabach MN, Levine JD (2013) Pigment-dispersing factor modulates pheromone production in clock cells that influence mating in *Drosophila*. Neuron 79:54–68. https://doi.org/10.1016/j.neuron.2013.05.019

Krupp JJ, Kent C, Billeter JC, Azanchi R, So AK, Schonfeld JA, Smith BP, Lucas C, Levine JD (2008) Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. Curr Biol 18:1373–1383. https://doi.org/10.1016/j.cub.2008.07.089

Levi-Zada A, Byers JA (2021) Circadian rhythms of insect pheromone titer, calling, emission, and response: a review. Naturwissenschaften 108:35. https://doi.org/10.1007/s00114-021-01746-w

Levine JD, Funes P, Dowse HB, Hall JC (2002) Resetting the circadian clock by social experience in *Drosophila melanogaster*. Science 298:2010–2012. https://doi.org/10.1126/science.1076008

Loboda A, Jazwa A, Grochot-Przeczek A, Rutkowski AJ, Cisowski J, Agarwal A, Jozkowicz A, Dulak J (2008) Heme oxygenase-1 and the vascular bed: from molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal 10:1767–1812. https://doi.org/10.1089/ars.2008.2043

Lukat R (1978) Circadian growth layers in the cuticle of behaviourally arrhythmic cockroaches (*Blaberus fuscus,* Ins., Blattoidea). Experientia 34:477. https://doi.org/10.1007/BF01935937

Lukat R, Weber F, Wiedenmann G (1989) Cyclic layer deposition in the cockroach (*Blaberus craniifer*) endocuticle: a decentral circadian "clock"? J Insect Physiol 35:321–329. https://doi.org/10.1016/0022-1910(89)90081-4

Merlin C, Francois MC, Queguiner I, Maibeche-Coisne M, Jacquin-Joly E (2006) Evidence for a putative antennal clock in *Mamestra brassicae*: molecular cloning and characterization of two clock genes -*period* and *cryptochrome*- in antennae. Insect Mol Biol 15:137–145. https://doi.org/10.1111/j.1365-2583.2006.00617.x

Merlin C, Gegear RJ, Reppert SM (2009) Antennal circadian clocks coordinate sun compass orientation in migratory monarch butterflies. Science 325:1700–1704. https://doi.org/10.1126/science.1176221

Merlin C, Lucas P, Rochat D, Francois MC, Maibeche-Coisne M, Jacquin-Joly E (2007) An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the moth *Spodoptera littoralis*. J Biol Rhythm 22:502–514. https://doi.org/10.1177/0748730407307737

Mizoguchi A, Ishizaki H (1982) Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. Proc Nat Acad of Sci USA 79:2726–2730. https://doi.org/10.1073/pnas.79.8.2726

Morioka E, Matsumoto A, Ikeda M (2012) Neuronal influence on peripheral circadian oscillators in pupal *Drosophila* prothoracic glands. Nat Commun 3:909. https://doi.org/10.1038/ncomms1922

Myers EM, Yu J, Sehgal A (2003) Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster*. Curr Biol 13:526–533. https://doi.org/10.1016/s0960-9822(03)00167-2

Neville AC (1963) Daily growth zones in insect skeletons. Acta Physiol Scand 59(Suppl 213): 107–108

Neville AC (1965a) Chitin lamellogenesis in locust cuticle. J Cell Sci s3-106:269–286. https://doi.org/10.1242/jcs.s3-106.75.269

Neville AC (1965b) Circadian organization of chitin in some insect skeletons. J Cell Sci s3-106: 315–325. https://doi.org/10.1242/jcs.s3-106.76.315

Neville AC (1967) A dermal light sense influencing skeletal structure in locusts. J Insect Physiol 13: 933–939. https://doi.org/10.1016/0022-1910(67)90056-X

Neville AC (1975) Biology of the arthropod cuticle. Springer, Berlin. https://doi.org/10.1007/978-3-642-80910-1

Neville AC (1983) Daily cuticular growth layers and the teneral stage in adult insects: a review. J Insect Physiol 29:211–219. https://doi.org/10.1016/0022-1910(83)90087-2

Ohguro C, Moriyama Y, Tomioka K (2021) The compound eye possesses a self-sustaining circadian oscillator in the cricket *Gryllus bimaculatus*. Zool Sci 38:82–89. https://doi.org/10.2108/zs200118

Page TL, Koelling E (2003) Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. J Insect Physiol 49:697–707. https://doi.org/10.1016/s0022-1910(03)00071-4

Plautz JD, Kaneko M, Hall JC, Kay SA (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278:1632–1635. https://doi.org/10.1126/science.278.5343.1632

Pyza E, Borycz J, Giebultowicz JM, Meinertzhagen IA (2004) Involvement of V-ATPase in the regulation of cell size in the fly's visual system. J Insect Physiol 50:985–994. https://doi.org/10.1016/j.jinsphys.2004.08.003

Pyza E, Cymborowski B (2001) Circadian rhythms in behaviour and in the visual system of the blow fly, *Calliphora vicina*. J Insect Physiol 47:897–904. https://doi.org/10.1016/S0022-1910(01)00062-2

Pyza E, Meinertzhagen IA (1993) Daily and circadian rhythms of synaptic frequency in the first visual neuropile of the housefly's (*Musca domestica L.*) optic lobe. Proc Biol Sci 254:97–105. https://doi.org/10.1098/rspb.1993.0133

Pyza E, Meinertzhagen IA (1995) Monopolar cell axons in the first optic neuropil of the housefly, *Musca domestica L.*, undergo daily fluctuations in diameter that have a circadian basis. J Neurosci 15:407–418. https://doi.org/10.1523/jneurosci.15-01-00407.1995

Pyza E, Meinertzhagen IA (1999) Daily rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*. J Neurobiol 40:77–88. https://doi.org/10.1002/(sici)1097-4695(199907)40:1<77::aid-neu7>3.0.co;2-0

Reddy P, Zehring WA, Wheeler DA, Pirrotta V, Hadfield C, Hall JC, Rosbash M (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell 38:701–710. https://doi.org/10.1016/0092-8674(84)90265-4

Riemann JG, Thorson BJ, Ruud RL (1974) Daily cycle of release of sperm from the testes of the mediterranean flour moth. J Insect Physiol 20:195–207. https://doi.org/10.1016/0022-1910(74)90133-4

Rymer J, Bauernfeind AL, Brown S, Page TL (2007) Circadian rhythms in the mating behavior of the cockroach, *Leucophaea maderae*. J Biol Rhythm 22:43–57. https://doi.org/10.1177/0748730406295462

Saifullah AS, Page TL (2009) Circadian regulation of olfactory receptor neurons in the cockroach antenna. J Biol Rhythm 24:144–152. https://doi.org/10.1177/0748730408331166

Sakura M, Takasuga K, Watanabe M, Eguchi E (2003) Diurnal and circadian rhythm in compound eye of cricket (*Gryllus bimaculatus*): changes in structure and photon capture efficiency. Zool Sci 20:833–840. https://doi.org/10.2108/zsj.20.833

Sauman I, Reppert SM (1996) Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. Neuron 17:889–900. https://doi.org/10.1016/s0896-6273(00)80220-2

Schuckel J, Siwicki KK, Stengl M (2007) Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. Cell Tissue Res 330:271–278. https://doi.org/10.1007/s00441-007-0471-x

Singh SS, Mittal AM, Chepurwar S, Shashank GN (2019) Olfactory systems in insects: similarities and differences between species. In: Picimbon JF (ed) Olfactory concepts of insect control - alternative to insecticides. Springer, Cham, pp 29–48. https://doi.org/10.1007/978-3-030-05165-5_2

Selcho M, Millán C, Palacios-Muñoz A, Ruf F, Ubillo L, Chen J et al (2017) Central and peripheral clocks are coupled by a neuropeptide pathway in *Drosophila*. Nat Commun 8:15563. https://doi.org/10.1038/ncomms15563

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA et al (1998) The cry^b mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692. https://doi.org/10.1016/s0092-8674(00)81638-4

Steel CGH, Vafopoulou X (2002) Physiology of circadian systems. In: Saunders DJ, Steel CGH, Vafopoulou X (eds) Insect clocks, 3rd edn. Elsevier, Amsterdam, pp 115–188. https://doi.org/10.1016/B978-044450407-4/50006-9

Suszczynska A, Kaniewska MM, Bebas P, Giebultowicz JM, Kotwica-Rolinska J (2017) Circadian regulation of caterpillar feeding and growth. J Insect Physiol 101:113–122. https://doi.org/10.1016/j.jinsphys.2017.07.009

Tanoue S, Krishnan P, Chatterjee A, Hardin PE (2008) G protein-coupled receptor kinase 2 is required for rhythmic olfactory responses in *Drosophila*. Curr Biol 18:787–794. https://doi.org/10.1016/j.cub.2008.04.062

Tanoue S, Krishnan P, Krishnan B, Dryer SE, Hardin PE (2004) Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. Curr Biol 14:638–649. https://doi.org/10.1016/j.cub.2004.04.009

Tobback J, Boerjan B, Vandersmissen HP, Huybrechts R (2012) Male reproduction is affected by rna interference of *period* and *timeless* in the desert locust *Schistocerca gregaria*. Insect Biochem Mol Biol 42:109–115. https://doi.org/10.1016/j.ibmb.2011.11.003

Tomioka K, Chiba Y (1982) Persistence of circadian erg rhythm in the cricket with optic tract severed. Naturwissenschaften 69:395–396. https://doi.org/10.1007/BF00396696

Tomioka K, Uryu O, Kamae Y, Umezaki Y, Yoshii T (2012) Peripheral circadian rhythms and their regulatory mechanism in insects and some other arthropods: a review. J Comp Physiol B 182: 729–740. https://doi.org/10.1007/s00360-012-0651-1

Uryu O, Tomioka K (2010) Circadian oscillations outside the optic lobe in the cricket *Gryllus bimaculatus*. J Insect Physiol 56:1284–1290. https://doi.org/10.1016/j.jinsphys.2010.04.010

Vafopoulou X, Steel CG (1996a) Circadian regulation of a daily rhythm of release of prothoracicotropic hormone from the brain retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. Gen Comp Endocrinol 102:123–129. https://doi.org/10.1006/gcen.1996.0053

Vafopoulou X, Steel CG (1996b) The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. Proc Natl Acad Sci U S A 93: 3368–3372. https://doi.org/10.1073/pnas.93.8.3368

Vafopoulou X, Steel CGH (1991) Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: evidence of a dual oscillator system. Gen Comp Endocrinol 83:27–34. https://doi.org/10.1016/0016-6480(91)90102-C

Vafopoulou X, Steel CGH (1998) A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. J Comp Physiol A 182:343–349. https://doi.org/10.1007/s003590050184

Vafopoulou X, Steel CGH (2001) Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine zeitgebers. J Insect Physiol 47:935–941. https://doi.org/10.1016/S0022-1910(01)00066-X

Vafopoulou X, Steel CGH (2014) Synergistic induction of the clock protein PERIOD by insulin-like peptide and prothoracicotropic hormone in *Rhodnius prolixus* (Hemiptera): implications for convergence of hormone signaling pathways. Front Physiol 5:41. https://doi.org/10.3389/fphys.2014.00041

Van Der Kooi CJ, Stavenga DG, Arikawa K, Belušič G, Kelber A (2021) Evolution of insect color vision: from spectral sensitivity to visual ecology. Annu Rev Entomol 66:435–461. https://doi.org/10.1146/annurev-ento-061720-071644

Versteven M, Ernst K-M, Stanewsky R (2020) A robust and self-sustained peripheral circadian oscillator reveals differences in temperature compensation properties with central brain clocks. iScience 23:101388. https://doi.org/10.1016/j.isci.2020.101388

Weber F (1985) Postmolt cuticle growth in a cockroach: *in vitro* deposition of multilamellate and circadian-like layered endocuticle. Experientia 41:398–400. https://doi.org/10.1007/BF02004527

Weber F (1995) Cyclic layer deposition in the cockroach (*Blaberus craniifer*) endocuticle: a circadian rhythm in leg pieces cultured *in vitro*. J Insect Physiol 41:153–161. https://doi.org/10.1016/0022-1910(94)00092-U

Wiedenmann G, Lukat R, Weber F (1986) Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? J Insect Physiol 32:1019–1027. https://doi.org/10.1016/0022-1910(86)90121-6

Wills SA, Page TL, Colwell CS (1985) Circadian rhythms in the electroretinogram of the cockroach. J Biol Rhythm 1:25–37. https://doi.org/10.1177/074873048600100105

Xu K, DiAngelo JR, Hughes ME, Hogenesch JB, Sehgal A (2011) The circadian clock interacts with metabolic physiology to influence reproductive fitness. Cell Metab 13:639–654. https://doi.org/10.1016/j.cmet.2011.05.001

Xu K, Zheng X, Sehgal A (2008) Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. Cell Metab 8:289–300. https://doi.org/10.1016/j.cmet.2008.09.006

Yan S, Liu YJ, Zhu JL, Cui WN, Zhang XF, Yang YH et al (2019) Daily expression of two circadian clock genes in compound eyes of *Helicoverpa armigera*: evidence for peripheral tissue circadian timing. Insect Sci 26:217–228. https://doi.org/10.1111/1744-7917.12541

Yan S, Zhu J, Zhu W, Zhang X, Li Z, Liu X, Zhang Q (2014) The expression of three opsin genes from the compound eye of *Helicoverpa armigera* (Lepidoptera: Noctuidae) is regulated by a circadian clock, light conditions and nutritional status. PLoS One 9:e111683. https://doi.org/10.1371/journal.pone.0111683

Yu R, Liu W, Li D, Zhao X, Ding G, Zhang M, Ma E, Zhu K, Li S, Moussian B, Zhang J (2016) Helicoidal organization of chitin in the cuticle of the migratory locust requires the function of the chitin deacetylase2 enzyme (LmCDA2). J Biol Chem 291:24352–24363. https://doi.org/10.1074/jbc.M116.720581

Zehring WA, Wheeler DA, Reddy P, Konopka RJ, Kyriacou CP, Rosbash M, Hall JC (1984) P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. Cell 39:369–376. https://doi.org/10.1016/0092-8674(84)90015-1

Zhang J, Li S, Li W, Chen Z, Guo H, Liu J et al (2021) Circadian regulation of night feeding and daytime detoxification in a formidable Asian pest *Spodoptera litura*. Commun Biol 4:286. https://doi.org/10.1038/s42003-021-01816-9

Chapter 7
Circabidian Rhythm

Sakiko Shiga

Abstract Two-day rhythms, referred to as circa"bi"dian rhythms, were first reported in humans. In insects, a circabidian rhythm has been reported in the flight activity of the cool-weather mosquito *Culiseta incidens* under constant darkness. In both humans and mosquitoes, the appearance of the circabidian rhythm is labile under constant conditions, and the rhythm does not continue for a long time. In contrast, the black chafer *Holotrichia parallela* exhibited a rigid 2-day circabidian rhythm under both field and laboratory conditions. Three characteristics of the biological rhythms, free-running, entrainment to the zeitgeber, and temperature compensation of the period, were observed in the circabidian rhythm in *H. parallela*. Phase responses to light pulses suggest that the circadian clock mechanism is involved in the circabidian rhythm. The results of the brain surgery experiments imply that the optic lobe-pars intercerebralis axis in the brain is involved in the circabidian rhythm of *H. parallela.* Molecular phylogeny and behavioral observations suggest that after separation into *Pedinotrichia*, including *H. parallela* and *Holotrichia picea*, and *Nigrotrichia*, the circabidian rhythm probably appeared once in the ancestral species of *Pedinotrichia*.

Keywords Circadian clock · *Holotrichia* · Optic lobe · Pars intercerebralis · Temperature compensation · Two-day rhythm

7.1 Introduction

It is widely considered that most organisms on Earth have circadian clocks with a period close to Earth's rotation cycle. The circadian clock is a physiological mechanism that measures approximately 24 h to drive circadian rhythms in behavior and physiology (Dunlap et al. 2004; Patke et al. 2020; Chap. 2). Organisms use rhythms to prepare in advance for daily changes in the physical environment, such as

S. Shiga (✉)
Graduate School of Science, Osaka University, Toyonaka, Japan
e-mail: shigask@bio.sci.osaka-u.ac.jp

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Table 7.1 Two-day periodicity reported in animals

Species	Phylum	Class	Phenomenon	Detection under[a]	References
Culiseta incidens	Arthropod	Insecta	Flight activity	DD	Clopton (1984)
Holotrichia parallela	Arthropod	Insecta	Pheromone gland size	Natural conditions	Leal et al. (1993)
			Emergence on the ground	LD, DD, natural conditions	Yoshioka and Yamasaki (1983), Kawasaki et al. (2017)
Holotrichia picea	Arthropod	Insecta	Emergence on the ground	LD, DD	Shiga et al. (2022)
Mercenaria mercenaria	Mollusc	Bivalvia	Shell growth	Natural conditions	Pannella and Macclintock (1968)
Comptopallium radula	Mollusc	Bivalvia	Shell striae formation	Natural conditions	Thébault et al. (2006)

[a]*DD* constant dark conditions, *LD* 12-h light/12h dark cycle conditions, *LL* constant light conditions

temperature, humidity, and illumination, and the resulting changes in the biological environment, such as food availability and predation risk (DeCoursey et al. 1997, 2000).

Periodicities with an integral multiple of ~24 h have been reported, although this period seems irrelevant to environmental cycles. A circaseptan rhythm with a period of approximately 7 days appears in unicellular marine algae: the growth rate of *Acetabularia mediterranea* (Chlorophyta) and the glow intensity of *Gonyaulax polyedra* (Dinoflagellata) (Cornelissen et al. 1986; Schweiger et al. 1986). In both cases, the amplitude of the diurnal rhythm changed over a period of approximately 7 days. However, their periodicity is mostly unclear, and it is debatable whether endogenous circaseptan rhythms actually exist (Piccione et al. 2004).

Another integral multiple of ~24 h is the 2-day periodicity (Table 7.1). When humans were isolated in a cave or underground bunker, a 2-day periodicity was observed in the sleep-wakefulness rhythm under constant dim light conditions, although the period of the body temperature rhythm was approximately 25 h (Aschoff et al. 1967; Colin et al. 1968). Endogenous 2-day periodicity is called circa"bi"dian rhythm, and in humans, different patterns were observed. For example, sleep and wakefulness times were approximately twice as long as normal; an extremely lengthened activity time interrupted by naps appeared with a relatively small increase in sleep time (Honma and Honma 1988; Wever 1979). These human circabidian rhythms were often unstable and subsequently returned to their circadian rhythm or became obscure. Circabidian rhythms are considered to result from internal desynchronization, in which uncoupling of multiple oscillators, such as an oscillator for activity and an oscillator for body temperature, occurs under constant conditions (Aschoff et al. 1967).

There are a few reports of biological rhythms with 2-day periodicity under natural conditions. In mollusks, shell pattern formation of the hard clam *Mercenaria mercenaria* and the tropical scallop *Comptopallium radula* exhibits 2-day periodicity (Table 7.1, Pannella and Macclintock 1968; Thébault et al. 2006). In *M. mercenaria*, daily shell layer growth commonly occurs in one thick increment, followed by a relatively thin one. Juvenile *C. radula* forms one stria every 2 days under natural daily environmental conditions. In the Pacific Ocean, some meteorological and oceanographic parameters have displayed 2-day variations (Kenyon 1996). For sea-level atmospheric pressure and wind velocity, the amplitude of the 2-day variations was larger than that of the diurnal variations. This may act as a zeitgeber to shell growth rhythm in these bivalves (Thébault et al. 2006). However, it remains unknown whether the 2-day periodicity is endogenous in these species.

Some insects also exhibit a 2-day periodicity or circabidian rhythms (Yoshioka and Yamasaki 1983; Kawasaki et al. 2017; Shiga et al. 2022), although any 2-day variation in the physical parameters of the terrestrial environment has rarely been reported. This chapter introduces the insect circabidian rhythm and discusses its mechanisms.

7.2 Circabidian Rhythm in the Mosquito *Culiseta incidens*

In insects, the circabidian rhythm was first reported in the flight activity of the cool-weather mosquito *Culiseta incidens* under constant darkness (DD) (Table 7.1, Clopton 1984). Adult *C. incidens* exhibits a circadian rhythm with activity in the subjective night of DD. Many individuals, but not all, displayed radical changes in the free-running period (τ) calculated from activity onset (τ_{on}, Fig. 7.1a, b). Circabidian rhythmicity is occasionally found in prolonged DD, as a highly variable phenomenon (Clopton 1984, 1985). τ_{on} doubled abruptly, with little or no prior period lengthening (Fig. 7.1b). As τ_{off}, calculated from the activity offset, and τ_{on} change differently in many individuals, it has been considered that a circadian pacemaker controlling flight activities consists of two mutually coupled oscillators, E and M, which predominantly control the evening (τ_{on}) and morning (τ_{off}) flight rhythms, respectively, as in the common house mosquito *Culex pipiens* (Jones 1982; Clopton 1984, 1985). The circabidian rhythm can be explained by the period of the E-oscillator lengthening to where it synchronizes with the M oscillator in two consecutive cycles of M: one cycle of the E mode (Clopton 1984). Clopton (1984) proposed that E and M oscillators may behave similarly to the human activity and temperature oscillators mentioned above (see Sect. 7.1). In contrast to the human model, circadian components were not observed during the circabidian period of *C. incidens.* After the τ of the E oscillator lengthens to a period longer than 24 h, E predominates over the M oscillator (Clopton 1984).

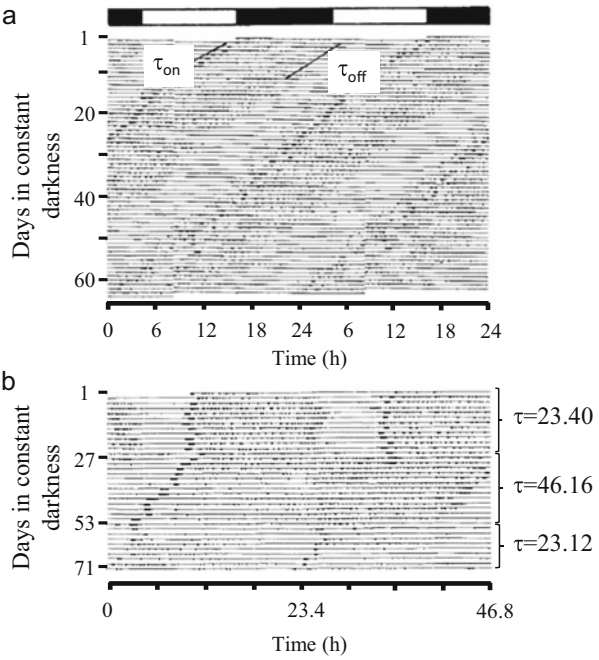

Fig. 7.1 Representative flight activity rhythm of the mosquito *Culiseta incidens*. (**a**, **b**) Double-plotted actogram of the flight activities under constant darkness. Previous light and dark schedule bar is shown in (**a**). τ_{on} and τ_{off} indicate free-running of the dusk (E) and dawn (M) activity rhythm, respectively (refer to the main text). (**b**) Under prolonged constant darkness, circabidian rhythm with the periodicity of 46.16 h occasionally occurs. Adapted from Clopton (1984), with permission from Springer

7.3 Circabidian Rhythm in *Holotrichia* Species

In both *C. incidens* and humans, the appearance of the circabidian rhythm is labile under constant conditions, and the rhythm does not continue for a long time (Fig. 7.1b). In contrast, some *Holotrichia* species (Insecta: Coleoptera: Scarabaeidae) exhibited a rigid 2-day periodicity under both field and laboratory conditions (Table 7.1). Yoshioka and Yamasaki (1983) originally reported that *H. parallela* populations appear on the ground every 2 days. Furthermore, measurements of pheromone titers in the pheromone glands of field-collected females suggest a 2-day periodicity in *H. parallela* (Leal et al. 1993). The genus *Holotrichia* includes serious pests of agricultural crops, such as *H. loochooana loochooana* of sugar canes and *H. parallela* of potatoes and glass roots in East Asia. Their behavior has been examined in different aspects of pest control, but their chronobiology has been unknown until recently.

7.3.1 Three Characteristics of Biological Rhythms in the Circabidian Rhythm of the Ground Emergence Activity

Under laboratory conditions, field-collected *H. parallela*, individually placed in a plastic container with soil, exhibited an approximately 2-day emergence rhythm on the ground. Under a 12 h light/12 h dark cycle (LD12:12) at 25 °C, male and female beetles appeared on the ground during the dark phase every 2 days (Fig. 7.2a). On the ground, beetles feed and walk around and remain underground for the rest of the time. Under LD12:12, the period of the ground emergence rhythm was 48.0 h in both males and females. Under DD, their emergence rhythm continued with a free-running period of slightly less than 48 h (Fig. 7.2a, Kawasaki et al. 2017). Thus, *H. parallela* exhibited a clear endogenous circabidian rhythm with a period of approximately 48 h under DD, and this rhythm was entrained to two cycles of LD 12:12 (Fig. 7.2a).

The circabidian rhythm might be due to developmental or physiological processes specific to *H. parallela*, such as gonadal development or the digestive system independent of the clock. Yoshioka and Yamasaki (1983) reported that *H. parallela* females deposit eggs in the daytime just before the night of adult emergence on the ground. A half-day feeding may require a long digestive time of 1.5 days. These physiological processes may suppress the behavioral output from the circadian clock every other day to produce a 2-day rhythmicity. If this is the case, the period of behavioral rhythm may become 24 h at higher temperatures because these physiological functions are temperature-dependent. Activity rhythms were recorded under DD conditions at different temperatures. Free-running periods did not differ at a range from 20.0 to 30.0 °C. The free-running period was 47.5 ± 0.5 h (mean ± S.D., $N = 11$) at 20 °C, 47.7 ± 0.2 h ($N = 11$) at 25 °C, and 47.4 ± 0.2 h ($N = 11$) at 30 °C. The average calculated temperature coefficient Q_{10} was 1.00 (Nakagawa and Shiga, unpublished Fig. 7.2b). This contradicts the hypothesis that the mechanism underlying 2-day rhythmicity involves some temperature-dependent physiological or developmental processes but suggests that the timekeeping mechanism in the circabidian rhythm solely involves a temperature-independent biological clock in *H. parallela*. This circabidian rhythm is also found in another species, *H. picea*, with some variation (Fig 7.2c, Shiga et al. 2022). Details are discussed in a later section (Sect. 7.4).

7.3.2 Phase Response of the Circabidian Rhythm to Light Pulses

There are two possibilities for the clock mechanism underlying circabidian rhythm: the circabidian rhythm is driven by the circadian clock or by the circabidian clock (Fig. 7.3a). Circabidian behavior may be driven by every two cycles of the circadian

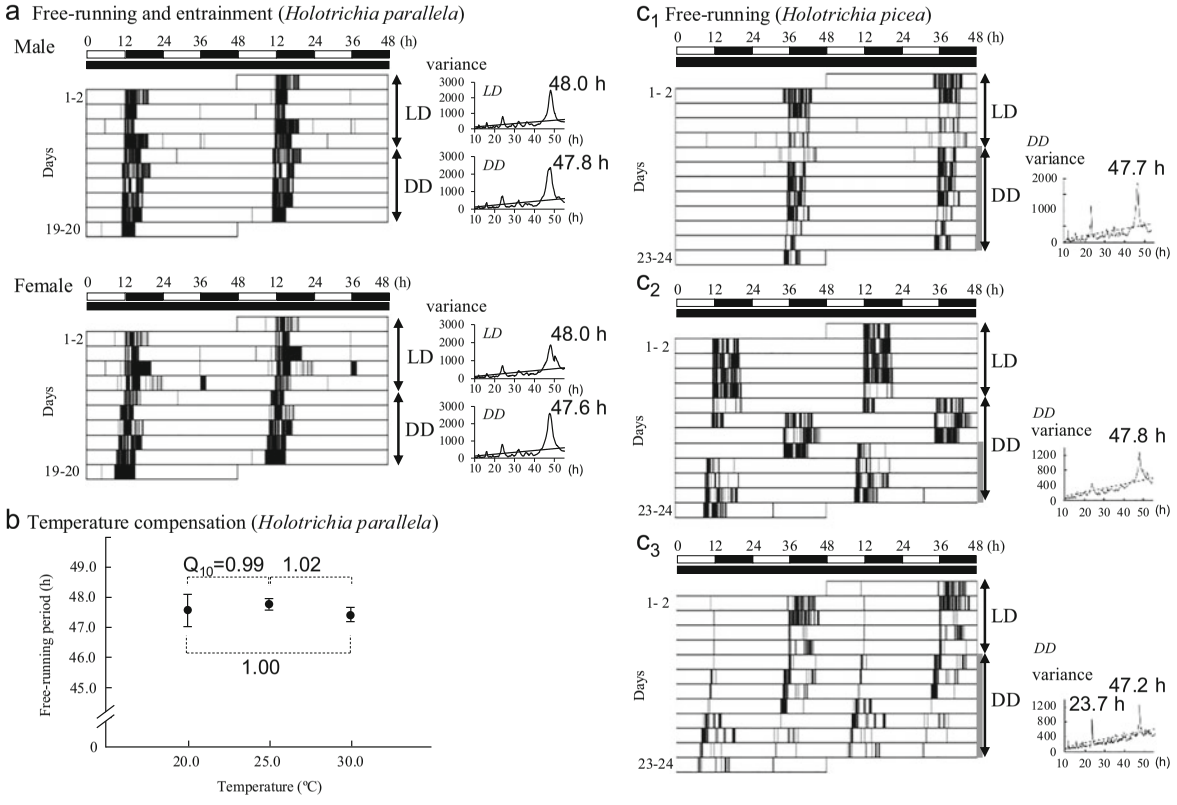

Fig. 7.2 Circabidian activity rhythm in *Holotrichia* species. (**a**) Representative actograms and chi-square periodogram of *H. parallela* under 12 h:12 h light: dark (LD) cycles and constant darkness (DD). (**b**) Average and SD of free-running periods under DD at different temperatures in *H. parallela*. Three calculated Q_{10} values are shown in the graph. (**c**) Representative actograms and chi-square periodogram of *H. picea* under LD cycles and DD. (**c_1**) and (**c_3**), female adults; **c_2**, sex undetermined. (**a**) Adapted from Kawasaki et al. (2017); (**b**) Issei Nakagawa and Sakiko Shiga (unpublished); (**c**) Adapted from Shiga et al. (2022), with permission from Zoological Science

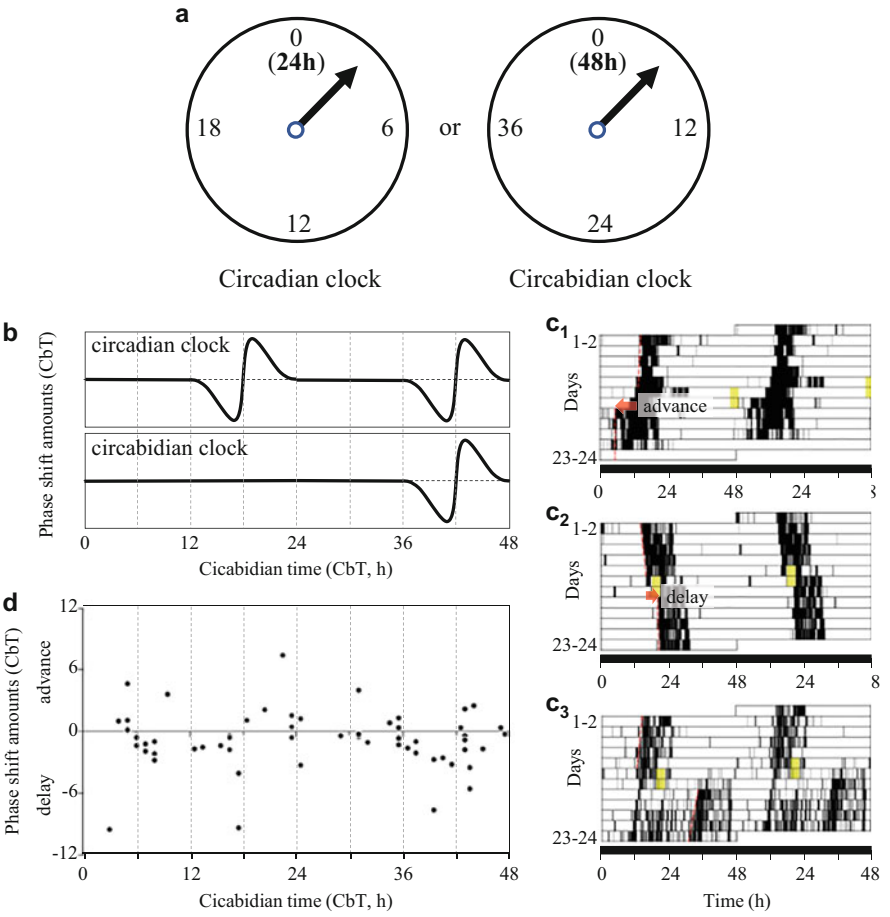

Fig. 7.3 Phase response of the circabidian rhythm to light pulses in *Holotrichia parallela*. (**a**) Two clock models underlying the circabidian rhythm. (**b**) Putative phase responses of the 24-h clock (upper) and of 48-h clock (lower). Advanced and delayed phase shift values are plotted as positive and negative values on the ordinate. (**c**) Representative actograms with light pulses (in yellow) twice. Phase changes are indicated by red arrows. (**d**) Phase responses to 3-h light pulses emitted at different circabidian times under constant darkness. (**c** and **d**) Adapted from Kawasaki et al. (2017)

clock. In this case, a mechanism doubling the circadian clock cycle must be present in the brain to produce a 2-day period. Alternatively, circabidian behavior might be driven by the cycle of the circabidian clock. One way to estimate the cycle of the clock that drives a rhythm is to examine the phase responses of the rhythm to zeitgeber stimuli. It is well known that a phase advance or delay of a rhythm occurs in response to a zeitgeber stimulus depending on the clock phase, which is a unique characteristic of oscillator-type clocks (Pittendrigh 1960; Chap. 3). Phase-response curves have been drawn for different biological rhythms. In the circadian clock, the clock phases are divided into subjective day and night periods. Circadian rhythms

driven by the circadian clock exhibit little or no response to light pulses during the subjective day but a delay in the first half and an advance in the last half of the subjective night (Okada et al. 1991). Because the *H. parallela* circabidian rhythm entrains to two LD 12:12 cycles, light must function as a zeitgeber. If the circabidian rhythm is driven by "a circabidian clock," phase delay and phase advance would occur once in a circabidian cycle. However, if the circabidian rhythm is driven by "a circadian clock," phase delay and advance would occur twice in the circabidian cycle (Fig. 7.3b). Based on this assumption, Kawasaki et al. (2017) examined the phase responses to light pulses. After the light pulse was applied, the onset of the activity phase was advanced, delayed, or unchanged depending on the light pulse phase (Fig. 7.3c_1, c_2). Although the shape of the response curve was not very clear, two sets of the less-responsive period (circabidian time, CbT 0–12, CbT 24–36) and more-responsive period (delay or advance CbT 12–24, CbT 36–48) appeared in one circabidian cycle (Fig. 7.3d). This suggests that the circabidian cycle is composed of two cycles of the circadian clock. Occasionally, circadian-like activity rhythms appeared after the light pulse (6.2%, $N = 65$, Fig. 7.3c_3, Kawasaki et al. 2017). This also suggested the presence of an oscillator with a period of approximately 24 h in *H. parallela*.

7.3.3 *The Optic Lobe-Pars Intercerebralis Axis in the Brain Is Involved in Circabidian Rhythm of* H. parallela

To discuss circadian clock involvement in the circabidian rhythm, brain regions necessary for the rhythm were examined. In insect brains, circadian clock cells are localized in the optic lobe of flies and cockroaches (King and Sehgal 2020; Shiga and Numata 2009; Reischig and Stengl 2003). Another brain region known to be involved in circadian rhythm output is a region called the pars intercerebralis (PI), where neurosecretory cells are concentrated. In *Drosophila melanogaster*, cells in the PI are connected to the circadian clock cells in the optic lobe through a polysynaptic circuit (Cavanaugh et al. 2014). Neuropeptides expressed in PI cells are required for locomotor activity rhythms (Cavanaugh et al. 2014; King and Sehgal 2020). The optic lobes and PI were examined to determine whether they are necessary for *H. parallela* circabidian rhythms. Adult beetles collected in the field were kept in the laboratory and subjected to removal of the optic lobes or PI (Watanabe and Shiga 2020).

7.3.3.1 Roles of the Optic Lobe

In *H. parallela*, the brain with bilateral optic lobes is located posteriorly in the head (Fig. 7.4). To remove the optic lobes, small cuticular openings were made just medial to the compound eyes, and the optic lobes were removed (Fig. 7.4 left). Most intact and sham-operated control beetles showed a clear circabidian rhythm,

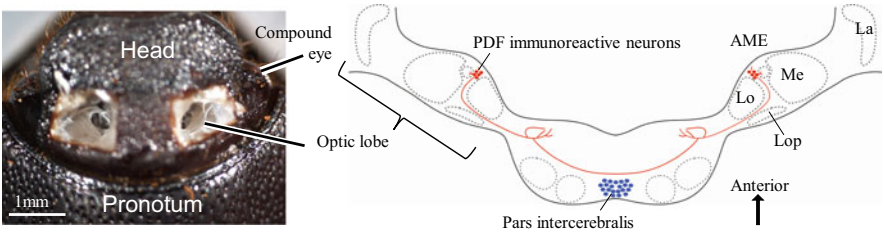

Fig. 7.4 Dorsal view of the head and brain of *Holotrichia parallela*. For optic lobe removal, bilateral small windows were opened (left). Distribution of pigment-dispersing factor (PDF) immunoreactive neurons and pars lateralis neurons (right). *La* lamina, *Lo* lobula, *Lop* lobula plate, *Me* medulla. Left, photo courtesy of Kohei Watanabe; right, redrawn from Hamanaka and Shiga et al. (2022)

although some stayed underground, probably because of winter dormancy in the late season of the experiment (Watanabe and Shiga 2020, Fig. 7.5a, b). When the bilateral optic lobes were removed, approximately half of the beetles exhibited arrhythmicity, and the other half never appeared on the ground. None of the beetles exhibited circabidian rhythms (Fig. 7.5a, b_1). After recording, beetles were dug up and their survival was confirmed. All beetles underground were active, similar to those in the intact and sham-operated groups. This suggests that the optic lobe itself, or the connection between the compound eye and midbrain, is involved in the formation of the circabidian rhythm and probably in emergence behavior on the ground. Clock protein PERIOD-immunoreactive cells have been found in the optic lobe of the beetle *Pachymorpha sexguttata* (Frisch et al. 1996). In *H. parallela*, a putative clock cell neuropeptide, pigment-dispersing factor, was observed in approximately 100 somata at the anterior base of the optic lobe medulla with medial fiber projections (Fig. 7.4 right, Hamanaka et al. 2022). Based on these findings, it is possible that circadian clock cells are located in the optic lobe of *H. parallela* and may be involved in the circabidian rhythm.

Questions such as the following, are both optic lobes (possibly bilateral clock systems) necessary for the circabidian rhythm and is it possible that an interaction between the two circadian clocks doubles the cycle of the 24-h clock?, still require exploration. If the two circadian clocks on the left and right sides are able to inhibit output signals from the contralateral clock to the premotor circuitry every 24 h and after the inhibition is released they become refractory to contralateral inhibition for 24 h, it might be possible to produce a 48-h cycle. This is called internal masking, in which the pacemaker output appears to be internally masked (Page 1989). Page (1989) introduced the circabidian rhythm of *C. incidens* and cockroaches as an example of internal masking.

For internal masking, unilateral optic lobe removal was performed. However, circabidian rhythm remained in all individuals (Fig. 7.5b_2), suggesting that the bilateral organization of the optic lobes, possibly containing the circadian clock, is not a prerequisite for circabidian rhythm. Interestingly, about one-fifth of circabidian beetles showed “day switching” (Fig. 7.5a, b_2). In day switching, beetles maintained

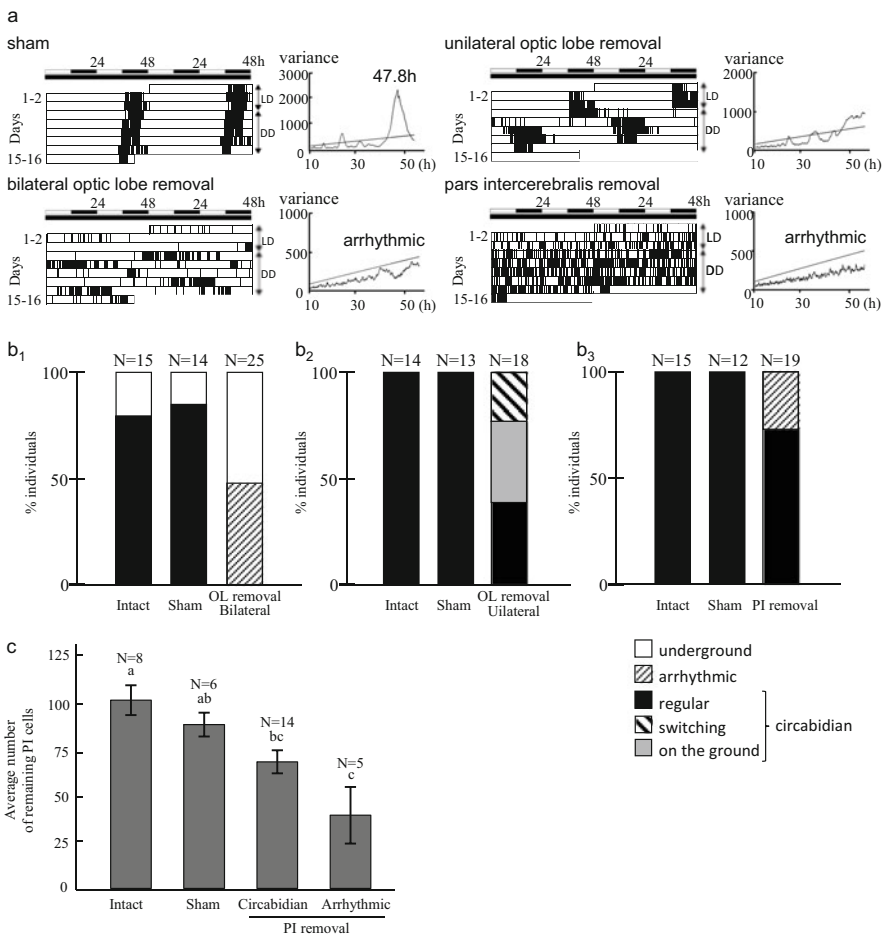

Fig. 7.5 Effects of brain surgery on the circadian rhythm in *Holotrichia parallela*. (**a**) Representative actograms under 12 h:12 h light/dark (LD) cycles and constant darkness (DD). Chi-square periodogram is shown for activities in DD. (**b**) Summary of effects of optic lobe (OL) removal (**b_1** and **b_2**) and pars intercerebralis (PI) removal (**b_3**). (**c**) The average number and SE of paraldehyde fuchsin-stained PI cells left. Arrhythmic beetles in the PI removal group had significantly reduced cell numbers compared to those in the control groups ($P < 0.05$, Tukey test). Adapted from Watanabe and Shiga (2020), with permission from Springer

a 2-day periodicity but changed their appearance night from odd to even days or vice versa by emerging on or skipping two serial nights (Kawasaki et al. 2017). Day switching has occasionally been observed in the field in *H. parallela* (Kawasaki et al. 2017). It occurs after heavy rain, suggesting some physiological mechanisms to suppress emergence on the ground under adverse conditions. The appearance of day switching by a single optic lobe may indicate that bilateral coupling between two optic lobes is necessary to maintain a regular 2-day rhythm, and weakening connections or decoupling between the right and the left circadian clock in the optic lobe

may cause day switching. In the cockroach *Rhyparobia* (*Leucophaea*) *maderae* and the cricket *Gryllus bimaculatus*, the circadian clocks in left and right optic lobes possess neural communication to allow synchronization (Page 1989; Tomioka 1993). This indicates that coupling between the bilateral optic lobe circadian clocks is responsible for the 24-h clock oscillation, and in *H. parallela*, decoupling of bilateral clocks or a solitary clock may weaken 24-h oscillation to cause an irregular pattern of the circabidian rhythm, that is, day switching. Clock coupling between the two optic lobes may be necessary for robustness of the 48-h rhythmic cycle.

In the unilateral optic lobe removal, another 40% of circabidian beetles did not go underground and remained aboveground throughout the day (Fig. 7.5b_2). Even beetles restricted above ground exhibited circabidian rhythms. Although underground behavior has not been shown, observation of their activities throughout the day on the ground raised the possibility that they are inactive underground and the total (above and below the ground) locomotor activity is circabidian.

7.3.3.2 Roles of the Pars Intercerebralis

In the PI of *H. parallela*, approximately 100 cells were positive for paraldehyde fuchsin, which stains a certain type of neurosecretory cell (Watanabe and Shiga 2020). Brain surgery experiments targeting the PI cells were performed, similar to optic lobe removal (Fig. 7.4). The surgery caused a reduction in paraldehyde fuchsin-stained cells in the PI (Watanabe and Shiga 2020). After this surgery, approximately one-fourth of the beetles showed arrhythmicity (Fig. 7.5b_3). The number of paraldehyde fuchsin-stained PI cells in the arrhythmic group was significantly reduced compared with that in the intact and sham-operated groups (Fig. 7.5c). Arrhythmic beetles walked and ate leaves randomly throughout the day, and their activity levels were higher than those of the control groups and PI-removed circabidian beetles (Watanabe and Shiga 2020). These results suggest that some paraldehyde fuchsin-stained PI cells are necessary for circabidian rhythm and the suppression or regulation of locomotor activity.

The results of these surgical experiments suggest that cells of the optic lobe and part of the PI are necessary for circabidian rhythms. The neural circuit of circadian clock cells in the optic lobe to the PI might be evolutionarily conserved for biological timing mechanisms in insects and may be involved in the generation of circabidian rhythms in *H. parallela*.

7.3.4 *Two-Day Rhythm of* H. parallela *in the Field*

Field observations by Kawasaki et al. (2017) showed male and female adults of *H. parallela* mostly appeared above ground a few hours after sunset from June to October and visited trees such as the Chinese elm *Ulmus parvifolia*. *H. parallela* remained on the tree throughout the night, and their appearance and disappearance

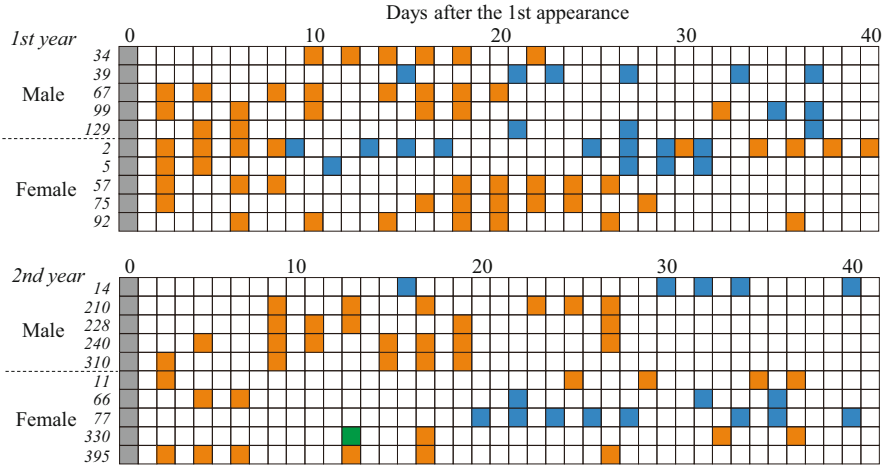

Fig. 7.6 Representative individual plots of *Holotrichia parallela* appearance in the field. The horizontal axis indicates the number of days from the first appearance. Orange cells and blue cells indicate an even and odd numbers of days, respectively, counted from the first appearance. Adapted from Kawasaki et al. (2017)

were mostly synchronized with sunset and sunrise, respectively (Kawasaki et al. 2017). At sunrise, they dug into the soil in an area of 15-m semidiameter around the tree where they stayed at night. A mark and recapture study showed that beetles repeatedly appeared on the same tree approximately every 2 nights (Kawasaki et al. 2017). However, the periodicity was not very rigid, and individuals often switched appearance days. Figure 7.6 shows individual plots of beetle appearance over 40 days in the field. Male no. 34 emerged on the same tree on days 10, 12, 14, 16, and 18 (every 2 days). In contrast, female no. 2 appeared on even days until day 8, but from day 9, she switched appearance day to odd days until day 29. From day 29, she appeared every day for 3 days and returned to an even-day appearance with 2-day periodicity (Fig. 7.6). Although a large amount of precipitation causes day switching in many beetles, day switching was sometimes observed without rain (Kawasaki et al. 2017).

The occurrence of day switching also supports the idea that the circabidian clock drives circabidian rhythm. If the circabidian rhythm was created by a hypothetical circabidian clock (Fig. 7.3a right), a phase shift of a half-cycle (∼24 h) of the clock would have had to occur to switch the appearance days. For an oscillator-type clock, it is difficult to make a half-period phase shift at one time (Benstaali et al. 2001), and it usually requires a transient period to complete a full shift. If day switching was adaptive (e.g., facultative avoidance of aversive conditions or increased population size), the beetles would have had to develop a mechanism to shift the appearance day without transients. If the circabidian rhythm is driven by the circadian clock system (Fig. 7.3a, left), an immediate switch in the appearance day may be possible. Circabidian output might be activated or suppressed every two circadian oscillations

by unknown mechanisms, such as counting two circadian cycles. If so, some environmental stimuli may provide an input signal to the cycle counting mechanism to produce an output after one or three circadian oscillations, thus resulting in day switching.

Phase responses to the light pulse, involvement of the optic lobe-PI axis, and day-switching characteristics suggest that the circabidian rhythm in *H. parallela* is driven by a ~ 24-h circadian clock. If this is the case, a novel function for the circadian clock that creates an integral multiple rhythm can be proposed. Circabidian rhythms may be produced by the release of an output signal from the circadian clock every two cycles to produce a 2-day rhythm. In future experiments, molecular and neuronal bases of the involvement of the circadian clock in circabidian rhythm should be elucidated.

7.4 Origin of the Circabidian Rhythm in the Genus *Holotrichia*

To elucidate the ancestral state of the circabidian rhythm in an evolutionary context, the activity rhythms of related species were examined (Shiga et al. 2022). *Holotrichia* (Coleoptera, Scarabaeidae, Melolonthinae, Rhizotrogina) is a large genus that includes heterogeneous species groups inhabiting Southeast and East Asia (Ward et al. 2002; Anitha et al. 2006; Matsumoto 2016). In addition to *H. parallela*, *Holotrichia picea* also exhibited a circabidian rhythm (Fig. 7.2c). In *H. picea*, three types of rhythms, including the regular circabidian pattern, circabidian patterns with day switching, and a circadian activity-like pattern, were observed under laboratory conditions. In the day-switching pattern, *H. picea* switched appearance from odd to even days, or vice versa, as did *H. parallela* (Fig. 7.2c_2). In the circadian-like activity patterns, major whole-night activity and minor dusk activity appeared alternately (Fig. 7.2c_3). The switching and circadian-like behavioral patterns in *H. picea* also support the idea that circabidian rhythms in *Holotrichia* species are driven by the circadian clock mechanism. *Holotrichia kiotonensis*, *Holotrichia convexopyga*, and *Holotrichia loochooana loochooana* exhibit a 24-h circadian rhythm (Shiga et al. 2022).

Two distinct clades were recognized in the phylogenetic trees – constructed using histone H3, cytochrome c oxidase subunit 1, and 16S ribosomal RNA – of the *Holotrichia* species (Shiga et al. 2022). This phylogenetic separation was in accordance with the subgeneric classification based on external morphology by Matsumoto (2015a, 2015b, 2016) and behavioral rhythms (Fig. 7.7a, b, Shiga et al. 2022). One clade included *Nigrotrichia* group members, *H. kiotonensis*, *H. convexopyga*, and *H. loochooana loochooana*, showing circadian rhythms, while the other clade included *Pedinotrichia* group members, *H. parallela* and *H. picea*, showing circabidian rhythms (Fig. 7.7). This suggests that after separation into *Nigrotrichia* and *Pedinotrichia* groups, the circabidian rhythm probably appeared once in the ancestral species of *Pedinotrichia*.

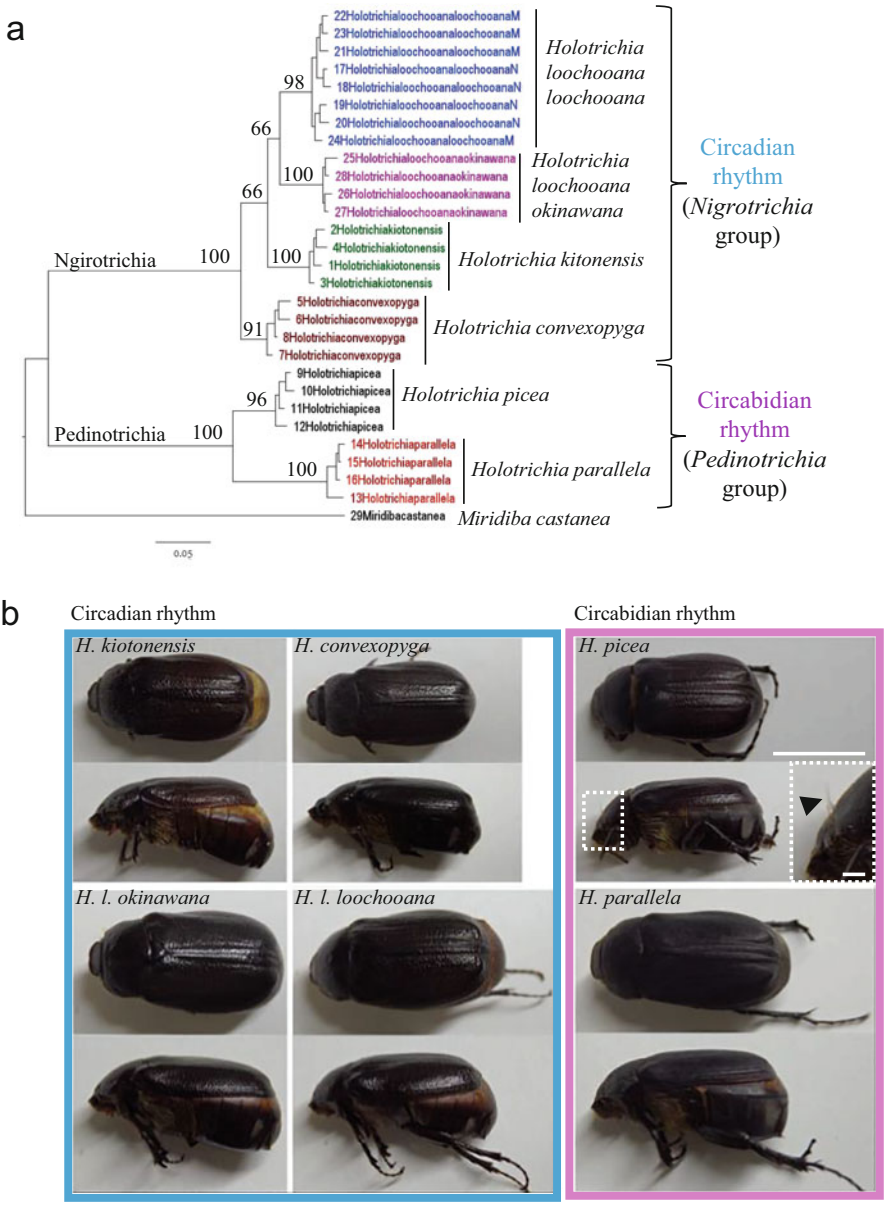

Fig. 7.7 Molecular phylogeny and behavioral analysis in *Holotrichia* species inhabiting Japan. (**a**) Histone H3, cytochrome c oxidase subunit 1, and 16S ribosomal RNA phylogenetic trees revealed two distinct clades: one clade (Nigrotrichia) including *Holotrichia kiotonensis*, *Holotrichia convexopyga*, and *Holotrichia loochooana loochooana* exhibiting a circadian rhythm, and the other clade (Pedinotrichia) including *H. parallela* and *Holotrichia picea* exhibiting circabidian rhythm. (**b**) Dorsal and lateral views of *Holotrichia* species (males). Adapted from Shiga et al. (2022), with permission from Zoological Science

7.5 Mechanism for Doubling the Circadian Clock Cycle

The occurrence of circadian-like activity patterns and day-switching patterns in *H. parallela* and *H. picea* suggests circadian clock involvement in the circabidian behavioral rhythm (Figs. 7.2c$_2$, c$_3$, 7.3c$_3$, and 7.5a). The phase-response patterns to light pulses (Fig. 7.3d) and the necessity of the optic lobes in *H. parallela* (Fig. 7.5b$_1$) also support this hypothesis. If this is the case, there must be some mechanism doubling the circadian clock cycle to achieve a 2-day periodicity. If *H. parallela* and *H. picea* possess circadian clock cells with a conventional transcriptional-translational negative feedback loop of clock genes, such as in *D. melanogaster* (Chap. 4), clock cycle doubling might occur in the clock cells intracellularly or intercellularly (Fig. 7.8).

Kinases may play an important role in intracellular mechanisms. In cultured mammalian cells, perturbation of phosphorylation by casein kinase 1ε (CK1ε) or CK1δ changes the period of clock gene expression rhythm from circadian (24 h) to circabidian (48 h) (Isojima et al. 2009). Kinases may be involved in the rate control of circadian oscillations. In clock cells of the circabidian species, some negative feedback loops (here, we say the first loop) of clock genes may exhibit a 24-h rhythm in their expression, and another loop (the second loop) may produce a 48-h rhythm by certain phosphorylation processes activated every two cycles of the first loop (Fig. 7.8a). In a single clock cell, clock-controlled genes for output signals that are under the control of the second loop may be able to produce a 2-day periodicity. The blind cavefish *Phreatichthys andruzzii*, which lives in perpetual darkness, does not show a clear circadian rhythm, and the clock gene expression of their cultured cells exhibits circabidian oscillation (Cavallari et al. 2011). These results suggested that circadian clock genes may be able to oscillate in a double period under certain circumstances.

Another possibility is that a 2-day periodicity is produced in the neuron network (Fig. 7.8b). Two-day periodicity can be produced by alternate suppression and activation of circadian clock cell output. Flip-flops and counters are sequential circuits with alternating on and off outputs. In moth olfactory processing systems, flip-flopping interneurons have been reported, and the flip-flop signal is thought to underlie locomotion occurring during pheromone-triggered orientation behavior (Olberg 1983; Namiki and Kanzaki 2016). This type of neuron switches back and forth between long-lasting high- and low-firing rates in response to repeated stimuli (Olberg 1983). A similar type of flip-flop interneuron might be incorporated into the clock network in *H. parallela* and *H. picea*. These neurons receive daily input from 24-h clock cells to alternate turning on and off of the postsynaptic neurons to produce 2-day cycles (Fig. 7.8b left). Another candidate is a network that contains counter neurons. Counter neurons count the number of circadian cycles; they do not activate (turn off) postsynaptic neurons when counting one cycle from the circadian clock but do (turn on) when signals for 2 days accumulate in them (Fig. 7.8b right). With this counter circuit, it is possible to produce other integral multiples of 24 h, such as 3- or 7-day rhythms.

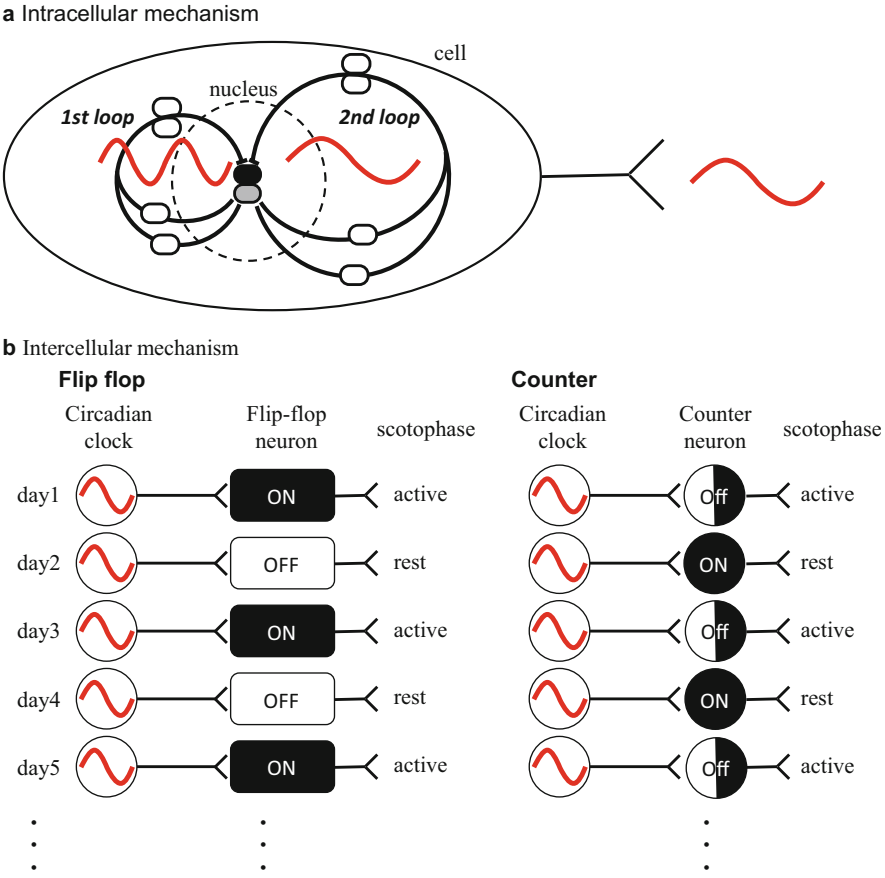

Fig. 7.8 Hypothetical mechanisms for doubling the circadian clock cycle. (**a**) In an intracellular mechanism, the first transcriptional-translational feedback loop produces conventional 24-h oscillation, and the second loop oscillates every two cycles of the first loop. Ovals indicate different types of clock proteins. (**b**) In intercellular mechanisms, neuronal circuitry containing flip-flop neurons or counter neurons may double the circadian clock cycle

When female bedbugs *Cimex lectularius* were fed on a 7-day cycle during juvenile development and allowed to feed and mate every 7 days after eclosion, the lysozyme-like activity gradually increased for the antibacterial immune response in the blood lymph in anticipation of mating in the next 7 days (Siva-Jothy et al. 2019). The male bedbug traumatically inseminates a freshly fed female, so it makes sense for the female to increase lysozyme activity prior to mating in order to prepare an immune response to bacteria entering the female's body during insemination. This report suggests that bedbugs learn the 7-day cycle of feeding and mating to produce a 7-day cycle of immune activity. This may involve some mechanism to learn an integral multiple of days and might support the presence of the clock-counter system.

7.6 Concluding Remarks

Several physiological data suggest the involvement of the circadian clock in circabidian rhythm. However, molecular evidence is missing. In the near future, oscillation patterns of circadian clock genes should be clarified, and their function in circabidian rhythm should be examined. Furthermore, the mechanism underlying clock cycle multiplication is fascinating. Using neuroanatomy and molecular genetics, it should be clarified whether the doubling of the circadian clock cycle occurs intracellularly or intercellularly in the brain.

Another important question is the ecological significance of the circabidian rhythm. In general, a reduction in the number of appearance days is unfavorable for feeding and mating opportunities. If predation pressure is high, it may make sense to reduce the number of emergence days. However, *H. parallela* remains on tree leaves during the night, and no active predators are known against *H. parallela* and *H. picea*. Therefore, it is unclear why these species exhibit circabidian rhythms. It is possible that some radical environmental changes have caused unfavorable conditions in a restricted region or era, and this may have benefited beetles in the *Pedinotrichia* group to emerge every 2 days, resulting in the appearance of the circabidian rhythm. However, if a few individuals return to the circadian rhythm when unfavorable environmental conditions disappear, beetles with circabidian rhythms may have lower reproductive fitness than those with circadian rhythms. Subsequently, the circabidian rhythm is lost. However, this rhythm has persisted in certain species, suggesting that the circabidian rhythm may have advantages that we are unaware of.

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References

Anitha V, Rodgers DJ, Wightman J, Ward A (2006) Distribution and abundance of white grubs (Coleoptera: Scarabaeidae) on groundnut in southern India. Crop Prot 25:732–740. https://doi.org/10.1016/j.cropro.2005.10.001

Aschoff J, Gerecke U, Wever R (1967) Desynchronization of human circadian rhythms. Jpn J Physiol 17:450–457. https://doi.org/10.2170/jjphysiol.17.450

Benstaali C, Mailloux A, Bogdan A, Auzéby A, Touitou Y (2001) Circadian rhythms of body temperature and motor activity in rodents their relationships with the light-dark cycle. Life Sci 68:2645–2656. https://doi.org/10.1016/s0024-3205(01)01081-5

Cavallari N, Frigato E, Vallone D, Fröhlich N, Lopez-Olmeda JF, Foà S et al (2011) A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. PLoS Biol 9:e1001142. https://doi.org/10.1371/journal.pbio.1001142

Cavanaugh DJ, Geratowski JD, Wooltorton JRA, Spaethling JM, Hector CE, Zheng X et al (2014) Identification of a circadian output circuit for rest:activity rhythms in *Drosophila*. Cell 157:689–701. https://doi.org/10.1016/j.cell.2014.02.024

Clopton JR (1984) Mosquito circadian and circa-bi-dian flight rhythms: a two oscillator model. J Comp Physiol A 155:1–12. https://doi.org/10.1007/BF00610925

Clopton JR (1985) Circa-bi-dian rhythmicity in the flight activity of the mosquito *Culiseta incidens*. Comp Biochem Physiol 80:469–475. https://doi.org/10.1016/0300-9629(85)90399-8

Colin J, Timbal J, Boutelier C, Houdas Y, Siffre M (1968) Rhythm of the rectal temperature during a 6-month free-running experiment. J Appl Physiol 25:170–176. https://doi.org/10.1152/jappl.1968.25.2.170

Cornelissen G, Broda H, Halberg F (1986) Does *Gonyaulax polyedra* measure a week? Cell Biophys 8:69–85. https://doi.org/10.1007/BF02788461

DeCoursey PJ, Krulas JR, Mele G, Holley DC (1997) Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure. Physiol Behav 62:1099–1108. https://doi.org/10.1016/S0031-9384(97)00263-1

DeCoursey PJ, Walker JK, Smith SA (2000) A circadian pacemaker in free-living chipmunks: essential for survival? J Comp Physiol A 186:169–180. https://doi.org/10.1007/s003590050017

Dunlap JC, Loros JJ, Decoursey PJ (2004) Chronobiology: biological timekeeping. Sinauer, Massachusetts

Frisch B, Fleissner G, Fleissner G, Brandes C, Hall JC (1996) Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock-gene product: labeling of cells with possible importance for the beetle's circadian rhythms. Cell Tissue Res 286:411–429. https://doi.org/10.1007/s004410050711

Hamanaka Y, Lu Z, Shiga S (2022) Morphology and synaptic connections of pigment-dispersing factor-immunoreactive neurons projecting to the lateral protocerebrum in the large black chafer, *Holotrichia parallela*. J Comp Neurol 530:2994–3010. https://doi.org/10.1002/cne.253

Honma K, Honma S (1988) Circabidian rhythm: its appearance and disappearance in association with a bright light pulse. Experientia 44:981–983. https://doi.org/10.1007/BF01939893

Isojima Y, Nakajima M, Ukai H, Fujishima H, Yamada RG, Masumoto K et al (2009) CKIε/δ-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. Proc Natl Acad Sci U S A 106:15744–15749. https://doi.org/10.1073/pnas.0908733106

Jones M (1982) Coupled oscillators controlling circadian flight activity in the mosquito *Culex pipiens quinquefasciatus*. Physiol Entomol 7:281–289. https://doi.org/10.1111/j.1365-3032.1982.tb00301.x

Kawasaki Y, Nishimura H, Shiga S (2017) Plausible link between circa'bi'dian activity rhythms and circadian clock systems in the large black chafer *Holotrichia parallela*. J Exp Biol 220:4024–4034. https://doi.org/10.1242/jeb.163253

Kenyon KE (1996) Bi-daily variation of meteorological properties at sea level across the Pacific along 35°N. Atmos Res 43:31–46. https://doi.org/10.1016/S0169-8095(96)00003-8

King AN, Sehgal A (2020) Molecular and circuit mechanisms mediating circadian clock output in the *Drosophila* brain. Eur J Neurosci 51:268–281. https://doi.org/10.1111/ejn.14092

Leal WS, Sawada M, Matsuyama S, Kuwahara Y, Hasegawa M (1993) Unusual periodicity of sex pheromone production in the large black chafer *Holotrichia parallela*. J Chem Ecol 19:1381–1391. https://doi.org/10.1007/BF00984883

Matsumoto T (2015a) Separation of *Amphitrichia* from *Holotrichia* (Scarabaeidae, Melolonthinae, Melolonthini) with description of a new species and new records of two known species. Kogane 17:11–17. (in Japanese) https://iss.ndl.go.jp/books/R100000002-I000000162723-00

Matsumoto T (2015b) Taxonomic notes on the genus *Eotrichia* Medvedev (Scarabaeidae, Melolonthinae, Melolonthini) with description of a new species and new records of two known species. Kogane 17:19–24. (in Japanese) https://iss.ndl.go.jp/books/R000000004-I026803277-00

Matsumoto T (2016) Three new genera of the subtribe Rhizotrogina (Scarabaeidae, Melolonthinae, Melolonthini). Kogane 18:5–14. (in Japanese) http://id.ndl.go.jp/bib/000000162723

Namiki S, Kanzaki R (2016) The neurobiological basis of orientation in insects: insights from the silkmoth mating dance. Curr Opin Insect Sci 15:16–26. https://doi.org/10.1016/j.cois.2016.02.009

Okada Y, Tomioka K, Chiba Y (1991) Circadian phase-response curves for light in nymphal and adult crickets, *Gryllus bimaculatus*. J Insect Physiol 37:583–590. https://doi.org/10.1016/0022-1910(91)90035-X

Olberg RM (1983) Pheromone-triggered flip-flopping interneurons in the ventral nerve cord of the silkworm moth, *Bombyx mori*. J Comp Physiol 152:297–307. https://doi.org/10.1007/BF00606236

Page T (1989) Masking in invertebrates. Chronobiol Int 6:13–11. https://doi.org/10.3109/07420528909059137

Pannella G, Macclintock C (1968) Biological and environmental rhythms reflected in molluscan shell growth. J Paleontol 42(S2):64–80. https://doi.org/10.1017/S0022336000061655

Patke A, Young MW, Axelrod S (2020) Molecular mechanisms and physiological importance of circadian rhythms. Nat Rev Mol Cell Biol 21:67–84. https://doi.org/10.1038/s41580-019-0179-2

Piccione G, Caola G, Refinetti R (2004) Feeble weekly rhythmicity in hematological, cardiovascular, and thermal parameters in the horse. Chronobiol Int 21:571–589. https://doi.org/10.1081/CBI-200026447

Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. Cold Spring Harb Symp Quant Biol 25:159–184. https://doi.org/10.1101/SQB.1960.025.01.015

Reischig T, Stengl M (2003) Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). J Exp Biol 206:1877–1886. https://doi.org/10.1242/jeb.00373

Schweiger HG, Berger S, Kretschmer B, Mörler H, Halberg E, Sothern RB et al (1986) Evidence for a circaseptan and a circasemiseptan growth response to light/dark cycle shifts in nucleated and enucleated *Acetabularia* cells, respectively. Proc Natl Acad Sci U S A 83:8619–8623. https://doi.org/10.1073/pnas.83.22.8619

Shiga S, Numata H (2009) Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, *Protophormia terraenovae*. J Exp Biol 212:867–877. https://doi.org/10.1242/jeb.027003

Shiga S, Omura Y, Kawasaki Y, Watanabe K (2022) Phylogenetic separation of *Holotrichia* species (Insecta, Coleoptera, Scarabaeidae) exhibiting circadian rhythm and circa'bi'dian rhythm. Zool Sci 39:227–235. https://doi.org/10.2108/zs210091

Siva-Jothy MT, Zhong W, Naylor R, Heaton L, Hentley W, Harney E (2019) Female bed bugs (*Cimex lectularius* L) anticipate the immunological consequences of traumatic insemination via feeding cues. Proc Natl Acad Sci U S A 116:14682–14687. https://doi.org/10.1073/pnas.1904539116

Thébault T, Chauvaud L, Clavier J, Fichez R, Morize E (2006) Evidence of a 2-day periodicity of striae formation in the tropical scallop *Comptopallium radula* using calcein marking. Mar Biol 149:247–267. https://doi.org/10.1007/s00227-005-0198-8

Tomioka K (1993) Analysis of coupling between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. J Comp Physiol A 172:401–408. https://doi.org/10.1007/BF00213522

Ward S, Moore C, Anitha V, Wightman J, Rogers DJ (2002) Identification of the sex pheromone of *Holotrichia reynaudi*. J Chem Ecol 28:515–522. https://doi.org/10.1023/a:1014535910946

Watanabe K, Shiga S (2020) The optic lobe-pars intercerebralis axis is involved in circa'bi'dian rhythm of the large black chafer *Holotrichia parallela*. J Comp Physiol A 206(6):819–829. https://doi.org/10.1007/s00359-020-01440-8

Wever R (1979) The circadian system of man. Springer, New York

Yoshioka K, Yamasaki Y (1983) Ecology of *Lachnosterna morosa* Waterhouse. I Behavior of the time of appearance on the ground and oviposition of adult insects. Jpn J Appl Entomol Zool 27:52–54. (in Japanese). https://doi.org/10.1303/jjaez.27.52

Chapter 8
Circadian Behavioral Rhythms in Social Insects

Taro Fuchikawa

Abstract The highly developed sociality of insects has been well studied from the perspectives of animal behavior, physiology, and ecology. However, less effort has been devoted to examining the chronobiology of social insects, likely because the lifestyle of most insects involves dense cohabitation of many individuals within small, dark places. This chapter reviews the circadian behavioral rhythms of social insects such as bees, ants, and termites, focusing first on the general features of circadian patterns in social hymenopterans and termites and then on insect entrainment to environmental cycles such as light and social cues, which are fundamental properties of circadian rhythms. Finally, the ontogeny and plasticity of insect circadian rhythms are discussed.

Keywords Circadian behavioral rhythms · Ontogeny of circadian rhythms · Plasticity of circadian rhythms · Social entrainment · Social insects

8.1 Introduction

Circadian rhythms are widely observed in organisms, from bacteria to vertebrates, allowing them to adapt to 24-h environmental changes. These changing environmental factors can be divided into abiotic (e.g., light, temperature, or humidity) and biotic factors that include both intraspecific interactions, such as sexual and nonsexual social interactions, and interspecific interactions, such as with predators, prey, and parasites (Sharma and Chandrashekaran 2005), including plant-pollinator interactions (Bloch et al. 2017). Abiotic factors, as well as some biotic factors, fluctuate as a direct result of the Earth's daily rotation, and these fluctuations modulate circadian systems. Many species modify circadian systems in response to biotic cues; for example, the rhythms of bees and flies are entrainable by social cues including pheromones (Bloch et al. 2013), and individual bees and ants switch

T. Fuchikawa (✉)
Graduate School of Science, Osaka Metropolitan University, Osaka, Japan
e-mail: tfuchi@omu.ac.jp

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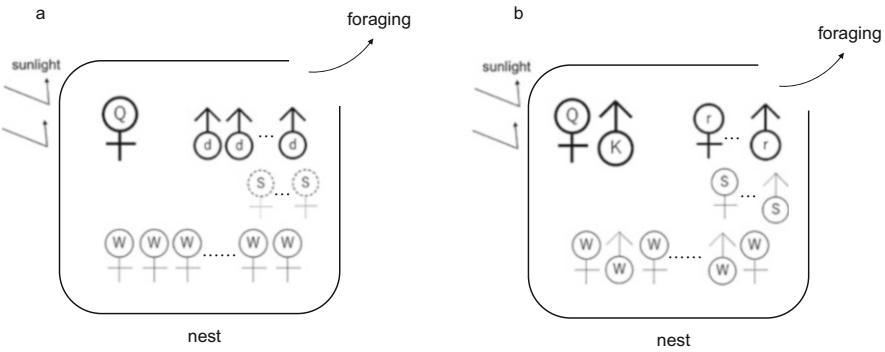

Fig. 8.1 Scheme of the simplified colony structure of social insects such as hymenopterans and termites. (**a**) Social hymenopterans such as bees and ants. (**b**) Termites. *Q* queen, *K* king, *w* worker, *d* drone, *s* soldier, *r* reproductive males and females. Bold symbols represent reproductive individuals. The presence/absence of soldiers is species-dependent in hymenopterans. Note that Q and K can be multiple, depending on the species

between rhythmic and arrhythmic behaviors in a socially mediated manner (Shemesh et al. 2010; Fujioka et al. 2017). Therefore, we cannot ignore the influence of the biotic environment on circadian systems, and social insects provide an attractive model for advancing our understanding of circadian system modifications in response to biotic cues.

Social insects live in colonies containing up to a few million individuals, most of which build protective nests that provide unique environmentally buffered habitats (Fig. 8.1). These protective nests may factor in the development or maintenance of eusociality, which is the highest level of sociality, typically with a division of labor between reproductive and nonreproductive castes, particularly in hymenopterans and termites (Howard and Thorne 2010). The nests of social insects play many roles, serving as a means of defense against enemies and insulating insects from a fluctuating environment, as well as a location for food storage and the sharing of information (Jeanne 2009). Because insect nests are constructed from materials such as soil and wood or excavated underground, they are usually dark, and fluctuations in temperature and other abiotic factors such as humidity are more moderate inside the nest than outside.

Another remarkable characteristic of social insects is the division of labor, in which different individuals specialize in subsets of tasks performed by the colony. Two different types of division of labor are seen in social insects: reproductive division of labor (a defining trait of eusociality) and division of labor among subgroups of workers according to morphology, size, or age (Beshers and Fewell 2001; Jeanne 2016). In both types, individuals engaged in different tasks tend to work in different places, as typically observed between an egg-laying queen and foraging workers or between nest workers and foraging workers. The combination of division of labor and nest isolation from the outside world means that individuals within the colony are exposed to different ranges of environmental fluctuations.

This chapter reviews the general features of circadian patterns in social hymenopterans and termites; the entrainment to environmental cycles, focusing on light and social cues; and the ontogeny and plasticity of circadian rhythms, which are remarkable characteristics of circadian systems in social insects.

8.2 Examples of Circadian Behavioral Rhythms in Social Insects

Social insects such as social hymenopterans and termites exhibit a suite of social behaviors including social foraging, group defense, and cooperative care of offspring or relatives (Robinson 1992; Robinson et al. 2008; Howard and Thorne 2010). To date, the daily rhythmicity of these behaviors has not been systematically investigated, with rare exceptions (e.g., Moore et al. 1998), although small subsets of behaviors such as foraging, aggression, and brood care of immature stages including eggs, larvae, and pupae have been studied separately in some species, as discussed below.

Among social bees and wasps, the European honeybee *Apis mellifera* is the most investigated insect for circadian rhythms, including foraging (Frisch and Aschoff 1987; Moore 2001) and various in-nest activities (Moore et al. 1998). The bumblebee *Bombus terrestris*, which is a primitively eusocial species, exhibits circadian foraging rhythms (Stelzer and Chittka 2010). Two eusocial wasps, *Polistes crinitus* and *Mischocyttarus phthisicus*, show circadian rhythms in their locomotor activity in the laboratory (Giannoni-Guzmán et al. 2014).

Many studies have revealed the foraging rhythms of ants (Hymenoptera: Formicidae), all extant species of which are eusocial, across a wide range of taxa including the subfamilies Formicinae, Myrmicinae, and Ponerinae (McCluskey 1987; Retana et al. 1992; Passera et al. 1994; Bochynek et al. 2017; Mildner and Roces 2017; Hoenle et al. 2019; Lei et al. 2019). The activity rhythms of ants range across diurnal, nocturnal, and arrhythmic scales (Narendra et al. 2017), which is in contrast to those of most bee species, which are active during the day (Wcislo and Tierney 2009). Only 1% of bees are nocturnal and able to fly at low light intensities (Liporoni et al. 2020). In the carpenter ants *Camponotus compressus* and *Camponotus rufipes*, isolated workers generally show circadian activity rhythms (Sharma et al. 2004c; Mildner and Roces 2017). Some ants have lost a clear resting period at night (*Anoplolepis gracilipes*, Chong and Lee 2009; *Diacamma*, Win et al. 2018; *Solenopsis invicta*, Lei et al. 2019).

All of the nearly 3,000 species of modern termites are eusocial, belonging to a monophyletic clade within the order Blattodea (Korb and Thorne 2017). Termites are classified as having single- or separate-site nesting and foraging habits (Korb and Thorne 2017). Single-site termites spend their entire lives nesting and feeding within a single tree or log, whereas separate-site termites keep nests separate from their multiple food sites. Separate-site termites can exhibit daily rhythmic behavior, as

they leave their nests daily to encounter the fluctuating environment during foraging (Hebrant 1970; Abushama and Al Houty 1989; Hinze and Leuthold 1999; Muradian et al. 1999). Single-site termites are less likely to exhibit daily rhythmic behavior because they feed and nest within the same tree or log. In the single-site termite *Incisitermes minor* (Kalotermitidae), apparent daily feeding rhythms are largely driven by temperature (Lewis et al. 2013). Similar temperature-driven daily feeding rhythms have been observed in field colonies of termites of the genus *Reticulitermes* (Fuchikawa et al. 2012), which has features of single-site termites but with separate-site habitats; they nest and feed in the same substrate but also forage in nearby substrates (Vargo and Husseneder 2009). In a laboratory experiment, *Reticulitermes* workers showed no daily rhythmic behavior (T. Fuchikawa, unpublished data).

8.3 Entrainment to Abiotic and Social Cycles

Entrainment, or synchronization, is a fundamental property of circadian systems, in which the period of the internal rhythm (τ) coincides with the period of an environmental cycle (T). For example, in animals, stimuli such as light, temperature, relative humidity, sound or vibration, food availability, social interactions, chemical substances, and forced activity (arousal) that synchronize biological rhythms have been described as zeitgebers (Sharma and Chandrashekaran 2005; Wams et al. 2017). In social insects, entrainment of activity rhythms to light/dark cycles has been observed in honeybees (Moore and Rankin 1985; Moore and Rankin 1993; Fuchikawa and Shimizu 2007, 2008), bumblebees (Stelzer et al. 2010; Chittka et al. 2013), and ants (North 1987; Roces 1995; Sharma et al. 2004a; Sharma et al. 2004b; Sharma et al. 2004c; Lone and Sharma 2011; Mildner and Roces 2017). In bumblebees, daily ultraviolet light cycles are sufficient for synchronization of foraging rhythms (Chittka et al. 2013). To the best of my knowledge, the role of ultraviolet light cycles in the synchronization/entrainment of circadian behavioral rhythms has not been investigated in other hymenopterans. In the separate-site termite *Anocanthotermes vagans*, Abushama and Al Houty (1989) failed to entrain the locomotor activity rhythm of workers to 12-h/12-h light/dark cycles under laboratory conditions. By contrast, the mate-search behavior in reproductive individuals (not workers) of the termite *Reticulitermes okinawanus* is synchronized with light/dark cycles; furthermore, the termite shows free-running rhythms with a period shorter than 24 h under constant darkness (Mizumoto et al. 2017).

Social interactions among individuals have long drawn the attention of researchers as zeitgeber candidates. An early study of honeybees conducted by Southwick and Moritz (1987) found that when two groups of 50 worker bees were allowed physical contact through metallic mesh under constant darkness, the two groups were able to synchronize their circadian metabolic rhythms with each other. Since then, several studies on social entrainment have been conducted in social insects. In early studies, workers were not classified into subgroups (i.e., nurse, forager, or newly emerged bees), which are called subcastes (Southwick and Moritz

1987, Frisch and Koeniger 1994, and Moritz and Kryger 1994 in honey bees and Lone and Sharma 2011 in ants), whereas recent studies on honeybees have classified workers into subcastes based on their behavior or age (Beer et al. 2016; Fuchikawa et al. 2016; Siehler et al. 2021a). Both types of study have produced results that support social entrainment. However, the effectiveness of social entrainment has not yet been compared among subcastes. To understand how worker bees inside and outside the hive synchronize their respective circadian rhythms with the environmental cycles outside the hive through both light entrainment and social entrainment, it is necessary to analyze such comparisons.

Potential cues mediating social entrainment in social insects have been analyzed in depth in a number of recent studies (e.g., Siehler and Bloch 2020; Siehler et al. 2021b). Earlier studies showed that honeybees can socially synchronize their activity rhythms without direct contact (Moritz and Kryger 1994; Beer et al. 2016; Fuchikawa et al. 2016). Consistent with these results, indirect factors such as vibrations and volatiles have been demonstrated to act as social entrainment cues in honeybee workers, notably including young nest bees (Siehler and Bloch 2020; Siehler et al. 2021a). These indirect cues have been demonstrated by focusing on behavioral rhythms at an individual level, using a well-established method for recording behavioral rhythms in the laboratory. In mammals, arousal-induced activity, sleep, and learning processes are thought to act as social entrainment cues (Reebs 1989; Amir and Stewart 1996; Mistlberger and Skene 2004). These factors have not yet been examined as zeitgebers in social insects.

Little is known about how social entrainment cues change the phase of animal rhythms. In entrainment by light, the phase and strength of photic stimuli affect the magnitude and direction of the phase shift of the rhythm (Golombek and Rosenstein 2010). In social entrainment, a study on rodents showed that the phase of exposure to social stimuli varied the phase shift of the rhythm (Mrosovsky 1988). These phase shifts have not yet been investigated in social insects. Whether the phase of exposure to vibration or volatiles influences the phase of the rhythm should be examined in social insects in future studies. In honeybee workers, a group with a larger number of individuals showed better behavioral rhythm synchronization, which implies that the amount of social stimuli generated by a social group alters the pattern of phase shifting, although no data were published to support this supposition (Siehler et al. 2021b).

It appears plausible that social entrainment has evolved in animals that exhibit high sociality. However, a number of studies do not support this view (see reviews by Castillo-Ruiz et al. 2012; Siehler et al. 2021b). The fruit fly *Drosophila melanogaster* is not a social insect, but shows social entrainment mediated by volatile pheromones detected by the olfactory system (Levine et al. 2002; Krupp et al. 2008). Conversely, the behavioral rhythms of the Mongolian gerbil *Meriones unguiculatus*, a highly social mammal whose reproduction is limited to a founder pair immediately after nest establishment, could not be entrained to the activity cycles of nearby conspecifics (Gattermann and Weinandy 1997). Despite several studies on honeybees and a formicine ant species, data on social entrainment in social insects and closely related solitary species remain scarce. Comparative studies

using closely related species are needed to ascertain whether these findings are consistent.

8.4 Social Influences on the Ontogeny of Circadian Rhythms

Insects exhibit daily behavioral rhythms over a wide range of developmental stages including egg hatching, molting, larval/nymphal or adult locomotion, and adult emergence (Saunders 2002). Although many studies on circadian rhythms have focused on each of these behaviors, only a few have focused on the ontogeny of circadian rhythms within single individuals. In *D. melanogaster*, the circadian clock begins to function during the embryonic stage (Zhao et al. 2019), and locomotor activity rhythm starts soon after eclosion (Sehgal et al. 1992). In hemimetabolous insects, nymphs of the cockroach *Rhyparobia* (*Leucophaea*) *maderae* show circadian rhythms in locomotor activity at 1–2 days after hatching (Page 1990). The cricket *Gryllus bimaculatus* maintains locomotor activity rhythms from the day on which imaginal molt occurs, accompanied by transition of the active phase from diurnal to nocturnal (Tomioka and Chiba 1982).

Among social insects, several species exhibit delayed onset of circadian behavioral rhythms after adult emergence. Three types of behavioral rhythm initiation have been observed in social insects (Eban-Rothschild and Bloch 2012): (1) onset of behavioral rhythms in individuals isolated from a colony following adult emergence, under laboratory conditions (Toma et al. 2000; Jong and Lee 2008; Giannoni-Guzman et al. 2020); (2) in workers separated from their brood, under laboratory conditions (Shemesh et al. 2007; Shemesh et al. 2010; Eban-Rothschild et al. 2011; Fujioka et al. 2017); and (3) when the task switches from nurse to forager in natural colonies (Crailsheim et al. 1996; Moore et al. 1998). These three types of behavioral rhythm initiation have been reported in bees and ants, but have yet to be investigated in termites.

Type 1 behavioral rhythm initiation must differ from types 2 and 3 in terms of their underlying mechanisms and represents, in a strict sense, the ontogeny of circadian behavioral rhythms during adult life. In type 1 initiation, young honeybee workers exhibit arrhythmic behavior immediately after emergence, even when the brood is in close proximity (i.e., under isolated conditions in the laboratory). Then the workers gradually initiate behavioral circadian rhythms regardless of the presence of the brood (Toma et al. 2000; Eban-Rothschild et al. 2012). At the cellular level, the number of neurons expressing pigment-dispersing factor (PDF), which plays a role in the circadian clock output pathway, increases with the development of behavioral circadian rhythms in honeybees (Beer and Helfrich-Förster 2020). In *Polyrhachis* workers, the initiation of circadian rhythms is gradual (Jong and Lee 2008), but the underlying mechanism has not been investigated in this species. In the bumblebee *B. terrestris*, circadian rhythm development has been analyzed separately

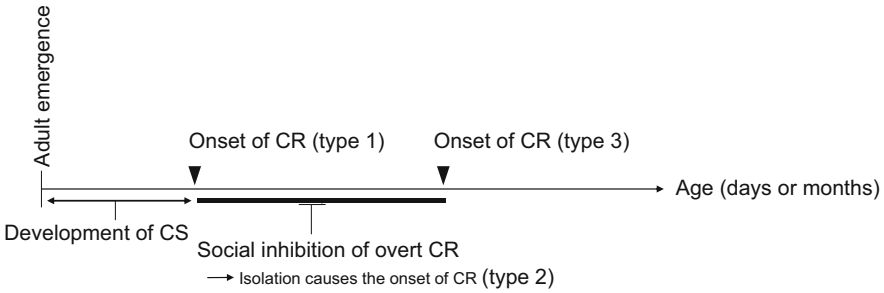

Fig. 8.2 Scheme of the ontogeny of circadian systems and rhythms in social hymenopterans. *CS* circadian system; *CR* circadian rhythm. Horizontal arrow indicates the timeline. Units vary (i.e., days or months) because ant workers live for several months, whereas bee workers sometimes live for as little as 1 month. Behavioral rhythm initiation types 1, 2, and 3 are defined in the text

in two sizes of worker groups, because the division of labor among workers depends on body size, with large workers typically performing foraging activities and small workers typically caring for the brood. Large bumblebee workers emerge without circadian locomotor activity and rapidly develop rhythms during the first few days after emergence, whereas small workers require more days to develop behavioral rhythms (Yerushalmi et al. 2006). At the cellular level, more PDF neurons are found in the brains of rhythmic large workers than in arrhythmic small workers (Weiss et al. 2009). However, the development of PDF-positive neurons has not been investigated in large rhythmic workers immediately after emergence. Comparisons of the development of PDF-positive neurons between large and small workers would contribute to our understanding of the mechanisms underlying the development of type 1 circadian rhythm initiation in social insects.

In social insects, behavioral rhythm initiation types 2 and 3 appear to share the same underlying mechanism (Fig. 8.2). For example, reverted nurse honeybees, honeybee workers that return from the foraging state to the nursing state, do not show circadian rhythms in their behavior (Bloch and Robinson 2001). Thus, the emergence and disappearance of circadian rhythms appear to be plastic, depending on the presence or absence of the brood. Type 2 behavioral rhythm initiation can be induced by experimental manipulation, whereas type 3 initiation occurs spontaneously in natural colonies. In honeybees, type 2 initiation occurs in workers when the brood is removed from the experimental cage (Shemesh et al. 2007; Shemesh et al. 2010). Type 3 has been documented in honeybees as follows: after adult emergence, workers do not show behavioral rhythms, even at ~10 days of age, and they care for the brood and perform other in-hive activities around the clock. Later (at ~20 days of age), they begin to work outside the hive with clear daily rhythmicity. Such brood deprivation experiments have not been performed in ants, but an experiment conducted by Fujioka et al. (2017) showed that the presence/absence of circadian rhythms depends on coexisting with the brood, which implies that brood deprivation would lead to the disappearance of circadian rhythms. The myrmicine ant *Pogonomyrmex occidentalis* shows age-related division of labor, in which old

foragers exhibit circadian rhythm but young nurses do not (Ingram et al. 2009); this species is speculated to experience the onset of circadian behavioral rhythm when the task switches from nurse to forager, an example of type 3 behavioral rhythm initiation.

8.5 Task- and Maternity-Related Circadian Rhythm Plasticity

The circadian rhythm plasticity seen in social insects is classified into two categories: that associated with division of labor among workers (category DOL) and that associated with reproductive state or maternity seen in reproductive individuals (category RS) (Table 8.1). DOL plasticity has been observed in bees and ants (Crailsheim et al. 1996; Moore et al. 1998; Shemesh et al. 2007; Shemesh et al. 2010; Nagari and Bloch 2012; Nagari et al. 2017). Honeybees and a few ant species exhibit a shift between clear circadian activity and activity around the clock, depending on whether they forage or nurse. In natural colonies of social insects that show age-related division of labor, young workers nurse the brood while old workers forage, thus shifting from arrhythmic to rhythmic behavior. In honeybees, reversion from rhythmic to arrhythmic behavior has been demonstrated in manipulated colonies from which nurse bees were removed (Bloch and Robinson 2001). Ants *Diacamma* sp. and *Polyrhachis dives* shifted from arrhythmic to circadian rhythmic behavior after coexisting with a brood in the laboratory (Jong and Lee 2008; Fujioka et al. 2017). Thus, it is reasonable to assume that their workers switch plastically between rhythmic and arrhythmic behavior, depending on the presence or absence of a brood.

Table. 8.1 Circadian rhythm plasticity in social insects

Category	Caste	State	Circadian rhythm	References
DOL	Worker/ nurse	With brood	Arrhythmic	Crailsheim et al. 1996, Moore et al. 1998, Bloch and Robinson 2001, Shemesh et al. 2007, Jong and Lee 2008, Shemesh et al. 2010, Nagari and Bloch 2012, Fujioka et al. 2017, Nagari et al. 2017
	Worker/ forager	Without brood	Rhythmic	
RS	Queen	Nonreproductive	Rhythmic	Free et al. 1992, Sharma et al. 2004a, Harano et al. 2007, Johnson et al. 2010
	Queen	Reproductive with brood	Arrhythmic	
	Queen	Reproductive without brood	Rhythmic	Eban-Rothschild et al. 2011

DOL Plasticity links to division of labor among workers
RS Plasticity links to reproductive state

Circadian rhythm plasticity related to reproductive state (category RS) has been reported in bees and ants (Sharma et al. 2004a; Eban-Rothschild et al. 2011). In honeybees, a queen was shown to perform the mating flight during a certain period of the day (Koeniger and Koeniger 2000). In a laboratory experiment, honeybee virgin queens exhibited circadian rhythms in locomotor activity (Harano et al. 2007), whereas in intensive observation studies, egg-laying queens exhibited around-the-clock activity (Free et al. 1992; Johnson et al. 2010). Similarly, in the ant *Camponotus compressus*, virgin queens exhibited circadian rhythms in locomotor activity in laboratory experiments, whereas queens exhibited around-the-clock activity during the egg-laying period (Sharma et al. 2004a). In a study on the bumblebee *B. terrestris*, virgin queens showed robust circadian rhythms in locomotor activity in the laboratory, whereas mated queens with a first batch of brood did not (Eban-Rothschild et al. 2011). These observations have given rise to the competing hypotheses that around-the-clock activity is caused either by interaction between reproductive individuals and a brood or by the reproductive state (i.e., the development of ovaries) of queens. The former hypothesis is supported by evidence that the presence/absence of brood is critical for circadian rhythm plasticity in bumblebee queens (Eban-Rothschild et al. 2011); in that study, ovariectomy did not affect circadian rhythm plasticity, contradicting the latter hypothesis in bumblebees. By contrast, in *Camponotus* ants, reproductive state appears to be critical for circadian rhythm plasticity (Sharma et al. 2004a). Further studies are required to determine whether there are different regulatory mechanisms for circadian rhythm plasticity in reproductive individuals among social insects of different taxa.

Whether circadian rhythm plasticity has different regulatory mechanisms in association with division of labor or reproductive state remains unknown. The critical effects of the presence of brood on circadian rhythm plasticity are commonly observed in reproductive individuals among bumblebees, honeybee workers, and some ant species. From an evolutionary perspective, corbiculate bees (e.g., honeybees and bumblebees) and ants (Formicidae) evolved eusociality independently (Peters et al. 2017). Thus, the links among division of labor, reproduction, and circadian organization may involve a mixture of common and differential regulatory machineries among taxa.

8.6 Closing Remarks

Social insects such as bees, wasps, ants, and termites exhibit clear circadian behavioral rhythms depending on their social environment that show broadly fundamental features including entrainment to light. In social insects, characteristic features of circadian rhythm, such as social entrainment, delayed behavioral rhythm ontogeny, and circadian rhythm plasticity, are observed in species with independent eusociality origins. Research on these features has advanced in honeybees but appears to be insufficient in ants and termites. Further research on ants and termites will show common and diverse regulatory mechanisms for the characteristic features of

circadian rhythms, which will elucidate the relationship between circadian systems and the evolution of sociality, which is among the most important topics in animal biology.

References

Abushama FT, Al Houty WA (1989) Diurnal activity rhythms of the subterranean termite *Anacanthotermes vagans* (Hagen) under laboratory and field conditions of the Kuwait desert. Int J Biomet 33:12–18. https://doi.org/10.1007/BF01045891

Amir S, Stewart J (1996) Resetting of the circadian clock by a conditioned stimulus. Nature 379: 542–545. https://doi.org/10.1038/379542a0

Beer K, Helfrich-Förster C (2020) Post-embryonic development of the circadian clock seems to correlate with social life style in bees. Front Cell Dev Biol 8:1325. https://doi.org/10.3389/fcell.2020.581323

Beer K, Steffan-Dewenter I, Härtel S, Helfrich-Förster C (2016) A new device for monitoring individual activity rhythms of honey bees reveals critical effects of the social environment on behavior. J Comp Physiol A 202:555–565. https://doi.org/10.1007/s00359-016-1103-2

Beshers SN, Fewell JH (2001) Models of division of labor in social insects. Annu Rev Entomol 46: 413–440. https://doi.org/10.1146/annurev.ento.46.1.413

Bloch G, Bar-Shai N, Cytter Y, Green R (2017) Time is honey: circadian clocks of bees and flowers and how their interactions may influence ecological communities. Phil Trans R Soc B. 372: 20160256. https://doi.org/10.1098/rstb.2016.0256

Bloch G, Herzog ED, Levine JD, Schwartz WJ (2013) Socially synchronized circadian oscillators. Proc R Soc B 280:20130035. https://doi.org/10.1098/rspb.2013.0035

Bloch G, Robinson GE (2001) Chronobiology - Reversal of honeybee behavioural rhythms. Nature 410:1048–1048. https://doi.org/10.1038/35074183

Bochynek T, Tanner JL, Meyer B, Burd M (2017) Parallel foraging cycles for different resources in leaf-cutting ants: a clue to the mechanisms of rhythmic activity. Ecol Entomol 42:849–852. https://doi.org/10.1111/een.12437

Castillo-Ruiz A, Paul MJ, Schwartz WJ (2012) In search of a temporal niche: Social interactions. In: Kalsbeek A, Merrow M, Roenneberg T, Foster RG (eds) Neurobiology of Circadian Timing. Elsevier, Amsterdam, pp 267–280

Chittka L, Stelzer RJ, Stanewsky R (2013) Daily changes in ultraviolet light levels can synchronize the circadian clock of bumblebees (*Bombus terrestris*). Chronobiol Int 30:434–442. https://doi.org/10.3109/07420528.2012.741168

Chong K-F, Lee C-Y (2009) Influences of temperature, relative humidity and light intensity on the foraging activity of field populations of the longlegged ant, *Anoplolepis gracilipes* (Hymenoptera: Formicidae). Sociobiology 54:531

Crailsheim K, Hrassnigg N, Stabentheiner A (1996) Diurnal behavioural differences in forager and nurse honey bees (*Apis mellifera carnica* Pollm). Apidologie 27:235–244. https://doi.org/10.1051/apido:19960406

Eban-Rothschild A, Belluci S, Bloch G (2011) Maternity-related plasticity in circadian rhythms of bumble-bee queens. Proc R Soc B 278:3510–3516. https://doi.org/10.1098/rspb.2011.0579

Eban-Rothschild A, Bloch G (2012) Social influences on circadian rhythms and sleep in insects. Adv Genet 77:1–32. https://doi.org/10.1016/B978-0-12-387687-4.00001-5

Eban-Rothschild A, Shemesh Y, Bloch G (2012) The colony environment, but not direct contact with conspecifics, influences the development of circadian rhythms in honey bees. J Biol Rhythms 27:217–225. https://doi.org/10.1177/0748730412440851

Free JB, Ferguson AW, Simpkins JR (1992) The Behavior of Queen Honeybees and Their Attendants. Physiol Entomol 17:43–55. https://doi.org/10.1111/j.1365-3032.1992.tb00988.x

Frisch B, Aschoff J (1987) Circadian rhythms in honeybees - Entrainment by feeding cycles. Physiol Entomol 12:41–49. https://doi.org/10.1111/j.1365-3032.1987.tb00722.x

Frisch B, Koeniger N (1994) Social synchronization of the activity rhythms of honeybees within a colony. Behav Ecol Sociobiol 35:91–98. https://doi.org/10.1007/BF00171498

Fuchikawa T, Eban-Rothschild A, Nagari M, Shemesh Y, Bloch G (2016) Potent social synchronization can override photic entrainment of circadian rhythms. Nat Commun 7. https://doi.org/10.1038/ncomms11662

Fuchikawa T, Matsubara K, Miyatake T, Matsuura K (2012) Acoustic emission monitoring of the effect of temperature on activity rhythms of the subterranean termite *Reticulitermes speratus*. Physiol Entomol 37:303–308. https://doi.org/10.1111/j.1365-3032.2012.00841.x

Fuchikawa T, Shimizu I (2007) Circadian rhythm of locomotor activity in the Japanese honeybee, *Apis cerana japonica*. Physiol Entomol 32:73–80. https://doi.org/10.1111/j.1365-3032.2006.00543.x

Fuchikawa T, Shimizu I (2008) Parametric and nonparametric entrainment of circadian locomotor rhythm in the Japanese honeybee *Apis cerana japonica*. Biol Rhythm Res 39:57–67. https://doi.org/10.1080/09291010701318188

Fujioka H, Abe MS, Fuchikawa T, Tsuji K, Shimada M, Okada Y (2017) Ant circadian activity associated with brood care type. Biol Lett 13:20160743. https://doi.org/10.1098/rsbl.2016.0743

Gattermann R, Weinandy R (1997) Lack of social entrainment of circadian activity rhythms in the solitary golden hamster and in the highly social Mongolian gerbil. Biol Rhythm Res 28:85–93. https://doi.org/10.1076/brhm.28.3.5.85.13122

Giannoni-Guzman MA, Aleman-Rios J, Moreno AMM, Hernandez GD, Torres MP, Lubriel D et al (2020) The role of temperature on the development of circadian rhythms in honey bee workers. bioRxiv. https://doi.org/10.1101/2020.08.17.254557

Giannoni-Guzmán MA, Avalos A, Perez JM, Loperena EJO, Kayım M, Medina JA et al (2014) Measuring individual locomotor rhythms in honey bees, paper wasps and other similar-sized insects. J Exp Biol 217:1307–1315. https://doi.org/10.1242/jeb.096180

Golombek DA, Rosenstein RE (2010) Physiology of circadian entrainment. Physiol Rev 90:1063–1102. https://doi.org/10.1152/physrev.00009.2009

Harano K, Sasaki M, Sasaki K (2007) Effects of reproductive state on rhythmicity, locomotor activity and body weight in the European honeybee, *Apis mellifera* queens (Hymenoptera, Apini). Sociobiology 50:189–200

Hebrant F (1970) Circadian rhythm of respiratory metabolism in whole colonies of the termite, *Cubitermes exiguus*. J Insect Physiol 16:1229–1235. https://doi.org/10.1016/0022-1910(70)90211-8

Hinze B, Leuthold RH (1999) Age related polyethism and activity rhythms in the nest of the termite *Macrotermes bellicosus* (Isoptera, Termitidae). Insectes Soc 46:392–397. https://doi.org/10.1007/s000400050162

Hoenle PO, Blüthgen N, Brückner A, Kronauer DJ, Fiala B, Donoso DA et al (2019) Species-level predation network uncovers high prey specificity in a Neotropical army ant community. Mol Ecol 28:2423–2440. https://doi.org/10.1111/mec.15078

Howard KJ, Thorne BL (2010) Eusocial evolution in termites and Hymenoptera. In: Bignell DE, Roisin Y, Lo N (eds) Biology of termites: a modern synthesis. Springer, Dordrecht, pp 97–132

Ingram KK, Krummey S, LeRoux M (2009) Expression patterns of a circadian clock gene are associated with age-related polyethism in harvester ants, *Pogonomyrmex occidentalis*. BMC Ecol 9:7. https://doi.org/10.1186/1472-6785-9-7

Jeanne RL (2009) Nest building. In: Resh VH, Cardé RT (eds) Encyclopedia of insects. Elsevier, Amsterdam, pp 688–691

Jeanne RL (2016) Division of labor is not a process or a misleading concept. Behav Ecol Sociobiol 70:1109–1112. https://doi.org/10.1007/s00265-016-2146-7

Johnson JN, Hardgrave E, Gill C, Moore D (2010) Absence of consistent diel rhythmicity in mated honey bee queen behavior. J Insect Physiol 56:761–773. https://doi.org/10.1016/j.jinsphys.2010.01.004

Jong JJ, Lee HJ (2008) Differential expression of circadian locomotor rhythms among castes of the gray-black spiny ant, *Polyrhachis dives* (Hymenoptera: Formicidae). Sociobiology 52:167–184
Koeniger N, Koeniger G (2000) Reproductive isolation among species of the genus *Apis*. Apidologie. 31:313–339. https://doi.org/10.1051/apido:2000125
Korb J, Thorne B (2017) Sociality in termites. In: Rubenstein D, Abbot P (eds) Comparative social evolution. Cambrigde Univeristy Press, Cambrigde, pp 124–153
Krupp JJ, Kent C, Billeter J-C, Azanchi R, So AK-C, Schonfeld JA et al (2008) Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. Curr Biol 18:1373–1383. https://doi.org/10.1016/j.cub.2008.07.089
Lei Y, Zhou Y, Lü L, He Y (2019) Rhythms in foraging behavior and expression patterns of the foraging gene in *Solenopsis invicta* (Hymenoptera: Formicidae) in relation to photoperiod. J Econ Entomol 112:2923–2930. https://doi.org/10.1093/jee/toz175
Levine JD, Funes P, Dowse HB, Hall JC (2002) Resetting the circadian clock by social experience in *Drosophila melanogaster*. Science 298:2010–2012. https://doi.org/10.1126/science.1076008
Lewis VR, Leighton S, Tabuchi R, Baldwin JA, Haverty MI (2013) Influence of environmental factors on activity patterns of *Incisitermes minor* (Isoptera: Kalotermitidae) in naturally infested logs. J Econ Entomol 106:338–346. https://doi.org/10.1603/ec12050
Liporoni R, Cordeiro GD, Prado PI, Schlindwein C, Warrant EJ, Alves-dos-Santos I (2020) Light intensity regulates flower visitation in Neotropical nocturnal bees. Sci Rep 10:1–11. https://doi.org/10.1038/s41598-020-72047-x
Lone SR, Sharma VK (2011) Timekeeping through social contacts: Social synchronization of circadian locomotor activity rhythm in the carpenter ant *Camponotus paria*. Chronobiol Int 28:862–872. https://doi.org/10.3109/07420528.2011.622676
McCluskey ES (1987) Circadian rhythm in the tropical ant *Ectatomma* (Hymenoptera: Formicidae). Psyche 94:245–251
Mildner S, Roces F (2017) Plasticity of daily behavioral rhythms in foragers and nurses of the ant *Camponotus rufipes*: influence of social context and feeding times. PLoS One 12:e0169244. https://doi.org/10.1371/journal.pone.0169244
Mistlberger RE, Skene DJ (2004) Social influences on mammalian circadian rhythms: animal and human studies. Biol Rev 79:533–556. https://doi.org/10.1017/S1464793103006353
Mizumoto N, Fuchikawa T, Matsuura K (2017) Pairing strategy after today's failure: unpaired termites synchronize mate search using photic cycles. Popul Ecol 59:205–211. https://doi.org/10.1007/s10144-017-0584-3
Moore D (2001) Honey bee circadian clocks: behavioral control from individual workers to whole-colony rhythms. J Insect Physiol 47:843–857. https://doi.org/10.1016/S0022-1910(01)00057-9
Moore D, Angel JE, Cheeseman IM, Fahrbach SE, Robinson GE (1998) Timekeeping in the honey bee colony: integration of circadian rhythms and division of labor. Behav Ecol Sociobiol 43:147–160. https://doi.org/10.1007/s002650050476
Moore D, Rankin MA (1985) Circadian locomotor rhythms in individual honeybees. Physiol Entomol 10:191–197. https://doi.org/10.1111/j.1365-3032.1985.tb00034.x
Moore D, Rankin MA (1993) Light and temperature entrainment of a locomotor rhythm in honeybees. Physiol Entomol 18:271–278. https://doi.org/10.1111/j.1365-3032.1993.tb00599.x
Moritz RFA, Kryger P (1994) Self-organization of circadian rhythms in groups of honeybees (*Apis mellifera* L). Behav Ecol Sociobiol 34:211–215. https://doi.org/10.1007/BF00167746
Mrosovsky N (1988) Phase response curves for social entrainment. J Comp Physiol A 162:35–46. https://doi.org/10.1007/BF01342701
Muradian R, Issa S, Jaffe K (1999) Energy consumption of termite colonies of *Nasutitermes ephratae* (Isoptera: Termitidae). Physiol Behav 66:731–735. https://doi.org/10.1016/S0031-9384(99)00011-6
Nagari M, Bloch G (2012) The involvement of the antennae in mediating the brood influence on circadian rhythms in "nurse" honey bee (*Apis mellifera*) workers. J Insect Physiol 58:1096–1103. https://doi.org/10.1016/j.jinsphys.2012.05.007

Nagari M, Brenner Y, Bloch G (2017) Nurse honeybee workers tend capped-brood, which does not require feeding, around-the-clock. J Exp Biol 220:4130–4140. https://doi.org/10.1242/jeb.166884

Narendra A, Kamhi JF, Ogawa Y (2017) Moving in dim light: behavioral and visual adaptations in nocturnal ants. Integr Comp Biol 57:1104–1116. https://doi.org/10.1093/icb/icx096

North R (1987) Circadian rhythm of locomotor activity in individual workers of the wood ant *Formica rufa*. Physiol Entomol 12:445–454. https://doi.org/10.1111/j.1365-3032.1987.tb00771.x

Page TL (1990) Circadian rhythms of locomotor activity in cockroach nymphs: free running and entrainment. J Biol Rhythms 5:273–289. https://doi.org/10.1177/074873049000500401

Passera L, Lachaud J-P, Gomel L (1994) Individual food source fidelity in the neotropical ponerine ant *Ectatomma ruidum* Roger (Hymenoptera Formicidae). Ethol Ecol Evol 6:13–21. https://doi.org/10.1080/08927014.1994.9523004

Peters RS, Krogmann L, Mayer C, Donath A, Gunkel S, Meusemann K et al (2017) Evolutionary history of the Hymenoptera. Curr Biol 27:1013–1018. https://doi.org/10.1016/j.cub.2017.01.027

Reebs SG (1989) Acoustical entrainment of circadian activity rhythms in house sparrows - Constant light is not necessary. Ethology 80:172–181. https://doi.org/10.1111/j.1439-0310.1989.tb00737.x

Retana J, Cerdá X, Espadaler X (1992) Coexistence of two sympatric ant species, *Pheidole pallidula* and *Tetramorium semilaeve* (Hymenoptera: Formicidae). Entomol Gen 17:029–040. https://doi.org/10.1127/entom.gen/17/1992/29

Robinson GE (1992) Regulation of division of labor in insect societies. Annu Rev Entomol 37:637–665. https://doi.org/10.1146/annurev.en.37.010192.003225

Robinson GE, Fernald RD, Clayton DF (2008) Genes and social behavior. Science 322:896–900. https://doi.org/10.1126/science.1159277

Roces F (1995) Variable thermal sensitivity as output of a circadian clock controlling the bimodal rhythm of temperature choice in the ant *Camponotus mus*. J Comp Physiol A 177:637–643. https://doi.org/10.1007/BF00207192

Saunders DS (2002) Insect Clocks, 3rd edn. Elsevier, Amsterdam

Sehgal A, Price J, Young MW (1992) Ontogeny of a biological clock in *Drosophila melanogaster*. Proc Natl Acad Sci USA 89:1423–1427. https://doi.org/10.1073/pnas.89.4.1423

Sharma VK, Chandrashekaran MK (2005) Zeitgebers (time cues) for biological clocks. Curr Sci 89:1136–1146. https://www.jstor.org/stable/24110966

Sharma VK, Lone SR, Goel A (2004a) Clocks for sex: loss of circadian rhythms in ants after mating? Naturwissenschaften 91:334–337. https://doi.org/10.1007/s00114-004-0526-8

Sharma VK, Lone SR, Goel A, Chandrashekaran M (2004b) Circadian consequences of social organization in the ant species *Camponotus compressus*. Naturwissenschaften 91:386–390. https://doi.org/10.1007/s00114-004-0544-6

Sharma VK, Lone SR, Mathew D, Goel A, Chandrashekaran MK (2004c) Possible evidence for shift work schedules in the media workers of the ant species *Camponotus compressus*. Chronobiol Int 21:297–308. https://doi.org/10.1081/CBI-120037817

Shemesh Y, Cohen M, Bloch G (2007) Natural plasticity in circadian rhythms is mediated by reorganization in the molecular clockwork in honeybees. FASEB J 21:2304–2311. https://doi.org/10.1096/fj.06-8032com

Shemesh Y, Eban-Rothschild A, Cohen M, Bloch G (2010) Molecular dynamics and social regulation of context-dependent plasticity in the circadian clockwork of the honey bee. J Neurosci 30:12517–12525. https://doi.org/10.1523/Jneurosci.1490-10.2010

Siehler O, Bloch G (2020) Colony volatiles and substrate-borne vibrations entrain circadian rhythms and are potential cues mediating social synchronization in honey bee colonies. J Biol Rhythms 35:246–256. https://doi.org/10.1177/0748730420913362

Siehler O, Wang S, Bloch G (2021a) Remarkable sensitivity of young honey bee workers to multiple non-photic, non-thermal, forager cues that synchronize their daily activity rhythms. Front Physiol 2249. https://doi.org/10.3389/fphys.2021.789773

Siehler O, Wang S, Bloch G (2021b) Social synchronization of circadian rhythms with a focus on honeybees. Phil Trans R Soc B 376:20200342. https://doi.org/10.1098/rstb.2020.0342

Southwick EE, Moritz RFA (1987) Social synchronization of circadian rhythms of metabolism in honeybees (*Apis mellifera*). Physiol Entomol 12:209–212. https://doi.org/10.1111/j.1365-3032.1987.tb00743.x

Stelzer RJ, Chittka L (2010) Bumblebee foraging rhythms under the midnight sun measured with radiofrequency identification. BMC Biol 8:93. https://doi.org/10.1186/1741-7007-8-93

Stelzer RJ, Stanewsky R, Chittka L (2010) Circadian foraging rhythms of bumblebees monitored by radio-frequency identification. J Biol Rhythms 25:257–267. https://doi.org/10.1177/0748730410371750

Toma DP, Bloch G, Moore D, Robinson GE (2000) Changes in period mRNA levels in the brain and division of labor in honey bee colonies. Proc Natl Acad Sci USA 97:6914–6919. https://doi.org/10.1073/pnas.97.12.6914

Tomioka K, Chiba Y (1982) Post-embryonic development of circadian rhythm in the cricket, *Gryllus bimaculatus*: a rhythm reversal. J Comp Physiol 147:299–304. https://doi.org/10.1007/BF00609663

Vargo EL, Husseneder C (2009) Biology of subterranean termites: insights from molecular studies of *Reticulitermes* and *Coptotermes*. Annu Rev Entomol 54:379–403. https://doi.org/10.1146/annurev.ento.54.110807.090443

Wams EJ, Riede SJ, Ivd L, Tt B, Hut RA (2017) Mechanisms of non-photic entrainment. In: Kumar V (ed) Biological timekeeping: clocks, rhythms and behaviour. Springer, New Delhi, pp 395–404

Wcislo WT, Tierney SM (2009) Behavioural environments and niche construction: the evolution of dim-light foraging in bees. Biol Rev 84:19–37. https://doi.org/10.1111/j.1469-185X.2008.00059.x

Weiss R, Dov A, Fahrbach SE, Bloch G (2009) Body size-related variation in pigment dispersing factor-immunoreactivity in the brain of the bumblebee *Bombus terrestris* (Hymenoptera, Apidae). J Insect Physiol 55:479–487. https://doi.org/10.1016/j.jinsphys.2009.01.016

Win AT, Machida Y, Miyamoto Y, Dobata S, Tsuji K (2018) Seasonal and temporal variations in colony-level foraging activity of a queenless ant, *Diacamma* sp., in Japan. J Ethol 36:277–282

Yerushalmi S, Bodenhaimer S, Bloch G (2006) Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees. J Exp Biol 209:1044–1051. https://doi.org/10.1242/jeb.02125

Zhao J, Warman GR, Stanewsky R, Cheeseman JF (2019) Development of the molecular circadian clock and its light sensitivity in *Drosophila melanogaster*. J Biol Rhythms 34:272–282. https://doi.org/10.1177/0748730419836818

Chapter 9
Environmental Adaptation and Evolution of Circadian Clocks

Chitrang Dani, Nisha N. Kannan, and Vasu Sheeba

Abstract In insects, circadian clocks regulate daily rhythmicity in behavior (e.g., activity, feeding, mating, and oviposition), physiological processes, and developmental events such as hatching, pupariation, and eclosion. While the abiotic environment poses risks such as death or sterility due to extreme temperatures or desiccation, interactions with the biotic environment also give rise to other stressors such as energy expenditure, starvation, and predation risk. In this chapter, we discuss studies, mostly on drosophilid species, in laboratory as well as natural environments, highlighting the impact of rhythmic light and temperature on clock evolution. We also examine how clocks modulate life-history traits and, conversely, how selection on life-history traits may alter circadian clock properties. We also present a few studies emphasizing the vast diversity in clock function across different insect taxa. Last, we draw attention to the consequences of a rapidly changing climate on insect physiology, specifically rhythms.

Keywords Adaptation · Circadian · *Drosophila* · Evolution · Insect · Rhythm

C. Dani
Chronobiology and Behavioural Neurogenetics Laboratory, Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India

Present Address: Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

N. N. Kannan
Chronobiology Laboratory, School of Biology, Indian Institute of Science Education and Research (IISER), Thiruvananthapuram, India

V. Sheeba (✉)
Chronobiology and Behavioural Neurogenetics Laboratory, Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India
e-mail: sheeba@jncasr.ac.in

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9.1 Introduction

Organisms face environmental challenges resulting from cyclic variations in light, temperature, humidity, etc. Thus, the need to effectively adapt to these environmental changes is hypothesized to have driven the evolution of highly conserved biological timekeeping systems. Circadian clocks provide an extrinsic advantage to organisms by improving their ability to anticipate environmental changes and to synchronize behavioral and physiological processes with daily environmental cycles (Enright 1980; Sharma 2003). During evolution, the circadian timing system may have also evolved the ability to govern the timing of endogenous processes and thus confer an additional intrinsic benefit (Pittendrigh 1993; Sharma 2003). Insects have served as important models for studies investigating the adaptive value of circadian clocks and yet have distinct attributes from other common model systems. Various forms of the term adaptation are commonly used in two contexts: as a process through which organisms become suited to their surroundings or as a trait giving organisms a higher fitness in a specific environment. This chapter uses "adapt" for the former and "adaptive/adaptation" for the latter context. Thus, adaptive traits for an organism in terms of survival and reproductive output in a given environment are likely to be favored by natural selection. Individuals with such beneficial traits can be thought of as having higher evolutionary fitness and contributing proportionally more to the gene pool.

Since insects are ectothermic and have a smaller body size than other animals, they are subjected to various environmental stressors with fatal or sublethal deleterious effects on physiology. Most insects exhibit active and inactive phases throughout the day, which is thought to help them cope with such stressors.

9.2 Light-Dark Cycles as a Selection Pressure for the Evolution of Endogenous Clocks

Environmental light-dark cycles with changes in the intensity and duration of light are believed to be the prime force of selection behind the evolution of circadian clocks (Roenneberg and Foster 1997; Woelfle et al. 2004). It is conceivable that circadian clocks segregated daytime and nighttime processes, and such temporal segregation of incompatible processes also minimized the harmful effects of the diurnal photooxidative environment on light-sensitive reactions (Pittendrigh 1993). Additionally, seasonal changes in day length at temperate and polar latitudes may have acted as additional constraints for the evolution of circadian clocks. Hence, clocks in species residing in divergent latitudes can be expected to evolve differential properties. Latitudinal clines refer to correlated phenotypic and/or genetic differences observed over a geographical area with a change in latitude. Since circadian clocks are believed to confer an adaptive advantage to their owners in natural environments, changes in circadian clock properties correlated with latitudinal changes have been investigated.

Several studies have surveyed circadian behavior and clock gene variation over large geographical areas, and latitudinal clines in behavior, physiology, gene frequencies, protein isoforms, etc. have been discovered. Surveys of 57 strains of *Drosophila littoralis* (30°N–70°N) and 12 strains of *Drosophila subobscura* (56° N–63°N) revealed latitude-dependent variation in the phase and period of the eclosion rhythm (Lankinen 1986, 1993). Four Japanese strains of *Drosophila auraria* (34.2°N–42.9°N) exhibit a significant latitudinal cline in phase, lability of the period, and amplitude of the phase response curve (PRC, a plot of the rhythm's shift in phase as a function of the phase of light pulse) of eclosion rhythm (Pittendrigh and Takamura 1989); high-latitude *D. auraria* strains showed a lower-amplitude PRC. A northern species, *Drosophila montana*, is widespread at high latitudes, and these flies completely lack morning activity. They maintain free-running periodicity better under constant light than under constant darkness and differ also in the number and location of PDF (pigment-dispersing factor)- and CRY (cryptochrome)-expressing neurons compared to *Drosophila melanogaster* (Kauranen et al. 2012). High-altitude species such as *Drosophila lummei*, *D. littoralis*, and *Drosophila ezoana* also exhibit similar features in their activity-rest rhythms that correlate with the difference in the neurochemistry of PDF and CRY in their circadian clock network. These are likely to be the specific adaptive features of the circadian clock that evolved in *Drosophila* species in winter environments to colonize polar regions (Kauranen et al. 2012; Menegazzi et al. 2017; Beauchamp et al. 2018). Another drosophilid fly, *Chymomyza costata*, found at latitudes above 40°N, also becomes behaviorally arrhythmic under constant darkness, whereas its molecular clock remains rhythmic and uncoupled from the behavioral output (Bertolini et al. 2019). Locomotor activity patterns and clock network neurochemistry are similar in distantly related *Drosophila* species colonized at low latitudes, suggestive of convergent evolution. In contrast, phylogenetically related species living at different latitudes exhibit differences in their clock organization and coupling (Bertolini et al. 2019). These studies suggest that in some *Drosophila* species, a *D. melanogaster*-like ancestral fly clock network evolved with altered PDF and CRY expression to adapt and colonize high-latitude environments (Beauchamp et al. 2018; Bertolini et al. 2019).

With respect to clines in clock gene variation and gene frequencies in *D. melanogaster*, the product of the *timeless* (*tim*) gene has two allelic forms, *ls-tim* and *s-tim*, varying in length due to the presence of a second start codon downstream. The presence of the *ls-tim* allele results in the formation of full-length LS-TIM and truncated S-TIM, while the presence of *s-tim* results in the formation of truncated S-TIM only. LS-TIM is less sensitive to light than S-TIM due to the weaker interaction with CRY (Sandrelli et al. 2007). Tauber et al. (2007) reported that natural populations of *D. melanogaster* in Europe show a latitudinal cline for this polymorphism, with the frequency of *ls-tim* increasing from north to south of Europe. The reduced light sensitivity of *ls-tim* flies prevents enhanced TIM degradation and arrhythmicity during prolonged exposure to light under northern long summer day length. In addition to lower light sensitivity, the *ls-tim* mutation induces earlier diapause in female flies during autumn. Thus, it appears that the latitudinal

cline in TIM polymorphism evolved in the circadian timekeeping system to adapt to the seasonal changes in the north (Kyriacou et al. 2008). This has been substantiated by a recent study showing that *ls-tim*, but not *s-tim* flies, can synchronize to temperature cycles under constant light and simulated northern summer conditions, and the expression of *ls-tim* in clock neurons is sufficient for this synchronization (Lamaze et al. 2022).

9.3 Impact of Temperature Cycles on Circadian Clocks

While light is considered the most potent time cue for the circadian timing system in almost all organisms, temperature has also been found to entrain the circadian clocks of various organisms, including *Drosophila* (Balzer and Hardeland 1988; Tomioka et al. 1998). Under lower temperatures, *D. melanogaster* schedules a large proportion of their activity to daytime, whereas under warmer temperatures, they exhibit increased nighttime activity with a pronounced midday siesta (Majercak et al. 1999). Differential splicing of *period* (*per*) (Majercak et al. 1999) and *tim* (Anduaga et al. 2019) is associated with seasonal redistribution of the activity pattern of *D. melanogaster*. *Drosophila simulans* also exhibit thermally sensitive alternate *per* splicing as an adaptation to summer in a temperate climate (Low et al. 2008).

In *D. melanogaster*, *per* encodes a continuous stretch of threonine-glycine (TG) repeats. A latitudinal cline exists in the length of the TG repeat number at the *per* locus in natural populations of Europe and North Africa. Two major alleles, *per* TG_{17} and *per* TG_{20}, comprise 90% of the variation observed, with the frequency of *per* TG_{20} decreasing, whereas that of *per* TG_{17} increases from north to south (Costa et al. 1992; Sawyer et al. 1997). Similarly, a cline in *per* TG_{20} frequency is observed in Australia, although it appears less robust than in Europe (Sawyer et al. 2006). The length of TG repeats is associated with temperature compensation based on a TG monomer's structural property to confer greater thermal stability (Castiglione-Morelli et al. 1995). Assessment of the functional significance of this repeat length polymorphism showed that TG_{17} may be suitable for thermally less variable environments, whereas the TG_{20} variant may be under selection for its better temperature compensatory ability under larger temperature fluctuations (Kyriacou et al. 2008). TG repeat length polymorphism is observed in other species, such as *D. simulans* and *D. pseudoobscura* (Costa et al. 1991; Rosato et al. 1994). Thus, there is compelling evidence in natural environments that in addition to light, temperature changes may have contributed to the genetic variance and evolution of the circadian timing system (Fig. 9.1).

In nature, animals experience varying light intensity and temperature throughout the day and seasons. It is crucial to understand the synergistic impact of such daily varying time cues on the evolution of the circadian clock and its adaptive significance. Recent studies have shown that the activity-rest rhythm and eclosion rhythm differ considerably under natural conditions from those observed under laboratory experiments (Vanin et al. 2012; De et al. 2013; Prabhakaran and Sheeba 2013;

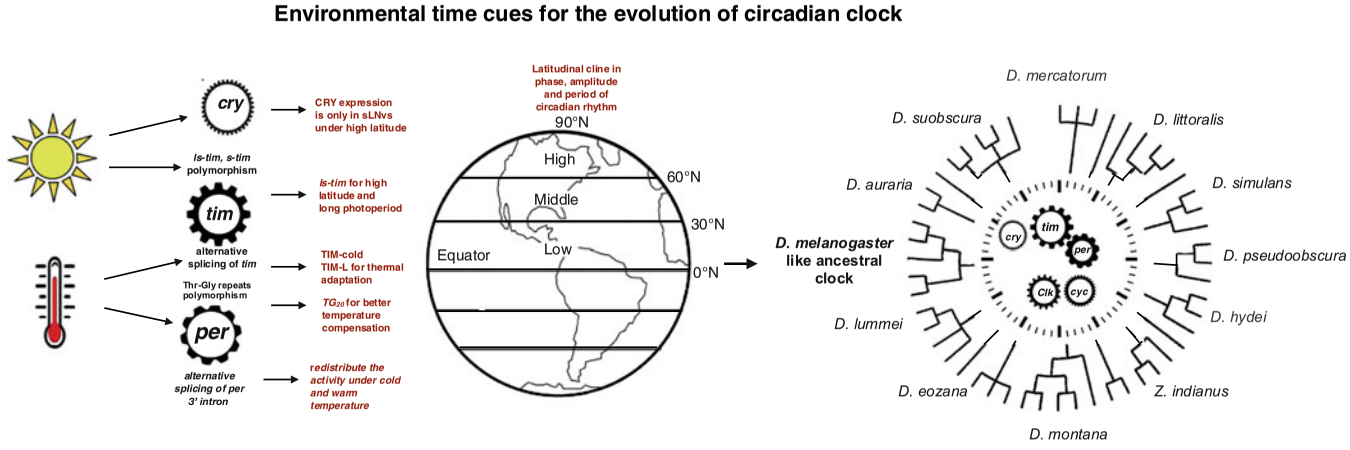

Fig. 9.1 Environmental time cues as a selection pressure for the evolution of circadian clocks. Extremely short and long photoperiods may have acted as selection pressure for the evolution of circadian clocks to adapt to different latitudes. Temperature changes modulate the intron splicing of *period* and *timeless* transcripts to reorganize activity patterns. Latitudinal clines have been observed in the phase and period of behavioral rhythm and threonine-glycine (TG) repeat number at the *per* locus of fly strains collected from different latitudes. In Europe, *D. melanogaster* shows a latitudinal cline in *timeless* polymorphism – frequency of *ls-tim* increasing from north to south. The *D. melanogaster*-like ancestral fly clock network may have evolved with alterations in neurons expressing PDF and CRY, allowing some *Drosophilid* species to adapt to high-latitude environments

Prabhakaran et al. 2013). Under laboratory conditions, *D. melanogaster* exhibits morning and evening peaks of activity with a siesta during the middle of the day. However, under seminatural conditions, an additional afternoon (A) peak of activity replaces the siesta (Vanin et al. 2012). This A peak is largely dependent on temperature and requires the temperature-sensitive transient receptor potential A1 ion channel (Das et al. 2015). PER levels change seasonally under seminatural conditions, whereas those of TIM remain somewhat constant (Menegazzi et al. 2013). The oscillation of these proteins is decoupled in summer conditions, and how it continues to drive rhythmic behavioral output has yet to be elucidated.

9.4 Evolution of Circadian Rhythms: Insights from Laboratory Selection Studies

The notion that circadian clocks are innate, having a genetic basis, paved the way for the idea of existent genetic variation for circadian clock-controlled behavior. Considering that it was intuitive to assume that circadian clocks evolved in response to geophysical cycles on earth, a laboratory selection approach was an attractive method to gain insights into how circadian clock properties respond to specific selection pressures. Indeed, several studies have used an experimental-evolution-based approach in insects to demonstrate various aspects of the evolution of traits such as longevity (Rose 1984), fecundity (Rose and Charlesworth 1981), development time (Zwaan et al. 1995), and starvation tolerance (Chippendale et al. 1996).

Conceptually, experimental-evolution studies are relatively straightforward. A series of replicated populations are exposed to a novel environment for many generations, while in parallel, a control set is kept under the ancestral environment. This makes alteration of any aspect of the ancestral population's abiotic or biotic environment or its demographic condition possible. For the sake of simplicity, only one environmental variable is usually changed. However, if the experimenter introduces a novel experimental environment, it is expected to exert selection pressure, promoting evolution. Depending on the study organism and selection regime, traits may evolve due to differential selection of variants from the existing genetic variation of populations. Alternately new genetic variants may emerge (via mutation or recombination) because they are differentially favored in altered conditions, ultimately resulting in differential reproduction and expansion of the favored genotypes within populations (Gibbs 1999).

As previously discussed, the intrinsic advantage hypothesis proposes that circadian clocks are necessary for maintaining internal synchrony among constituent oscillators within an organism. However, it is also believed that having a biological clock in constant conditions could be unnecessary, if not harmful, because rhythmically active organisms in such environments will be more likely to miss foraging opportunities that could be aperiodic (Poulson and White 1969). Thus, functional circadian clocks in aperiodic environments, along with the possibility of having an

intrinsic advantage, may also confer an apparent "extrinsic disadvantage." In such a scenario, the persistence of rhythmicity in constant environments indicates fitness benefits due to internal synchrony, possibly overriding a fitness cost due to missed foraging opportunities or predator avoidance.

9.4.1 Evolution of Clocks Under Aperiodic Environments

Previous laboratory selection studies using *Drosophila* have shown that it is common to find that traits providing no fitness advantage to the organism under the given culture conditions become affected by random genetic drift relatively quickly within 100–200 generations (Service et al. 1988). When the specific trait has an evolutionary cost, the regression can be even faster, with mean values reverting to those of control populations in a span of ~20 generations (Teotónio and Rose 2001). If populations are allowed to evolve in the absence of any daily time cues, for a sufficiently long time, one can examine whether the ability to measure time cues is retained or lost. A laboratory selection approach showed that populations of *D. melanogaster* reared under constant light for more than 600 generations (LL populations) exhibited the persistence of both the population eclosion rhythm, as well as individual-level oviposition, and locomotor activity-rest under DD (constant darkness) and LD (light-dark cycles) (Sheeba et al. 1999b, 2001, 2002b) (Fig. 9.2a). The persistence of circadian rhythms in DD implied that their underlying clocks had not regressed over time, whereas the behavior in LD indicated that such clocks were capable of entrainment. Along with these observations, Sheeba et al. (2001) also found a significant difference between the free-running periods of eclosion, activity-rest, and oviposition rhythms. Furthermore, the ability to entrain to a wide range of LD, LD 10:10 (10 h light:14 h darkness), LD 12:12, and LD 14:14, was also retained (Paranjpe et al. 2003).

Another group carried out studies on *D. melanogaster* "dark-fly" stocks reared under constant darkness for ~1300 generations (Imafuku and Haramura 2011). These were initially established in 1954 and maintained as a culture consisting of 50–200 individuals. These flies were adapted to dark conditions, reflected in higher fecundity in constant darkness compared to control lines, while they did not differ under constant light (Izutsu et al. 2012). Additionally, the same group also showed a nonsense mutation in the R7 photoreceptor gene of the dark-fly culture via genome sequencing, suggesting that dark-raised flies may lose a light-input channel to the circadian clock due to being reared under DD for many generations (Saint-Charles et al. 2016). A recent study investigating relaxed selection on dark-flies under normal lighting conditions found a simultaneous trade-off between vision and olfaction, with the size of the optic lobes increasing and antennal lobes decreasing at the first and 65th generations compared to controls (Özer and Carle 2020). Dark-flies have also shown differences from control flies in several other phenotypes, such as photokinesis, olfactory response, and head bristle elongation (Fuse et al. 2014).

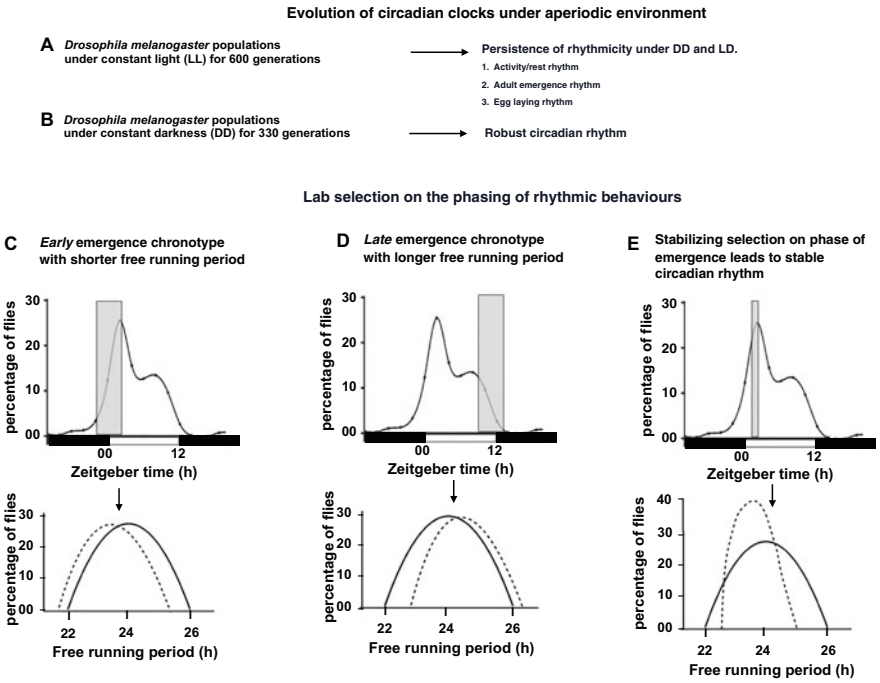

Fig. 9.2 Evolution of circadian clocks: Insights from the laboratory selection studies from JNCASR, India. (**a**) Populations of *Drosophila melanogaster* reared under constant light (LL) for more than 600 generations exhibited persistence of rhythmicity in adult emergence rhythm, activity-rest rhythms, and oviposition rhythm under constant darkness (DD) and light-dark cycles (LD). (**b**) Rearing populations under constant darkness for more than 330 generations lead to the evolution of robustness of the rhythm. (**c**), (**d**) The *Early* and *Late* populations of *D. melanogaster* derived by selecting for individuals who emerged during the morning or the evening hours only under LD 12: 12. After 70 generations of selection, the free-running periodicities of the activity-rest rhythm of the *Early* flies were significantly shorter than those of the controls, whereas those of the *Late* flies were significantly longer than those of the controls. (**e**) Populations of *D. melanogaster* selected for accuracy of emergence were derived by selecting for individuals who emerged during a narrow window of 1 h that coincides with the peak of adult emergence. This stabilizing selection for 80 generations led to the evolution of stable circadian clocks with reduced interindividual variation in the free-running period. Solid and dashed lines in (**c**–**e**) indicate the flies from control and selected populations, respectively

While the former set of studies by Sheeba et al. (1999b, 2001, 2002b) used large population sizes (>1500 flies), discrete generations, and multiple replicate populations, the latter carried out on the dark-fly culture had inbred origins (Oregon-R-S) and a relatively small population size. Hence, while in Sheeba et al. (1999b, 2001, 2002b), the results have to be interpreted with respect to selection on standing genetic variation of populations, in the case of studies on dark-flies, evolution by mutation is the primary driver of evolutionary change. Even with a small effective population size (~90 individuals), concluding about the occurrence and fixation of a gene for arrhythmia, if beneficial for evolutionary fitness, will

require approximately 3000 generations (Imafuku and Haramura 2011) and thus warrants future investigation.

The above-mentioned studies on the long-term LL populations (Sheeba et al. 1999b, 2001, 2002b) also have a major shortcoming: the lack of relevant control populations kept in a rhythmic environment such as LD 12:12 to deduce if the proportions of individuals having persisting rhythms despite being raised in LL have changed at all. Furthermore, the question of an intrinsic advantage arises only if circadian clocks were ticking under LL conditions. Previous research demonstrated that for *Drosophila* and many other organisms under LL, most behaviors and the underlying molecular clock become arrhythmic (Marrus et al. 1996). For the LL populations, if the challenge of sustaining internal synchronization did not arise, why do rhythms persist in these populations under constant darkness? One explanation is that perhaps under LL, certain unknown and light-insensitive components of the circadian clock still exhibit rhythms. Another possibility is that molecular clock components may have pleiotropic functions that prevent their regression despite being in an arrhythmic state even after several hundred generations. It is also possible that not enough generations have passed to indicate any notable circadian clock regression.

To overcome these drawbacks posed by the lack of control populations, two additional sets of populations were created from the $LL_{1–4}$ populations that were subsequently maintained under DD ($DD_{1–4}$ populations) and under LD 12:12 ($T24_{1–4}$ populations). A study after more than 330 generations under the above regimes on all 3 sets of populations found the persistence of rhythms in behaviors such as eclosion, activity-rest, and egg-laying. The power of the activity-rest rhythm was also higher for the DD populations (Shindey et al. 2016) (Fig. 9.2b). The evolution of robustness of the rhythm in DD populations may be indicative of the necessity for rhythm orchestration of internal physiology and metabolism. This is considered to be the selection pressure for the DD populations, as hypothesized for organisms in aperiodic habitats (Beale et al. 2016). Notably, a follow-up study by Shindey et al. (2017) found that in comparison with the LL populations, the DD populations showed lower anticipation to lights-on of the eclosion rhythm and more oviposition during the light phase. Thus, despite having more robust rhythms under DD, DD populations seem to exhibit poorer entrainment to LD 12:12 than LL populations, perhaps due to being reared in darkness for several hundred generations.

9.4.2 Evolution of Clocks Under Periodic Environments

While persistence of rhythms in aperiodic environments is an interesting question, another aspect of clocks that fascinates chronobiologists is the control on the timing of behavior and whether/how it evolves. Since most organisms on Earth encounter some form of daily cycling environmental cues, it is thought that circadian clocks evolved in response to selection pressures imposed by daily cycles and not a constant

environment. Thus, it is reasonable to assume that selection pressures acted on the phasing of rhythmic behaviors driving the evolution of underlying circadian clock properties. Several laboratory selection studies have examined whether the phasing of rhythms changes in response to periodic selection pressures and their effects on circadian clock properties and evolutionary fitness components. An early study by Pittendrigh (1967) using *D. pseudoobscura* suggested that the circadian period coevolved as a result of artificial selection on the phase of eclosion. A later series of studies using *D. melanogaster* from the group of VK Sharma (Kumar et al. 2007; Vaze et al. 2012b; Nikhil et al. 2015, 2016b) clearly demonstrated that evolution of the circadian phase resulted in changes in many aspects of circadian clock phenotypes, including differential circadian light sensitivity, amplitude, lability, and changes in underlying molecular circadian clocks. Additionally, they also diverged in temperature sensitivity and nonclock-driven light responses/masking (Abhilash et al. 2019, 2020; Ghosh et al. 2021).

Several studies have shown that individuals exhibiting deviant phases of activity, possessing dysfunctional circadian clocks, or exposed to exogenous cycle mismatches usually suffer fitness consequences (DeCoursey et al. 1997; Knutsson 2003; Horn et al. 2019). Maintaining a stable phase angle in cyclic conditions may be critical for an organism's survival and reproduction (Cloudsley-Thompson 1960). As a result, the idea of circadian clocks evolving higher stability was intriguing, as was the question of what other characteristics of circadian clocks may coevolve to aid such stability.

A long-term selection study was initiated from large outbreeding *D. melanogaster* populations by selecting for individuals emerging in a narrow window of time, i.e., 1 h (Kannan et al. 2012c). In response to selection, after ~80 generations, the number of flies eclosing in the selection window in the selected populations increased by approximately 10% compared to controls (Kannan et al. 2012c). Selection for accuracy also resulted in the evolution of lower inter- and intrapopulation variance in eclosion as an associated response, revealing that circadian clocks can acquire better stability in response to selection on the timing of eclosion. These "accurate" populations also evolved a shorter free-running period with less interindividual variation than the control populations, which is a demonstration of the complex link between clock properties exhibited under entrained and constant conditions (Fig. 9.2e). Furthermore, such stability resulting in overall robustness of the circadian system for the "accurate" populations was prevalent not only for the eclosion rhythm (under selection directly) but also for the activity-rest rhythm (Kannan et al. 2012a).

The same set of populations has provided valuable insights into how light sensitivity of the clock may evolve. A systematic set of experiments varying the lights-on timing showed that compared to controls, populations selected for high accuracy exhibited less masking to light, especially when the light was provided outside the eclosion gate, suggestive of tight gating of eclosion by the circadian clock (Varma et al. 2019). The "accurate" populations also showed increased delay phase shifts to light pulses, possibly acting via CRYPTOCHROME, and higher activity under orange light-dark cycles, perhaps mediated by compound eyes (Varma

2018). Several life-history-related changes also occurred in these populations, which we will discuss later.

Studies with large outbreeding *D. melanogaster* populations have also been carried out in seminatural conditions outside the laboratory. An investigation using the *Early* and *Late* populations showed increased divergence in the phasing of chronotypes under seminatural conditions (Vaze et al. 2012a). The emergence waveforms also appeared to be more consolidated under seminatural conditions than the phenotype observed in the laboratory. This was proposed to be a combined effect of multiple zeitgebers and/or twilight zones, both of which were absent in the laboratory. Similarly, populations selected for accuracy of emergence in the laboratory showed an enhanced peak and narrower gate width when assayed under seminatural conditions (Kannan et al. 2012b). Another study compared the eclosion rhythms of three closely related drosophilids – *D. melanogaster*, *D. malerkotliana*, and *D. ananassae* – under seminatural conditions, which had previously shown differences in the phasing of eclosion under standard laboratory conditions (Prabhakaran et al. 2013). Surprisingly, there was no difference in the phase of eclosion even across different seasons, which led them to conclude that these species showed a dissimilar phase of entrainment only in the presence of a light cycle. This also indicates that there is no certainty of obtaining an enhanced circadian phenotype in complex naturalistic environments compared to laboratory regimes.

To ascertain the relative importance of time cues under seminatural conditions, an experimental-evolution approach was initiated by rearing *D. melanogaster* populations under seminatural conditions (NT24) in an outdoor enclosure in southern India and maintaining their ancestral control populations (T24) under standard laboratory conditions (Dani and Sheeba 2022). Assays of their eclosion rhythms showed that while the outdoor-reared NT24 populations did not differ from the laboratory-reared T24 populations in periodicity and phasing under laboratory DD and LD, they exhibited a season-dependent advance in phase compared to controls under seminatural conditions. Further analysis showed that the NT24 populations did not merely track the phasing of a specific environmental variable across seasons but instead were responsive to a change in the magnitude of temperature cycle variables. This is interesting, as seasonal circadian variation in nature is thought to be driven mainly by photoperiodic variation, despite most insects residing in tropical habitats (Denlinger et al. 2017). Conducting such studies under several ecologically distinct conditions is likely to reveal more insights into the regulation of rhythmic behavior in nature.

9.5 Influence of Circadian Clocks on Life-History Traits

Even though evolutionary biology and chronobiology remained separate investigatory fields for the longest time, there was good reason to suspect the involvement of circadian clocks in shaping the life-history traits of organisms (Sharma and Joshi 2002). Most investigations at this interface have also chosen *Drosophila* as their

model and have shown that the circadian clock influences several aspects of the *Drosophila* life cycle. For instance, in *D. melanogaster*, a rhythmic environment such as LD 12:12 has been shown to affect several traits, such as adult lifespan (Pittendrigh and Minis 1972; Klarsfeld and Rouyer 1998; Sheeba et al. 2000), preadult developmental duration (Sheeba et al. 1999a), lifetime fecundity (Sheeba et al. 2000), and larval growth rate (Sheeba et al. 2002a). Environmental factors and their timing can induce stage-specific effects on insect developmental programs (Nijhout 2003; Smith-Gill 1983). For example, variation in pupation height is speculated to be an adaptation decreasing the risk of predation, heat, or desiccation (Markow 1979; Manning and Markow 2014). Paranjpe et al. (2004) examined the possible involvement of circadian clocks using various daily durations of light, which were expected to give rise to pupation heights ranging from lowest in LL to highest under DD. Contrary to expectations, regimes of LD 12:12 and LD 14:14 resulted in lower pupation heights than LL, suggesting that this behavior is influenced by complex interactions between the specific regime of development and circadian clocks.

Most studies in this context have been targeted toward verifying the adaptive value of circadian clocks concerning the circadian resonance hypothesis (Pittendrigh and Minis 1972). While this has been empirically validated in cyanobacteria (Ouyang et al. 1998), studies using insect models have been limited and inconclusive. When lifespans of per^{0}, per^{T} (short period), and per^{L} flies were compared with wild-type flies, wild-type flies were observed to live only marginally longer under LD 12:12 cycles (Klarsfeld and Rouyer 1998). There were no differences among their lifespans under an LD 8:8 cycle resonating with the free-running period of per^{T}.

On the other hand, ambiguous evidence of circadian clocks conferring some advantage in blow flies (Von Saint and Aschoff 1978), pitcher plant mosquitoes (Emerson et al. 2008), and ants (Lone et al. 2010) made it difficult to refute the resonance hypothesis. Recently, a long-term study using fruit flies spanning 2 years and more than 50 generations quantified several fitness components, such as fertility, mating success, preadult survival, and reproductive output, for wild-type and clock mutant flies (Horn et al. 2019). This study showed that in a competition assay, wild-type flies had a clear fitness advantage over per^{0} flies, but this advantage also persisted in LL conditions where even wild-type flies were rendered arrhythmic. Furthermore, the resonance hypothesis was partly confirmed, as per^{L} mutants outcompeted wild-type flies in a longer T-cycle; however, per^{S} mutants were unable to outcompete wild-type flies under short T-cycles. This indicated that variables other than timing also contribute to the competitive fitness advantage of wild-type flies. Contradictory evidence from the jewel wasp, *Nasonia vitripennis*, is clearer. When jewel wasps were subjected to light-dark T-cycles ranging from 20 to 28 h, no differences in longevity occurred despite differences in the phase of entrainment (Floessner et al. 2019). This result is thought to be a consequence of the broad range of entrainment of jewel wasps. Along with the other results, it suggests that circadian resonance, which has been clearly demonstrated in cyanobacteria, may not be such a strong player in shaping the evolution of clocks in complex organisms.

Preadult development time and activity-rest rhythm were linked in a study using *D. melanogaster per* mutant, with homozygous individuals of the short-period allele of *per* (per^S) exhibiting shorter development time than wild-type flies and individuals homozygous for the long-period allele (per^L) exhibiting longer development time (Kyriacou et al. 1990). However, since inbred mutant fly lines were used here, conclusions about evolutionary fitness are limited. Interestingly, a later study using large, outbred populations of *D. melanogaster* under two constant conditions (LL and DD) and three symmetric light-dark cycles (10:10, 12:12, and 14:14) showed the influence of an entraining regime on development time (Paranjpe et al. 2005). *D. melanogaster* developed fastest under LL, followed by LD 10:10, DD, LD 12:12, and LD 14:14 regimes, demonstrating the involvement of circadian clocks in appropriately timing adult emergence within a favorable "gate" depending on periodicity and environmental conditions (Paranjpe et al. 2005).

Recently, populations of *D. melanogaster* with the per^S and per^L alleles were used to investigate the role of circadian clocks and the external cyclic environment on the speed of preadult development (Srivastava et al. 2018). While per^S and per^+ flies did not differ, per^L flies took longer to develop in DD and LL, suggesting a nonclock influence. Long and short T-cycles were also used to understand the influence of the external environment's period on the internal pacemaker and its role in determining development time. Under long T-cycles, the developmental rate of per^L flies was slower than that of per^S and per^+; under short T-cycles, per^S was faster to develop than per^+ and per^L, while no genotype-based difference was seen under LD 12:12, establishing that the circadian clock influences preadult development such that slow or fast circadian clocks also delay or speed up developmental pathways.

The mechanistic link of clock control, at least over the final stages of development, has been recently discovered. Recently, it was shown that the *Drosophila* circadian clock imposes rhythmicity on eclosion by controlling the timing of the final steps of metamorphosis (Mark et al. 2021). However, this study shows control of the timing of eclosion to occur within a suitable gate; the mechanistic underpinnings of how intrinsic period influences the rate of development are yet to be unearthed. A similar result has been observed with two tropical ant species, the night-active *Camponotus compressus* and the day-active *Camponotus paria*, which also develop slowly under DD compared to LL and LD (Lone and Sharma 2008). Interestingly, recent data from monarch butterflies shows that individuals reared under constant conditions (LL and DD) exhibit longer larval development times than LD (Adams et al. 2021), with pupal development being longer in LL than DD and LD. Thus, it is clear that findings from the *Drosophila* model are not generalizable, putting forward the requirement for more research on other insect species.

In addition to the free-running period, could other clock properties also be associated with life history? Once again, *D. melanogaster* populations selected for stability of the phase of eclosion revealed greater coherence in emergence time despite having no difference in mean development time (Varma et al. 2014). Additionally, females of "accurate" populations exhibited a shorter lifespan than controls. It was also observed that such sex-specific differences were attributable to

the phasing of emergence. Morning emerging females had shorter lifespans than their evening emerging counterparts; however, this was compensated by higher midlife fecundity (Varma et al. 2014). Since these populations were under selection for phase stability (eclosion occurring in a tight morning window), one can view the evening emerging flies as those exhibiting less phase stability. The results observed in terms of life history, while interesting, may not be generalizable. When the previously described *Early* and *Late* populations of *D. melanogaster* were assayed for changes in life history under LD and DD conditions, *Late* flies exhibited a longer duration of preadult development than *Early* flies (Nikhil et al. 2016a). Surprisingly, the longer preadult duration in the *Late* flies did not result in higher body mass at pupariation or eclosion; however, *Late* females had higher fecundity and lived significantly shorter than *Early* females. Again, both of these studies were carried out with *D. melanogaster* reared under crowded conditions, which are known to have profound effects on life history (Mueller et al. 1993; Joshi and Mueller 1997). Hence, more studies with a range of insect models investigating how chronotype relates to life history will significantly enhance our understanding. Conversely, how selection on life-history traits might alter circadian phenotypes is also of evolutionary significance (Abhilash and Sharma 2016) but is beyond the scope of this review.

9.6 Diversity in Circadian Clock Function in Insects and Further Considerations

The previous sections have discussed the evolution of clock properties and their links to key aspects of life-history traits. We will now highlight the multilevel diversity observed in the roles of the circadian clock across organisms. Honey bees and fruit flies were among the earliest used insect models in chronobiology. Over the years, they have provided many insights into the behavioral, physiological, genetic, and neuronal bases of circadian rhythms (Beer and Helfrich-Förster 2020a, 2020b). Apart from the contrasting nature of their sociality, both of these models also differ in aspects of clock function: in honey bee *Apis mellifera*, the circadian clock is known to play a role in time-place learning, memory, and solar compass navigation, less so for the commonly studied fruit fly *D. melanogaster*. Such diversity in clock function means that these species can scarcely represent Hymenoptera and Diptera. In Hymenoptera, apart from eusocial honey bees, there are primitively social, facultatively social, and solitary bees that have been shown to have diversity in clock-controlled behavior (Shell and Rehan 2018). Bumblebees show plasticity in rhythmic behavior similar to honey bees; however, the determinant of plasticity is not age but size (Yerushalmi et al. 2006; Eban-Rothschild et al. 2011). On the other hand, the solitary bee *Osmia bicornis* displays rhythmic locomotor behavior and has a mature circadian system at emergence (Beer and Helfrich-Förster 2020b). This has been attributed to its emergence from small nests in the spring season, where it experiences environmental changes.

In *Drosophila*, the variation in rhythmicity, photoperiodic response, and incidence of diapause has shown that cosmopolitan species such as *D. melanogaster* may not be the best choice for studying response to photoperiod and diapause incidence. There is a need to conduct research on typical nonmodel insects, which are perhaps better suited for addressing questions on the circadian clock's role in specific behaviors associated with seasonal environmental changes. For example, the pea aphid, *Acyrthosiphon pisum*, is an emerging model whose reproductive strategy varies across the year in response to photoperiodic change. The pea aphids adopt viviparous parthenogenesis during the warmer months of spring and summer, and with the advent of shorter day length in autumn, the reproductive strategy becomes sexual, which results in the production of fertile eggs. These aphid eggs survive the harsh winter to give rise to new parthenogenetic females (Hardie and Vaz Nunes 2001). Recently, it was also shown that clock neurons in pea aphids neuroanatomically connect to the pars intercerebralis and the corpora allata complex, supporting the possibility of a direct link between the circadian clock and photoperiodic response to mediate hormone release (Colizzi et al. 2021). Similarly, several new perspectives have been gained by studying the role of the circadian clock and diapause induction in nonmodel insects such as butterflies, moths, and wasps (Denlinger et al. 2017).

An additional factor to consider here is the frequent overbearing effect of ecology on clock function. Antarctic midges are an excellent example of this: due to the extreme environment, *Belgica antarctica* only has a short period of time during the year with temperatures permissive for development. As a result, these midges remain active throughout the day, and despite possessing circadian clock genes, there is no cyclic pattern of expression seen in similar species living in temperate regions (Kobelkova et al. 2015). The absence of persistent rhythmicity in extreme conditions might not be as baffling as the exact opposite. *Ridgeia piscesae*, a tubeworm typically found near hydrothermal vents with extremely high temperatures, has exhibited fluctuations in density at the population level with circadian and ultradian periodicities (Cuvelier et al. 2014). Several such examples exist (reviewed in Abhilash et al. 2017), and while an intrinsic advantage is often hypothesized for such cases, it will be interesting to see the results of future studies addressing such questions that move beyond speculation. On the other hand, studies using cosmopolitan species across environments have largely convinced us of potential environmental factors shaping rhythms and clock function (Adrion et al. 2015). This subfield would benefit tremendously by (a) tracing variation in genes of interest and genomic variation brought about by the environment as well as gene × environment interactions and (b) studies conducted under a multitude of differing seminatural conditions for verification of genetic correlations reported.

Another layer of variation in the clock function of individuals occurs by interspecific interactions that are specific to their ecology, which adds to the already existing complexity in circadian behavior. This is a relatively understudied field at the interface of chronobiology and ecology in which interactions related to predation, food availability, competition, parasitism, etc. have been linked to the influence of rhythms (Kronfeld-Schor et al. 2017). For instance, in deer ticks, detachment from

diurnal hosts such as hamsters has been shown to occur synchronously late in the day, which concentrates ticks in the nests of their nocturnal mouse hosts, possibly enhancing the transmission of pathogens. Similarly, for two nonpermanent ticks, *Ixodes arboricola* and *Ixodes ricinus*, detachment from their common host *Parus major* (great tit) appears to be temporally coordinated. Detachment of *I. ricinus* occurs when tits are most active during daytime, while detachment of *I. arboricola* occurs during the night when the birds sleep in tree holes (Heylen and Matthysen 2010). Temporal avoidance of competition may also be beneficial, as exemplified by the solitary bee *Proxylocopa olivieri*. This bee forages maximally at dawn and dusk, thereby avoiding a temporal overlap with other bees, such as *A. mellifera*, which show unimodal foraging during the day (Gottlieb et al. 2005). In cohabitating dung beetle guilds, such temporal avoidance of superior competitors has been observed (Krell-Westerwalbesloh et al. 2004). Apart from these, research on important disease vectors, such as mosquitoes *Aedes aegypti* and *Anopheles gambiae*, has shown over the years that several behaviors important for disease spread, such as biting, mating, and flight activity, are under the control of the circadian clock (Jones et al. 1967; Yee and Foster 1992; Rund et al. 2012). Whether these behaviors also have interspecific influences might be worth investigating. These recent advances have only revealed the void in our understanding of the regulation of circadian behavior in an ecologically realistic scenario. Hence, future research on such interspecific effects will enhance our understanding of circadian behavior in model and nonmodel insects.

9.7 Evolutionary Consequences of Climate Change on Insect Clocks and Future Perspectives

As with other organisms, insects are also subject to a wide variety of environmental cues, which can dramatically affect their endogenous circadian clocks in addition to various other systems (Fig. 9.3). These may modulate physiology and behavior across generations and become differentially affected by selection pressures to produce significant shifts in the biodiversity of insect forms. By extension, one can view climate change and urbanization as potential challenges for circadian clocks as if they were natural experiments on the adaptability and plasticity of circadian clocks on a global scale. The day length-temperature relationship, providing valuable input to circadian systems, has remained relatively consistent, in which shorter day length is often associated with lower temperatures and vice versa. However, this relationship may become inconsistent with global warming, resulting in temperature shifts without accompanying photoperiodic change (Walker et al. 2019). Indeed, it has been observed that population peaks for certain insects have advanced in response to increasing spring temperatures, ultimately affecting the food chain (Visser et al. 1998). The rise in mean temperature over land is marked by a pattern of diurnal asymmetry, with larger tendencies of night warming than day warming (Alexander et al. 2006), as well as an increase in the incidence, intensity,

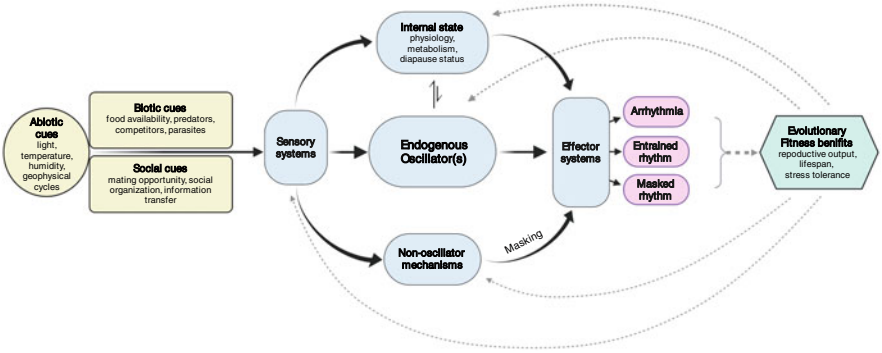

Fig. 9.3 Schematic depicting a generalized conceptual framework of sensory integration, regulation of rhythm output, and natural selection applicable across organisms. The ecological niche with environmental cues, abiotic, biotic, and social, is perceived by sensory systems, and this information is relayed to internal systems. The output by effector systems can be either arrhythmia, an entrained rhythmic output, or a masked rhythmic output based on the state and entrainment range of endogenous oscillator(s) and permissive conditions. Mechanisms for masking may be specific to time cues and sensory systems, e.g., the photoreception pathway for light. Ultimately, over several generations of a population, if the output (arrhythmic/rhythmic/masked) results in evolutionary fitness benefits, underlying systems of the organism, including endogenous oscillators, giving rise to the beneficial output are likely to increase in frequency (dotted arrows) (created with BioRender.com)

and length of warm weather and spatial changes in water availability (Tabari 2020). Warmer temperatures at night have been shown to have a nontrivial effect on several aspects of insect life history, such as development, fecundity, and survival (Zhao et al. 2014). Another aspect worthy of consideration is thermal extremes. Minor changes in maximal temperature are often overlooked but may have nontrivial effects on organismal demography and fitness (Ma et al. 2015). Moreover, the global average increase in temperature is not representative of local change, as the effects of global warming are not the same everywhere. Thus, even though general changes due to global warming can be predicted, the realized effects on local climate and their impact on insect behavior, life history, and rhythms in local habitats are not understood. In an overall ecological context, the importance and impact of insects is vastly underestimated and overlooked. More data and targeted studies, as well as dissemination of our understanding to the general public and policymakers, will be needed for appropriate measures to be taken to slow down the speed with which our environments are being altered.

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References

Abhilash L, Ghosh A, Sheeba V (2019) Selection for timing of eclosion results in co-evolution of temperature responsiveness in *Drosophila melanogaster*. J Biol Rhythm 34:596–609. https://doi.org/10.1177/0748730419877315

Abhilash L, Kalliyil A, Sheeba V (2020) Responses of activity rhythms to temperature cues evolve in *Drosophila* populations selected for divergent timing of eclosion. J Exp Biol 223:11. https://doi.org/10.1242/jeb.222414

Abhilash L, Sharma VK (2016) On the relevance of using laboratory selection to study the adaptive value of circadian clocks. Physiol Entomol 41:293–306. https://doi.org/10.1111/phen.12158

Abhilash L, Shindey R, Sharma VK (2017) To be or not to be rhythmic? A review of studies on organisms inhabiting constant environments. Biol Rhythm Res 48:677–691. https://doi.org/10.1080/09291016.2017.1345426

Adams KL, Sun EF, Alaidrous W, De Roode JC (2021) Constant light and frequent schedule changes do not impact resistance to parasites in monarch butterflies. J Biol Rhythm 36:286–296. https://doi.org/10.1177/0748730420985312

Adrion JR, Hahn MW, Cooper BS (2015) Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. Trends Genet 31:434–444. https://doi.org/10.1016/j.tig.2015.05.006

Alexander LV, Zhang X, Peterson TC, Caesar J, Gleason B, Klein Tank AMK (2006) Global observed changes in daily climate extremes of temperature and precipitation. J Geophys Res Atmos 111:1–22. https://doi.org/10.1029/2005JD006290

Anduaga AM, Evanta N, Patop IL, Bartok O, Weiss R, Kadener S (2019) Thermosensitive alternative splicing senses and mediates 2 temperature adaptation in *Drosophila*. eLife 8:1–31. https://doi.org/10.7554/eLife.44642

Balzer I, Hardeland R (1988) Influence of temperature on biological rhythms. Int J Biometeorol 32:231–241. https://doi.org/10.1007/BF01080021

Beale AD, Whitmore D, Moran D (2016) Life in a dark biosphere: a review of circadian physiology in "arrhythmic" environments. J Comp Physiol B Biochem Syst Environ Physiol 186:947–968. https://doi.org/10.1007/s00360-016-1000-6

Beauchamp M, Bertolini E, Deppisch P, Steubing J, Menegazzi P, Helfrich-Förster C (2018) Closely related fruit fly species living at different latitudes diverge in their circadian clock anatomy and rhythmic behavior. J Biol Rhythm 33:602–613. https://doi.org/10.1177/0748730418798096

Beer K, Helfrich-Förster C (2020a) Model and non-model insects in chronobiology. Front Behav Neurosci 14:1–23. https://doi.org/10.3389/fnbeh.2020.601676

Beer K, Helfrich-Förster C (2020b) Post-embryonic development of the circadian clock seems to correlate with social life style in bees. Front Cell Develop Biol 8:1–9. https://doi.org/10.3389/fcell.2020.581323

Bertolini E, Schubert FK, Zanini D, Sehadová H, Helfrich-Förster C, Menegazzi P (2019) Life at high latitudes does not require circadian behavioral rhythmicity under constant darkness. Curr Biol 29:3928–3936.e3. https://doi.org/10.1016/j.cub.2019.09.032

Castiglione-Morelli MA, Guantieri V, Villani V, Kyriacou CP, Costa R, Tamburro AM (1995) Conformational study of the Thr-Gly repeat in the *Drosophila* clock protein, PERIOD. Proc R Soc B 260:155–163. https://doi.org/10.1098/rspb.1995.0073

Chippendale AK, Chu TJF, Rose MR (1996) Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. Evolution 50:753–766. https://doi.org/10.1111/j.1558-5646.1996.tb03885.x

Cloudsley-Thompson JL (1960) Adaptive functions of circadian rhythms. Cold Spring Harb Symp Quant Biol 25:345–355. https://doi.org/10.1101/SQB.1960.025.01.035

Colizzi FS, Beer K, Cuti P, Deppisch P, Martínez Torres D et al (2021) Antibodies against the clock proteins period and Cryptochrome reveal the neuronal organization of the circadian clock in the pea aphid. Front Physiol 12:988. https://doi.org/10.3389/fphys.2021.705048

Costa R, Peixoto AA, Barbujani G, Kyriacou CP (1992) A latitudinal cline in a *Drosophila* clock gene. Proc R Soc B 250:43–49. https://doi.org/10.1098/rspb.1992.0128

Costa R, Peixoto AA, Thackeray JR, Dalgleish R, Kyriacou CP (1991) Length polymorphism in the threonine-glycine-encoding repeat region of the period gene in *Drosophila*. J Mol Evol 32:238–246. https://doi.org/10.1007/BF02342746

Cuvelier D, Legendre P, Laes A, Sarradin PM, Sarrazin J (2014) Rhythms and community dynamics of a hydrothermal tubeworm assemblage at main endeavour field - a multidisciplinary deep-sea observatory approach. PLoS One 9:e96924. https://doi.org/10.1371/journal.pone.0096924

Dani C, Sheeba V (2022) *Drosophila* populations reared under tropical semi-natural conditions evolve season-dependent differences in timing of eclosion. Front Physiol 13:954731. https://doi.org/10.3389/fphys.2022.954731

Das A, Holmes TC, Sheeba V (2015) dTRPA1 modulates afternoon peak of activity of fruit flies *Drosophila melanogaster*. PLoS One 10:1–21. https://doi.org/10.1371/journal.pone.0134213

De J, Varma V, Saha S, Sheeba V, Sharma VK (2013) Significance of activity peaks in fruit flies, *Drosophila melanogaster*, under semi-natural conditions. Proc Natl Acad Sci U S A 110:8984–8989. https://doi.org/10.1073/pnas.1220960110

DeCoursey PJ, Krulas JR, Mele G, Holley DC (1997) Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure. Physiol Behav 62:1099–1108. https://doi.org/10.1016/S0031-9384(97)00263-1

Denlinger DL, Hahn DA, Merlin C, Holzapfel CM, Bradshaw WE (2017) Keeping time without a spine: what can the insect clock teach us about seasonal adaptation? Philos Trans R Soc B 372:20160257. https://doi.org/10.1098/rstb.2016.0257

Eban-Rothschild A, Belluci S, Bloch G (2011) Maternity-related plasticity in circadian rhythms of bumble-bee queens. Proc R Soc B 278:3510–3516. https://doi.org/10.1098/rspb.2011.0579

Emerson KJ, Bradshaw WE, Holzapfel CM (2008) Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. Evolution 62:979–983. https://doi.org/10.1111/j.1558-5646.2008.00324.x

Enright JT (1980) The timing of sleep and wakefulness. Springer, Berlin

Floessner TSE, Boekelman FE, Druiven SJ, De Jong M, Rigter PM, Beersma DG et al (2019) Lifespan is unaffected by size and direction of daily phase shifts in *Nasonia*, a hymenopteran insect with strong circadian light resetting. J Insect Physiol 117:103896. https://doi.org/10.1016/j.jinsphys.2019.103896

Fuse N, Kitamura T, Haramura T, Arikawa K, Imafuku M (2014) Evolution in the dark: adaptation of *Drosophila* in the laboratory. Springer, Tokyo

Ghosh A, Sharma P, Dansana S, Sheeba V (2021) Evidence for co-evolution of masking with circadian phase in *Drosophila melanogaster*. J Biol Rhythm 36:254–270. https://doi.org/10.1177/0748730421997262

Gibbs AG (1999) Laboratory selection for the comparative physiologist. J Exp Biol 202:2709–2718. https://doi.org/10.1242/jeb.202.20.2709

Gottlieb D, Keasar T, Shmida A, Motro U (2005) Possible foraging benefits of bimodal daily activity in *Proxylocopa olivieri* (Lepeletier) (Hymenoptera: Anthophoridae). Environ Entomol 34:417–424. https://doi.org/10.1603/0046-225X-34.2.417

Hardie J, Vaz Nunes M (2001) Aphid photoperiodic clocks. J Insect Physiol 47:821–832. https://doi.org/10.1016/S0022-1910(01)00055-5

Heylen DJA, Matthysen E (2010) Contrasting detachment strategies in two congeneric ticks (Ixodidae) parasitizing the same songbird. Parasitology 137:661–667. https://doi.org/10.1017/S0031182009991582

Horn M, Mitesser O, Hovestadt T, Yoshii T, Rieger D, Helfrich-Förster C (2019) The circadian clock improves fitness in the fruit fly, *Drosophila melanogaster*. Front Physiol 10:1–18. https://doi.org/10.3389/fphys.2019.01374

Imafuku M, Haramura T (2011) Activity rhythm of *Drosophila* kept in complete darkness for 1300 generations. Zool Sci 28:195–198. https://doi.org/10.2108/zsj.28.195

Izutsu M, Zhou J, Sugiyama Y, Nishimura O, Aizu T, Toyoda A, Fujiyama A, Agata K, Fuse N (2012) Genome features of "dark-fly", a *Drosophila* line reared long-term in a dark environment. PLoS One 7:e33288. https://doi.org/10.1371/journal.pone.0033288

Jones MD, Hill M, Hope AM (1967) The circadian flight activity of the mosquito *Anopheles gambiae*: phase setting by the light régime. J Exp Biol 47:503–511. https://doi.org/10.1242/jeb.47.3.503

Joshi A, Mueller LD (1997) Adult crowding effects on longevity in *Drosophila melanogaster*: increase in age-independent mortality. Curr Sci 72:255–260. https://www.jstor.org/stable/24098593

Kannan NN, Mukherjee N, Sharma VK (2012a) Robustness of circadian timing systems evolves in the fruit fly *Drosophila melanogaster* as a correlated response to selection for adult emergence in a narrow window of time. Chronobiol Int 29:1312–1328. https://doi.org/10.3109/07420528.2012.728550

Kannan NN, Varma V, De J, Sharma VK (2012b) Stability of adult emergence and activity/rest rhythms in fruit flies *Drosophila melanogaster* under semi-natural conditions. PLoS One 7:e50379. https://doi.org/10.1371/journal.pone.0050379

Kannan NN, Vaze KM, Sharma VK (2012c) Clock accuracy and precision evolve as a consequence of selection for adult emergence in a narrow window of time in fruit flies *Drosophila melanogaster*. J Exp Biol 215:3527–3534. https://doi.org/10.1242/jeb.074534

Kauranen H, Menegazzi P, Costa R, Helfrich-Förster C, Kankainen A, Hoikkala A (2012) Flies in the north: locomotor behavior and clock neuron organization of *Drosophila montana*. J Biol Rhythm 27:377–387. https://doi.org/10.1177/0748730412455916

Klarsfeld A, Rouyer F (1998) Effects of circadian mutations and LD periodicity on the life span of *Drosophila melanogaster*. J Biol Rhythm 13:471–478. https://doi.org/10.1177/074873098129000309

Knutsson A (2003) Health disorders of shift workers. Occup Med 53:103–108. https://doi.org/10.1093/occmed/kqg048

Kobelkova A, Goto SG, Peyton JT, Ikeno T, Lee RE Jr, Denlinger DL (2015) Continuous activity and no cycling of clock genes in the Antarctic midge during the polar summer. J Insect Physiol 81:90–96. https://doi.org/10.1016/j.jinsphys.2015.07.008

Krell-Westerwalbesloh S, Krell FT, Linsenmair KE (2004) Diel separation of Afrotropical dung beetle guilds - avoiding competition and neglecting resources (Coleoptera: Scarabaeoidea). J Nat Hist 38:2225–2249. https://doi.org/10.1080/00222930310001618921

Kronfeld-Schor N, Visser ME, Salis L, van Gils JA (2017) Chronobiology of interspecific interactions in a changing world. Philos Trans R Soc B 372:20160248. https://doi.org/10.1098/rstb.2016.0248

Kumar S, Kumar D, Paranjpe DA, Akarsh CR, Sharma VK (2007) Selection on the timing of adult emergence results in altered circadian clocks in fruit flies *Drosophila melanogaster*. J Exp Biol 210:906–918. https://doi.org/10.1242/jeb.001354

Kyriacou CP, Oldroyd M, Wood J, Sharp M, Hill M (1990) Clock mutations alter developmental timing in *Drosophila*. Heredity 64:395–401. https://doi.org/10.1038/hdy.1990.50

Kyriacou CP, Peixoto AA, Sandrelli F, Costa R, Tauber E (2008) Clines in clock genes: fine-tuning circadian rhythms to the environment. Trends Genet 24:124–132. https://doi.org/10.1016/j.tig.2007.12.003

Lamaze A, Chen C, Leleux S, Xu M, George R, Stanewsky R (2022) A natural *timeless* polymorphism allowing circadian clock synchronization in "white nights". Nat Commun 13:1–12. https://doi.org/10.1038/s41467-022-29293-6

Lankinen P (1986) Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis*. J Comp Physiol A 159:123–142. https://doi.org/10.1007/BF00612503

Lankinen P (1993) North-south differences in circadian eclosion rhythm in European populations of *Drosophila subobscura*. Heredity 71:210–218. https://doi.org/10.1038/hdy.1993.126

Lone SR, Ilangovan V, Murugan M, Sharma VK (2010) Circadian resonance in the development of two sympatric species of *Camponotus* ants. J Insect Physiol 56:1611–1616. https://doi.org/10.1016/j.jinsphys.2010.05.023

Lone SR, Sharma VK (2008) Exposure to light enhances pre-adult fitness in two dark-dwelling sympatric species of ants. BMC Dev Biol 11:1–11. https://doi.org/10.1186/1471-213X-8-113

Low KH, Lim C, Ko HW, Edery I (2008) Natural variation in the splice site strength of a clock gene and species-specific thermal adaptation. Neuron 60:1054–1067. https://doi.org/10.1016/j.neuron.2008.10.048

Ma G, Rudolf VHW, Ma C (2015) Extreme temperature events alter demographic rates, relative fitness, and community structure. Glob Chang Biol 21:1794–1808. https://doi.org/10.1111/gcb.12654

Majercak J, Sidote D, Hardin PE, Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron 24:219–230. https://doi.org/10.1016/S0896-6273(00)80834-X

Manning M, Markow TA (2014) Light-dependent pupation site preferences in *Drosophila simulans*. Behav Genet 11:557–563. https://doi.org/10.1007/BF01065790

Mark B, Bustos-González L, Cascallares G, Conejera F, Ewer J (2021) The circadian clock gates *Drosophila* adult emergence by controlling the timecourse of metamorphosis. Proc Natl Acad Sci U S A 118:e2023249118. https://doi.org/10.1073/pnas.2023249118

Markow TA (1979) A survey of intra- and interspecific variation for pupation height in *Drosophila*. Behav Genet 9:209–217. https://doi.org/10.1007/BF01071301

Marrus SB, Zeng H, Rosbash M (1996) Effect of constant light and circadian entrainment of perS flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*. EMBO J 15:6877–6886. https://doi.org/10.1002/j.1460-2075.1996.tb01080.x

Menegazzi P, Dalla Benetta E, Beauchamp M, Schlichting M, Steffan-Dewenter I, Helfrich-Förster C (2017) Adaptation of circadian neuronal network to photoperiod in high-latitude European drosophilids. Curr Biol 27:833–839. https://doi.org/10.1016/j.cub.2017.01.036

Menegazzi P, Vanin S, Yoshii T, Rieger D, Hermann C, Dusik V et al (2013) *Drosophila* clock neurons under natural conditions. J Biol Rhythm 28:3–14. https://doi.org/10.1177/0748730412471303

Mueller LD, Graves JL, Rose MR (1993) Interactions between density-dependent and age-specific selection in *Drosophila melanogaster*. Funct Ecol 7:469–479. https://doi.org/10.2307/2390034

Nijhout HF (2003) Development and evolution of adaptive polyphenisms. Evol Dev 5:9–18. https://doi.org/10.1046/j.1525-142X.2003.03003.x

Nikhil KL, Ratna K, Sharma VK (2016a) Life-history traits of *Drosophila melanogaster* populations exhibiting early and late eclosion chronotypes. BMC Evol Biol 16:1–14. https://doi.org/10.1186/s12862-016-0622-3

Nikhil KL, Vaze KM, Ratna K, Sharma VK (2016b) Circadian clock properties of fruit flies *Drosophila melanogaster* exhibiting early and late emergence chronotypes. Chronobiol Int 33:22–38. https://doi.org/10.3109/07420528.2015.1108981

Nikhil KL, Vaze KM, Sharma VK (2015) Late emergence chronotypes of fruit flies *Drosophila melanogaster* exhibit higher accuracy of entrainment. Chronobiol Int 32:1477–1485. https://doi.org/10.3109/07420528.2015.1105251

Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. Proc Natl Acad Sci U S A 95:8660–8664. https://doi.org/10.1073/pnas.95.15.8660

Özer I, Carle T (2020) Back to the light, coevolution between vision and olfaction in the "dark-flies" (*Drosophila melanogaster*). PLoS One 15:1–15. https://doi.org/10.1371/journal.pone.0228939

Paranjpe DA, Anitha D, Chandrashekaran MK, Joshi A, Sharma VK (2005) Possible role of eclosion rhythm in mediating the effects of light-dark environments on pre-adult development in *Drosophila*. BMC Dev Biol 5:1–6. https://doi.org/10.1186/1471-213X-5-5

Paranjpe DA, Anitha D, Kumar S, Kumar D, Verkhedkar K, Chandrashekaran MK et al (2003) Entrainment of eclosion rhythm in *Drosophila melanogaster* populations reared for more than 700 generations in constant light environment. Chronobiol Int 20:977–987. https://doi.org/10.1081/CBI-120025247

Paranjpe DA, Anitha D, Sharma VK, Joshi A (2004) Circadian clocks and life-history related traits: is pupation height affected by circadian organization in *Drosophila melanogaster*? J Genet 83:73. https://doi.org/10.1007/BF02715831

Pittendrigh CS (1967) Circadian systems. I. the driving oscillation and its assay in *Drosophila pseudoobscura*. Proc Natl Acad Sci U S A 58:1762–1767. https://doi.org/10.1073/pnas.58.4.1762

Pittendrigh CS (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol 55:17–54. https://doi.org/10.1146/annurev.physiol.55.1.17

Pittendrigh CS, Minis DH (1972) Circadian systems: longevity as a function of circadian resonance in *Drosophila melanogaster*. Proc Natl Acad Sci U S A 69:1537–1539. https://doi.org/10.1073/pnas.69.6.1537

Pittendrigh CS, Takamura T (1989) Latitudinal clines in the properties of a circadian pacemaker. J Biol Rhythm 4:105–123. https://doi.org/10.1177/074873048900400209

Poulson TL, White WB (1969) The cave environment: limestone caves provide unique natural laboratories for studying biological and geological processes. Science 165:971–981. https://doi.org/10.1126/science.165.3897.971

Prabhakaran PM, De J, Sheeba V (2013) Natural conditions override differences in emergence rhythm among closely related drosophilids. PLoS One 8:1–9. https://doi.org/10.1371/journal.pone.0083048

Prabhakaran PM, Sheeba V (2013) Insights into differential activity patterns of drosophilids under semi-natural conditions. J Exp Biol 216:4691–4702. https://doi.org/10.1242/jeb.092270

Roenneberg T, Foster RG (1997) Twilight times: light and the circadian system. Photochem Photobiol 66:549–561. https://doi.org/10.1111/j.1751-1097.1997.tb03188.x

Rosato E, Peixoto AA, Barbujani G, Costa R, Kyriacou CP (1994) Molecular polymorphism in the period gene of *Drosophila simulans*. Genetics 138:693–707. https://doi.org/10.1093/genetics/138.3.693

Rose MR (1984) Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution 38:1004–1010. https://doi.org/10.2307/2408434

Rose MR, Charlesworth B (1981) Genetics of life history in *Drosophila melanogaster* II Exploratory selection experiments. Genetics 97:187–196. https://doi.org/10.1093/genetics/97.1.187

Rund SSC, Lee SJ, Bush BR, Duffield GE (2012) Strain- and sex-specific differences in daily flight activity and the circadian clock of *Anopheles gambiae* mosquitoes. J Insect Physiol 58:1609–1619. https://doi.org/10.1016/j.jinsphys.2012.09.016

Saint-Charles A, Michard-Vanhēe C, Alejevski F, Chélot E, Boivin A, Rouyer F (2016) Four of the six *Drosophila* rhodopsin-expressing photoreceptors can mediate circadian entrainment in low light. J Comp Neurol 524:2828–2844. https://doi.org/10.1002/cne.23994

Sandrelli F, Tauber E, Pegoraro M, Mazzotta G, Cisotto P, Landskron J et al (2007) A molecular basis for natural selection at the *timeless* locus in *Drosophila melanogaster*. Science 316:1898–1900. https://doi.org/10.1126/science.1138426

Sawyer LA, Hennessy JM, Peixoto AA, Rosato E, Parkinson H, Costa R et al (1997) Natural variation in a *Drosophila* clock gene and temperature compensation. Science 278:2117–2120. https://doi.org/10.1126/science.278.5346.2117

Sawyer LA, Sandrelli F, Pasetto C, Peixoto AA, Rosato E, Costa R et al (2006) The period gene Thr-Gly polymorphism in Australian and African *Drosophila melanogaster* populations: implications for selection. Genetics 174:465–480. https://doi.org/10.1534/genetics.106.058792

Service PM, Hutchinson EW, Rose MR (1988) Multiple genetic mechanisms for the evolution of senescence in *Drosophila melanogaster*. Evolution 42:708. https://doi.org/10.2307/2408862

Sharma VK (2003) Adaptive significance of circadian clocks. Chronobiol Int 20:901–919. https://doi.org/10.1081/CBI-120026099

Sharma VK, Joshi A (2002) Clocks, genes and evolution: the evolution of circadian organization. In: Kumar V (ed) Biological rhythms. Springer, Berlin, pp 5–23. https://doi.org/10.1007/978-3-662-06085-8_2

Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK (2001) Persistence of oviposition rhythm in individuals of *Drosophila melanogaster* reared in an aperiodic environment for several hundred generations. J Exp Zool 290:541–549. https://doi.org/10.1002/jez.1098

Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK (2002a) Developmental plasticity of the locomotor activity rhythm of *Drosophila melanogaster*. J Insect Physiol 48:25–32. https://doi.org/10.1016/S0022-1910(01)00139-1

Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK (2002b) Locomotor activity rhythm in *Drosophila melanogaster* after 600 generations in an aperiodic environment. Naturwissenschaften 89:512–514. https://doi.org/10.1007/s00114-002-0360-9

Sheeba V, Sharma VK, Chandrashekaran MK, Joshi A (1999a) Effect of different light regimes on pre-adult fitness in *Drosophila melanogaster* populations reared in constant light for over six hundred generations. Biol Rhythm Res 30:424–433. https://doi.org/10.1076/brhm.30.4.424.1416

Sheeba V, Sharma VK, Chandrashekaran MK, Joshi A (1999b) Persistence of eclosion rhythm in *Drosophila melanogaster* after 600 generations in an aperiodic environment. Naturwissenschaften 86:448–449. https://doi.org/10.1007/s001140050651

Sheeba V, Sharma VK, Shubha K, Chandrashekaran MK, Joshi A (2000) The effect of different light regimes on adult life span in *Drosophila melanogaster* is partly mediated through reproductive output. J Biol Rhythm 15:380–392. https://doi.org/10.1177/074873000129001477

Shell WA, Rehan SM (2018) Behavioral and genetic mechanisms of social evolution: insights from incipiently and facultatively social bees. Apidologie 49:13–30. https://doi.org/10.1007/s13592-017-0527-1

Shindey R, Varma V, Nikhil KL, Sharma VK (2016) Evolution of robust circadian clocks in *Drosophila melanogaster* populations reared in constant dark for over 330 generations. Sci Nat 103:1–11. https://doi.org/10.1007/s00114-016-1399-3

Shindey R, Varma V, Nikhil KL, Sharma VK (2017) Evolution of circadian rhythms in *Drosophila melanogaster* populations reared in constant light and dark regimes for over 330 generations. Chronobiol Int 34:537–550. https://doi.org/10.1080/07420528.2016.1195397

Smith-gill SJ (1983) Developmental plasticity: developmental conversion versus phenotypic modulation. Integr Comp Biol 23:47–55. https://doi.org/10.1093/icb/23.1.47

Srivastava M, James A, Varma V, Sharma VK, Sheeba V (2018) Environmental cycles regulate development time via circadian clock mediated gating of adult emergence. BMC Dev Biol 18:1–10. https://doi.org/10.1186/s12861-018-0180-6

Tabari H (2020) Climate change impact on flood and extreme precipitation increases with water availability. Sci Rep 10:1–10. https://doi.org/10.1038/s41598-020-70816-2

Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C et al (2007) Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science 316:1895–1898. https://doi.org/10.1126/science.1138412

Teotónio H, Rose MR (2001) Perspective: reverse evolution. Evolution 55:653–660. https://doi.org/10.1111/j.0014-3820.2001.tb00800.x

Tomioka K, Sakamoto M, Harui Y, Matsumoto N, Matsumoto A (1998) Light and temperature cooperate to regulate the circadian locomotor rhythm of wild type and period mutants of *Drosophila melanogaster*. J Insect Physiol 44:587–596. https://doi.org/10.1016/S0022-1910(98)00046-8

Vanin S, Bhutani S, Montelli S, Menegazzi P, Green EW, Pegoraro M et al (2012) Unexpected features of *Drosophila* circadian behavioural rhythms under natural conditions. Nature 484:371–375. https://doi.org/10.1038/nature10991

Varma V (2018) Evaluating the role of circadian clock properties and developmental processes in the evolution of accurate eclosion rhythms in *Drosophila melanogaster*. PhD Thesis. Jawaharlal Nehru Cent. Adv. Sci. Res. http://lib.jncasr.ac.in:8080/jspui/handle/10572/2625

Varma V, Kannan NN, Sharma VK (2014) Selection for narrow gate of emergence results in correlated sex-specific changes in life history of *Drosophila melanogaster*. Biol Open 3:606–613. https://doi.org/10.1242/bio.20147906

Varma V, Krishna S, Srivastava M, Sharma VK, Sheeba V (2019) Accuracy of fruit-fly eclosion rhythms evolves by strengthening circadian gating rather than developmental fine-tuning. Biol Open 8:bio042176. https://doi.org/10.1242/bio.042176

Vaze KM, Kannan NN, Abhilash L, Sharma VK (2012a) Chronotype differences in *Drosophila* are enhanced by semi-natural conditions. Naturwissenschaften 99:967–971. https://doi.org/10.1007/s00114-012-0978-1

Vaze KM, Nikhil KL, Abhilash L, Sharma VK (2012b) Early-and late-emerging *Drosophila melanogaster* fruit flies differ in their sensitivity to light during morning and evening. Chronobiol Int 29:674–682. https://doi.org/10.3109/07420528.2012.680557

Visser ME, Van Noordwijk AJ, Tinbergen JM, Lessells CM (1998) Warmer springs lead to mistimed reproduction in great tits (Parus major). Proc R Soc B Biol Sci 265:1867–1870. https://doi.org/10.1061/9780784479926.035

Von Saint Paul U, Aschoff J (1978) Longevity among blowflies Phormia terraenovae R.D. kept in non-24-hour light-dark cycles. J Comp Physiol A 127:191–195. https://doi.org/10.1007/BF01350109

Walker WH, Meléndez-Fernández OH, Nelson RJ, Reiter RJ (2019) Global climate change and invariable photoperiods: a mismatch that jeopardizes animal fitness. Ecol Evol 9:10044–10054. https://doi.org/10.1002/ece3.5537

Woelfle MA, Ouyang Y, Phanvijhitsiri K, Johnson CH (2004) The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. Curr Biol 14:1481–1486. https://doi.org/10.1016/j.cub.2004.08.023

Yee WL, Foster WA (1992) Diel sugar-feeding and host-seeking rhythms in mosquitoes (Diptera: Culicidae) under laboratory conditions. J Med Entomol 29:784–791. https://doi.org/10.1093/jmedent/29.5.784

Yerushalmi S, Bodenhaimer S, Bloch G (2006) Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees. J Exp Biol 209:1044–1051. https://doi.org/10.1242/jeb.02125

Zhao F, Zhang W, Hoffmann AA, Ma CS (2014) Night warming on hot days produces novel impacts on development, survival and reproduction in a small arthropod. J Anim Ecol 83:769–778. https://doi.org/10.1111/1365-2656.12196

Zwaan B, Bijlsma R, Hoekstra RF (1995) Artificial selection for developmental time in *Drosophila melanogaster* in relation to the evolution of aging: direct and correlated responses. Evolution 49:635–648. https://doi.org/10.1111/j.1558-5646.1995.tb02300.x

Part II
Other Types of Insect Rhythms and Photoperiodsim

Chapter 10
Lunar and Tidal Rhythms and Clocks

Jule Neumann and Tobias S. Kaiser

Abstract The presence of the moon results in tidal and lunar cycles that particularly affect life in the intertidal zone through tidal motion. A few insect species managed to colonize this dynamic interface between marine and terrestrial habitats, displaying tidal and lunar rhythms. These rhythms are caused by endogenous time-keeping mechanisms, namely, circatidal and circalunar clocks, but to date, the molecular mechanisms of lunar and tidal time-keeping remain largely unknown. Extensive chronobiological behavioral experiments conducted in a few insect species have identified the basic properties of circatidal and circalunar clocks, such as the free-running period, zeitgebers for entrainment, phase response curves, and temperature compensation. First molecular insights have been obtained for the circatidal clock of the mangrove cricket. Further development of molecular manipulation methods, as well as of genetic screens and omics experiments, will pave the way to unravel the mechanisms of both circatidal and circalunar clocks.

Keywords *Apteronemobius asahinai* · Circalunar clock · Circatidal clock · *Clunio*

10.1 Introduction

The moon has been our planet's most loyal companion. Its presence inevitably shaped the appearance of Earth, most evidently through tidal motion – the greatest synchronized movement of matter on our planet (Bowers and Roberts 2019). Both the 29.5-day lunar cycle and the 12.4-h tidal cycle are induced by the moon and influence life in the oceans. Consequently, lunar and tidal rhythms are found in the life cycles and synchronized reproduction of many marine species. This is especially true for coastal habitats, where the intertidal zone alternates between marine and terrestrial conditions.

J. Neumann · T. S. Kaiser (✉)
Max Planck Institute for Evolutionary Biology, Plön, Germany
e-mail: kaiser@evolbio.mpg.de

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Historically, causal relationships between lunar and tidal cycles and their related biological rhythms have been difficult to demonstrate by experimental verification. At the same time, a plethora of pseudoscientific publications debase the authenticity of such phenomena. Today, not only the existence but also the endogenous nature of circalunar and circatidal clocks have been experimentally confirmed in organisms across eukaryotes. Such prevalence underlines the ecological and evolutionary significance of moon-related biological rhythms. Even though only a few insect species have adapted to the marine environment, they provide us with the unique opportunity to study the acquisition of circalunar and circatidal clocks in organisms which colonized the ocean secondarily. Today, we are only at the beginning of an exciting journey to unravel the molecular mechanisms of lunar and tidal time-keeping, their operating principles, and their underlying genes.

10.1.1 The Moon Causes Environmental Cycles with Different Periods

The combined gravitational pull of the moon and sun on large water bodies, along with the rotation of the Earth, results in the phenomenon of tides, i.e., the periodic rise and fall of the water surface at a particular place (Fig. 10.1a, b). Along most coasts, tides rise and fall twice a day (every 12.4 h) and are called semidiurnal tides (Fig. 10.1b). In a few locations, tides occur only once a day (every 24.8 h) and are therefore referred to as diurnal tides. When sun, moon, and Earth align every 14.77 days during full and new moon, their gravitational pull interferes constructively, and the tidal amplitude is highest, resulting in so-called spring tides (Fig. 10.1a). The synodic lunar cycle is completed when all visible phases of the moon in relation to the Earth and sun have passed after 29.53 days (Fig. 10.1c). There are two spring tide events in a full synodic lunar cycle, i.e., spring tides occur in a semilunar rhythm (Fig. 10.1c). Tidal (12.4 h), semilunar (14.77 days), and lunar (29.53 days) rhythms are to be found in many marine organisms inhabiting the intertidal zone, including insects.

10.1.2 Organisms Anticipate Moon-Related Cycles by Endogenous Circasemilunar, Circalunar, and Circatidal Clocks

Lunar and tidal rhythms could either be merely induced by external stimuli or could result from an endogenous biological clock. This is a fundamental difference, and hence, the first question with respect to an observed biological rhythm is always: Is there an endogenous (molecular) time-keeping mechanism that can tell time even in the absence of environmental cycles? The matter has been a long-standing debate in

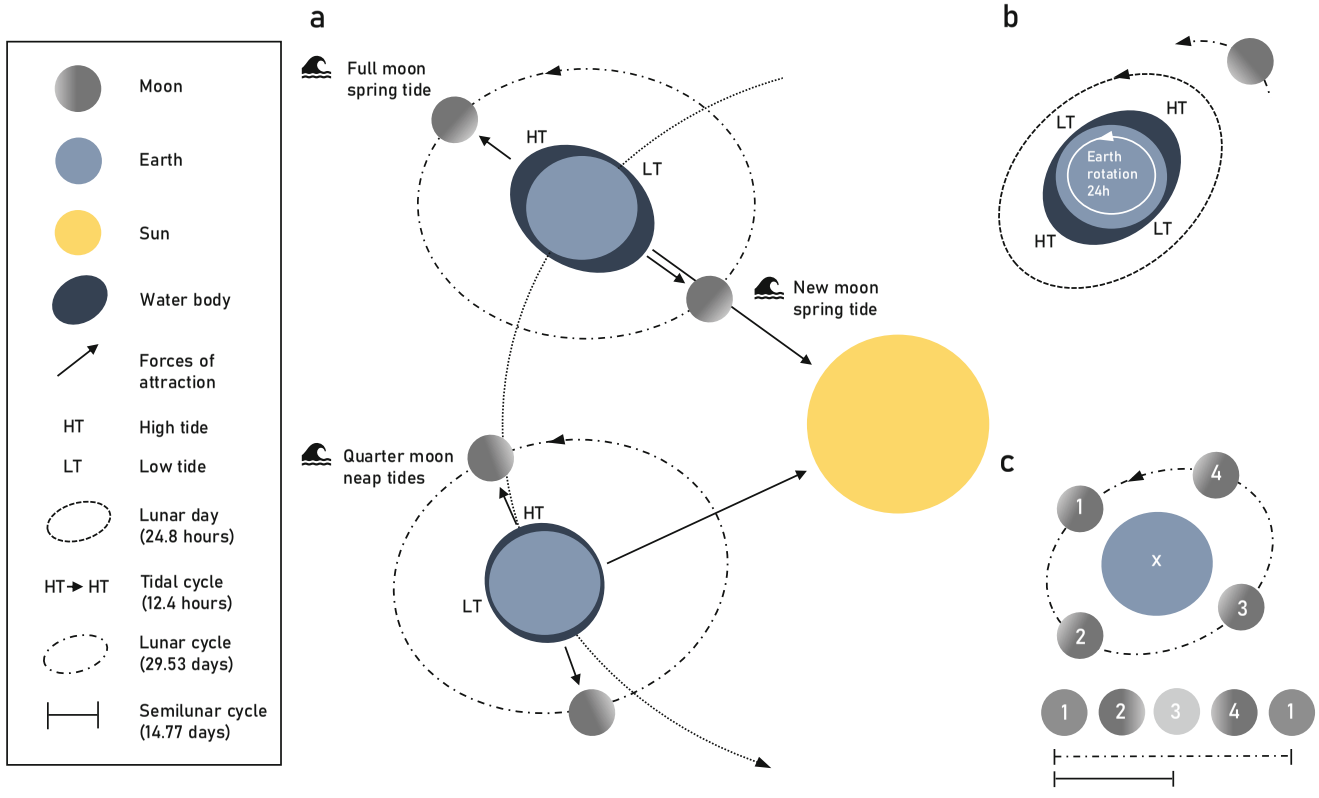

Fig. 10.1 The interaction of the sun, moon, and Earth results in tidal and lunar cycles. (**a**) Forces of attraction between the sun, moon, and Earth on Earth's water body result in the occurrence of tides. When all celestial bodies align during the full and new moon, the forces add up, and the tidal amplitude is highest (spring tides), while when the sun and moon pull on Earth's water body at a 90° angle, forces cancel each other out, and the tidal amplitude is lowest (neap tides). (**b**) The moon orbits Earth in the same direction as Earth rotates around its own axis. To regain its exact position in relation to the moon, every location on Earth has to

chronobiology. Today, the criteria characterizing an endogenous biological clock are well defined: The clock must be free-running, be temperature-compensated, and be subject to entrainment by specific stimuli (Pittendrigh 1993). The following section will explain these basic features.

Biological clocks are described in the terminology of physics and mathematics. They are viewed as oscillatory systems (limit-cycle oscillators) with a natural, endogenous period τ which is slightly different from the period T of the corresponding exogenous environmental cycle. To test for the existence of an internal oscillator with period τ, the organism displaying the rhythm of interest is transferred into constant conditions (e.g., for a tidal rhythm, none of the environmental conditions should have a 12.4-h period). If the organism still displays rhythmicity with τ close to T over several cycles in this constant environment, the rhythm is free-running. Furthermore, the free-running period of the rhythm must be temperature-compensated, meaning that within a given range of temperatures, the period τ does not change. This is in stark contrast to most other chemical and metabolic processes, which increase at a rate of 2 to 3 times with every 10 °C increase in temperature (Q_{10} temperature coefficient = 2–3). Temperature compensation is important for reliably keeping track of time independent of the naturally fluctuating temperature of the environment. Finally, it must be possible to synchronize the endogenous biological clock to the exogenous environmental cycle via specific environmental cues, which are called zeitgebers. The process of synchronization is called entrainment, during which the observed biological rhythm assumes a specific phase relationship to the environmental cycle. Zeitgebers therefore set the phase of the displayed rhythm. For lunar and tidal rhythms, tidal vibration, tidal temperature cycles, and moonlight have all been identified as zeitgebers for entraining the circatidal, circasemilunar, and circalunar clocks in insects (Table 10.1).

10.1.3 Evolutionary Significance of Circatidal, Circasemilunar, and Circalunar Clocks: Why Should We Study Lunar and Tidal Rhythms in Insects?

Studying the evolutionary causes and history of circatidal and circalunar clocks will be valuable to understand biological temporal organization. In this section, we will explore three evolutionary concepts:

Fig. 10.1 (continued) catch up with the 50-min discrepancy between both trajectories. Therefore, a lunar day from Earth's perspective is 24 h and 50 min. Hence, a location experiences two low and high tides per day, resulting in a tidal cycle of 12.4 h. (**c**) From Earth's perspective (x), the moon passes through all lunar phases every 29.53 days (synodic lunar cycle). A synodic semilunar cycle corresponds to half a synodic lunar cycle (14.77 days)

Table 10.1 Insects displaying lunar and tidal rhythms for which endogenous biological clock criteria have been experimentally examined

Species	Behavioral rhythm	(1) Free-run demonstrated in the laboratory	(2) Entrainment		(3) Temperature compensation
			Identified cue (zeitgeber)	Phase response curve	
Circatidal rhythms in hexapods					
Anurida maritima (Collembola)	Locomotor activity, aggregation behavior, phototaxis	[1] LL [2] LL, [2] LL, [3]	– – –	– – –	– – –
Thalassotrechus barbarae (Coleoptera)	Locomotor activity	[4] LL	–	–	–
Callytron inspecularis (Coleoptera)	Burrow plugging	[5] LL	[5] TSu	–	–
Apteronemobius asahinai (Orthoptera)	Locomotor activity	[6] DD [9] LL	[7] TSu [8] Wa	[7] [8]	–
Circalunar rhythms in insects					
Clunio marinus (Diptera)	Semilunar or lunar adult emergence	[10] LD [11] LL	[10] ML [12] TVi [13] TTe	–	–
Clunio tsushimensis (Diptera)	Semilunar adult emergence	[14] DD	[14] ML	[15]	[14]
Pontomyia oceana (Diptera)	Semilunar adult emergence	[16] LD, LL, and DD	[16] ML	–	[16]

ML moonlight, *TVi* tidal vibration, *TTe* tidal temperature, *TSu* tidal submergence, *DD* constant darkness, *LL* constant light, *LD* light-dark cycle, *Wa* water stimulus
[1] Foster and Moreton 1981, [2] Mcmeechan et al. 2000, [3] Manica et al. 2000, [4] Evans 1976, [5] Satoh et al. 2006, [6] Satoh et al. 2008, [7] Satoh et al. 2009, [8] Sakura and Numata 2017, [9] Satoh 2017, [10] Neumann 1966, [11] Neumann 1976, [12] Neumann 1978, [13] Neumann and Heimbach 1984, [14] Neumann 1988, [15] Kaiser and Neumann 2021, [16] Soong et al. 2011

(a) The adaptive benefits of circalunar and circatidal clocks: Why did circatidal and circasemilunar clocks evolve? In other words, what is the ultimate or evolutionary advantage for keeping track of the tides and the lunar cycle?
(b) The diversity of time-keeping mechanisms: Do organisms find different solutions for circalunar and circatidal time-keeping mechanisms? This will help to understand how tidal and lunar biological rhythms evolved or how plastic time-keeping can be organized.
(c) The temporal organization within an organism: How do organisms track different geophysical cycles simultaneously?

10.1.3.1 The Adaptive Benefits of Circalunar and Circatidal Clocks

The intertidal zone is defined as the area lying within the tidal range. This means that during low tides, the seafloor is exposed, while at high tides, it is submerged. Changes in the water level are accompanied by considerable differences in temperature, salinity, UV exposure, nutrient availability, or predator abundance. While the adaptive value of the circadian clock has been demonstrated (Abhilash and Sharma 2016), such evidence is entirely lacking for circatidal or circa(semi)lunar clocks. However, there are two main hypotheses for the adaptive benefits of these moon-related rhythms. The first is that they may synchronize important life cycle events, such as reproduction or hatching, as well as locomotor and feeding behavior, with favorable tidal conditions. This is known as the extrinsic advantage hypothesis (Naylor 2010). Second, they may serve to synchronize these same life cycle events within a population (Naylor 1976). In particular, lunar rhythms may serve to synchronize reproduction within a population, especially in marine or tropical habitats where seasonality is often not very pronounced (Hartland-Rowe 1958). In a sparse population, it will be harder to find a suitable mate, and therefore, reproductive success decreases, which is known as the Allee effect (Courchamp et al. 1999). Precise synchronization of reproduction reduces the Allee effect. Despite the lack of experimental evidence, the omnipresence of (semi)lunar and tidal rhythms across the tree of life suggests that moon-related rhythms are adaptive for many organisms, particularly in marine environments.

10.1.3.2 Diversity of Circatidal and Circalunar Clocks

As insects first evolved in terrestrial habitats, all marine insect species must have colonized the ocean secondarily (Andersen 1999; Misof et al. 2014). These insects provide us with an excellent opportunity to study recently acquired endogenous timing systems as adaptations to the tidal environment. The principles of these timing mechanisms can then be compared to those that have evolved in primordial marine organisms, like those of marine annelids. First evidence suggests that the circa(semi)lunar clocks of annelids, insects, and algae may follow different functional principles (Kaiser and Neumann 2021). Unraveling these principles and the molecular clockwork of circatidal and circa(semi)lunar clocks across the tree of life will help to fully understand their divergent mechanisms and multiple evolutionary origins (Rock et al. 2022).

10.1.3.3 Temporal Organization on Different Time Scales

Organisms are simultaneously exposed to a variety of environmental cycles of different periods. The circadian clock might time the feeding behavior of an organism, while a circalunar clock times reproduction, and a photoperiodic response

regulates overwinter dormancy. The interplay of time-keeping mechanisms, their interrelations, coordination, and dependencies are ultimately what ensures survival of an organism and is subject to selection. It remains an exciting challenge to unravel the complex interplay of time-keeping mechanisms. Only organisms that show robust phenotypes on all different time scales will allow us to understand their overall temporal organization. Insects displaying tidal and (semi)lunar rhythms are a starting point for addressing such complex questions.

10.2 Circatidal Clocks in Hexapods

Tides and lunar cycles predominantly impact life in the ocean where insects are rare. Approximately 50% of all insect orders contain marine species, most notably the orders Collembola (springtails), Hemiptera (true bugs), Coleoptera (beetles), and Diptera (true flies) (Cheng 2009). The vast majority of marine insects inhabit the intertidal zone, and some of them display behavioral rhythms related to tides. For example, hemipteran bugs of the genus *Halovelia* and members of the family Hermatobatidae leave their refuge of the upper tidal zone to forage during low tide (Cheng 2009). The common tiger beetle *Cicindela* inhabits sandy shores and populates the intertidal zone when the tide recedes. Larvae of the beetles plug their burrow entrances shortly before the tide returns to avoid flooding of their refuge (Cheng 2009). Similar burrow-plugging behavior was also observed in the mangrove forests of Japan for the tiger beetle *Callytron yuasai* (Satoh and Hayaishi 2007). Interestingly, a circaseptan activity rhythm was recorded in the laboratory for the beach beetle *Chaerodes trachyscelides* (Meyer-Rochow and Brown 1998). The authors speculate that such weekly rhythms could be an adaptation to the position of the wrack line, which is altered by weekly alternation of spring and neap tides and may serve as shelter and foraging grounds.

Despite these observations, experimental confirmation of circatidal clocks in insects is scarce (Table 10.1). The criteria for endogenous circatidal rhythms have been investigated in only four hexapod species: the springtail *Anurida maritima* (Collembola) (Foster and Moreton 1981), the beetles *Callytron inspecularis* and *Thalassotrechus barbarae* (Coleoptera) (Evans 1976; Satoh et al. 2006), and the mangrove cricket *Apteronemobius asahinai* (Orthoptera) (Satoh et al. 2008). It appears that tidal rhythms generally dampen rapidly under constant conditions, which complicates their study in the laboratory. With respect to zeitgebers, artificial submergence given in a tidal pattern has been identified to entrain *C. inspecularis* and *A. asahinai* (Satoh et al. 2006; Satoh et al. 2009). Temperature compensation of the tidal rhythm has never been experimentally verified in any of the examined insect species (see Table 10.1). This may be due to the quick dampening, as well as a lack of efficient culturing procedures for most intertidal species.

10.2.1 Collembola: Anurida maritima

The small Collembola *Anurida maritima* inhabits rocky intertidal zones and salt marshes (Cheng 2009). During high tide, *A. maritima* can be found hiding among rocks, while during low tide, they come out to forage. This circatidal locomotor rhythm has been demonstrated to be free-running under constant light (LL) in the laboratory for at least 7 days (Mcmeechan et al. 2000; Foster and Moreton 1981). Early anticipation of the approaching tide is essential for survival of the Collembola because it physically cannot outrun the water (Foster and Moreton 1981). Some individuals inhabit the higher intertidal zone, which is not exposed to high tides every day. Interestingly, *A. maritima* there still displays a tidal activity rhythm, providing a rare opportunity to investigate an apparently free-running rhythm under field conditions (Foster and Moreton 1981). When *A. maritima* moves toward the upper shore around high tide, they often aggregate with hundreds of individuals (Joosse 1966). This aggregation behavior also persists rhythmically under LL, matching the phase of lowest locomotor activity (Mcmeechan et al. 2000). Additionally, it has been reported that in the field, the peak of activity occurring during the night low tide in *A. maritima* can be suppressed (Foster and Moreton 1981). This raises the question as to whether the circadian clock modulates the tidal activity pattern or whether darkness or temperature instantly suppresses the behavior (Foster and Moreton 1981; Mcmeechan et al. 2000). Interestingly, *A. maritima* is usually negatively phototactic, meaning they move away from light, consistent with the shelter searching behavior in the field observed during high tide. However, for 2–7 h after high tide, most of the population becomes positively phototactic, coinciding with *A. maritima* leaving their refuge to feed (Manica et al. 2000). This change in phototactic behavior has been shown to follow a circatidal rhythm, potentially using the horizon brightness as a guidepost (Manica et al. 2000). Water reflects light better than soil, and thus, the horizon toward the water is brighter, attracting *A. maritima* during their foraging excursions (Manica et al. 2000).

10.2.2 Coleoptera: Thalassotrechus barbarae *and* Callytron inspecularis

The carabid beetle *Thalassotrechus barbarae* was the first insect for which a circatidal locomotor rhythm had been investigated in the laboratory. The beetles inhabit the rocky tidal shore along the American continent. Adults forage during the night, but just at low tide. Hence, they display a lunidian (lunar-day) locomotor activity rhythm (24.8 h) during night tides. Consistently, under LD (light:dark) 15:9 in the laboratory, beetles were active only during the dark phase, suggesting either a circadian or circalunidian rhythm, which could not be distinguished statistically (Evans 1976). However, under LL, a significant correlation between the amplitude of the corresponding field night tide and the amount of activity was demonstrated for

the first 3 days after beetles were brought to the laboratory (Evans 1976). Accordingly, it has been proposed that a circatidal oscillator might suppress the beetle's activity during high tide (Evans 1976). It should be noted, however, that there was never a follow-up publication investigating the endogenous nature of the tidal rhythm in *T. barbarae.*

Larvae of the tiger beetle *Callytron inspecularis* reside in sand burrows, which they plug shortly before high tide. It was found that this burrow-plugging behavior continues under LL in the laboratory (Satoh et al. 2006). When one larva was subjected to an artificial tidal cycle of submergence, the burrow-plugging rhythm was entrained and, after cessation of the cue, was free-running with a period of 12.45 ± 0.51 h (Satoh et al. 2006). Interestingly, larvae only start the plugging behavior after their burrows have been submerged by one to three high tides (Satoh et al. 2006). When approaching neap tide, the tides, and therefore tidal submergence, cannot reach the beetle's habitat, and the burrow-plugging behavior eventually ceases (Satoh et al. 2006). It has been hypothesized that the circatidal oscillator is entrained by tidal submergence but dampened with subsiding tides, in accordance with the observation that burrow plugging in the field has not been observed during neap tides.

10.2.3 Orthoptera: Apteronemobius asahinai

10.2.3.1 The Circatidal Activity Rhythm in the Mangrove Cricket Free-Runs Under LL and DD

By far, the best studied circatidal rhythm of an insect is found in the mangrove cricket *Apteronemobius asahinai*. These crickets are active during low tide but remain inactive around high tide, residing on mangrove roots. The locomotor rhythm of field-caught crickets has a free-running period of approximately 12.6 h under constant darkness (DD) (Satoh et al. 2008) as well as under LL (Satoh 2017). Notably, the recorded activity levels differed in amplitude under DD, alternating between strong and weak activity peaks (Fig. 10.2a, a'). This can be interpreted as a diel modulation of the activity, with the crickets being less active during the night. Such diel modulation might be controlled by a circadian clock. Indeed, the difference in activity levels was absent in LL, and it has been hypothesized that under LL, the circadian modulation diminishes because of the suspension of the circadian clock (Satoh 2017). Under DD, the circadian oscillations can persist, and hence, the circadian modulation of the circatidal rhythm is reflected in an unequal activity rhythm.

To further disentangle circatidal and circadian rhythms, locomotor activity of crickets that never experienced a natural tidal cue (raised from eggs in the laboratory) was monitored under dim red light (Sakura and Numata 2021). Some crickets showed significant circatidal rhythmicity for more than 20 days, while others showed circatidal rhythmicity only within the first 10 days but switched to diurnal

rhythmicity afterward. Additionally, some crickets displayed a circatidal rhythm in the first 10 days but became arrhythmic thereafter. To investigate whether the diel rhythm was controlled by a circadian clock, the crickets were subjected to LL, as well as light-dark cycles (LD) with phase advances and delays. However, no consistent responses could be obtained, and more experiments are required to investigate whether the diel activity rhythm is controlled by a circadian clock.

10.2.3.2 Inundation Stimuli Entrain the Circatidal Rhythm in the Mangrove Cricket

Artificial inundation was found to be a zeitgeber for the circatidal clock of *Apteronemobius asahinai* and served to obtain the only circatidal phase response curve for insects (Satoh et al. 2009). Field-caught crickets were exposed to DD for approximately 10 days, and free-running circatidal locomotor rhythm was recorded. When a 30-min inundation stimulus was given at different times of the tidal cycle, the onset of activity was shifted depending on when the cue was given. If the stimulus was given during subjective high tide, only a small phase advance could be invoked, while a stimulus given during the subjective low tide resulted in noticeable phase delays (first half) and advances (second half).

Although inundation was shown to entrain the circatidal clock in the mangrove crickets, the particular sensory stimulus could not be clarified from the experiment. As a follow-up, it was tested whether contact with water was the cue (Sakura and Numata 2017). In cold-anesthetized and immobilized crickets, a water stimulus given during the middle phase of the subjective low tide resulted in a delay of locomotor rhythm, while a stimulus given during the later phase of the subjective low tide advanced the rhythm (Sakura and Numata 2017). Only a slight phase shift was observed when the water stimulus was given during the subjective high tide. The authors proposed that water receptors could perceive the water stimulus, as they have been described in the legs of another cricket, *Gryllus bimaculatus* (Kanou et al. 2007).

10.2.4 Involvement of the Circadian Clock in Circatidal Time-Keeping

It has been a long-standing debate whether the circatidal clock in one way or the other involves the circadian clock's molecular machinery. There are three main hypotheses. Enright suggested that a single clock could govern both circatidal and circadian rhythms and be entrained by both tidal and circadian zeitgebers (Enright 1976). Palmer proposed the presence of two 24.8-h oscillators that run in antiphase and produce a rhythm that appears to be only 12.4 h apart, known as the circalunidian clock hypothesis (Palmer 1995; Palmer and Williams 1986).

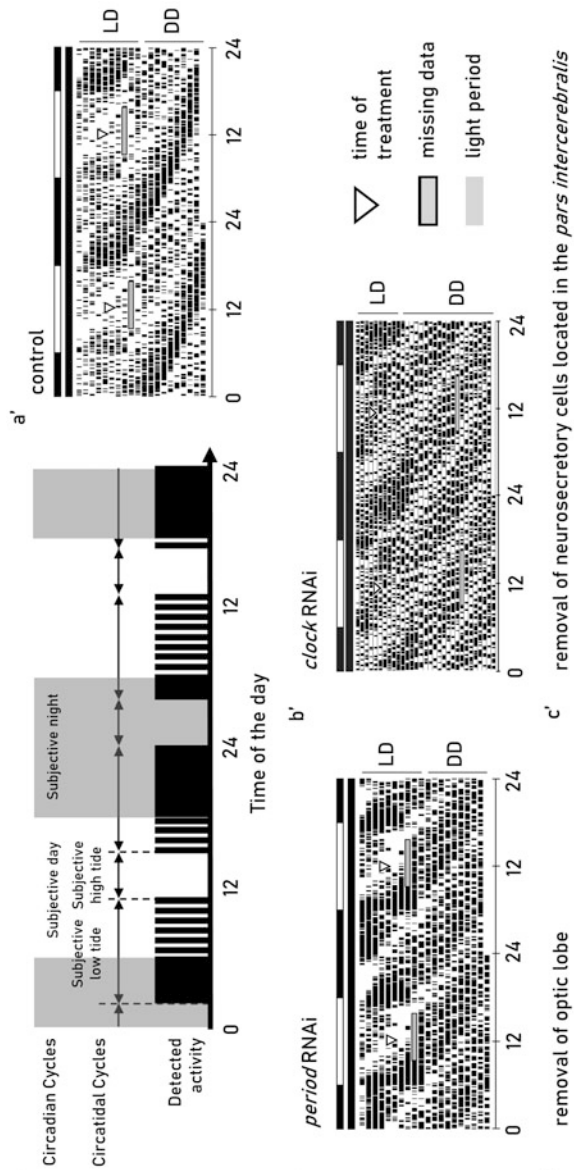

Fig. 10.2 (**a**, **a'**) The circadian clock modulates the circatidal activity rhythm of the mangrove cricket *Apteronemobius asahinai*. Crickets are active during subjective low tide, but this activity remains suppressed by the circadian clock during the subjective day. Schematic figure **a** modified from Goto and Takekata (2015), Copyright (2015), with permission from Elsevier. Experimental figure **a'** used with permission of The Royal Society from Takekata et al. (2012),

Circalunidian refers to the period between consecutive moonrises, equaling 24.8 h (Fig. 10.1b). Given the period close to 24 h, such circalunidian oscillators could be based on the circadian clock machinery. Finally, Naylor proposed an independent circatidal clock mechanism with its own molecular machinery (Naylor 1996; Naylor 1958).

Given the inability of the circadian clockwork to adopt periods that deviate significantly from 24 h, Enright's idea of a single clock would only work if the overt 12.4-h tidal rhythm is generated as a submultiple of a 24.8-h circalunidian oscillator (Enright 1976; Goto and Takekata 2015). Additionally, the circalunidian instance of such a single multipurpose clockwork must not respond to light entrainment, as then night and day should force it to a 24-h period (Palmer 1995). There are no observations in insects that would support such a single clockwork mechanism, but it was demonstrated for fish (Gibson 1973) and crustaceans (Akiyama 1997).

Interestingly, it is well documented that in *Drosophila* the morning (M) and evening (E) activity peaks are generated by two oscillators in antiphase (Helfrich-Förster 2009; Yoshii et al. 2012). Considering that marine insects evolved from terrestrial ancestors, it might be that by extending the M-E interval, an adaptation to the tides (a 12.4-h rhythm) could have been achieved (Zhang et al. 2013). The burrow-plugging behavior of the tiger beetle *Callytron inspecularis* has been hypothesized to be explained by the circalunidian hypothesis because the time intervals between two consecutive burrow-plugging events vary in the field (Satoh et al. 2006). This suggests that the two oscillators are slightly out of phase. In line with that, in free-run experiments with crabs, the two daily activity peaks sometimes seem to run with different free-running periods (Palmer 2000; Palmer and Williams 1986). However, based on experiments in *Carcinus maenas* (Naylor 1996; Naylor 1958), Naylor argued that the observed patterns under free-run conditions can rather be explained by the interplay of independent circatidal and circadian oscillators. Indeed, for the mangrove cricket, an independent circatidal clock has been proposed based on molecular knockdown of core circadian clock genes (see next paragraph). Additionally, recent molecular findings in the crustacean *Eurydice pulchra* suggest that the circatidal oscillator may use some molecules of the circadian clock, but not

Fig. 10.2 (continued) Copyright (2012), permission conveyed through Copyright Clearance Center, Inc. (**b**, **b'**) RNAi of the circadian clock gene *period* (**b**) and *clock* (**b'**) abolishes the circadian modulation of the circatidal activity rhythm, indicating that both genes are essential for the circadian but not the circatidal clock. (**b**) used with permission of The Royal Society from Takekata et al. (2012), Copyright (2012), permission conveyed through Copyright Clearance Center, Inc. (**b'**) Reprinted from Takekata et al. (2014b), Copyright (2014), with permission from Elsevier. (**c**) Removal of the optic lobe results in a circatidal activity rhythm without circadian modulation. Reprinted from Takekata et al. (2014a), Copyright (2014), with permission from SAGE. (**c'**) More than half of the crickets display an arrhythmic activity pattern after removal of the neurosecretory cells in the pars intercerebralis (PI), indicating that this region is important for both circadian and circatidal time-keeping. Adapted from Takekata et al. (2018), with permission from Springer Nature: Copyright (2018)

all of them (Zhang et al. 2013). This finding might support an independent circatidal oscillator with some circadian clock components reused.

10.2.4.1 Molecular and Neurophysiological Data Support an Independent Circatidal Pacemaker in the Mangrove Cricket

The circadian clock is by far the best studied biological clock at the molecular level. As a consequence, involvement of the circadian clock in other biological time-keeping systems – including photoperiodism, circatidal, circalunar, and circannual clocks – has been the focus of research for a long time. Knocking down known core circadian clock genes using RNA interference (RNAi) is one approach for investigating the role of the circadian clock. If the circadian phenotype is disrupted but the tidal, lunar, or photoperiodic rhythm persists, the underlying clock is assumed to run independently of the circadian system. Knockdown methods are often the only available tool for non-model species displaying circatidal, circalunar, or circannual rhythms. In the long run, not only knockdown but also knockout and gain of function studies need to be established and conducted to clarify the involvement of the circadian clock.

In the mangrove cricket *Apteronemobius asahinai*, knockdown experiments using RNAi against the core clock gene *period* and *clock* support the independence of the circatidal and circadian clocks (Takekata et al. 2012; Takekata et al. 2014b). The circadian modulation of the tidal locomotor rhythm was disrupted, while the tidal rhythm persisted (Fig. 10.2b, b'). Neurophysiological ablation experiments were also conducted in the mangrove cricket to identify the pacemaker center of the circatidal clock. It is known that the optic lobe is the location of the circadian clock pacemaker in crickets (Shiga et al. 1999; Tomioka and Chiba 1992). Removal of the optic lobe in the mangrove cricket disrupted circadian but not circatidal rhythmicity, indicating that this region is not important for circatidal time-keeping (Fig. 10.2c) (Takekata et al. 2014a). When the pars intercerebralis (PI, another region relevant to the circadian clock (Sokolove and Loher 1975)) and/or surrounding regions were removed, the tidal rhythm was perturbed, i.e., more than half of the crickets became tidally arrhythmic. This suggests that the PI is important not only for the circadian clock but also for the circatidal clock (Fig. 10.2c') (Takekata et al. 2018).

The possibility of global genomic and transcriptomic screens offered by next-generation sequencing methods can be key for getting hold of yet unknown components of circatidal and circalunar clocks. Unfortunately, such screens are still scarce for organisms with robust tidal rhythms. A pilot study (only one replicate per time point) analyzed RNA-sequencing (RNA-Seq) data over two tidal cycles sampled every 3 h under DD of the mangrove cricket's head (Satoh and Terai 2019). A total of 206 genes were found cycling with a circadian period. Arrhythmic expression of *clock* is in accordance with Takekata et al. (2012), in which mRNA of *clock* was found not cycling under LD in crickets with β-lactamase RNAi. However, the RNA-Seq data could not recapture the cycling pattern of *period*

mRNA, probably because one replicate did not give enough power to detect all cycling transcripts. Nevertheless, more than 284 genes were found to cycle tidally in the cricket brain, with most of them peaking around either subjective high or low tide, indicating that their transcript abundance might be correlated with the tidal cycle. Moreover, genes involved in metabolic processes and molecular chaperons were upregulated at low tide (Satoh and Terai 2019). Because only half of all tidally cycling genes could be annotated, the authors speculated that some of them could potentially be noncoding RNAs. More sophisticated RNA-Seq experiments are required to understand tidal gene regulation in insects. As an important step on the way, an annotated genome has been recently published for the mangrove cricket (Satoh et al. 2021).

10.2.5 Tidal Non-oscillatory Timing Mechanisms

Although a biological clock has been traditionally defined as an oscillatory system, it is worth noting that other ways of tidal time-keeping exist. Adults of an Arctic population of the marine midge *Clunio marinus* emerge every low tide during midsummer. The temperature increase associated with exposure of the substrate during low tide causes the start of an 11- to 13-h hourglass timer, which allows the midges to emerge during the subsequent low tide (Pflüger 1973). In the laboratory, no free-running emergence rhythm was observed, consistent with the idea that a non-oscillatory or highly dampened oscillatory system times emergence of the midge (Pflüger 1973).

10.3 Circalunar Clocks in Insects

10.3.1 Lunar Rhythms of Insects in Terrestrial Habitats

Lunar periodicity of abundance in insects has been observed for various insect orders, including Ephemeroptera, Trichoptera, and Diptera (Hartland-Rowe 1955; Corbet 1958), as well as Lepidoptera and Coleoptera (Nowinszky et al. 2010). Lunar periodicity in flight behavior has also been observed for a few species of Hymenoptera, Heteroptera, Isoptera, and Orthoptera (Danthanarayana 1986). It should be noted, however, that some controversy among authors exists. Lunar phase and rhythmicity have been inconsistently reported for the same insect species (Danthanarayana 1986; Nowinszky 2004). Danthanarayana (1986) provides an extensive list of insect species for which flight activity has been correlated to lunar phases, noting that not only aquatic but also entirely terrestrial species show clear lunar rhythmicity in abundance. It is accepted that changes in insect abundance in relation to the lunar phase can be attributed to a reduction in the effectiveness of the trap (light of the trap essentially competes with moonlight) (Williams et al. 1956).

However, some species truly vary in abundance due to changes in the lunar phase (Bowden and Church 1973). It has been hypothesized that such lunar periodicity in emergence may be an adaptation to wind-assisted dispersal (migration) (Danthanarayana 1976).

However, to date, there is no fully convincing evidence for an endogenous lunar time-keeping mechanism in a terrestrial insect. Ito et al. (1993) found that the catch size of adult Malayan black rice bugs *Scotinophara coarctata* was highest around full moon. However, when second instar nymphs were isolated and monitored in the laboratory, adult emergence was not synchronized with the lunar cycle. The mayfly *Povilla adusta* shows a lunar rhythm in emergence, peaking around full moon (Hartland-Rowe 1955). Although it was shown that this emergence peak persists in DD and artificial LD (Hartland-Rowe 1955; Hartland-Rowe 1958), the very small number of individuals examined (< 10) in a short time frame (< 1 month) disqualifies the use of these experiments as evidence for an endogenous lunar free-run in the mayfly. The pit volume of the ant lion *Myrmeleon obscurus* is highest around full moon and was observed for two peaks subjected to daylight conditions as well as in DD in the laboratory (Youthed and Moran 1969). An interesting example of a semilunar rhythm comes from the cockroach *Periplaneta americana*, for which neurotransmitter-like substances in the central nervous system reach their highest levels around full and new moon (Rounds 1981).

10.3.2 Lunar Rhythms of Insects in Marine Habitats

Lunar rhythms are prevalent in the marine environment across the tree of life, and free-run criterion has been experimentally validated in many species, including algae (Bünning and Müller 1961), annelids (Hauenschild 1960; Franke 1985), mollusks (Yoshioka 1989), crustaceans (Saigusa 1980; Enright 1972), and fish (Hsiao and Meier 1992). Interesting cases of lunar rhythms can be found in marine midges of the genera *Clunio* and *Pontomyia* (Diptera, Chironomidae), which have extremely short adult life spans of only 1–2 h. Adult emergence is precisely timed to the lowest low tides around full and new moon by a circalunar clock. For both *Pontomyia* and *Clunio*, free-run, entrainment, and temperature compensation are well documented, making them attractive systems to study the circalunar clock (Table 10.1).

10.3.2.1 The Case of *Clunio*

In marine nonbiting midges of the genus *Clunio*, development is tightly coupled with the lunar cycle (Neumann and Spindler 1991; Krüger and Neumann 1983). Chevrel (1894) was the first to notice that adults of *Clunio marinus* only swarm around full and new moon for mating. Enduring most of their life as larvae, adult emergence is restricted to only a few days around full and new moon, precisely matching the spring tides. *Clunio marinus* can be found along the entire rocky Atlantic coast of

Europe from Portugal to Norway, wherever spring tides expose the seafloor and larval substrate becomes available to oviposition of the sticky egg clutches. To achieve this precise synchronization to the lunar cycle and the tides, a developmental waiting stage (CDA, circalunar developmental arrest) occurs during the early fourth instar in *Clunio* (Neumann and Spindler 1991). This developmental stage experiences a brief increase in ecdysone titer followed by temperature-compensated development (Neumann and Spindler 1991). It is the circa(semi)lunar clock that controls the developmental switching point in the fourth instar and ultimately synchronizes development of the marine midge with the lunar cycle. During the 1960s, Dietrich Neumann established a culturing protocol for *Clunio* which allowed to systematically study endogenous clock criteria in the laboratory (Neumann 1966).

The Semilunar Emergence Rhythm of *Clunio* Is Free-Running and Temperature-Compensated

The (semi)lunar adult emergence rhythm of *Clunio* persists under constant conditions (LD, but no lunar time cues), verifying the endogenous nature of the rhythm (Neumann 1966; Neumann 1976). Free-running lunar and semilunar emergence rhythms also persist in DD (Neumann 1988) as well as in LL (Neumann 1976). Additionally, the free-running semilunar period has been found to be temperature-compensated between 14 °C and 24 °C in the Japanese midge *Clunio tsushimensis* (Fig. 10.3) (Neumann 1988).

Moonlight, Tidal Turbulence, and Temperature Cycles Can Entrain the Circalunar Clock of *Clunio*

When artificial moonlight (0.3 lux) was given for four consecutive nights every 30 days, a semilunar or lunar emergence rhythm can be evoked in southern populations of *Clunio marinus* (Neumann 1966) and in *Clunio tsushimensis* (Neumann 1988). Importantly, only light perceived during the subjective night synchronizes the phase of the lunar rhythm (Neumann 1995). It was shown that the circadian clock is important to set such a nocturnal light sensitivity window (Neumann 1995). As the rising and setting of the moon changes with the lunar cycle, moonlight is only available throughout the night around full moon. Moonlight is detected when the presence of moonlight during the night-time low tide and the nocturnal light sensitivity window coincide (Kaiser et al. 2011); hence, this is an instance of coincidence detection.

At higher latitudes, the moon stays close to the horizon, and days become very long during summer, making it difficult to perceive "light at night" as a distinct cue (Neumann 1995). Correspondingly, the (semi)lunar rhythm of northern populations of *Clunio marinus* is often more precisely entrained by vibration (Neumann and Heimbach 1979) or temperature (Neumann and Heimbach 1984) given in a tidal pattern (Neumann 1968; Neumann 1978). Such tidal cycles of vibration or

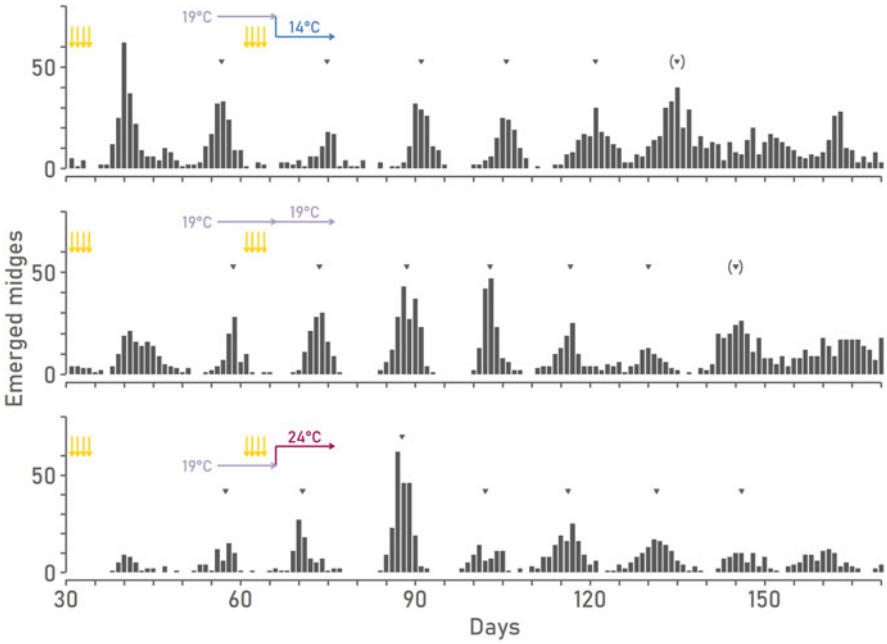

Fig. 10.3 The free-running semilunar emergence rhythm of the marine midge *Clunio tsushimensis* is temperature-compensated. After entrainment by moonlight (four nights every 30 days; yellow arrows), the semilunar rhythm free-runs for several months with a period of approximately 15 days. From Kaiser and Neumann (2021) licensed under CC BY 4.0

temperature can be used as a semilunar time cue because – just as the rising and setting of the moon – the 12.4-h tidal cycle advances every day by approximately 50 min relative to the 24-h LD. This unique phase relationship between LD and the tidal cycle recurring every 15 days serves as a semilunar cue. It is assumed that the detection of these tidal cues is also based on a coincidence detection mechanism, very similar to the detection of moonlight (Neumann 1995; Neumann and Heimbach 1985).

A lunar phase response curve has been recently published for *Clunio tsushimensis* (Kaiser and Neumann 2021). Interestingly, there is a linear phase response without transient cycles, suggesting an immediate resetting of the circalunar clock. This implies a tight coupling of the overt rhythm to the pacemaker. *Clunio's* lunar PRC is different from that of the marine annelid *Syllis prolifera*, which has a nonlinear phase response and characteristic transient cycles, suggesting that the two species may rely on different circalunar clock mechanisms (Franke 1986).

10.3.2.2 The Case of *Pontomyia*

Midges of the genus *Pontomyia* inhabit tidal pools and shallow waters of the Western Pacific (Huang and Cheng 2011). The Taiwanese midge *Pontomyia oceana* has a well-studied semilunar rhythm of adult emergence (Soong et al. 1999). Midges emerge around full and new moon in the field. The semilunar rhythm has been shown to persist under LD as well as in DD in the laboratory for at least two peaks. Hence, it is free-running (Soong et al. 2011). The semilunar emergence rhythm is also temperature-compensated between 24 °C and 30 °C, although the Q_{10} value of 0.86 is lower than the expected value of 1 (Soong et al. 2011).

10.3.3 The Role of the Circadian Clock in Circalunar Time-Keeping

Bünning and Müller proposed three hypotheses on how a lunar or semilunar period could be evoked by the circalunar clock (Bünning and Müller 1961). First, an independent semilunar or lunar oscillator of yet unknown molecular nature could run with a period of 15 or 30 days, respectively. Second, the beat hypothesis assumes superposition of a circadian rhythm (24 h) with either a circalunidian (24.8 h) or a circatidal rhythm (12.4 h). This would result in a beat wave with an amplitude changing in a lunar or semilunar rhythm. Third, there could be a counter mechanism based on either counting endogenous circadian oscillations or LD. Importantly, a circadian system is indispensable for the beat hypothesis as well as the counter hypothesis. In contrast, a circa(semi)lunar oscillator could hypothetically run independently of the circadian system.

When exposing *Pontomyia* midges to different T-cycles between LD 11:11 and LD 14:14, the period of the semilunar emergence rhythm was found to change linearly with the change in LD, i.e., the free-running semilunar emergence rhythm has a shorter period under short T-cycles and a longer period under longer T-cycles (Soong and Chang 2012). However, the semilunar emergence rhythm was unchanged under multiples of 24 h (LD 3:3, LD 6:6, and LD 12:36). These experiments suggest that *Pontomyia* uses a mechanism based on counting endogenous circadian oscillations.

Persistence of rhythmic emergence of *Clunio* in experiments conducted in DD (Neumann 1988) and LD (Neumann 1976) has been taken as evidence that *Clunio's* circalunar clock is an independent self-sustained oscillator. However, the existence of a counter mechanism based on endogenous circadian oscillations cannot be excluded. When reanalyzing the circadian phenotype under DD (Neumann 1988), a significant circadian period of approximately 25 h persisted for up to 17 days. This is long enough to explain the observed persistence of the lunar rhythm in DD, and thus, a counting mechanism might also exist in *C. marinus*. The fact that the marine midge *Pontomyia oceana* was already found to count circadian cycles for lunar time-

keeping (Soong and Chang 2012) allows one to speculate that marine midges in general could use a counter mechanism for lunar time-keeping. In an evolutionary context, this makes sense: Diptera evolved on land (Misof et al. 2014) and marine midges secondarily colonized the ocean. Many terrestrial insects possess a photoperiodic counting mechanism. It seems possible that marine insects coopted the photoperiodic counter for circalunar time-keeping, i.e., as a circalunar counter.

10.3.4 Molecular and Genetic Data on Circalunar Time-Keeping in Insects

To understand the mechanisms of lunar time-keeping, a combination of molecular and genetic methods is indispensable. Considering that *Clunio* and *Pontomyia* – the only insects with described robust lunar rhythms – are still non-model organisms, molecular methods often need to be established from scratch. Even annotated reference genomes cannot be taken for granted. Generating these resources will be necessary to unravel the pathways and genes underlying lunar rhythmicity.

As a major asset, *Clunio marinus* has a highly complete and well-annotated reference genome on chromosomal scale (Kaiser et al. 2016). Additionally, there are many laboratory strains of *C. marinus* that differ in various timing phenotypes and can hence serve for comparative molecular analysis and genetic dissection of the circa(semi)lunar clockworks (Fig. 10.4a). First, populations of *Clunio marinus* differ in whether they emerge only around full moon, only around new moon, or during both full and new moon (Fig. 10.4b). The phase of emergence within the lunar cycle is genetically determined (Kaiser et al. 2011) (Fig. 10.4c). Through crossing experiments and quantitative trait locus (QTL) mapping, it was found that the lunar emergence phase is determined by two major QTLs (Kaiser et al. 2016; Kaiser and Heckel 2012). Second, populations of *Clunio marinus* differ in whether they respond to moonlight or tidal cycles of vibration. These phenotypes offer a starting point to understand the entrainment pathways. The shielding pigment transparency changes over the lunar cycle in larval ocelli of *Clunio* (Fleissner et al. 2008; Falkenberg et al. 2013). Therefore, the ocelli might play a crucial role in the perception of moonlight. Finally, Arctic and Baltic populations of *C. marinus* have lost the lunar rhythm altogether, as the lunar rhythm has no adaptive value in the absence of tides in the Baltic Sea and detection of the lunar time cues is not possible during the polar day in the Arctic. Recent genome screens that compared the lunar-arrhythmic Baltic ecotype to the lunar-rhythmic Atlantic ecotype suggest that lunar arrhythmicity is primarily dependent on circadian clock genes as well as genes involved in nervous system development (Fuhrmann et al. 2023). These findings are in line with the involvement of the circadian clock in the perception of lunar zeitgebers (Neumann 1995; Neumann and Heimbach 1985; Neumann 1989), as well as a possible counting mechanism based on circadian clock cycles.

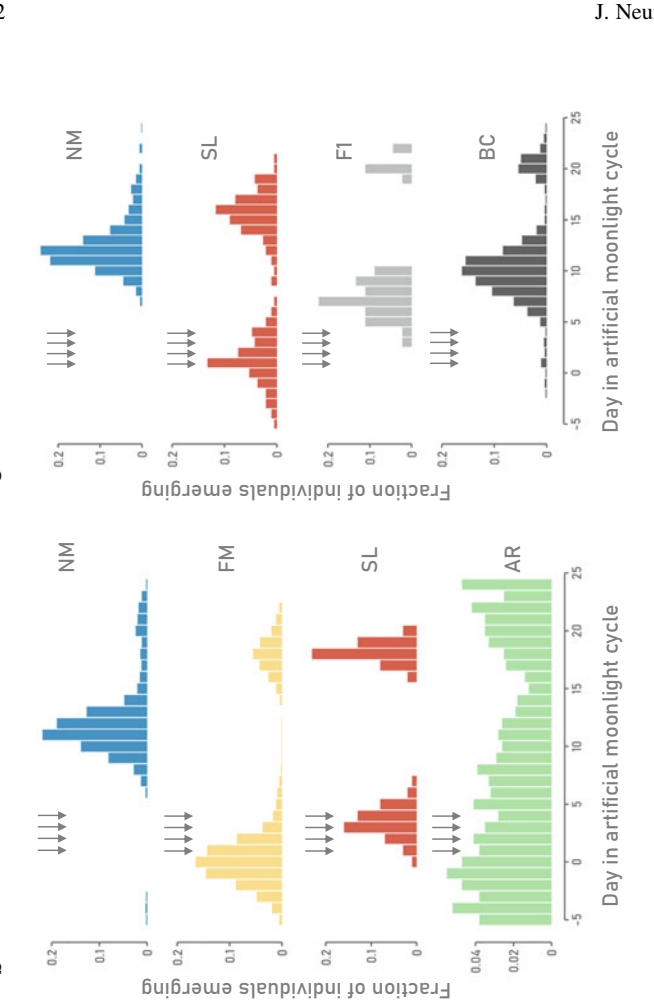

Fig. 10.4 Lunar rhythmicity, as well as phase and period are genetically determined in the marine midge *Clunio marinus* and differ between populations. *Clunio marinus* is therefore an attractive model to study the genetic architecture of lunar time-keeping using quantitative trait locus (QTL) mapping and genome-wide association screens (GWAS). (**a**) *Clunio marinus* inhabits the intertidal zone along the rocky Atlantic coast of Europe. Dots mark sampling locations from which lab strains were derived. Color coding denotes the emergence phenotype. (**b**) Under artificial moonlight (arrows, four nights of dim night light), populations emerge either around the new moon (NM) or the full moon (FM), both at the full and new moon (semilunar, SL) or arrhythmically (AR). **c** The phase of the lunar rhythm is genetically determined. When crossing a semilunar strain (Por) and a new moon strain (Jean), the F1 generation emerges in an intermediate phase and a semilunar period with unequal peaks. Based on Kaiser et al. (2011) licensed under CC BY 2.0

10.4 Conclusions

Despite their importance for numerous organisms, circalunar and circatidal clocks are still scarcely addressed in the field of chronobiology. Studying lunar and tidal rhythms provides us with the unique opportunity to understand time-keeping on multiple time scales as well as its evolution under multiple geophysical cycles. Tidal and lunar rhythms remain hard to study because they are displayed by marine non-model organisms which are often difficult to culture in the laboratory and for which molecular methods usually still need to be established. However, insects might be the key to addressing such a task. They have adapted multiple times to the marine environment and are a very diverse group providing us with the opportunity to study potentially different tidal and lunar clock mechanisms. Knowledge about lunar and tidal rhythms is as old as the field of chronobiology, but advances in understanding them at the molecular level lag far behind those of the circadian clock. Thus, the fascinating world of lunar and tidal rhythms still offers ample opportunities for discovery.

References

Abhilash L, Sharma VK (2016) On the relevance of using laboratory selection to study the adaptive value of circadian clocks. Physiol Entomol 41:293–306. https://doi.org/10.1111/phen.12158

Akiyama T (1997) Tidal adaptation of a circadian clock controlling a crustacean swimming behavior. Zool Sci 14:901–906. https://doi.org/10.2108/zsj.14.901

Andersen NM (1999) The evolution of marine insects: phylogenetic, ecological and geographical aspects of species diversity in marine water striders. Ecography 22:98–111. https://doi.org/10.1111/j.1600-0587.1999.tb00458.x

Bowden J, Church B (1973) The influence of moonlight on catches of insects in light-traps in Africa. Part II. The effect of moon phase on light-trap catches. Bull Entomol Res 63:129–142. https://doi.org/10.1017/S0007485300050938

Bowers DG, Roberts EM (2019) Tides: A very short introduction. Oxford University Press, Oxford

Bünning E, Müller D (1961) Wie messen Organismen lunare Zyklen? Z Naturforsch B 16:391–395. https://doi.org/10.1515/znb-1961-0609

Cheng L (2009) Marine insects. In: Resh VH, Cardé RT (eds) Encyclopedia of insects. Elsevier, pp 600–604. https://doi.org/10.1016/B978-0-12-374,144-8.00167-3

Chevrel R (1894) Sur un diptère marin du genre *Clunio* Haliday. Arch Zool Exp Gen 3:583–598

Corbet PS (1958) Lunar periodicity of aquatic insects in Lake Victoria. Nature 182:330–331. https://doi.org/10.1038/182330a0

Courchamp F, Clutton-Brock T, Grenfell B (1999) Inverse density dependence and the Allee effect. Ecol Evol 14:405–410. https://doi.org/10.1016/S0169-5347(99)01683-3

Danthanarayana W (1976) Diel and lunar flight periodicities in the light brown apple moth, *Epiphyas postvittana* (Walker) (Tortricidae) and their possible adaptive significance. Aust J Zool 24:65–73. https://doi.org/10.1071/ZO9760065

Danthanarayana W (1986) Lunar periodicity of insect flight and migration. Insect flight,. Springer, Berlin, Heidelberg, pp 88–119. https://doi.org/10.1007/978-3-642-71,155-8_7

Enright J (1972) A virtuoso isopod. J Comp Physiol 77:141–162. https://doi.org/10.1007/BF00693603

Enright J (1976) Plasticity in an isopod's clockworks: shaking shapes form and affects phase and frequency. J Comp Physiol 107:13–37. https://doi.org/10.1007/BF00663916

Evans WG (1976) Circadian and circatidal locomotory rhythms in the intertidal beetle *Thalassotrechus barbarae* (Horn): Carabidae. J Exp Mar Biol Ecol 22:79–90. https://doi.org/10.1016/0022-0981(76)90110-6

Falkenberg G, Fleissner G, Neumann D, Wellenreuther G, Alraun P, Fleissner G (2013) Moonlight receptor of the "1-h-midge" *Clunio marinus* studied by micro-XRF. J Phys Conf Ser 463: 012016. https://doi.org/10.1088/1742-6596/463/1/012016

Fleissner G, Schuchardt K, Neumann D, Bali G, Falkenberg G, Fleissner G (2008) A lunar clock changes shielding pigment transparency in larval ocelli of *Clunio marinus*. Chronobiol Int 25: 17–30. https://doi.org/10.1080/07420520801904008

Foster W, Moreton R (1981) Synchronization of activity rhythms with the tide in a saltmarsh collembolan *Anurida maritima*. Oecologia 50:265–270. https://doi.org/10.1007/BF00348049

Franke H-D (1985) On a clocklike mechanism timing lunar-rhythmic reproduction in *Typosyllis prolifera* (Polychaeta). J Comp Physiol A 156:553–561. https://doi.org/10.1007/BF00613979

Franke H-D (1986) Resetting a circalunar reproduction rhythm with artificial moonlight signals: Phase-response curve and 'moon-off' effect. J Comp Physiol A 159:569–576. https://doi.org/10.1007/BF00604176

Fuhrmann N, Prakash C, Kaiser TS (2023) Polygenic adaptation from standing genetic variation allows rapid ecotype formation. eLife:e82824. https://doi.org/10.7554/eLife.82824

Gibson R (1973) Tidal and circadian activity rhythms in juvenile plaice, *Pleuronectes platessa*. Mar Biol 22:379–386. https://doi.org/10.1007/BF00391398

Goto SG, Takekata H (2015) Circatidal rhythm and the veiled clockwork. Curr Opin Insect Sci 7: 92–97. https://doi.org/10.1016/j.cois.2014.12.004

Hartland-Rowe R (1955) Lunar rhythm in the emergence of an Ephemeropteran. Nature 176:657. https://doi.org/10.1038/176657a0

Hartland-Rowe R (1958) The biology of a tropical mayfly *Povilla adusta* Navas (Ephemeroptera, Polymitarcidae) with special reference to the lunar rhythm of emergence. Rev Zool Bot Afr 58: 185–202

Hauenschild C (1960) Lunar periodicity. In: Cold spring harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press, pp 491–497. https://doi.org/10.1101/SQB.1960.025.01.051

Helfrich-Förster C (2009) Does the morning and evening oscillator model fit better for flies or mice? J Biol Rhythms 24:259–270. https://doi.org/10.1177/0748730409339614

Hsiao S-M, Meier AH (1992) Freerunning circasemilunar spawning rhythm of *Fundulus grandis* and its temperature compensation. Fish Physiol Biochem 10:259–265. https://doi.org/10.1007/BF00004519

Huang D, Cheng L (2011) The flightless marine midge *Pontomyia* (Diptera: Chironomidae): ecology, distribution, and molecular phylogeny. Zool J Linn Soc 162:443–456. https://doi.org/10.1111/j.1096-3642.2010.00680.x

Ito K, Sugiyama H, Min CP (1993) Effects of lunar phase on light trap catches of the Malayan black rice bug, *Scotinophara coarctata* (Heteroptera: Pentatomidae). Bull Entomol Res 83:59–65. https://doi.org/10.1017/S000748530004178X

Joosse EN (1966) Some observations on the biology of *Anurida maritima* (Guérin), (Collembola). Z Morphol Oekol Tiere 57:320–328. https://doi.org/10.1007/BF00407599

Kaiser TS, Heckel DG (2012) Genetic architecture of local adaptation in lunar and diurnal emergence times of the marine midge *Clunio marinus* (Chironomidae, Diptera). PLoS One 7: e32092. https://doi.org/10.1371/journal.pone.0032092

Kaiser TS, Neumann D, Heckel DG (2011) Timing the tides: Genetic control of diurnal and lunar emergence times is correlated in the marine midge *Clunio marinus*. BMC Genet 12:49. https://doi.org/10.1186/1471-2156-12-49

Kaiser TS, Neumann J (2021) Circalunar clocks - Old experiments for a new era. BioEssays 43: 2100074. https://doi.org/10.1002/bies.202100074

Kaiser TS, Poehn B, Szkiba D, Preussner M, Sedlazeck FJ, Zrim A et al (2016) The genomic basis of circadian and circalunar timing adaptations in a midge. Nature 540:69–73. https://doi.org/10.1038/nature20151

Kanou M, Morita S, Matsuura T, Yamaguchi T (2007) Morphology and electrophysiology of water receptors on legs of the cricket *Gryllus bimaculatus*. Zool Sci 24:953–958. https://doi.org/10.2108/zsj.24.953

Krüger M, Neumann D (1983) Die Temperaturabhängigkeit semilunarer und diurnaler Schlüpfrhythmen bei der intertidalen Mücke *Clunio marinus* (Diptera, Chironomidae). Helgoländer Meeresuntersuchungen 36:427. https://doi.org/10.1007/BF01983459

Manica A, Mcmeechan FK, Foster WA (2000) Orientation in the intertidal salt-marsh collembolan *Anurida maritima*. Behav Ecol Sociobiol 47:371–375. https://doi.org/10.1007/s002650050679

Mcmeechan FK, Manica A, Foster WA (2000) Rhythms of activity and foraging in the intertidal insect *Anurida maritima*: coping with the tide. J Mar Biolog Assoc UK 80:189–190. https://doi.org/10.1017/S0025315499001770

Meyer-Rochow VB, Brown PJ (1998) Possible natural circaseptan rhythm in the beach beetle *Chaerodes trachyscelides*. White Acta Neurobiol Exp 58:287–290

Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C et al (2014) Phylogenomics resolves the timing and pattern of insect evolution. Science 346:763–767. https://doi.org/10.1126/science.1257570

Naylor E (1958) Tidal and diurnal rhythms of locomotory activity in *Carcinus maenas*. J Exp Biol 35:602–610. https://doi.org/10.1242/jeb.35.3.602

Naylor E (1976) Rhythmic behaviour and reproduction in marine animals. In: Newell RC (ed) Adaptation to environment: essays on the physiology of marine animals. Butterworth & Co Ltd., Belfast, pp 393–418. https://doi.org/10.1016/B978-0-408-70,778-7.50010-X

Naylor E (1996) Crab clockwork: the case for interactive circatidal and circadian oscillators controlling rhythmic locomotor activity of *Carcinus maenas*. Chronobiol Int 13:153–161. https://doi.org/10.3109/07420529609012649

Naylor E (2010) Chronobiology of marine organisms. Cambridge University Press. https://doi.org/10.1017/CBO9780511803567

Neumann D (1966) Die lunare und tägliche Schlüpfperiodik der Mücke *Clunio*. Z Vergl Physiol 53:1–61. https://doi.org/10.1007/BF00343045

Neumann D (1968) Die Steuerung einer semilunaren Schlüpfperiodik mit Hilfe eines künstlichen Gezeitenzyklus. Z vgl Physiol 60:63–78. https://doi.org/10.1007/BF00737095

Neumann D (1976) Entrainment of a semilunar rhythm. In: De Coursey PJ (ed) Biological rhythms in the marine environment. University of South Carolina Press, Columbia, South Carolina, pp 115–127

Neumann D (1978) Entrainment of a semilunar rhythm by simulated tidal cycles of mechanical disturbance. J Exp Mar Biol Ecol 35:73–85. https://doi.org/10.1016/0022-0981(78)90091-6

Neumann D (1988) Temperature compensation of circasemilunar timing in the intertidal insect *Clunio*. J Comp Physiol A 163:671–676. https://doi.org/10.1007/BF00603851

Neumann D (1989) Circadian components of semilunar and lunar timing mechanisms. J Biol Rhythms 4:173–182. https://doi.org/10.1177/074873048900400213

Neumann D (1995) Physiologische Uhren von Insekten. Zur Ökophysiologie lunarperiodisch kontrollierter Fortpflanzungszeiten. Sci Nat 7:310–320. https://doi.org/10.1007/BF01131527

Neumann D, Heimbach F (1979) Time cues for semilunar reproduction rhythms in European populations of *Clunio marinus*. I. The influence of tidal cycles of mechanical disturbance. In: Cyclic phenomena in marine plants and animals. Elsevier, Amsterdam, pp 423–433. https://doi.org/10.1016/B978-0-08-023217-1.50061-8

Neumann D, Heimbach F (1984) Time cues for semilunar reproduction rhythms in European populations of *Clunio marinus* II. The influence of tidal temperature cycles. Biol Bull 166:509–524. https://doi.org/10.2307/1541158

Neumann D, Heimbach F (1985) Circadian range of entrainment in the semilunar eclosion rhythm of the marine insect *Clunio marinus*. J Insect Physiol 31:549–557. https://doi.org/10.1016/0022-1910(85)90111-8

Neumann D, Spindler KD (1991) Circasemilunar control of imaginal disc development in *Clunio marinus*: Temporal switching point, temperature-compensated developmental time and ecdysteroid profile. J Insect Physiol 37:101–109. https://doi.org/10.1016/0022-1910(91)90095-H

Nowinszky L (2004) Nocturnal illumination and night flying insects. Appl Ecol Environ Res 2:17–52

Nowinszky L, Petranyi G, Puskas J (2010) The relationship between lunar phases and the emergence of the adult brood of insects. Appl Ecol Environ Res 8:51–62

Palmer JD (1995) Review of the dual-clock control of tidal rhythms and the hypothesis that the same clock governs both circatidal and circadian rhythms. Chronobiol Int 12:299–310. https://doi.org/10.3109/07420529509057279

Palmer JD (2000) The clocks controlling the tide-associated rhythms of intertidal animals. Bioessays 22:32–37. https://doi.org/10.1002/(SICI)1521-1878(200001)22:1<32::AID-BIES7>3.0.CO;2-U

Palmer JD, Williams BG (1986) Comparative studies of tidal rhythms. II. The dual clock control of the locomotor rhythms of two decapod crustaceans. Marine Freshwater Behav Phy 12:269–278. https://doi.org/10.1080/10236248609378653

Pflüger W (1973). Die Sanduhrsteuerung der gezeitensynchronen Schlüpfrhythmik der Mücke *Clunio marinus* im arktischen Mittsommer (Hour-Glass Control of the tidal rhythm of *Clunio marinus* (Chironomidae) in adaptation to Arctic conditions). Oecologia 113–150. https://doi.org/10.1007/BF00345128.

Pittendrigh CS (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol 55:17–54. https://doi.org/10.1146/annurev.ph.55.030193.000313

Rock A, Wilcockson D, Last K (2022) Towards an understanding of circatidal clocks. Front Physiol 319. https://doi.org/10.3389/fphys.2022.830107

Rounds HD (1981) Semi-lunar cyclicity of neurotransmitter-like substances in the CNS of *Periplaneta americana* (L.). Comp Biochem Physiol Part C: Toxicol Pharmacol 69:293–299. https://doi.org/10.1016/0306-4492(81)90141-6

Saigusa M (1980) Entrainment of a semilunar rhythm by a simulated moonlight cycle in the terrestrial crab, *Sesarma haematocheir*. Oecologia 46:38–44. https://doi.org/10.1007/BF00346963

Sakura K, Numata H (2017) Contact with water functions as a Zeitgeber for the circatidal rhythm in the mangrove cricket *Apteronemobius asahinai*. Biol Rhythm Res 48:887–895. https://doi.org/10.1080/09291016.2017.1319639

Sakura K, Numata H (2021) Locomotor activity rhythms in laboratory-reared adults of the mangrove cricket, *Apteronemobius asahinai*. Entomol Sci 24:247–255. https://doi.org/10.1111/ens.12470

Satoh A (2017) Constant light disrupts the circadian but not the circatidal rhythm in mangrove crickets. Biol Rhythm Res 48:459–463. https://doi.org/10.1080/09291016.2016.1275392

Satoh A, Hayaishi S (2007) Microhabitat and rhythmic behavior of tiger beetle *Callytron yuasai okinawense* larvae in a mangrove forest in Japan. Entomol Sci 10:231–235. https://doi.org/10.1111/j.1479-8298.2007.00218.x

Satoh A, Momoshita H, Hori M (2006) Circatidal rhythmic behaviour in the coastal tiger beetle *Callytron inspecularis* in Japan. Biol Rhythm Res 37:147–155. https://doi.org/10.1080/09291010500429939

Satoh A, Takasu M, Yano K, Terai Y (2021) De novo assembly and annotation of the mangrove cricket genome. BMC Res Notes 14:1–3. https://doi.org/10.1186/s13104-021-05798-z

Satoh A, Terai Y (2019) Circatidal gene expression in the mangrove cricket *Apteronemobius asahinai*. Sci Rep 9:1–7. https://doi.org/10.1038/s41598-019-40,197-2

Satoh A, Yoshioka E, Numata H (2008) Circatidal activity rhythm in the mangrove cricket *Apteronemobius asahinai*. Biol Lett 4:233–236. https://doi.org/10.1098/rsbl.2008.0036

Satoh A, Yoshioka E, Numata H (2009) Entrainment of the circatidal activity rhythm of the mangrove cricket, *Apteronemobius asahinai*, to periodic inundations. Anim Behav 78:189–194. https://doi.org/10.1016/j.anbehav.2009.04.018

Shiga S, Numata H, Yoshioka E (1999) Localization of the photoreceptor and pacemaker for the circadian activity rhythm in the band-legged ground cricket, *Dianemobius nigrofasciatus*. Zool Sci 16:193–201. https://doi.org/10.2108/zsj.16.193

Sokolove P, Loher W (1975) Role of eyes, optic lobes, and pars intercerebralis in locomotory and stridulatory circadian rhythms of *Teleogryllus commodus*. J Insect Physiol 21:785–799. https://doi.org/10.1016/0022-1910(75)90009-8

Soong K, Chang Y-H (2012) Counting circadian cycles to determine the period of a circasemilunar rhythm in a marine insect. Chronobiol Int 29:1329–1335. https://doi.org/10.3109/07420528.2012.728548

Soong K, Chen G-F, Cao J-R (1999) Life history studies of the flightless marine midges *Pontomyia* spp. (Diptera: Chironomidae). Zool Stud Taipei 38:466–473

Soong K, Lee Y-J, Chang I-H (2011) Short-lived intertidal midge *Pontomyia oceana* have semilunar eclosion rhythm entrained by night light. Mar Ecol Prog Ser 433:121–130. https://doi.org/10.3354/meps09181

Takekata H, Matsuura Y, Goto SG, Satoh A, Numata H (2012) RNAi of the circadian clock gene *period* disrupts the circadian rhythm but not the circatidal rhythm in the mangrove cricket. Biol Lett 8:488–491. https://doi.org/10.1098/rsbl.2012.0079

Takekata H, Numata H, Shiga S (2014a) The circatidal rhythm persists without the optic lobe in the mangrove cricket *Apteronemobius asahinai*. J Biol Rhythms 29:28–37. https://doi.org/10.1177/0748730413516309

Takekata H, Numata H, Shiga S (2018) Effects of pars intercerebralis removal on circatidal rhythm in the mangrove cricket, *Apteronemobius asahinai*. J Comp Physiol A 204:801–810. https://doi.org/10.1007/s00359-018-1281-1

Takekata H, Numata H, Shiga S, Goto SG (2014b) Silencing the circadian clock gene Clock using RNAi reveals dissociation of the circatidal clock from the circadian clock in the mangrove cricket. J Insect Physiol 68:16–22. https://doi.org/10.1016/j.jinsphys.2014.06.012

Tomioka K, Chiba Y (1992) Characterization of an optic lobe circadian pacemaker by in situ and in vitro recording of neural activity in the cricket, *Gryllus bimaculatus*. J Comp Physiol A 171:1–7. https://doi.org/10.1007/BF00195955

Williams C, Singh B, Ziady SE (1956) An investigation into the possible effects of moonlight on the activity of insects in the field. In: Proc R Soc Lond Ser A Gen Ent. Wiley Online Library, pp 135–144. https://doi.org/10.1111/j.1365-3032.1956.tb00197.x

Yoshii T, Rieger D, Helfrich-Förster C (2012) Two clocks in the brain: an update of the morning and evening oscillator model in *Drosophila*. Prog Brain Res 199:59–82. https://doi.org/10.1016/B978-0-444-59,427-3.00027-7

Yoshioka E (1989) Phase shift of semilunar spawning periodicity of the chiton *Acanthopleura japonica* (Lischke) by artificial regimes of light and tide. J Exp Mar Biol Ecol 129:133–140. https://doi.org/10.1016/0022-0981(89)90052-X

Youthed G, Moran V (1969) The lunar-day activity rhythm of Myrmeleontidae larvae. J Insect Physiol 15:1259–1271. https://doi.org/10.1016/0022-1910(69)90235-2

Zhang L, Hastings MH, Green EW, Tauber E, Sladek M, Webster SG et al (2013) Dissociation of circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. Curr Biol 23:1863–1873. https://doi.org/10.1016/j.cub.2013.08.038

Chapter 11
Circannual Rhythms

Yosuke Miyazaki

Abstract Circannual rhythms corresponding to annual fluctuations in the environment have been reported in various taxa. Although the physiological mechanism producing the circannual rhythms in insects is lesser known than the circadian clock, some remarkable properties have been revealed in the pupation rhythm of the varied carpet beetle, *Anthrenus verbasci.* The period length of circannual rhythm of *A. verbasci* is approximately 40 weeks and is barely affected by constant temperature and nutrition. Daylength and temperature act as zeitgebers for this circannual rhythm. Responsiveness to zeitgebers was similar to that of circadian rhythms. Based on these facts, it has been concluded that a biological rhythm of approximately 1 year is produced by the circannual clock in *A. verbasci*, and the physiological properties are extremely similar to those of the circadian clock. Moreover, since the mechanism for measuring the daylength for entrainment of the circannual rhythm in *A. verbasci* involves a circadian clock, similar to many other insects, it is thought to have a common feature with conventional photoperiodism. Although research into the circannual rhythm of insects is still limited, other insects with a life cycle of 1 year or longer may adapt to seasonal changes using similar circannual rhythms.

Keywords *Anthrenus verbasci* · Circannual clock · Phase response curve · Photoperiodism · Temperature change · Temperature compensation

11.1 Circannual Rhythms Corresponding to an Annual Cycle

Organisms have adopted various strategies to improve survival and reproduction efficiency under annual seasonal changes. Because most species on Earth display seasonal behaviors and reactions, under natural conditions, organisms exhibit

Y. Miyazaki (✉)
Faculty of Management Education, Ashiya University, Ashiya, Japan
e-mail: ymiyazaki@ashiya-u.ac.jp

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rhythms with an annual period (52 weeks). Such annual rhythms of organisms may be caused primarily by exogenous factors or by endogenous mechanisms. In the former case, they are the results of growth and behavior of organisms directly promoted or suppressed by environmental factors such as temperature, water, and food. In the latter case, they are induced by physiological mechanisms responding to seasonal information such as daylength and temperature (Goldman et al. 2004; Paul et al. 2008).

Most organisms have an internal circadian rhythm that corresponds to environmental changes with 1-day periodicity. Under constant conditions, this rhythm persists with a period of approximately 24 h (Johnson et al. 2004). Similarly, some annual rhythms are considered to result from endogenous rhythms with an approximately 1-year periodicity and are called "circannual rhythms" (Gwinner 1986; Goldman et al. 2004; Helm and Lincoln 2017). Because an organism can show an annual rhythm by directly reacting to changes in temperature and daylength without a circannual rhythm, carefully investigating whether the organism has a circannual rhythm is necessary. To confirm this, an experiment under constant photoperiod and temperature conditions without information on annual periodicity is necessary. If the rhythm persists under seasonally constant conditions for at least two cycles, with endogenous periods deviating from 12 months, it is regarded as strong evidence for a circannual rhythm (Gwinner 1986). For example, the golden-mantled ground squirrel, *Callospermophilus lateralis*, when maintained for 2 years under constant photoperiod and temperature, showed changes in body weight, food consumption, and hibernation with a periodicity shorter than 12 months (Pengelley and Fisher 1957, 1963). Apart from vertebrates, circannual rhythms have also been reported in invertebrates, higher plants, brown algae, and unicellular dinoflagellates. The endogenous periods of circannual rhythms range from approximately 6–16 months and are often shorter than 1 year. The inter- and intraindividual variability of the period is higher in circannual rhythms than in circadian rhythms, even within a single species (Gwinner 1986; Helm and Lincoln 2017).

Circannual rhythms require a much longer research period than circadian rhythms and thus are less studied. In addition, although it is accepted that organisms have an internal mechanism producing a circannual rhythm, little is known about its working and how many of its properties are shared among different species. However, there have been attempts to approach the true nature of the mechanism behind circannual rhythms (Helm and Lincoln 2017; Lincoln 2019).

Although most insect species have life cycles of less than a year, there are some that live for a year or more, and some of these show a circannual rhythm (Saunders 2010; Miyazaki et al. 2014). The properties of insect circannual rhythms have been particularly well elucidated in the varied carpet beetle, *Anthrenus verbasci*. Table 11.1 summarizes the key properties and representative studies of circannual rhythm in *A. verbasci*. In this chapter, the circannual rhythm of *A. verbasci* and the nature of its underlying mechanism are explained. In addition, the long-term biological rhythms of other insect species are also discussed.

Table 11.1 Key properties and representative studies in the circannual rhythm of *Anthrenus verbasci*

1. Self-sustainability under constant conditions	
1.1. Rhythmicity in constant darkness	Blake (1958, 1959)
1.2. Rhythmicity under constant 24-h photoperiods	Nisimura and Numata (2001, 2003), Miyazaki et al. (2009a), and Matsuno et al. (2013a)
1.3. Rhythmicity under constant photoperiods slightly different from 24 h	Nisimura and Numata (2002)
1.4. Rhythmicity in constant light	Miyazaki and Numata (2010) and Matsuno et al. (2013b)
2. Temperature compensation of the period	Blake (1958, 1959) and Nisimura and Numata (2001)
3. Entrainability to zeitgebers	
3.1. Entrainment to naturally changing daylength	Blake (1960), Nisimura and Numata (2003), and Miyazaki et al. (2006)
3.2. Response to a photoperiodic transfer	Nisimura and Numata (2001) and Matsuno et al. (2013a)
3.3. Phase response curves for photoperiod	Miyazaki et al. (2005, 2007)
3.4. Phase response curves for temperature	Miyazaki et al. (2016)
3.5. Response to the Nanda-Hamner protocol	Miyazaki et al. (2009b)

11.2 Pupation Rhythm of the Varied Carpet Beetle

11.2.1 Early Reports

The circannual rhythm of insects was first reported in a British population of *A. verbasci* (Blake 1958). This small beetle, approximately 3 mm in adult body length, is widely distributed in temperate zones worldwide and is famous as a household pest. Adults are observed on white flowers from spring to early summer and consume pollen and nectar. Larvae feed on woolen clothing, dried food, and animal specimens. The larval period is long; larvae that hatch in early summer grow slowly while feeding and overwinter. They pupate at the beginning of spring when they are fully grown, and after 2–4 weeks, they emerge as adults and reproduce. As the rate of larval development depends on the temperature (Griswold 1941; Kiritani 1958), the life cycle of *A. verbasci* takes 1 year in central Japan and 2 years in southern England, where the average annual temperature is lower than that in central Japan (Kuwana 1951; Blake 1958). However, even in central Japan, the life cycle occasionally takes 2 or more years under poor nutritional conditions (Kiritani 1958).

Although the hatching, pupation, and eclosion of insects occur once in a lifetime, periodicity can be observed in a population with variable development if the timing is determined by an endogenous periodic mechanism. For example, in some species, eclosion is timed by the circadian clock and, under constant conditions, exhibits a rhythm with an approximately 24-h periodicity (Saunders 2002). A similar rhythm was observed in the pupation of a population of *A. verbasci* on a circannual rather

than a circadian scale. Blake (1958, 1959) kept the larvae of *A. verbasci* in constant darkness (DD) at various constant temperatures and found two peaks of pupation at 17.5 and 20 °C. The interval between the first and second peaks was approximately 40 weeks at both temperatures. The same result was observed when the experiment commenced at different times of the year and using the next generation obtained by reproducing individuals from the second pupation peak. These results rule out the influence of annually fluctuating unknown environmental factors and the mixing of different strains. Blake (1958, 1959) suggested that *A. verbasci* has a physiological mechanism with a period of approximately 40 weeks to indicate the timing of pupation. This mechanism signals the arrival of spring even under seasonally constant conditions, and if the larvae develop sufficiently by that time, pupation will occur. However, if the larvae develop insufficiently, owing to relatively low temperatures or other factors, they will not pupate and will spend time as larvae until the mechanism signals spring again. Blake (1958) reported that not only the pupation phase but also the alternation of the active (molting) and resting (diapause) periods in the larvae was repeated under constant conditions. However, detailed analyses of the circannual rhythm of *A. verbasci* and the physiological mechanisms producing the rhythm had not been attempted 30 or more years.

11.2.2 Stability of Circannual Period

Nisimura and Numata (2001) found that the Osaka population of *A. verbasci* in Japan also showed a circannual rhythm with up to three pupation peaks, when the larvae were reared on dried bonito under 12-h light and 12-h darkness (LD 12:12) at 20 °C and 66% relative humidity (Fig. 11.1a) within 1 week of hatching. The results of the past 15 experiments on the Osaka population under these conditions show that the interval between the first and second pupation peaks was 38.9 ± 2.4 weeks (mean ± standard deviation) (Matsuno et al. 2013a). The period of this circannual rhythm was relatively stable among the geographic populations investigated in Japan (Matsuno et al. 2013a).

It is possible to suppress larval development and delay pupation in *A. verbasci* by a year or more by feeding them low-nutrition diets (Kiritani 1958). When pigeon feathers, which are less nutritious than dried bonito, were used as feed, the circannual period for the species was nearly unchanged. However, the number of pupae in the first peak of the rhythm proportionally decreased and that in the second and third peaks increased because of a considerable effect on the growth rate of larvae (Miyazaki et al. 2009a). Therefore, the endogenous period of the circannual rhythm of *A. verbasci* is largely independent of the degree of development and the quality of food.

In biological rhythms, the period is stable regardless of temperature, which is known as temperature compensation (Saunders 2002; Johnson et al. 2004). In a few small hibernating mammals, such as *C. lateralis*, the circannual period hardly changes at different temperatures (Pengelley and Fisher 1963; Gwinner 1986;

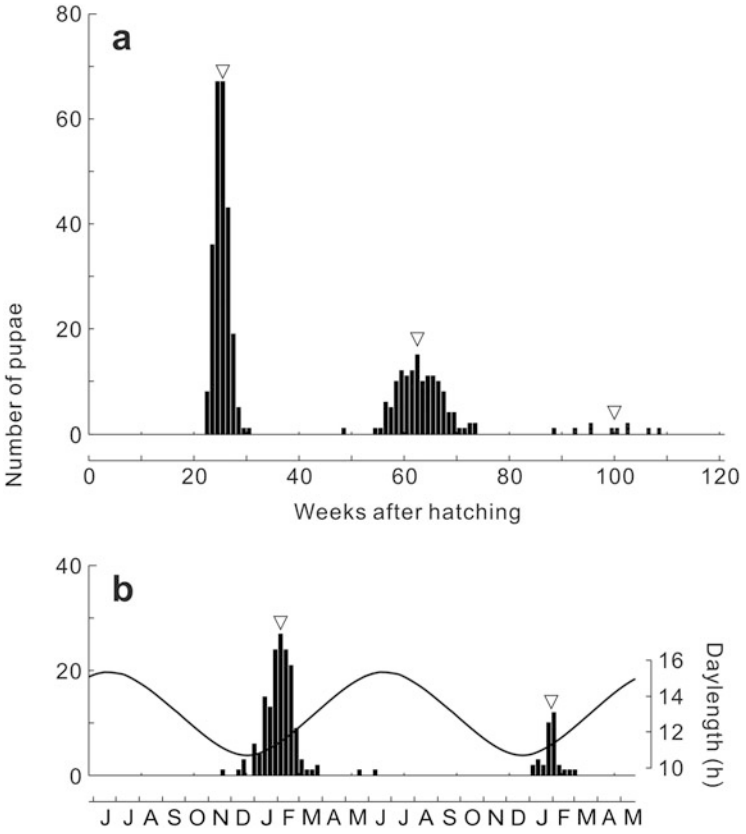

Fig. 11.1 Pupation under light/dark (LD) 12:12 (**a**) and under naturally changing daylength (**b**) at 20 °C in *Anthrenus verbasci*. The triangle indicates the median of each pupation group. The solid line indicates the natural daylength, including 1 h of twilight in Osaka, Japan (35°N). (**a**) Adapted from Nisimura and Numata (2001), with permission from Springer Nature. (**b**) Adapted from Miyazaki et al. (2006), with permission from The Zoological Society of Japan

Andjus et al. 2000). However, poikilotherms, including insects, are more suitable than homeotherms to study temperature compensation. Nisimura and Numata (2001) kept the larvae of *A. verbasci* under LD 12:12 at various constant temperatures between 17.5 and 27.5 °C and found that neither the pupation times in the first and second peaks nor the periods of circannual rhythm were largely affected by temperature (Fig. 11.2). This result is partly consistent with that reported by Blake (1958, 1959) and suggests temperature compensation in the period of the pupation rhythm of *A. verbasci*. Larvae of *A. verbasci*, like those of other insects, grow faster at higher temperatures (Griswold 1941; Blake 1958; Kiritani 1958). However, the timing of pupation does not result in a distinct temperature dependence because it is determined by a temperature-compensated circannual rhythm.

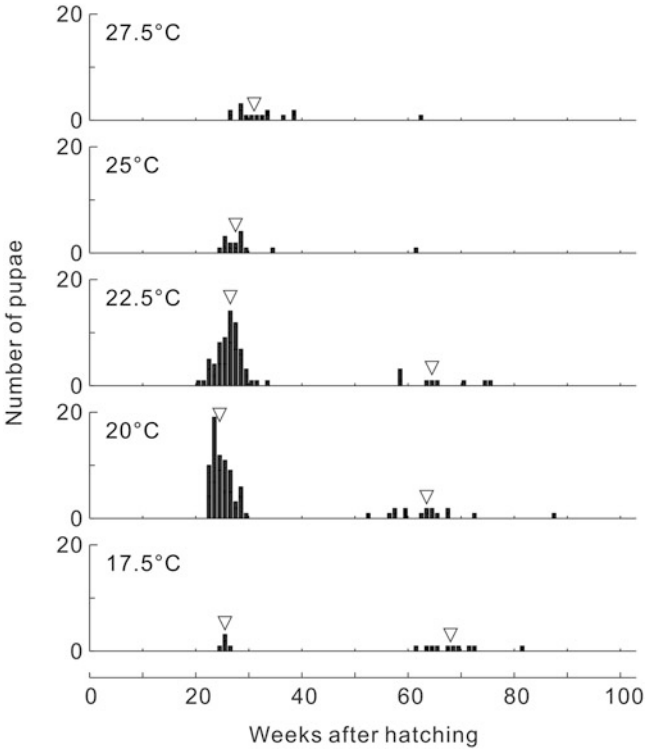

Fig. 11.2 Pupation in *Anthrenus verbasci* under LD 12:12 at various constant temperatures. The triangle indicates the median of each pupation group. Adapted from Nisimura and Numata (2001), with permission from Springer Nature

In contrast to the effects of nutrition and temperature, the lengths of the photophase and scotophase of a day markedly influence the period length and degree of persistence of this rhythm (Nisimura and Numata 2003). Under LD 13:11, the circannual period was slightly longer than that under LD 12:12; under LD 16:8 and 15:9, the pupation rhythm became unclear. Under LD 14:10, there was no periodicity, and pupation occurred for more than a year. The effects of photoperiods on the period length and clarity of the periodicity of circannual rhythms have also been reported in other species, including vertebrates and kelps (Gwinner 1986; Schaffelke and Lüning 1994).

11.2.3 Entrainment to a Natural Annual Cycle

Circadian rhythms with a period of approximately 24 h must be entrained to exactly 24 h by a zeitgeber, such as natural light-dark and temperature cycles (Saunders 2002; Johnson et al. 2004). Similarly, circannual rhythms with a period of

approximately 1 year must be entrained to exactly 1 year by the cycle of the seasonal signal as a zeitgeber in the natural environment. While the appropriate zeitgeber to be used for circannual rhythms of organisms living in the tropics is still under investigation (Goymann and Helm 2014), for organisms in temperate zones, the most common zeitgeber for entrainment of circannual rhythms to the natural year is the change in photoperiod (Gwinner 1986; Goldman et al. 2004; Paul et al. 2008; Helm and Lincoln 2017). The photoperiod shows clear annual changes, except in the equatorial zone, and is highly reliable as seasonal information because there are no annual differences.

The circannual pupation rhythm of *A. verbasci* can also be entrained to 1 year by natural changes in the photoperiod. Blake (1960) kept the larvae of *A. verbasci* at a constant temperature of 20 °C under naturally changing daylength in southern England and found that pupation occurred in January and February in both the first and second years. Similar experiments were performed in the Osaka population, with similar results (Fig. 11.1b; Nisimura and Numata 2003; Miyazaki et al. 2006). Thus, a pupation rhythm, which under seasonally constant conditions had an approximately 40-week period, showed an accurate 1-year (52-week) periodicity in synchronization with natural change in daylength.

Although full-grown larvae of *A. verbasci* were ready to pupate in February (Kuwana 1951; Kiritani 1958), the outdoor temperature at that time was too low to allow pupation. Therefore, larval development stopped before pupation. In the study by Nisimura and Numata (2003), larvae pupated synchronously in the spring, especially in April in Osaka, when the air temperature was sufficient for larval development.

11.2.4 Phase Resetting by Photoperiodic Change

Entrainment is achieved by a zeitgeber, which induces a phase shift (phase advance or delay) of the biological rhythm. In the study of circadian rhythms, it is assumed that light-on and light-off act as dawn and dusk signals, respectively. To investigate entrainment patterns in circadian rhythms to the light-dark cycle, researchers have observed how the rhythm changed after changing the timing of light-on or light-off (Saunders 2002; Johnson et al. 2004). Applying this to the study of circannual rhythm, it is assumed that a transfer from short-day to long-day conditions acts as a spring signal and a transfer from long-day to short-day conditions acts as an autumn signal. The pattern of entrainment of the circannual rhythm can be investigated by changing the timing of the photoperiodic transfers. Although the natural daylength changes continuously, even a one-step photoperiodic transfer in the laboratory from long-day to short-day conditions or vice versa can reset the phase of the circannual rhythm (Gwinner 1986).

Nisimura and Numata (2001) transferred *A. verbasci* larvae reared under LD 16:8 to LD 12:12 at different times. In a photoperiodic transfer at any time of year, the median time of pupation was approximately half a year (21–26 weeks) after the

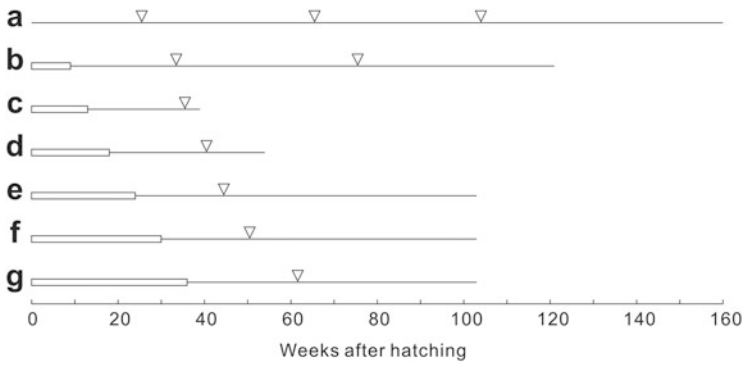

Fig. 11.3 Effects of a transfer on the pupation time from LD 16:8 (empty bars) to LD 12:12 (solid lines) at 0 weeks (**a**), 9 weeks (**b**), 13 weeks (**c**), 18 weeks (**d**), 24 weeks (**e**), 30 weeks (**f**), or 36 weeks (**g**) after hatching at 20 °C in *Anthrenus verbasci*. The triangle indicates the median of each pupation group. Based on Nisimura and Numata (2001)

transfer (Fig. 11.3). When larvae were transferred 9 weeks after hatching, the second peak was observed 42 weeks after the first peak (Fig. 11.3b). This result implies that even one photoperiodic transfer is effective in resetting the circannual rhythm, affecting both the first and second cycles. When the larvae recognize the change from long to short days, the rhythm is likely reset to the circannual phase of late summer or autumn, and subsequently, half a year later, around the phase of late winter, the larvae are ready to pupate (Nisimura and Numata 2001, 2003).

Matsuno et al. (2013a) transferred larvae of *A. verbasci* of different geographic populations in Japan to LD 12:12 from LD 13:11, LD 13.5:10.5, LD 14:10, LD 15:9, or LD 16:8, 12 weeks after hatching. The critical daylength for resetting by photoperiodic transfer was 13.2 h in the Osaka population, and there was a small correlation between critical daylength and habitat latitude.

11.2.5 Phase Response Curve and Phase Singularity

For circadian rhythms under DD, a single short light pulse can act as both a light-on (dawn) and light-off (dusk) signal and reset the phase. The manner in which the pulse resets its phase depends on the phase at which the pulse is given. If the light pulse is given on a subjective day, there is almost no phase shift. However, a light pulse given in the first half of the subjective night acts as a dusk signal and induces a phase delay, while a light pulse given in the latter half of the subjective night acts as a dawn signal and induces a phase advance. A phase response curve (PRC) can be constructed by plotting the magnitude of the phase shift against the phase of the pulse which is here shown in terms of angle degrees (0°–360°, the subjective day is 0°–180°, and the subjective night is 180°–360°) (Fig. 11.4a, b). The PRC not only suggests the manner in which the rhythm is entrained to the environmental cycle but

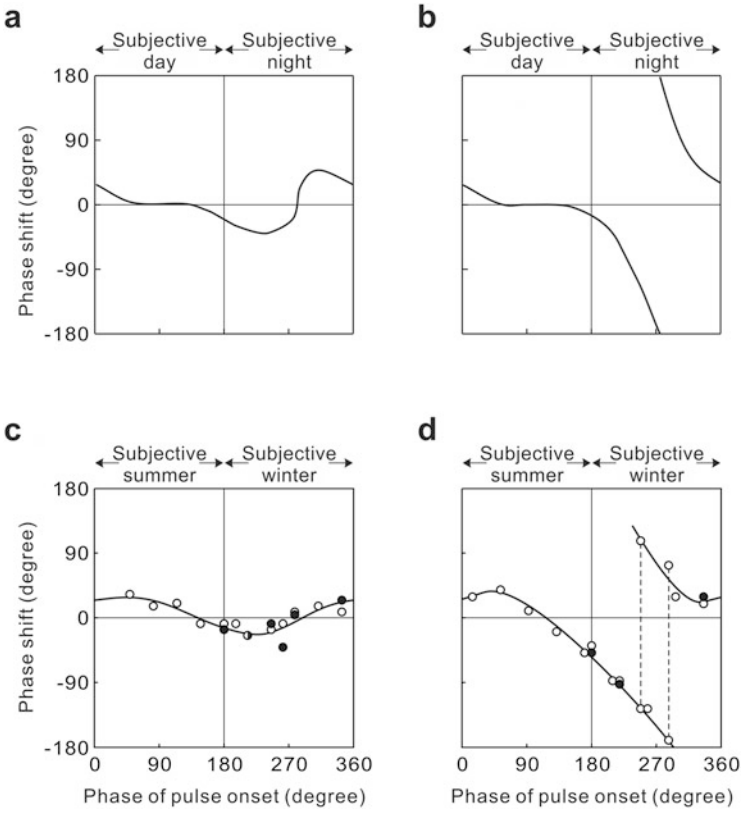

Fig. 11.4 Comparison of phase response curves for circadian and circannual rhythms. (**a**, **b**) Phase response curves in circadian rhythms, Type 1 (**a**) and Type 0 (**b**). (**c**, **d**) Phase response curves in the circannual pupation rhythm of *Anthrenus verbasci*, a curve to 2-week long-day pulses (**c**) and a curve to 4-week long-day pulses (**d**). Larvae were kept under LD 12:12 at 20 °C within 1 week of hatching and exposed to LD 16:8 for 2 (**c**) or 4 (**d**) weeks at various phases in the circannual rhythm. The circannual period under continuous LD 12:12 (37 weeks) is shown in terms of angle degrees (0°–360°), and the initial phase under LD 12:12, i.e., the beginning of this experiment, is represented as 180°. The median pupation time in the control is 0° in phase shift. Phase advance and delay are shown with the positive and negative values in phase shift, respectively. Open and closed circles represent the phase shifts in the first and second pupation group after pulse perturbation, respectively. Broken lines in (**d**) show the split into advanced and delayed groups. (**c**) Adapted from Miyazaki et al. (2007), with permission from Springer Nature. (**d**) Adapted from Miyazaki et al. (2005), with permission from Springer Nature

also exhibits diverse features of endogenous oscillation (Saunders 2002; Johnson et al. 2004; Numata et al. 2015). The magnitude of the phase shift changes depending on the focal species, rhythm examined, and strength of the applied stimulus; therefore, the amplitude of the PRC also changes. PRCs with a relatively small amplitude are classified as Type 1 (Fig. 11.4a), and those with a relatively large amplitude as Type 0 (Fig. 11.4b). Furthermore, in Type 0, the curve is discontinuous

at the phase where the phase shift is most remarkable (Saunders 2002; Johnson et al. 2004; Numata et al. 2015).

As the change in photoperiod acts as a zeitgeber in the circannual rhythm of *A. verbasci*, a circannual PRC can be constructed by superimposing long-day conditions for several weeks on constant short-day conditions. Miyazaki et al. (2005) examined the effects of 4-week exposure to LD 16:8 (4-week long-day pulse) by applying the pulse at various time points to larvae kept under LD 12:12. Consequently, the long-day pulse delayed or advanced the pupation, depending on the circannual phase in which the long-day pulse was applied. Based on the results, a PRC for the circannual rhythm of *A. verbasci* was constructed (Fig. 11.4d), where the period of the rhythm under continuous LD 12:12 (37 weeks) is shown in terms of angle degrees (0°–360°, the subjective summer is 0°–180°, and the subjective winter is 180°–360°). This PRC is similar in shape to that obtained for circadian rhythms. The phase shift was small when a long-day pulse is given in the subjective summer and large in the subjective winter. In the first half of the subjective winter (180°–270°), the long-day pulse was recognized as a signal of decreasing daylength in autumn, delaying the phase of the circannual rhythm. In the latter half of the subjective winter (270°–360°), the long-day pulse was recognized as a signal of increasing daylength in spring, advancing the phase of the circannual rhythm. This PRC is classified as Type 0, in which the curve has a point of discontinuous transition from the phase delay to advance near the center of the subjective winter, and a large phase shift is obtained before and after that point (Miyazaki et al. 2005). Moreover, Miyazaki et al. (2007) found that Type 1 PRC with a relatively small amplitude could be obtained by providing a 2-week long-day pulse in this circannual rhythm (Fig. 11.4c).

In circadian rhythms, in the phase near the discontinuity of Type 0 PRC, a light pulse of a certain intensity or duration may cause a loss of periodicity, known as the phase singularity of the circadian rhythm (Saunders 2002; Johnson et al. 2004). According to theoretical explanations for the existence of Type 1 and Type 0 PRCs and phase singularity, the circadian rhythm oscillates with two or more state variables, similar to the oscillation of a pendulum with two variables of position and momentum. These variables can be moved by a stimulus, and the phase shift of a circadian rhythm is subsequently established. A pulse causing the loss of periodicity moves state variables into a phase singularity, and the rhythm is driven to a phaseless state, similar to a swinging pendulum coming to a complete stop (Lakin-Thomas 1995; Johnson et al. 2004). In the circannual rhythm of *A. verbasci*, the periodicity disappeared when a 4-week long-day pulse was given in the phase near the discontinuity of Type 0 PRC (Miyazaki et al. 2007). The two types of PRCs and the existence of phase singularity in the circannual rhythm show that this rhythm also oscillates with circannual variations in two or more state variables. Thus, there are many similarities between circadian and circannual rhythms with regard to the mode of entrainment to the environmental cycle and characteristics of the periodic mechanisms generating the rhythm (Miyazaki et al. 2007, 2012; Numata et al. 2015).

11.2.6 Effects of Low Temperature as a Zeitgeber

In addition to photoperiod, annual changes in temperature, daytime light intensity, rainfall, food and water availability, and social factors can alter seasonal development and behavior in various species and have been suggested as potential zeitgebers for circannual rhythms (Gwinner 1986; Paul et al. 2008; Goymann and Helm 2014). However, the roles of these seasonal cues in circannual rhythms have not been sufficiently examined. Researchers must note that such cues often induce so-called masking effects, which directly drive or suppress the seasonal development and behavior of organisms without entrainment of the circannual rhythm (Paul et al. 2008).

As shown in Sect. 11.2.3, in Osaka, larvae of *A. verbasci* exposed to a natural photoperiod at a constant temperature of 20 °C pupate in January and February, whereas at outdoor temperatures, pupation occurs concentratedly in April (Nisimura and Numata 2003). Low temperatures during winter and early spring likely suppress pupation until April. However, it is possible that the change in temperature also functions as a zeitgeber in this circannual rhythm. In fact, in circannual rhythms of hibernating mammals, temperature has been suggested to act as a zeitgeber (Pengelley and Fisher 1963; Gwinner 1986; Andjus et al. 2000). Temperature change may have more meaningful effects on the entrainment of circannual rhythm in poikilotherms, including *A. verbasci* (Blake 1960; Brock 1979). However, low temperatures during winter would cause the direct suppression of development and the induction or termination of winter diapause, even in insects without a circannual rhythm (Danks 1987). Therefore, exposure to low temperatures can impact the physiological mechanisms associated with stimulation and suppression of pupation without affecting the phase of the circannual rhythm. Because of the masking effects of temperature on rhythm, the pupation of *A. verbasci* observed immediately after cold treatment may not reflect the phase shift of the circannual rhythm.

Miyazaki et al. (2016) examined the effects of low-temperature pulses on the circannual rhythm of *A. verbasci* by exposing larvae reared under LD 12:12 at 20 °C to low temperatures for 8 or 12 weeks at different phases. These pulses resulted in not only the direct suppression of development but also the induction of pupation in sufficiently grown larvae within 10 weeks of returning to 20 °C. This result was probably attributed to the masking effect of temperature on the circannual rhythm. However, further long-term observations demonstrated the existence of a phase-dependent phase shift in the circannual pupation rhythm as a result of low-temperature pulses. A PRC with 8 weeks of low-temperature pulses (10 °C) has large phase shifts and is categorized as Type 0 (Fig. 11.5). Thus, a 10 °C pulse could act as a winter signal and strongly reset the circannual rhythm of *A. verbasci*. Such phase shifts may be particularly important for adjusting the phase of the circannual cycle following exposure to the pulse.

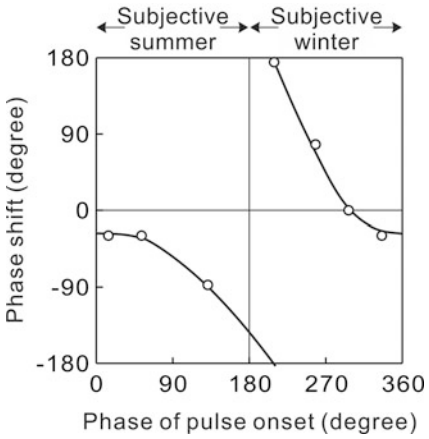

Fig. 11.5 A phase response curve to low-temperature pulses in the circannual pupation rhythm of *Anthrenus verbasci*. Larvae were kept under LD 12:12 at 20 °C within 1 week of hatching and exposed to 10 °C for 8 weeks at various phases in the circannual rhythm more than 8 weeks after hatching. The circannual period under a constant temperature of 20 °C (37 weeks) is shown in terms of angle degrees (0°–360°), and the initial phase under LD 12:12 is represented as 180°. The median pupation time in the control is 0° in phase shift. Phase advance and delay are shown with the positive and negative values in phase shift, respectively. Open circles represent the phase shifts in the pupation group more than 10 weeks after pulse exposure. Adapted from Miyazaki et al. (2016), with permission from John Wiley and Sons

11.2.7 Mechanisms Producing Circannual Rhythms

The circadian clock producing a circadian rhythm has been clarified at the molecular level (Patke et al. 2020); however, the internal mechanism behind the circannual rhythm has not been clarified in any organism. The simplest assumption is that a circannual rhythm is produced by a circannual clock, a biological clock with a period of approximately 1 year with characteristics similar to that of the circadian clock. The most troublesome aspect of this assumption is the premise that organisms have evolved a physiological process that requires very long elapsed times (Gwinner 1986). However, there are several ideas that explain the generation of circannual rhythms without assuming the existence of a circannual clock.

It seems possible to know a period of 1 year if there is a mechanism to count the experienced light-dark cycles every day, similar to turning pages of a daily calendar. However, the circannual rhythm can be observed in some species, including *A. verbasci*, under DD and constant light (LL) (Blake 1958; Gwinner 1986; Miyazaki and Numata 2010; Helm and Lincoln 2017). This indicates that daily light-dark cycles are not necessarily required to generate a circannual rhythm.

Gwinner (1973) noted that the oscillation of a circadian clock can continue in DD and LL and proposed a frequency demultiplication hypothesis (FDH) for producing a circannual rhythm without a circannual clock. According to FDH, circannual rhythms are derived from circadian rhythms through a process of frequency

demultiplication to transform the periodicity of approximately 1 day to a periodicity of approximately 365 days. This transformation is analogous to the generation of low-frequency rhythms from high-frequency rhythms in the electric clock, producing a 24-h rhythm by dividing the 50 or 60 Hz frequency of the commercial electrical current. FDH is a plausible alternative, as the circadian clock is involved in the physiology of a wide range of organisms. This hypothesis requires that the period of the circannual rhythm is proportional to the period of the circadian rhythm entrained to a light-dark cycle. Therefore, rigorous FDH tests can be performed by exposing individuals to light-dark cycles of different periods. Nisimura and Numata (2002) reared larvae of *A. verbasci* under a light-dark cycle with periods of 20, 24, and 26 h, to which circadian rhythms can usually be entrained. However, there was no correlation between the period of the light-dark cycle and that of the circannual rhythm. Similar experiments have been performed on the circannual rhythms of several vertebrates, but there is no evidence supporting the FDH (Gwinner 1986; Budki et al. 2014).

The relationship between circadian and circannual oscillations has also been examined in another study, in which the endogenous periods of circadian and circannual rhythms were compared for each individual under constant illumination (Gwinner 1973; Kenagy 1981). In *A. verbasci*, Matsuno et al. (2013b) investigated whether there was a correlation between the pupation time determined by the circannual rhythm and the period of the circadian rhythm of adult locomotor activity. However, no correlation between these two rhythms was observed in this experiment. These results suggest the independence of the mechanism producing the circannual rhythm of *A. verbasci* from the circadian clock.

As described in Sect. 11.2.5, the existence of Type 1 and Type 0 PRCs and phase singularity in the circannual rhythm of *A. verbasci* suggests that the mechanism generating this rhythm shares the theoretical oscillation background with the circadian clock and oscillates with circannual variations in two or more state variables. Therefore, the mechanism generating the circannual rhythm of *A. verbasci* is considered to be a circannual clock with some parallels to the circadian clock, with a period of approximately 1 year (Miyazaki et al. 2007, 2012; Numata et al. 2015). In current circannual rhythm research, including studies on species other than *A. verbasci*, there is a strong belief that the circannual rhythm is generated by a circannual clock. However, the state variables and specific mechanisms involved in the rhythm, the organ or tissue in which the circannual clock is located, and whether there are multiple clocks in an organism are still unclear, although some models have been proposed (Stevenson and Lincoln 2017; Lincoln 2019). Moreover, little is known at this time how such long-period biological clocks evolved and whether they were of heterogeneous origin in different groups of organisms.

11.2.8 Circadian Clock in Photoperiodic Time Measurement for Circannual Rhythms

Many insects adapt to seasons through photoperiodism. Because the circannual clock is entrained by changes in the photoperiod, it is highly likely that the physiological mechanism common to conventional photoperiodism is adopted before inputting photoperiodic information to the circannual clock. The conventional photoperiodism of insects without a circannual rhythm is carried out through a photoreceptor to receive light, photoperiodic time measurement system to measure the day (or night) length, photoperiodic counter to count the experienced photoperiods, and an endocrine system (output system) (Saunders 2002, 2010). Many studies support the idea that the circadian clock is involved in the photoperiodic time measurement system. The Nanda-Hamner protocol has frequently been used to clarify the involvement of the circadian clock in the photoperiodic time measurement system. In the Nanda-Hamner protocol, organisms are subjected to light-dark cycles with a fixed short photophase followed by a variable scotophase to give cycle lengths (T) up to 72 h or more. If short-day responses are induced when T is a multiple of 24 h, but not when T is not a multiple of 24 h, a circadian clock is considered to be involved in photoperiodic time measurements (Saunders 2002, 2010; Goldman et al. 2004).

The circannual rhythm of *A. verbasci* may involve steps of photoreception, measurement of the photoperiod, and counting of the experienced photoperiods, followed by the input of photoperiodic information to the circannual clock, reset of the clock, and output by the endocrine system (Fig. 11.6). To examine whether the circadian clock is involved in the photoperiodic time measurement system for the circannual rhythm of *A. verbasci*, Miyazaki et al. (2009b) exposed larvae to the Nanda-Hamner protocol (LD 12:12, LD 12:24, LD 12:36, LD 12:48, and LD 12:60)

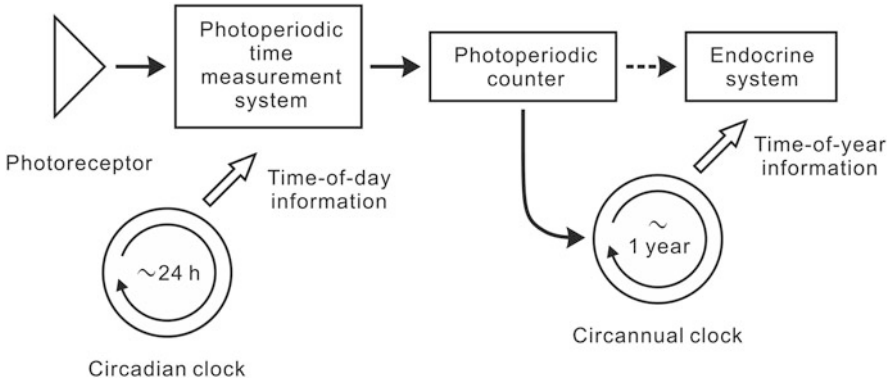

Fig. 11.6 A schematic of the physiological components related to the photoperiodic entrainment of circannual rhythm and the pathways between them. A dashed arrow represents the pathway assumed by conventional photoperiodism without going through the circannual clock

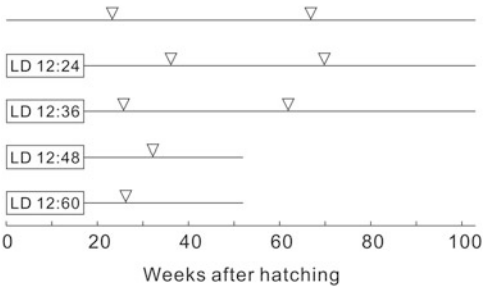

Fig. 11.7 Effects of exposure to the Nanda-Hamner protocol for 120 days on pupation in *A. verbasci* at 20 °C. Larvae were transferred to LD 12:12 (solid lines) after exposure to various photoperiods in the Nanda-Hamner protocol (boxes). The triangle indicates the median of each pupation group. Based on Miyazaki et al. (2009b)

for the first 120 days and then recorded pupation under LD 12:12. The result was similar to that under LD 12:12 when the length of the light-dark cycle was a multiple of 24 h, such as 48 h (LD 12:36) or 72 h (LD 12:60), but differed when it was a multiple of 24 h + 12 h, such as 36 h (LD 12:24) or 60 h (LD 12:48) (Fig. 11.7). Because of these results, the circadian clock is considered to be involved in photoperiodism to reset the circannual clock in *A. verbasci* (Miyazaki et al. 2009b, 2012).

11.3 Pupation Rhythms of Other Carpet Beetles

There are a few reports on circannual rhythms in insects other than *A. verbasci*. The Guernsey carpet beetle, *Anthrenus sarnicus*, has a life cycle and circannual pupation rhythm similar to those of *A. verbasci*. The interval between pupation peaks was approximately 40 weeks at 15 and 25 °C in DD (Coombs and Woodroffe 1983; Armes 1990). The timing of pupation was modulated by naturally changing daylengths (Armes 1990). These results suggest the existence of temperature compensation in the circannual period and the role of photoperiodic change as a zeitgeber in this rhythm.

Other temperate carpet beetles that have a life cycle similar to that of *A. verbasci* and *A. sarnicus* may also have a circannual pupation rhythm. For example, Griswold (1941) recorded two pupation times with an interval of approximately 32 weeks under almost constant conditions in the black carpet beetle, *Attagenus unicolor*. Baker (1983) showed no significant difference in larval duration under DD at constant temperatures ranging from 20 to 30 °C, which may represent the presence of a temperature-compensated circannual pupation rhythm, although a second peak was not observed. Monthly records under various constant conditions ranging from 15 to 30 °C for the Japanese subspecies *A. unicolor japonicus* also displayed roughly the same timing of pupation at 20, 25, and 30 °C. Pupation at 15 °C was observed approximately 8 months later, which may be due to the retardation of larval

development by the lower temperature and timing of pupation by the temperature-compensated circannual rhythm (Dobashi et al. 1980).

11.4 Pupation Rhythm of the Carpenter Moth

A temperature-compensated circannual pupation rhythm may exist in the carpenter moth *Cossus insularis* (Nakanishi et al. 2017). This species is suggested to have a generation time of 2 years or more in Japan. Larvae hatching in late summer overwintered at least once and pupated the following summer. Nakanishi et al. (2017) reared larvae under LD 15:9 conditions at different temperatures. At 25 °C, two clear pupation peaks with an interval of approximately 28 weeks were observed. Although only a single pupation peak occurred at 30 °C, it was almost synchronous with the first peak at 25 °C. At 20 °C, which is less suitable for pupation, three individuals pupated approximately 28 weeks after the second peak at 25 °C. Similar to *A. verbasci*, the rate of larval development likely differs considerably among individuals in *C. insularis*. The circannual clock may play an important role in enabling larvae with variable development to pupate during the appropriate season each year (Miyazaki et al. 2014).

11.5 Oviposition Rhythms of Queen Ants

The circannual rhythm, which is also observed at the individual level, has been reported for the oviposition of queen ants. Some queen ants have a lifespan of several years or more. Kipyatkov and Shenderova (1990) maintained field colonies of the red wood ants *Formica aquilonia* and *F. polyctena* under laboratory conditions. They reported that the egg laying time and the time when no eggs were laid alternated periodically under constant conditions. The period of this oviposition rhythm showed considerable variability (90–525 days for *F. aquilonia* and 60–345 days for *F. polyctena*) over several years; however, the average periods (212 days for *F. aquilonia* and 179 days *F. polyctena*) were largely unaffected by constant temperatures between 17 and 30 °C. In addition, there was almost no difference in the average periods of the oviposition rhythm between the long-day and short-day conditions (Kipyatkov and Shenderova 1990).

Other ant species also show an annual rhythm in the queen's oviposition under natural conditions, and some of them have an endogenous long-term rhythm even under constant conditions, similar to the aforementioned species (Kipyatkov 1993, 1995). However, it is difficult to determine whether a rhythm with a period shorter than half a year (182.5 days), such as the oviposition rhythm of *F. polyctena*, can be considered as a circannual rhythm (Gwinner 1986). In ant seasonality, changes in temperature often act as a more important environmental factor than changes in photoperiod. The long-term oviposition rhythms of ants may exhibit an accurate

1-year periodicity under natural conditions, mainly by synchronizing with changes in temperature (Kipyatkov 1993, 1995).

Kuroki et al. (2018) recorded the number of eggs in the queens of the garden ant *Lasius japonicus* kept in an isolated condition just after nuptial flight under LD 12:12 or constant light. Even in conditions where social factors were eliminated as much as possible, a temperature-compensated endogenous oviposition rhythm was observed, with relatively slight individual differences. However, the average period was 130–150 days, which is much shorter than a year.

11.6 Conclusions

Studies on *A. verbasci* have revealed that similar to circadian clocks, the circannual clock is self-sustainable under constant conditions, can be temperature-compensated in the period, and can be entrained to environmental changes (Table 11.1). Since PRCs and phase singularities similar to those reported in circadian rhythms can be obtained, it is considered that the theoretical background in the mechanism for producing circannual rhythms is similar to that of circadian clocks (Miyazaki et al. 2007, 2012). Such a clock may also work in the seasonal physiological mechanisms used by other carpet beetles, moths, and ants that live for over a year, although detailed research has not yet been conducted in these species.

It is likely that many other insects that live for 1 or more years also use circannual rhythms for seasonal adaptation (Saunders 2010; Miyazaki et al. 2014); however, the numbers are unclear. Despite the fact that circannual rhythms of *A. verbasci* were discovered over 60 years ago, there are several possible reasons for the paucity of research on this phenomenon in insects. To investigate circannual rhythms, it is necessary to maintain long-lived species for 2 or more years under constant conditions to record developmental or behavioral data, but such long-term experiments are not easy. Even if a species has a circannual rhythm of pupation, like *A. verbasci* and *C. insularis*, most individuals may pupate in the first cycle, and the second cycle may not be detectable under temperature and nutritional conditions suitable for larval development (Blake 1958; Nakanishi et al. 2017). It is also possible that the circannual rhythm is not found due to photoperiodic conditions under which periodicity is not expressed, such as LD 14:10 for the pupation rhythm in *A. verbasci* (Nisimura and Numata 2003).

To complicate matters, even if two peaks with an interval of a few months or more in insect metamorphosis are observed during group rearing under constant conditions, they are not necessarily circannual or other endogenous long-term rhythms. For example, when nymphal development of the cricket *Modicogryllus siamensis* was examined under DD at 25 °C, the pattern of adult emergence showed two peaks with an interval of 80–90 days (Sakamoto and Tomioka 2007). However, this bimodal pattern is unlikely to be caused by long-term biological rhythms because the strain investigated is bivoltine in the field, and the timing of adult emergence is altered in a temperature-dependent manner (Sakamoto and Tomioka 2007; Miki

et al. 2020). It is possible that the early and late peaks of *M. siamensis* are the result of long-day-like early and short-day-like late development, respectively, with both developmental types revealed in DD (Sakamoto and Tomioka 2007). To clearly determine whether the two peaks observed under constant conditions are produced by a circannual rhythm, careful investigation of temperature compensation of the period under different constant temperature conditions and the life cycle and seasonal adaptation strategies in the field of species of interest would be required, as done by Blake (1958) 65 years ago.

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References

Andjus RK, Živadinović D, Marjanović M (2000) Circannual rhythms in European ground squirrels during nine years of entrainment. In: Heldmaier G, Klingenspor M (eds) Life in the cold. Springer, Berlin, pp 233–240. https://doi.org/10.1007/978-3-662-04162-8_25

Armes NJ (1990) The biology of *Anthrenus sarnicus* Mroczkowski (Coleoptera: Dermestidae): I. Egg and larval development. J Stored Prod Res 26:11–22. https://doi.org/10.1016/0022-474X(90)90033-O

Baker JE (1983) Temperature regulation of larval size and development in *Attagenus megatoma* (Coleoptera: Dermestidae). Ann Entomol Soc Am 76:752–756. https://doi.org/10.1093/aesa/76.4.752

Blake GM (1958) Diapause and the regulation of development in *Anthrenus verbasci* (L.) (Col., Dermestidae). Bull Entomol Res 49:751–775. https://doi.org/10.1017/S0007485300054006

Blake GM (1959) Control of diapause by an 'internal clock' in *Anthrenus verbasci* (L.) (Col., Dermestidae). Nature 183:126–127. https://doi.org/10.1038/183126a0

Blake GM (1960) Decreasing photoperiod inhibiting metamorphosis in an insect. Nature 188:168–169. https://doi.org/10.1038/188168a0

Brock MA (1979) Differential sensitivity to temperature steps in the circannual rhythm of hydranth longevity in the marine cnidarian, *Campanularia flexuosa*. Comp Biochem Physiol 64A:381–390. https://doi.org/10.1016/0300-9629(79)90458-4

Budki P, Malik S, Rani S, Kumar V (2014) Circadian rhythms are not involved in the regulation of circannual reproductive cycles in a sub-tropical bird, the spotted munia. J Exp Biol 217:2569–2579. https://doi.org/10.1242/jeb.100651

Coombs CW, Woodroffe GE (1983) The effect of temperature upon the longevity, fecundity and circannual development of *Anthrenus sarnicus* Mroczkowski (Coleoptera: Dermestidae). J Stored Prod Res 19:111–115. https://doi.org/10.1016/0022-474X(83)90042-5

Danks HV (1987) Insect dormancy: an ecological perspective. Biological Survey of Canada, Ottawa

Dobashi A, Ozawa S, Kuwana Z (1980) The effect of the environmental temperature and photoperiodism upon the length of developmental period of *Attagenus piceus* (Oliver). J Bunka Women's Univ 11:121–128. (in Japanese)

Goldman B, Gwinner E, Karsch FJ, Saunders D, Zucker I, Gall GF (2004) Circannual rhythms and photoperiodism. In: Dunlap JC, Loros JJ, DeCoursey PJ (eds) Chronobiology—biological timekeeping. Sinauer, Sunderland, pp 107–142

Goymann W, Helm B (2014) Seasonality of life histories in tropical birds: circannual rhythms and *Zeitgeber*. In: Numata H, Helm B (eds) Annual, lunar, and tidal clocks: patterns and mechanisms of nature's enigmatic rhythms. Springer, Tokyo, pp 247–275. https://doi.org/10.1007/978-4-431-55261-1_13

Griswold GH (1941) Studies on the biology of four common carpet beetles. Part I. The black carpet beetle (*Attagenus piceus* Oliv.), the varied carpet beetle (*Anthrenus verbasci* L.), and the furniture carpet beetle (*Anthrenus vorax* Waterh.). Mem Cornell Univ Agric Exp Stn 240:5–57, 70–75

Gwinner E (1973) Circannual rhythms in birds: their interaction with circadian rhythms and environmental photoperiod. J Reprod Fertil Suppl 19:51–65

Gwinner E (1986) Circannual rhythms. Springer, Berlin

Helm B, Lincoln GA (2017) Circannual rhythms anticipate the Earth's annual periodicity. In: Kumar V (ed) Biological timekeeping: clocks, rhythms and behavior. Springer, New Delhi, pp 545–569. https://doi.org/10.1007/978-81-322-3688-7_26

Johnson CH, Elliott J, Foster R, Honma KI, Kronauer R (2004) Fundamental properties of circadian rhythms. In: Dunlap JC, Loros JJ, DeCoursey PJ (eds) Chronobiology—biological timekeeping. Sinauer, Sunderland, pp 67–105

Kenagy GJ (1981) Endogenous annual rhythm of reproductive function in the non-hibernating desert ground squirrel *Ammospermophilus leucurus*. J Comp Physiol A 142:251–258. https://doi.org/10.1007/BF00605743

Kipyatkov VE (1993) Annual cycles of development in ants: diversity, evolution, regulation. In: Kipyatkov VE (ed) Proceedings of the colloquia on social insects, vol 2. Socium, St Petersburg, pp 25–48

Kipyatkov VE (1995) Role of endogenous rhythms in regulation of annual cycles of development in ants (Hymenoptera, Formicidae). Entomol Rev 74:1–15

Kipyatkov VE, Shenderova SS (1990) Endogenous rhythm of reproductive activity of red wood ant queens (*Formica rufa* group). Entomol Rev 69:137–149

Kiritani K (1958) Factors influencing the development of *Anthrenus verbasci* L. Botyu-Kagaku 23: 137–146. (in Japanese with English summary)

Kuroki I, Tagawa J, Nakamura K (2018) Endogenous periodicity in egg-number fluctuation in *Lasius japonicus* (Formicidae). Biol Rhythm Res 49:872–882. https://doi.org/10.1080/09291016.2018.1427600

Kuwana Z (1951) Temperature-effect as a factor for the pupation of *Anthrenus verbasci* (Coleoptera, Dermestidae). J Seric Sci Jpn 20:202–207. (in Japanese with English summary)

Lakin-Thomas PL (1995) A beginner's guide to limit cycles, their uses and abuses. Biol Rhythm Res 26:216–232. https://doi.org/10.1080/09291019509360337

Lincoln G (2019) A brief history of circannual time. J Neuroendocrinol 31:e12694. https://doi.org/10.1111/jne.12694

Matsuno T, Kawasaki Y, Numata H (2013a) Small geographic variation in photoperiodic entrainment of the circannual rhythm in the varied carpet beetle, *Anthrenus verbasci*. Zool Sci 30:304–310. https://doi.org/10.2108/zsj.30.304

Matsuno T, Miyazaki Y, Muramatsu N, Numata H (2013b) Circannual pupation timing is not correlated with circadian period in the varied carpet beetle *Anthrenus verbasci*. Biol Rhythm Res 44:849–855. https://doi.org/10.1080/09291016.2013.770293

Miki T, Shinohara T, Chafino S, Noji S, Tomioka K (2020) Photoperiod and temperature separately regulate nymphal development through JH and insulin/TOR signaling pathways in an insect. Proc Natl Acad Sci USA 117:5525–5531. https://doi.org/10.1073/pnas.1922747117

Miyazaki Y, Numata H (2010) Exhibition of circannual rhythm under constant light in the varied carpet beetle *Anthrenus verbasci*. Biol Rhythm Res 41:441–448. https://doi.org/10.1080/09291010903411443

Miyazaki Y, Nisimura T, Numata H (2005) A phase response curve for circannual rhythm in the varied carpet beetle *Anthrenus verbasci*. J Comp Physiol A 191:883–887. https://doi.org/10.1007/s00359-005-0012-6

Miyazaki Y, Nisimura T, Numata H (2006) Phase responses in the circannual rhythm of the varied carpet beetle, *Anthrenus verbasci*, under naturally changing day length. Zool Sci 23:1031–1037. https://doi.org/10.2108/zsj.23.1031

Miyazaki Y, Nisimura T, Numata H (2007) Phase resetting and phase singularity of an insect circannual oscillator. J Comp Physiol A 193:1169–1176. https://doi.org/10.1007/s00359-007-0270-6

Miyazaki Y, Nisimura T, Numata H (2009a) Circannual pupation rhythm in the varied carpet beetle *Anthrenus verbasci* under different nutrient conditions. Entomol Sci 12:370–375. https://doi.org/10.1111/j.1479-8298.2009.00349.x

Miyazaki Y, Nisimura T, Numata H (2009b) A circadian system is involved in photoperiodic entrainment of the circannual rhythm of *Anthrenus verbasci*. J Insect Physiol 55:494–498. https://doi.org/10.1016/j.jinsphys.2008.12.003

Miyazaki Y, Nisimura T, Numata H (2012) Circannual rhythm in the varied carpet beetle, *Anthrenus verbasci*. In: Kalsbeek A, Merrow M, Roenneberg T, Foster RG (eds) Progress in brain research, vol 199., The neurobiology of circadian timing. Elsevier, Amsterdam, pp 439–456. https://doi.org/10.1016/B978-0-444-59427-3.00025-3

Miyazaki Y, Nisimura T, Numata H (2014) Circannual rhythms in insects. In: Numata H, Helm B (eds) Annual, lunar, and tidal clocks: patterns and mechanisms of nature's enigmatic rhythms. Springer, Tokyo, pp 333–350. https://doi.org/10.1007/978-4-431-55261-1_16

Miyazaki Y, Watari Y, Numata H (2016) Resetting of the circannual rhythm of the varied carpet beetle *Anthrenus verbasci* by low-temperature pulses. Physiol Entomol 41:390–399. https://doi.org/10.1111/phen.12172

Nakanishi T, Kaneda T, Nakamuta K (2017) Effects of temperature on the development and circannual control of pupation in the carpenter moth, *Cossus insularis* (Lepidoptera: Cossidae), reared on an artificial diet. Appl Entomol Zool 52:29–35. https://doi.org/10.1007/s13355-016-0450-3

Nisimura T, Numata H (2001) Endogenous timing mechanism controlling the circannual pupation rhythm of the varied carpet beetle *Anthrenus verbasci*. J Comp Physiol A 187:433–440. https://doi.org/10.1007/s003590100215

Nisimura T, Numata H (2002) Evaluation of the frequency demultiplication hypothesis of circannual pupation rhythm in the varied carpet beetle *Anthrenus verbasci* (Coleoptera: Dermestidae). Biol Rhythm Res 33:255–260. https://doi.org/10.1076/brhm.33.3.255.8264

Nisimura T, Numata H (2003) Circannual control of the life cycle in the varied carpet beetle *Anthrenus verbasci*. Funct Ecol 17:489–495. https://doi.org/10.1046/j.1365-2435.2003.00753.x

Numata H, Miyazaki Y, Ikeno T (2015) Common features in diverse insect clocks. Zoological Lett 1:10. https://doi.org/10.1186/s40851-014-0003-y

Patke A, Young MW, Axelrod S (2020) Molecular mechanisms and physiological importance of circadian rhythms. Nat Rev Mol Cell Biol 21:67–84. https://doi.org/10.1038/s41580-019-0179-2

Paul MJ, Zucker I, Schwartz WJ (2008) Tracking the seasons: the internal calendars of vertebrates. Philos Trans R Soc London B Biol Sci 363:341–361. https://doi.org/10.1098/rstb.2007.2143

Pengelley ET, Fisher KC (1957) Onset and cessation of hibernation under constant temperature and light in the golden-mantled ground squirrel, *Citellus lateralis*. Nature 180:1371–1372. https://doi.org/10.1038/1801371b0

Pengelley ET, Fisher KC (1963) The effect of temperature and photoperiod on the yearly hibernating behavior of captive golden-mantled ground squirrels (*Citellus lateralis tescorum*). Can J Zool 41:1103–1120. https://doi.org/10.1139/z63-087

Sakamoto T, Tomioka K (2007) Effects of unilateral compound-eye removal on the photoperiodic responses of nymphal development in the cricket *Modicogryllus siamensis*. Zool Sci 24:604–610. https://doi.org/10.2108/zsj.24.604

Saunders DS (2002) Insect clocks, 3rd edn. Elsevier, Amsterdam. https://doi.org/10.1016/B978-0-444-50407-4.X5000-9

Saunders DS (2010) Photoperiodism in insects: migration and diapause responses. In: Nelson RJ, Denlinger DL, Somers DE (eds) Photoperiodism: the biological calendar. Oxford University Press, New York, pp 218–257. https://doi.org/10.1093/acprof:oso/9780195335903.003.0010

Schaffelke B, Lüning K (1994) A circannual rhythm controls seasonal growth in the kelps *Laminaria hyperborea* and *L. digitata* from Helgoland (North Sea). Eur J Phycol 29:49–56. https://doi.org/10.1080/09670269400650471

Stevenson T, Lincoln GA (2017) Epigenetic mechanisms regulating circannual rhythms. In: Kumar V (ed) Biological timekeeping: clocks, rhythms and behavior. Springer, New Delhi, pp 607–623. https://doi.org/10.1007/978-81-322-3688-7_29

Chapter 12
General Features of Photoperiodism

Hideharu Numata

Abstract Photoperiodism controls both dimorphic and continuous phenotypes in insects, and threshold and quantitative responses to photoperiod have been reported. Both responses coexist in some insects, and some threshold responses also show quantitative properties. Moreover, there are responses to gradual changes in photoperiod without crossing the threshold value. To explain all these responses, the mechanism of photoperiodism must retain quantitative information of the photoperiod, and then it is converted into all-or-none information with a threshold. Although various theoretical models have been proposed to explain photoperiodic time measurement, no model can comprehensively explain all experimental results obtained in insects. However, it is now unequivocal that insect photoperiodism commonly uses the circadian clock.

Keywords Quantitative response · Circadian clock · Bünning's hypothesis · Hourglass · Internal coincidence model · External coincidence model

12.1 Classification of Photoperiodism

12.1.1 Photoperiodism Controlling Dimorphic and Continuous Phenotypes

Photoperiodism, an organism's response to the length of the light or dark period in a day, controls various phenotypes in insects, which are classified into two groups: one is a discrete dimorphic phenotype, one of two discontinuous states in each individual, and the other is a continuous phenotype (Fig. 12.1).

The first report of insect photoperiodism was on photoperiodic determination of parthenogenetic and bisexual morphs of the strawberry aphid, *Aphis forbesi*, and the second one was on the maternal induction of embryonic diapause in the domestic

H. Numata (✉)
Institute for the Future of Human Society, Kyoto University, Kyoto, Japan
e-mail: numata.hideharu.8r@kyoto-u.jp

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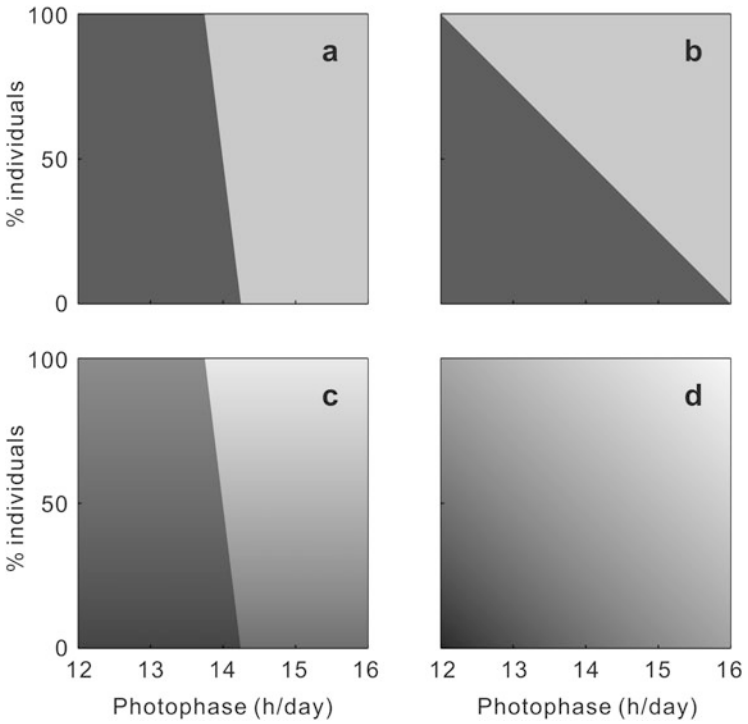

Fig. 12.1 Schematic representation of photoperiodism for a dimorphic phenotype (**a**, **b**) and a continuous phenotype (**c**, **d**). (**a**) and (**c**) are threshold responses, whereas (**b**) and (**d**) are quantitative responses to photoperiod. The shade of gray represents the phenotype

silkmoth, *Bombyx mori* (Marcovitch 1923; Kogure 1933). Both of the phenotypes are dimorphic because there is no intermediate phenotype between parthenogenetic and bisexual morphs and between diapause and nondiapause embryos. Then, photoperiodic determination of seasonal morphs was shown in the nymphalid butterfly *Araschnia levana*. The surface of the wing of an adult found in spring has a mottled pattern of black and orange, whereas that of an adult found in summer has a thick white belt on a black background. Due to this distinct difference, the two have long been treated as different species. However, it was shown that they are actually dimorphisms within a single species, determined by the photoperiod experienced during the larval stage (Danilevskii 1948; Müller 1955). In the swallowtail butterfly *Papilio xuthus*, which has spring and summer morphs, a detailed examination of the proportion of orange scales characteristic of the spring morph revealed that the two types are discontinuous, and usually the intermediate type does not occur (Endo and Funatsu 1985). Photoperiodic induction of diapause has been reported in many species with diapause at all developmental stages, e.g., the nymph in the emperor dragonfly, *Anax imperator*; the larva in the European corn borer, *Ostrinia nubilalis*; the prepupa in the European pine sawfly, *Neodiprion sertifer*; the pupa in the

bright-line brown-eye moth, *Lacanobia oleracea*; and the adult in the Colorado potato beetle, *Leptinotarsa decemlineata* (Corbet 1956; Beck 1962; Sullivan and Wallace 1965; Way et al. 1949; De Wilde et al. 1959; see Danks 1987 for review). Phenotypes related to embryonic and pupal diapauses are dimorphic, and an individual stops morphogenesis or not. In adult diapause, phenotypes are also dimorphic, and an adult stops reproduction or not, although an exception was shown in a parasitoid wasp, *Ooencyrtus nezarae*, in which a low reproductive state, intermediate between diapause and a high reproductive state, is induced by photoperiod (Numata 1993).

Although the occurrence of diapause is a dimorphic phenotype in principle, the intensity of diapause and the rate of diapause development are continuous, e.g., in a green lacewing, *Chrysoperla carnea*; fruit flies, *Drosophila auraria* species complex; two zygaenid moths, *Pryeria sinica* and *Elcysma westwoodii*; and the bean bug, *Riptortus pedestris* (Tauber and Tauber 1972, 1973; Kimura 1988; Ishii 1988; Gomi and Takeda 1992; Nakamura and Numata 2000). Danilevskii (1961) reported that larvae of a univoltine moth, *Eurois occulta*, increased their body weight more slowly and had a longer developmental period under shorter days, even though he did not regard this elongation of the larval period as diapause. Photoperiodic elongation of the larval or nymphal period is often not accompanied by a complete stop in morphogenesis, e.g., in the Emma field cricket, *Teleogryllus emma*; the southwestern corn borer, *Diatraea grandiosella*; a trigonidiid cricket, *Pteronemobius nitidus*; a subtropical cockroach, *Opisoplatia orientalis*; and a fruit fly, *Drosophila montana* (Masaki 1967; Chippendale and Yin 1973; Tanaka 1978; Zhu and Tanaka 2004; Salman et al. 2012). In these insects, the phenotype controlled by photoperiod is not dimorphic but continuous. Moreover, whereas the seasonal morphs in adult butterflies are dimorphic, body coloration controlled by photoperiod is continuous in two stink bugs (Kobayashi and Numata 1993; Numata and Kobayashi 1994).

12.1.2 Threshold and Quantitative Responses

Photoperiodism controlling dimorphic phenotypes usually has a threshold in the duration of the light period, i.e., a critical daylength. Here, I call such photoperiodism the threshold response (Fig. 12.1a). The critical daylength is frequently very abrupt, indicating the accuracy of the system measuring the daylength (Saunders 2002). Moreover, because the critical daylength cannot be determined in an individual but in a population, its steepness also shows a small genetic variation of the threshold in the population.

Photoperiodism controlling continuous phenotypes also can show a threshold response (Fig. 12.1c). The leafhopper *Euselis plebejus* has seasonal morphs, which are different in body size, relative wing length, and, interestingly, the shape and length of the aedeagus of the male genitalia (Müller 1960). This phenotype is not dimorphic but continuous even though they were divided into seven morphs, several

of which were once described as different species (Müller 1960). Under long and short days, spring and summer morphs were obtained, respectively. Only under a narrow range between them, intermediate morphs were produced, and a critical daylength does exist. Therefore, it can be regarded as a threshold response in a continuous phenotype, as pointed out by Zaslavski (1988).

Photoperiodism controlling continuous phenotypes sometimes does not show a threshold response but quantitatively responds to the daylength (Fig. 12.1d). Here, I call such photoperiodism the quantitative response. Zaslavski (1988) called it the gradual response and emphasized its importance in insect seasonal development. Although the rate of diapause development is a continuous phenotype, quantitative responses in diapause development do not play important roles in winter diapause because winter diapause is mostly not terminated by long days but by exposure to low temperatures or a simple passage of time (Danks 1987). However, summer diapause is often terminated by short days, and the rate of diapause development is quantitative to the daylength, e.g., in a crane fly, *Tipula subnodicornis*, and two zygaenid moths, *P. sinica* and *E. westwoodii* (Butterfield 1976; Ishii 1988; Gomi and Takeda 1992). The photoperiodic elongation of the larval or nymphal period is often quantitative to the duration of the light period, e.g., in *E. occulta*, *T. emma*, *O. orientalis*, and *D. montana* (Danilevskii 1961; Masaki 1967; Zhu and Tanaka 2004; Salman et al. 2012).

It is difficult to prove the existence of a quantitative response in photoperiodism controlling a dimorphic phenotype (Fig. 12.1b). For example, the proportion of diapause adults gradually decreases from a typical short day of LD 12:12 (12-h light and 12-h darkness) to a typical long day of LD 18:6 at 25 °C in the blow fly *Protophormia terraenovae* (Numata and Shiga 1995). However, I cannot conclude from such results whether the diapause incidence is stochastically determined in a quantitative response or each individual has a distinct threshold response but the population has a large genetic variation in the threshold.

12.1.3 Long-Day and Short-Day Responses

When insects grow and reproduce under long days and enter diapause under short days, the response is called "a long-day response." Long-day responses are common in insects in the temperate and frigid zones, where low temperature in winter is generally unsuitable for growth and reproduction for ectotherms (Danilevskii 1961; Beck 1980; Danks 1987; Zaslavski 1988; Saunders 2002).

When the daylength and the proportion of individuals with a certain phenotype are put on the horizontal and vertical axes, respectively, we call the line on the graph a photoperiodic response curve. Figure 12.2a shows an example of a photoperiodic response curve for a long-day response. *R. pedestris* overwinter as adults in diapause, of which the induction is controlled by photoperiod (Kobayashi and Numata 1993). Under long days, adults promptly reproduce after emergence, whereas under short days they enter diapause with suppression of development of reproductive

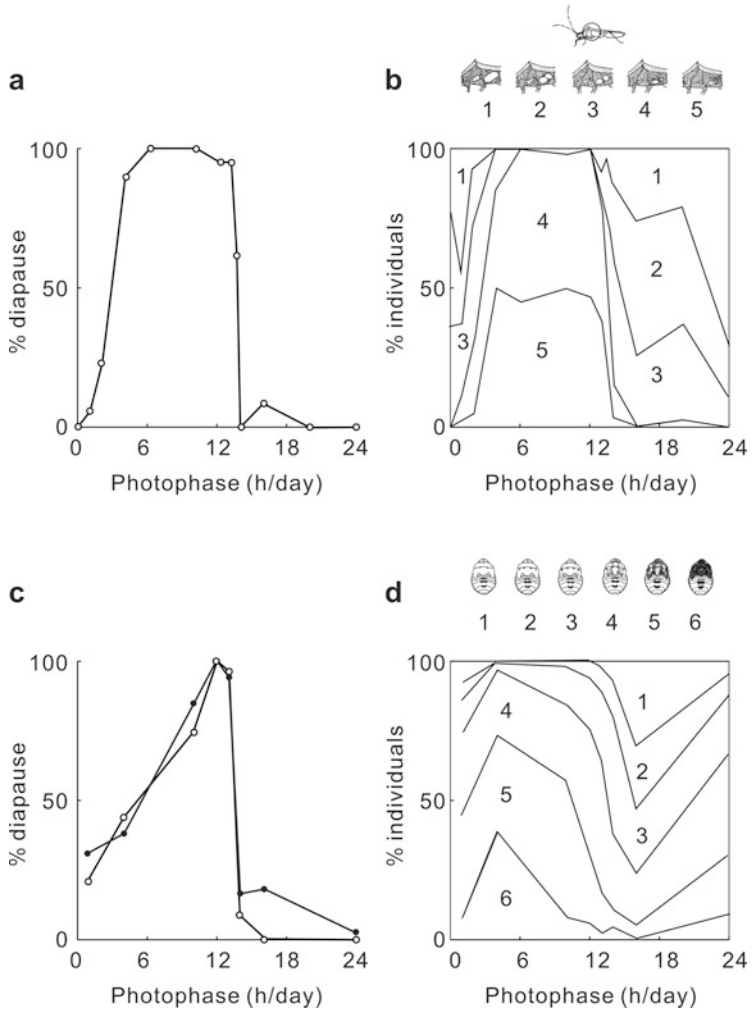

Fig. 12.2 Photoperiodic response curves for the induction of diapause and the determination of adult body coloration at 25 °C in two heteropterans. (**a**) Adult diapause; (**b**) adult body coloration in males of *Riptortus pedestris*. (**c**) Adult diapause (open circles, females; closed circles, males); (**d**) body coloration in the fifth (final) instar nymphs in *Plautia stali*. (**a**, **b**) Adapted from Kobayashi and Numata (1993) with permission from the Zoological Society of Japan, (**c**, **d**) adapted from Numata and Kobayashi (1994) with permission from Springer Nature

organs in both sexes. Under very short days and constant darkness, moreover, adults did not enter diapause. This is frequently observed in long-day responses (Danilevskii 1961; Beck 1980; Danks 1987; Zaslavski 1988; Saunders 2002). In such cases, there are two critical daylengths. Danilevskii (1961) pointed out that a photoperiodic response curve includes responses both within and out of the range of photoperiods under natural conditions. The critical daylength in the range of natural

conditions that has ecological significance is approximately 13.5 h in *R. pedestris*. In general, the curve showing diapause incidence changes steeply near the critical daylength in the range of natural conditions. This is because the daylength measurement should be accurate and the genetic individual variation should be small to assure the production and survival of offspring. On the other hand, the critical daylength in the range of extremely short days is approximately 3 h, and the curve changes more gently in *R. pedestris*. This part of the curve has no ecological significance but may reflect the physiological nature of the daylength measurement (see Sect. 12.2). The selection pressure is not applied out of the natural range of daylength, and therefore the daylength measurement in this range should not be accurate, a large individual variation is allowed, or both.

A smaller number of species grow and reproduce under short days and enter diapause under long days, and the response is called "a short-day response." Short-day responses are shown by insects with summer diapause (Danilevskii 1961; Beck 1980; Danks 1987; Zaslavski 1988; Saunders 2002). In a bivoltine strain (Shohaku) of *B. mori*, however, the induction of winter embryonic diapause is controlled by a short-day response (Kogure 1933). This is because the diapause stage is in the embryo and the stage sensitive to photoperiod is also in the embryo but of the maternal generation. Because there is one generation interval between the sensitive and diapause stages, spring short days and summer long days prevent and induce winter diapause, respectively (Kogure 1933). Most likely the short-day response in *B. mori*, which does not inhabit the field, has been selected during domestication, because the wild mulberry silkmoth, *Bombyx mandarina*, the wild ancestor of *B. mori*, shows a long-day response at the maternal larval stage for the induction of embryonic diapause, similar to many temperate insects (Kobayashi 1990).

A few insects enter diapause in all photoperiods except for a narrow range. The response was called "an intermediate response" and discussed in relation to the evolution of univoltine life cycles (Danilevskii 1961; Zaslavski 1988; Saunders 2002). However, such a response can be shown when there are summer and winter diapauses at the same developmental stage in a species (see Danks 1987). For example, when the Oriental green stink bug, *Nezara antennata*, was reared under stationary photoperiods at 25 °C, only a small proportion of adults averted diapause at intermediate photoperiods, and the others entered diapause (Fig. 12.3). When adults raised under long days were transferred to shorter days of LD 14:10 or LD 13:11 at adult emergence, however, most adults started reproduction. Therefore, this apparently intermediate response is produced by coexistence of summer and winter diapauses. The former is induced by long days and averted or terminated by transfer to shorter days, whereas the latter is induced by short days. Under natural conditions, this response assures the bivoltine life cycle of *N. antennata*: adults of the first generation enter summer diapause, and the production of the second generation is delayed until the daylength becomes shorter in late summer.

Conversely, a few other insects enter diapause only in a narrow range of photoperiods. However, this response can be explained as a long-day response with lower diapause incidence in the unnatural short-day range (Fig. 12.2c).

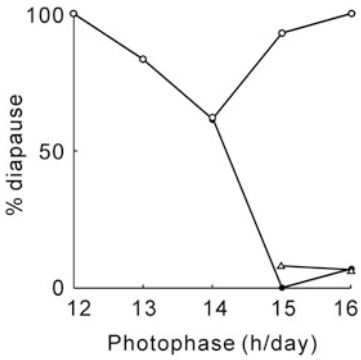

Fig. 12.3 Photoperiodic response curves for the induction of adult diapause in *Nezara antennata*. Adults were collected in Kyoto, Japan, and their progeny were reared under different photoperiods at 25 °C. Female adults that had not laid eggs for 30 days and had no mature eggs 30 days after emergence were regarded as in diapause. Open circles, under stationary photoperiods; closed circles, transfer to LD 13:11 at adult emergence; triangles, transfer to LD 14:10 at adult emergence. $N = 24–30$

12.1.4 Threshold Response in Dimorphic and Continuous Phenotypes

In addition to the photoperiodic control of a dimorphic phenotype, adult diapause, *R. pedestris* responds to photoperiod for a continuous phenotype, adult body coloration (Kobayashi and Numata 1993; Fig. 12.2b). The body color of male adults was classified into five grades, with regard to the number and area of the white spots on the side thorax. However, adults with intermediate color between two adjacent types appeared, and therefore the phenotype was continuous contrary to the seasonal morphs of butterflies. Under long days, very short days, and constant darkness, adults with bright body color (grades 1–3) appeared, whereas under typical short days, adults with dark body color (grades 4 and 5) appeared. The portion showing diapause adults in Fig. 12.2a and the portion of dark adults in Fig. 12.2b almost overlap with the two critical daylengths of 3 and 13.5 h. Thus, the photoperiodism that determines the continuous phenotype of adult body coloration was not a quantitative response but a threshold response.

Some other insects with photoperiodism both in dimorphic and continuous phenotypes show threshold responses with common critical daylength(s) (the pitcher-plant mosquito, *Wyeomyia smithii*, Bradshaw and Lounibos 1972; the cabbage moth, *Mamestra brassicae*, Tyshchenko et al. 1977; the white-spotted tussock moth, *Orgyia thyellina*, Kimura and Masaki 1977; *P. xuthus*, Endo and Murakami 1985). Both the critical daylength in the natural range and that in the extremely short range are common between a dimorphic phenotype of pupal diapause and a continuous phenotype of pupal body weight in *M. brassicae* (Tyshchenko et al. 1977). These results support the view that the physiological mechanism of photoperiodism outputs information on whether the daylength is longer than the critical value.

12.1.5 Coexistence of Threshold and Quantitative Responses

The brown-winged green stink bug, *Plautia stali*, also has dimorphic and continuous phenotypes (Numata and Kobayashi 1994). The induction of adult diapause, a dimorphic phenotype, was a long-day response, although the diapause incidence gradually decreased from a typical natural short day of LD 12:12 to shorter daylengths (Fig. 12.2c). The critical daylength in the natural range was 13.5 h as in *R. pedestris*. The body coloration of the fifth (final) instar nymphs in *P. stali* was also affected by photoperiod. The body color was classified into six grades, between the totally bright green grade 1 and the dark-colored grade 6. However, nymphs with intermediate color between two adjacent types appeared, and therefore the phenotype was continuous as in the adults of *R. pedestris*. Contrary to the threshold response in *R. pedestris*, the photoperiodism for the determination of nymphal coloration was a quantitative response in *P. stali* (Fig. 12.2d). In the range between LD 4:20 and LD 16:8, the longer the light period is, the higher the proportion of bright-colored larvae, and a single critical daylength cannot be determined. The results under an extreme short day of LD 1:23 and constant light were intermediate between those under LD 4:20 and LD 16:8. Therefore *P. stali* shows both threshold and quantitative responses and responds differently to the daylength for dimorphic and continuous phenotypes.

12.1.6 Quantitative Properties in Threshold Responses

In some threshold responses, quantitative properties were also detected. In Sect. 12.1.4, I regarded the photoperiodic determination of the adult body coloration in *R. pedestris* as a threshold response. However, under LD 13:11, most adults entered diapause but approximately 20% bright color adults (grades 1–3) emerged. Under LD 14:10 no adults entered diapause but approximately 15% dark color adults (grades 4 and 5) emerged. Thus, this insect responds quantitatively to photoperiod to some extent in a range close to the critical daylength of 13.5 h (Kobayashi and Numata 1993; Fig. 12.2b).

An English population of the large cabbage white, *Pieris brassicae*, shows a long-day threshold response for the induction of pupal diapause with a critical daylength of approximately 15 h (Spieth and Sauer 1991). Spieth and Sauer (1991) showed a quantitative measurement of the daylength behind this apparent threshold response by cleverly devised experiments. When the first to fourth (penultimate) instar larvae are reared under a typical long day of LD 16:8 and placed under various short days only in the fifth (final) instar, the shorter the daylength was, the higher was the diapause incidence in the range between LD 11:13 and LD 14:10. Moreover, when larvae were reared under a typical short day of LD 12:12 and exposed to various long days for only 3 days in the fourth or fifth instar, the longer the daylength was, the lower was the diapause incidence in the range between LD 15.5:8.5 and LD 19:5

(Spieth and Sauer 1991). Similar results were obtained in photoperiodic induction of adult diapause in the *D. auraria* species complex and photoperiodic determination of reproductive morphs (viviparae, oviparae, and males) in the vetch aphid, *Megoura viciae* (Kimura 1990; Hardie 1990).

The water strider *Aquarius paludum* shows two threshold responses in dimorphic phenotypes, i.e., induction of adult diapause and determination of wing forms, of which the sensitive period is in the nymphal stage (Harada and Numata 1993). However, the critical daylength was shorter by approximately 45 min in the former than in the latter response. This cannot be explained by a mechanism that outputs only information about whether the daylength is longer than a certain value. Therefore, quantitative measurement of photoperiod exists behind apparent threshold responses.

12.1.7 Response to Changes in Photoperiod

I have discussed based on the results obtained under stationary photoperiods. Under natural conditions, however, photoperiods gradually change. Responses to changes in photoperiod have also been reported (Danilevskii 1961; Danks 1987; Zaslavski 1988; Saunders 2002). Zaslavski (1988) listed 61 insect species with such responses. A greater proportion of them show a combination of two threshold responses: Sequential exposure to long and short days or vice versa is necessary to produce a certain phenotype. This response is useful to discriminate spring and autumn with a similar photoperiod (e.g., the heather ladybird, *Chilocorus bipustulatus*, Zaslavski 1970; a carabid beetle, *Pterostichus nigrita*, Ferenz 1977; *N. antennata*, Fig. 12.3). On the other hand, some insects respond to the direction in photoperiodic changes without crossing the critical daylength determined under stationary photoperiods for fine-tuning of the seasonal life cycles (e.g., *C. carnea*, Tauber and Tauber 1970; *M. viciae*, Fomenko and Zaslavski 1978; water striders, *Gerris odontogaster* and *A. paludum*, Vepsäläinen 1974; Harada and Numata 1993).

12.2 Theoretical Models for Photoperiodic Time Measurement

Bünning (1936) proposed the idea that time measurement in photoperiodism is determined by the relationship between the phase of an endogenous rhythm, which is now called the circadian rhythm, and light (see Bünning 1960 also). In this hypothesis, later named Bünning's hypothesis by Pittendrigh (1960), there are distinct scotophil (dark-requiring) and photophil (light-requiring) sections of the rhythm, and a photoperiodic effect is triggered or not according to whether light falls in the scotophil. This hypothesis successfully explains the results of night

Table 12.1 Typical examples of photoperiodism, of which the mechanism of time measurement has been intensively examined

		Experiment			
Species	Response	Nanda-Hamner	Bünsaw	Adapted model	References
Nasonia vitripennis	Maternal induction of larval diapause	Positive[a]	Positive	Internal coincidence	Saunders (1970, 1974)
Sarcophaga argirostoma	Induction of pupal diapause	Positive	Positive	External coincidence	Saunders (1973b, 1979)
Tetranychus urticae	Induction of adult diapause	Positive	Positive	Hourglass/circadian clock	Veerman and Vaz Nunes (1980) and Vaz Nunes and Veerman (1982)
Mamestra brassicae	Induction of pupal diapause	Positive/negative[b]	Negative	Hourglass/circadian clock	Kimura and Masaki (1993)
Drosophila triauraria	Induction of adult diapause	Positive/negative	Negative	Desynchronizing circadian clocks	Yoshida and Kimura (1993)
Megoura viciae	Determination of reproductive morphs	Negative	Negative	Hourglass	Lees (1966, 1973)

[a]A positive effect shows the involvement of the circadian clock
[b]Positive and negative effects were obtained at different temperatures

interruption experiments in which interruption of the long scotophase by a short light pulse produces long-day effects in various organisms including insects (e.g., *P. brassicae*, Bünning and Joerrens 1960). Thereafter various theoretical models have been proposed to explain photoperiodic time measurements, based on results obtained under skillfully devised light-dark schedules that differ from the natural solar day (Vaz Nunes and Saunders 1999). However, no model can explain all experimental results obtained in various species, and trials to construct a universal model have made the model more complex. In this chapter, I show two representative experimental schedules, i.e., the Nanda-Hamner and Bünsaw protocols, and the typical results in five insects and a mite (Table 12.1).

In the Nanda-Hamner protocol, organisms are exposed to cycles of a constant duration of the photophase (e.g., 8, 12 h) and various durations of the scotophase (Nanda and Hamner 1958). If an inducible effect is high in cycles with a period of approximately 24 h and its multiples, entrainment of a circadian clock is necessary for the photoperiodism (Saunders 2002; Saunders et al. 2004). Saunders (2002) listed the results of the Nanda-Hamner protocol in insects and mites. Although the results are different depending on temperature, strain, or both within a species, 16 species showed positive effects in at least 1 condition, and 8 did not show positive effects in any condition examined.

In the Bünsaw protocol, organisms are exposed to cycles of a constant photophase and an extended scotophase (e.g., LD 12:36, LD 12:60) with systematic interruption of the scotophase by a short (e.g., 1-h, 2-h) light pulse (Bünsow

1960). If a long-day effect of a light pulse appears with approximately 24-h intervals in the scotophase, a circadian free-running rhythm in the sensitivity of light and, therefore, the involvement of a circadian clock in the photoperiodism are shown (Saunders 2002; Saunders et al. 2004). Saunders (2002) listed the results of the Bünsaw protocol in insects and mites; 10 and 4 species show positive and negative effects, respectively.

The results of the Nanda-Hamner, Bünsaw, and other protocols varied among species, and many authors have proposed different theoretical models based on the results of their own subject species. Vaz Nunes and Saunders (1999) listed 13 clock models for photoperiodic time measurement in insects and mites. One is the hourglass model by Lees (1973) based on the results of his extensive experiments in *M. viciae*. The other 12 models assume the involvement of the circadian clock (Vaz Nunes and Saunders 1999).

Bünning's hypothesis was later expanded to explain the photoperiodic induction of diapause in the pink bollworm, *Pectinophora gossypiella*, with reference to a phase response curve of the circadian eclosion rhythm in *Drosophila pseudoobscura* (Pittendrigh and Minis 1964). This model retains Bünning's proposition of photoperiodic time measurement with a single circadian clock and two roles of light, i.e., entrainment of the clock and photoinduction. The circadian clock is reset to a constant phase (circadian time 12, CT12) at the end of a long photophase, and the coincidence of light with the photoinducible phase (φ_i) in the subjective night results in long-day effects (Pittendrigh and Minis 1964). This model explains the phenomenon frequently observed in night interruption experiments in which a light pulse produces long-day effects in two positions in the scotophase (Pittendrigh and Minis 1964). This model was later named the external coincidence model because the photoperiod is measured by coincidence of a phase in an internal rhythm with a phase of an external cycle (Pittendrigh 1972). Although the original external coincidence model could not explain long-day effects under very short days, Lewis and Saunders (1987) later modified it and explained the response. The photoperiodic induction of pupal diapause in the flesh fly *Sarcophaga argyrostoma* is unequivocally explained by the external coincidence model with φ_i located late in the subjective night, CT 21.5 h (Saunders 1973b, 1979).

Pittendrigh (1960) suggested that the photoperiod can be recognized by the phase angle of two oscillators. Tyshchenko (1966) proposed a model in which two circadian clocks are involved: the phase of one clock is reset by light-off (dusk) and that of the other by light-on (dawn) (see Danilevsky et al. 1970). In this model, light plays a single role, entrainment. This model was later named the internal coincidence model because the photoperiod is measured by the coincidence of phases in two internal rhythms (Pittendrigh 1972). This model simply explains long-day effects under very short days (Danilevsky et al. 1970). Saunders (1974) obtained evidence of the involvement of two circadian clocks entrained to light-off and light-on by Nanda-Hamner protocols with different lengths of the photophase in maternal induction of larval diapause in the jewel wasp, *Nasonia vitripennis*, and concluded that the photoperiodic time measurement is explained by the internal

coincidence mode. The response to thermoperiod in constant darkness in *N. vitripennis* also supported this conclusion (Saunders 1973a).

In photoperiodic induction of adult diapause in the two-spotted spider mite, *Tetranychus urticae*, and *Drosophila triauraria* and pupal diapause in *M. brassicae*, the positive effect of the Nanda-Hamner protocol at least at one temperature showed the involvement of the circadian clock (Veerman and Vaz Nunes 1980; Yoshida and Kimura 1993; Kimura and Masaki 1993). *T. urticae* also showed a positive effect in the Bünsaw protocol. However, the results of some other experiments did not support the measurement of the night length by a circadian clock (Vaz Nunes and Veerman 1982). *M. brassicae* showed a negative effect in the Bünsaw protocol. In these two species, it was proposed that the measurement of the night length is performed by an hourglass and that a circadian clock is involved in a later process (Vaz Nunes and Veerman 1982; Kimura and Masaki 1993). *D. triauraria* also showed a negative effect in the Bünsaw protocol, and multiple circadian clocks that are desynchronized in darkness were proposed for measuring the night length (Yoshida and Kimura 1993).

Although in many insects it has been shown that the circadian clock is involved in photoperiodism as described above, one typical exception was *M. viciae*. In this species, reproductive morphs are determined exclusively by the length of the scotophase, and no sign of the involvement of the circadian clock had been shown by systematic experiments including the Nanda-Hamner and Bünsaw protocols (Lees 1966, 1973). However, Vaz Nunes and Hardie (1993) showed that the night length is measured repeatedly in a prolonged scotophase by exposing *M. viciae* to various sequences of long scotophases with a 12-h photophase, suggesting the involvement of the circadian clock in *M. viciae* (Fig. 12.4). In the original Bünning's hypothesis, dumping of the circadian clock under constant darkness was already assumed (Bünning 1936), and later Bünning (1969) pointed out that hourglass-like responses can be attributed to circadian clocks dampening promptly under constant darkness. I agree with the view of Saunders (2002, 2011) that insect photoperiodism commonly uses the circadian clock, and the hourglass is one extent of continuum in the damping rate of the clock.

Vaz Nunes and Saunders (1999) concluded that insects and mites determine the night length in a quantitative manner, even though most models listed by them are for threshold responses, and only two models can explain quantitative responses (Zaslavski 1988; Vaz Nunes 1998). Taking into account the existence of quantitative responses (Sects. 12.1.2 and 12.1.5), quantitative properties in threshold responses (Sect. 12.1.6), and response to gradual change in photoperiod (Sect. 12.1.7), I also conclude that the mechanism of photoperiodism retains quantitative information of photoperiod in principle, and then it is converted, if necessary, into all-or-none information with a threshold. Moreover, all the proposed models assume that the waveform of the output from the circadian clock is constant in different photoperiods. In the cricket *Gryllus bimaculatus*, however, photoperiods modulate the circadian waveform of the locomotor rhythm and the optic lobe electrical activity in an experience-dependent manner, and the modulated waveform continues for a long period (Koga et al. 2005). Although *G. bimaculatus* shows no photoperiodism,

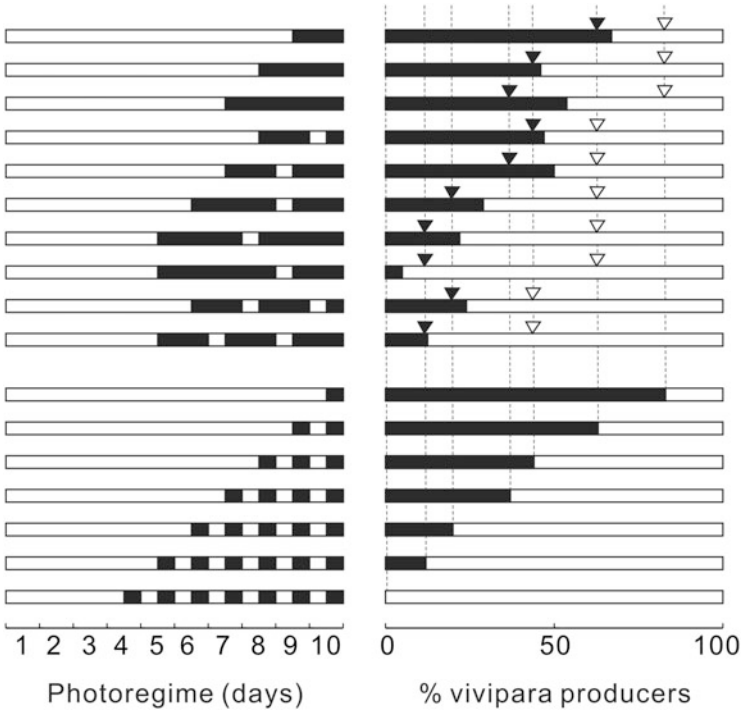

Fig. 12.4 Comparison of the effect on vivipara production in various sequences of a long scotophase with a 12-h photophase following continuous light at 15 °C in *Megoura viciae*. Open and closed triangles, proportions of vivipara producers if the photoperiodic time measurement is achieved by an hourglass and a circadian clock, respectively, estimated from control experiments (below). Based on Vaz Nunes and Hardie (1993)

Koga et al. (2005) pointed out the possibility that the photoperiodic modulation of the circadian clock plays a role in photoperiodism.

12.3 Photoreceptors for Photoperiodism

It had been believed that external photoreceptors such as the compound eye and ocellus are not involved in photoreception for photoperiodism for many years. However, evidence of the role of the compound eyes and other photoreceptor organs has been accumulated (Numata et al. 1997; Goto et al. 2010). Goto et al. (2010) listed 19 insect species in which the photoreceptor for photoperiodism had been localized. In adults, the compound eye and the brain are the predominant photoreceptors for photoperiodism in six and four species, respectively. In nymphs of two hemimetabolous insects, the compound eye is the predominant photoreceptor for photoperiodism. In larvae and pupae of six holometabolous insects, the brain is the

predominant photoreceptor for photoperiodism. The carabid beetle *Leptocarabus kumagaii* uses the stemma as the photoreceptor for photoperiodic induction of larval winter diapause, whereas after adult emergence it uses the compound eye for photoperiodic termination of adult summer diapause (Shintani et al. 2009).

Insects have two types of photopigments, opsins and cryptochrome. Opsins are sensitive to various wavelengths as visual pigments, whereas cryptochrome has an action peak at 450 nm (blue light) with no sensitivity to light of 500 nm or longer (Van Der Kooi et al. 2021; Hoang et al. 2008). Saunders (2012) listed 21 insects and 2 mites in which spectral sensitivity in photoperiodism had been examined. In many of them, light of 400–550 nm wavelength (violet to green light) is effective, and only six insects can respond to light of longer wavelength (orange to red light).

In the Nanda-Hamner protocol, *T. urticae* shows oscillation in diapause incidence with a period of approximately 20 h (Veerman and Vaz Nunes 1980). Under LD 8:12 and LD 12:8, of which the period was 20 h, the mite entered and averted diapause, respectively, either in white or orange-red light (Veerman and Veenendaal 2003). Under LD 12:12, of which the period was much longer than the period of the Nanda-Hamner rhythm, however, *T. urticae* entered diapause in white light but not in orange-red light. Veerman and Veenendaal (2003) interpreted the results that the Nanda-Hamner rhythm is entrained with a photoreceptor that cannot respond to orange-red light, and the night length is measured by an hourglass with a photoreceptor that can respond to orange-red light. This species needs vitamin A or its derivatives for photoperiodism (Veerman and Helle 1978), and the eyes are necessary for photoperiodism (Hori et al. 2014). One possible explanation is that the Nanda-Hamner rhythm is entrained to light-dark cycles by cryptochrome and the night length is measured by opsins in the eye.

The long-day response for the induction of pupal diapause is well explained by the external coincidence model in the flesh fly *Sarcophaga similis*, as in the congeneric species *S. argyrostoma* (Goto and Numata 2009). There were two light-sensitive points in the scotophase for preventing diapause. The earlier point was sensitive to light of 470 nm or shorter in wavelength, but not to light of 583 nm or longer. In contrast, the latter point, which is assumed to be φ_i, was sensitive to light of broad wavelengths, ranging from 395 to 660 nm (Fig. 12.5). Goto and Numata (2009) hypothesized that the circadian clock is entrained to light-dark cycles by cryptochrome and photoinduction at φ_i is brought by opsins.

12.4 Conclusions

In the twentieth century, information on the general features of insect photoperiodism has been accumulated as shown above. The results are not uniform but vary with species and phenotypes controlled by photoperiod. The circadian clock is assumed to originate from ancestral insects and the fundamental framework of the mechanism might be common (Chaps. 4 and 5). In contrast, photoperiodism may have evolved independently in various insects, and therefore, a large variation in the mechanism is

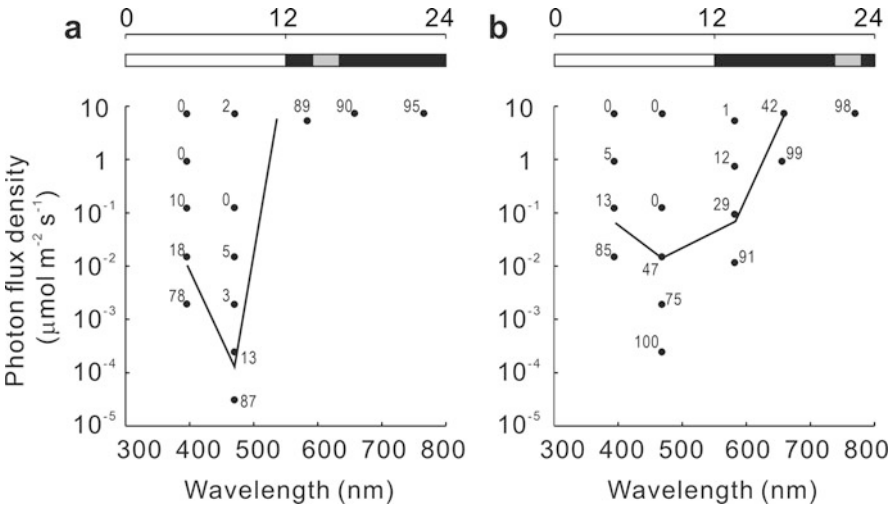

Fig. 12.5 Effect of wavelength of night interruption for the photoperiodic induction of pupal diapause in *Sarcophaga similis*. Diapause-destined wandering larvae were exposed to monochromatic light of 395 nm (UV), 470 nm (blue), 583 nm (yellow), 660 nm (red), and 730 nm (far red) at an early (**a**) or a late (**b**) point of scotophase. Closed circles indicate the photon flux densities that were used in the experiments, and the numbers at the circles indicate diapause incidence. Lines indicate estimates of the photon flux density at which 50% of individuals enter diapause. Adapted from Goto and Numata (2009) with permission from Elsevier

not surprising. Nevertheless, there is no doubt that the circadian clock provides the essential clockwork for photoperiodic timing (Saunders 2002, 2011). Classic techniques such as rearing insects under complex light-dark schedules have brought this conviction, leaving the molecular and neural mechanisms in a black box (Saunders et al. 2004). On the other hand, the molecular and neural mechanisms of the circadian clock in insects have been clarified by novel techniques since the 1990s (Chaps. 4 and 5). As Saunders et al. (2004) pointed out, we have been a stage to open the black box already for two decades. Studies on the molecular and neural mechanisms of photoperiodism have recently advanced (Chaps. 13 and 14), and I expect that the mechanisms will be clarified at the same levels as the current knowledge on the circadian clock within a decade.

References

Beck SD (1962) Photoperiodic induction of diapause in an insect. Biol Bull 122:1–12. https://doi.org/10.2307/1539316

Beck SD (1980) Insect photoperiodism, 2nd edn. Academic Press, New York

Bradshaw WE, Lounibos LP (1972) Photoperiodic control of development in the pitcher-plant mosquito, *Wyeomyia smithii*. Can J Zool 50:713–719. https://doi.org/10.1139/z72-098

Bünning E (1936) Die endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion. Ber Dtsch Bot Ges 54:590–607
Bünning E (1960) Circadian rhythms and the time measurement in photoperiodism. Cold Spring Harb Symp Quant Biol 25:249–256. https://doi.org/10.1101/SQB.1960.025.01.026
Bünning E (1969) Common features of photoperiodism in plants and animals. Photochem Photobiol 9:219–228. https://doi.org/10.1111/j.1751-1097.1969.tb07286.x
Bünning E, Joerrens G (1960) Tagesperiodische antagonistische Schwankungen der Blauviolett und Gelbrot-Empfindlichkeit als Grundlage der photoperiodischen Diapause-Induktion bei *Pieris brassicae*. Z Naturforsch 15b:205–213
Bünsow RC (1960) The circadian rhythm of photoperiodic responsiveness in Kalanchoe. Cold Spring Harb Symp Quant Biol 25:257–260. https://doi.org/10.1101/SQB.1960.025.01.027
Butterfield J (1976) Effect of photoperiod on a winter and on a summer diapause in two species of cranefly (Tiplidae). J Insect Physiol 22:1443–1446. https://doi.org/10.1016/0022-1910(76)90207-9
Chippendale GM, Yin C-M (1973) Endocrine activity retained in diapause insect larvae. Nature 246:511–512. https://doi.org/10.1038/246511a0
Corbet PS (1956) Environmental factors influencing the induction and termination of diapause in the emperor dragonfly, *Anax imperator* Leach (Odonata: Aeshnidae). J Exp Biol 33:1–14. https://doi.org/10.1242/jeb.33.1.1a
Danilevskii AS (1948) The photoperiodic reaction of insects in condition of artificial light. Dokl Akad Nauk SSSR 60:481–484. (In Russian)
Danilevskii AS (1961) Photoperiodism and seasonal development of insects. Leningrad State University, Leningrad. (in Russian)
Danilevsky AS, Goryshin NI, Tyshchenko VP (1970) Biological rhythms in terrestrial arthropods. Annu Rev Entomol 15:201–244
Danks HV (1987) Insect dormancy: an ecological perspective. Biological Survey of Canada, Ottawa
De Wilde J, Duintjer CS, Mook L (1959) Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say)—I The photoperiod as a controlling factor. J Insect Physiol 3:75–85. https://doi.org/10.1016/0022-1910(59)90022-8
Endo K, Funatsu S (1985) Hormonal control of seasonal morph determination in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae). J Insect Physiol 31:669–674. https://doi.org/10.1016/0022-1910(85)90045-9
Endo K, Murakami Y (1985) Photoperiodic control of the determination of three different seasonal phenomena of the swallowtail butterfly, *Papilio xuthus* L. Zool Sci 2:755–760. https://doi.org/10.34425/zs000185
Ferenz H-J (1977) Two-step photoperiodic and hormonal control of reproduction in the female beetle, *Pterostichus nigrita*. J Insect Physiol 23:671–676. https://doi.org/10.1016/0022-1910(77)90082-8
Fomenko RB, Zaslavski VA (1978) Stepwise photoperiodic reactions in the aphid *Megoura viciae* Buckt. Trudy Zool Inst Acad Nauk SSSR 69:102–109. (in Russian)
Gomi T, Takeda M (1992) A quantitative photoperiodic response terminates summer diapause in the tailed zygaenid moth, *Elcysma westwoodii*. J Insect Physiol 38:665–670. https://doi.org/10.1016/0022-1910(92)90048-I
Goto SG, Numata H (2009) Possible involvement of distinct photoreceptors in the photoperiodic induction of diapause in the flesh fly *Sarcophaga similis*. J Insect Physiol 55:401–407. https://doi.org/10.1016/j.jinsphys.2008.11.008
Goto SG, Shiga S, Numata H (2010) Photoperiodism in insects: perception of light and the role of clock genes. In: Nelson RJ, Denlinger DL, Somers DE (eds) Photoperiodism: the biological calendar. Oxford University Press, Oxford, pp 258–286
Harada T, Numata H (1993) Two critical day lengths for the determination of wing forms and the induction of adult diapause in the water strider, *Aquarius paludum*. Naturwissenschaften 80:430–432. https://doi.org/10.1007/BF01168342

Hardie J (1990) The photoperiodic counter, quantitative day-length effects and scotophase timing in the vetch aphid *Megoura viciae*. J Insect Physiol 36:939–949. https://doi.org/10.1016/0022-1910(90)90082-Q

Hoang N, Schleicher E, Kacprzak S, Bouly J-P, Picot M, Wu W, Berndt A, Wolf E, Bittl R, Ahmad M (2008) Human and *Drosophila* cryptochromes are light activated by flavin photoreduction in living cells. PLoS Biol 6:e160. https://doi.org/10.1371/journal.pbio.0060160

Hori Y, Numata H, Shiga S, Goto SG (2014) Both the anterior and posterior eyes function as photoperiodic receptors for photoperiodic termination of diapause in the two-spotted spider mite. J Comp Physiol A 200:161–167. https://doi.org/10.1007/s00359-013-0872-0

Ishii M (1988) Phenology in univoltine insects. In: Nakasuji F (ed) Entomological seminar III. Life cycle and behavior. Toju-sha, Tokyo, pp 66–108. (in Japanese)

Kimura MT (1988) Interspecific and geographic variation of diapause intensity and seasonal adaptation in the *Drosophila auraria* species complex (Diptera: Drosophilidae). Funct Ecol 2: 177–183

Kimura MT (1990) Quantitative response to photoperiod during reproductive diapause in the *Drosophila auraria* species-complex. J Insect Physiol 36:147–152. https://doi.org/10.1016/0022-1910(90)90115-V

Kimura T, Masaki S (1977) Brachypterism and seasonal adaptation in *Orgyia thyellina* Butler (Lepidoptera, Lymantriidae). Kontyû 45:97–106

Kimura Y, Masaki S (1993) Hourglass and oscillator expressions of photoperiodic diapause response in the cabbage moth *Mamestra brassicae*. Physiol Entomol 18:240–246. https://doi.org/10.1111/j.1365-3032.1993.tb00594.x

Kobayashi J (1990) Effect of the photoperiod on the induction of egg diapause of tropical races of the domestic silkworm, *Bombyx mori*, and the wild silkworm, *B. mandarina*. JARQ 23:202–205

Kobayashi S, Numata H (1993) Photoperiodic responses controlling the induction of adult diapause and the determination of seasonal form in the bean bug, *Riptortus clavatus*. Zool Sci 10:983–990. https://doi.org/10.34425/zs001165

Koga M, Ushirogawa H, Tomioka K (2005) Photoperiodic modulation of circadian rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 51:681–690. https://doi.org/10.1016/j.jinsphys.2005.01.006

Kogure M (1933) The influence of light and temperature on certain characters of the silkworm, *Bombyx mori*. J Dept Agric Kyushu Imp Univ 4:1–93. https://doi.org/10.5109/22568

Lees AD (1966) Photoperiodic timing mechanisms in insects. Nature 210:986–989. https://doi.org/10.1038/210986a0

Lees AD (1973) Photoperiodic time measurement in the aphid *Megoura viciae*. J Insect Physiol 19: 2279–2316. https://doi.org/10.1016/0022-1910(73)90237-0

Lewis RD, Saunders DS (1987) A damped circadian oscillator model of an insect photoperiodic clock. I. Description of the model based on a feedback control system. J Theor Biol 128:47–59. https://doi.org/10.1016/S0022-5193(87)80030-9

Marcovitch S (1923) Plant lice and light exposure. Science 58:537–538. https://doi.org/10.1126/science.58.1513.537-a

Masaki S (1967) Geographic variation and climatic adaptation in a field cricket (Orthoptera: Gryllidae). Evolution 21:725–741

Müller HJ (1955) Die Saisonformenbildung von *Arachnia levana*, ein photoperiodisch gesteueter Diapause-Effekt. Naturwissenschaften 42:134–135. https://doi.org/10.1007/BF00589421

Müller HJ (1960) Die Bedeutung der Photoperiode im Lebensablauf der Insekten. Z Amgew Entomol 47:7–24. https://doi.org/10.1111/j.1439-0418.1960.tb02820.x

Nakamura K, Numata H (2000) Photoperiodic control of the intensity of diapause and diapause development in the bean bug, *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae). Eur J Entomol 97:19–23. https://doi.org/10.14411/eje.2000.004

Nanda KK, Hamner KC (1958) Studies on the nature of the endogenous rhythm affecting photoperiodic response of Biloxi soybean. Bot Gaz 120:14–25. https://doi.org/10.1086/335992

Numata H (1993) Induction of adult diapause and of low and high reproductive states in a parasitoid wasp, *Ooencyrtus nezarae*, by photoperiod and temperature. Entomol Exp Appl 66:127–134. https://doi.org/10.1111/j.1570-7458.1993.tb00700.x

Numata H, Kobayashi S (1994) Threshold and quantitative photoperiodic responses exist in an insect. Experientia 50:969–971. https://doi.org/10.1007/BF01923489

Numata H, Shiga S (1995) Induction of adult diapause by photoperiod and temperature in *Protophormia terraenovae* (Diptera: Calliphoridae) in Japan. Environ Entomol 24:1633–1636. https://doi.org/10.1093/ee/24.6.1633

Numata H, Shiga S, Morita A (1997) Photoperiodic receptors in arthropods. Zool Sci 14:187–197. https://doi.org/10.2108/zsj.14.187

Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. Cold Spring Harb Symp Quant Biol 25:59–184. https://doi.org/10.1101/SQB.1960.025.01.015

Pittendrigh CS (1972) Circadian surfaces and the diversity of possible roles of circadian organization in photoperiodic induction. Proc Natl Acad Sci USA 69:2734–2737. https://doi.org/10.1073/pnas.69.9.2734

Pittendrigh CS, Minis DH (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. Am Nat 98:261–294. https://doi.org/10.1086/282327

Salman TS, Vesala L, Hoikkala A (2012) Photoperiodic regulation of life-history traits before and after eclosion: egg-to-adult development time, juvenile body mass and reproductive diapause in *Drosophila montana*. J Insect Physiol 58:1541–1547. https://doi.org/10.1016/j.jinsphys.2012.09.007

Saunders DS (1970) Circadian clock in insect photoperiodism. Science 168:601–603. https://doi.org/10.1126/science.168.3931.601

Saunders DS (1973a) Thermoperiodic control of diapause in an insect: theory of internal coincidence. Science 181:358–160. https://doi.org/10.1126/science.181.4097.358

Saunders DS (1973b) The photoperiodic clock in the flesh-fly, *Sarcophaga argyrostoma*. J Insect Physiol 19:1941–1954. https://doi.org/10.1016/0022-1910(73)90188-1

Saunders DS (1974) Evidence for 'dawn' and 'dusk' oscillators in the *Nasonia* photoperiodic clock. J Insect Physiol 20:77–88. https://doi.org/10.1016/0022-1910(74)90125-5

Saunders DS (1979) External coincidence and the photoinducible phase in the *Sarcophaga* photoperiodic clock. J Comp Physiol 132:179–189. https://doi.org/10.1007/BF00610722

Saunders DS (2002) Insect clocks, 3rd edn. Elsevier, Amsterdam

Saunders DS (2011) Unity and diversity of the insect photoperiodic mechanism. Entomol Sci 14:235–244. https://doi.org/10.1111/j.1479-8298.2011.00463.x

Saunders DS (2012) Insect photoperiodism: seeing the light. Physiol Entomol 37:207–218. https://doi.org/10.1111/j.1365-3032.2012.00837.x

Saunders DS, Lewis RD, Warman GR (2004) Photoperiodic induction of diapause: opening the black box. Physiol Entomol 29:1–15. https://doi.org/10.1111/j.1365-3032.2004.0369.x

Shintani Y, Shiga S, Numata H (2009) Different photoreceptor organs are used for photoperiodism in the larval and adult stages of a carabid beetle, *Leptocarabus kumagaii*. J Exp Biol 212:3651–3655. https://doi.org/10.1242/jeb.034033

Spieth HR, Sauer KP (1991) Quantitative measurement of photoperiods and its significance for the induction of diapause in *Pieris brassicae* (Lepidoptera, Pieridae). J Insect Physiol 37:231–238. https://doi.org/10.1016/0022-1910(91)90073-9

Sullivan CR, Wallace DR (1965) Interaction of temperature and photoperiod in the induction of prolonged diapause in *Neodiprion sertifer*. Can Entomol 99:834–850. https://doi.org/10.4039/Ent99834-8

Tanaka S (1978) Effects of changing photoperiod on nymphal development in *Pteronemobius nitidus* Boliver (Orthoptera, Gryllidae). Kontyû 46:135–151

Tauber MJ, Tauber CA (1970) Photoperiodic induction and termination of diapause in an insect: response to changing day lengths. Science 167:170. https://doi.org/10.1126/science.167.3915.170

Tauber MJ, Tauber CA (1972) Geographic variation in critical photoperiod and in diapause intensity of *Chrysopa carnea* (Neuroptera). J Insect Physiol 18:25–29. https://doi.org/10.1016/0022-1910(72)90061-3

Tauber MJ, Tauber CA (1973) Quantitative response to daylength during diapause in insects. Nature 244:296–297. https://doi.org/10.1038/244296a0

Tyshchenko VP (1966) Two-oscillatory model of the physiological mechanism of insect photoperiodic reaction. Zh Obshch Biol 27:209–222. (in Russian)

Tyshchenko VP, Lanevich VP, Gusanov O (1977) On correlations of quantitative and qualitative display of photoperiodism in *Barathra brassicae* L. (Lepidoptera). Zh Obshch Biol 38:264–276. (in Russian with English summary)

Van Der Kooi CJ, Stavenga DG, Arikawa K, Belušič G, Kelber A (2021) Evolution of insect color vision: from spectral sensitivity to visual ecology. Annu Rev Entomol 66:435–461. https://doi.org/10.1146/annurev-ento-061720-071644

Vaz Nunes M (1998) A double circadian oscillator model for quantitative photoperiodic time measurement in insects and mites. J Theor Biol 194:299–311. https://doi.org/10.1006/jtbi.1998.0767

Vaz Nunes M, Hardie J (1993) Circadian rhythmicity is involved in photoperiodic time measurement in the aphid *Megoura viciae*. Experientia 49:711–713. https://doi.org/10.1007/BF01923957

Vaz Nunes M, Saunders D (1999) Photoperiodic time measurement in insects: a review of clock models. J Biol Rhythm 14:84–104. https://doi.org/10.1177/074873049901400202

Vaz Nunes M, Veerman A (1982) Photoperiodic time measurement in the spider mite *Tetranychus urticae*: a novel concept. J Insect Physiol 28:1041–1053. https://doi.org/10.1016/0022-1910(82)90011-7

Veerman A, Helle W (1978) Evidence for the functional involvement of carotenoids in the photoperiodic reaction of spider mites. Nature 275:234. https://doi.org/10.1038/275234a0

Veerman A, Vaz Nunes M (1980) Circadian rhythmicity participates in the photoperiodic determination of diapause in spider mites. Nature 287:140–141. https://doi.org/10.1038/287140a0

Veerman A, Veenendaal RL (2003) Experimental evidence for a non-clock role of the circadian system in spider mite photoperiodism. J Insect Physiol 49:727–732. https://doi.org/10.1016/S0022-1910(03)00097-0

Vepsäläinen K (1974) Lengthening of illumination period is a factor in averting diapause. Nature 247:385–386. https://doi.org/10.1038/247385a0

Way MJ, Hopkins B, Smith PM (1949) Photoperiodism and diapause in insects. Nature 164:615. https://doi.org/10.1038/164615a0

Yoshida T, Kimura MT (1993) The photoperiodic clock of *Drosophila triauraria*: involvement of a circadian oscillatory system. J Insect Physiol 39:223–228. https://doi.org/10.1016/0022-1910(93)90092-6

Zaslavski VA (1970) Geographical races of *Chilocorus bipustulatus* L. (Coleoptera, Coccinellidae). I. Two types of photoperiodical reaction controlling the imaginal diapause in the northern race. Zool Zh 49:1354–1365. (in Russian with English summary)

Zaslavski VA (1988) Insect development: photoperiodic and temperature control. Springer, Berlin

Zhu DH, Tanaka S (2004) Summer diapause and nymphal growth in a subtropical cockroach: response to changing photoperiod. Physiol Entomol 29:78–83. https://doi.org/10.1111/j.1365-3032.2004.0368.x

Chapter 13
Molecular Mechanisms of Photoperiodism

Shin G. Goto

Abstract This chapter closely focuses on the molecular elements involved in each physiological module in photoperiodism, i.e., photoreceptors, a photoperiodic time measurement system, circadian clocks, and a counter. Multiple opsins are the photopigments in photoperiodism. *Drosophila*-type cryptochrome also acts as a photopigment, but further experiments are necessary. These photopigments may play distinct roles. A circadian clock consisting of circadian clock genes, such as *period*, *timeless*, *cycle*, *Clock*, and *mammalian-type cryptochrome*, controls the photoperiodic time measurement system. However, circadian clocks or each circadian clock gene itself also plays a critical role in other photoperiodic processes, which reside downstream of photoperiodic time measurement, and therefore careful interpretation is necessary. Possible neuropeptides and neurotransmitters are proposed as circadian output molecules. Pigment-dispersing factor (PDF), short neuropeptide F (sNPF), and glutamate are promising candidates. In contrast to recent advances in our understanding of the circadian clock, the molecular mechanisms of photoperiodic time measurement are still largely unknown. The counter system may be operated by biogenic amines, but this has been investigated in only a few species. Further extensive studies are awaited.

Keywords Circadian clock · Clock gene · Diapause · Photoperiodic response · Photoperiodic time measurement · Photopigment

13.1 Introduction

Insect photoperiodism comprises a sequence of several physiological modules (see Fig. 14.1). Photoreception is the first process in photoperiodism. Insects must be equipped with photoreceptors to receive environmental light. A circadian clock sets its phase based on the photic information from the photoreceptor and conveys the

S. G. Goto (✉)
Graduate School of Science, Osaka Metropolitan University, Osaka, Japan
e-mail: shingoto@omu.ac.jp

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temporal information to a photoperiodic time measurement system, which assesses the length of day or night. Simultaneously, insects count the number of long days and/or short days. This is operated by a photoperiodic counter. To be more precise, insects evaluate the photoperiod quantitatively, and therefore, they do not count the photoperiod qualitatively. When the hypothetical diapause titer exceeds an internal threshold in the counter system, organisms activate or inactivate endocrine effectors to induce or avert photoperiodic events. The most well-known photoperiodic phenotype is diapause, which is genetically and hormonally regulated developmental arrest or suspension. The key hormones regulating diapause are the diapause hormone, juvenile hormone, ecdysteroids, and insulin (or insulin-like peptide). It is beyond the scope of this review to describe the endocrine effectors in detail, so only one important reference is given here (Denlinger 2022). In this chapter, I introduce the molecular elements involved in each event, with special emphasis on the role of circadian clock genes, which have received much attention in the last decade.

13.2 Photoreceptors

Insects use two types of photopigments: opsins and a cryptochrome (see Chap. 3). Opsin comprises an opsin protein and vitamin A-based chromophore retinal or 3-hydroxyretinal. Opsins in the visual system mainly contribute to color vision (Van Der Kooi et al. 2021); however, some opsins are also found in the nonvisual system and play a role other than color vision, such as photic entrainment of the circadian clock (Senthilan et al. 2019). Spectral sensitivity is determined by specific amino acid side chains in the opsin protein. Insects have multiple opsins covering a broad range of wavelengths (Van Der Kooi et al. 2021). There are two cryptochrome genes in the genomes of most insect species; one cryptochrome acts as a photoreceptor, but the other does not. The photosensitive cryptochrome is called the *Drosophila*-type cryptochrome (abbreviated as CRY-d, dCRY, or CRY1). CRY-d absorbs light of a short wavelength from UV to blue (Berndt et al. 2007; Song et al. 2007) and is the major photopigment that resets the circadian clock in *Drosophila melanogaster* (Emery et al. 1998, 2000). The cryptochrome insensitive to light is called the mammalian-type cryptochrome (abbreviated as CRY-m, mCRY, or CRY2). It acts as a transcriptional repressor in the circadian clocks (Yuan et al. 2007; Tokuoka et al. 2017). Higher dipterans, including *D. melanogaster*, lost the *cry-m* gene, while hymenopterans and coleopterans lost the *cry-d* gene during the course of their evolution (Kotwica-Rolinska et al. 2022a).

Classic dietary-deficient experiments suggest the involvement of a vitamin A-based pigment, i.e., opsin, in photoperiodism [see Goto et al. (2010) for review]. Genetic approaches also support this assumption. Four albino mutants in the two-spotted spider mite *Tetranychus urticae*, in which uptake and oxidative metabolism of carotenoids are blocked, failed to enter diapause irrespective of photoperiod (Veerman 1980). However, partial restoration of the photoperiodic response was obtained after the addition of β-carotene to the diet, and full restoration was observed

after the addition of vitamin A (Bosse and Veerman 1996). The bulked segregant analysis with the high-throughput genomic sequencing data revealed that mutations in a single gene encoding phytoene desaturase, which is involved in carotenoid biosynthesis, result in complete albinism in *T. urticae* and the citrus red mite, *Panonychus citri*. Furthermore, genome editing revealed that the gene is essential for photoperiodic induction in *T. urticae* (Bryon et al. 2017). The loss-of-function mutant of *neither inactivation nor after potential B* (*ninaB1*), which encodes the rate-limiting enzyme converting carotenoids into retinaldehyde, was established in the monarch butterfly, *Danaus plexippus.* The null mutant females lost the ability to respond to short days; they developed their ovaries irrespective of photoperiod (Iiams et al. 2019).

RNAi directed to opsin genes was performed in the cricket *Modicogryllus siamensis.* The compound eyes are the site of photoperiodic photoreception in this species (Sakamoto and Tomioka 2007). RNAi directed to three opsins (UV-, blue-, and long wavelength-sensitive opsins), which are expressed in the compound eyes, resulted in partial disruption of the long-day response in the duration of the nymphal period and the number of molting until adult emergence. Interestingly, RNAi of UV-sensitive opsins also disrupted the short-day response in these characteristics, whereas RNAi of other opsins did not show distinct effects (Tamaki et al. 2013). These results indicate that multiple opsins are photopigments in the photoperiodic response, but they may play different roles.

The role of CRY-d in the photoperiodic response was also examined in *M. siamensis* via RNAi (Ueda et al. 2018). RNAi directed to *cry-d* partially prevented the long-day response. In contrast, unexpectedly, the same RNAi enhanced the short-day response under short days (Ueda et al. 2018). It is important to note that the function of CRY-d in crickets appears to be different from that in other insect species. The cricket CRY-d is considered to be the core element of the circadian clock as well as the photopigment playing a minor role in photoentrainment (Tokuoka et al. 2017; Kutaragi et al. 2018). Thus, it is still unknown how CRY-d is involved in the photoperiodic response in *M. siamensis*. Additional experiments in the species of which CRY-d plays a major role in light perception are needed.

13.3 Photoperiodic Time Measurement

Bünning (1936) first proposed that photoperiodic time measurement is one of the functions of the circadian clock. The circadian clock is a biological time-keeping system that controls biological rhythms with a period of approximately 24 h. Involvement of the circadian clock in photoperiodic time measurement is now widely accepted not only in insects but also in other organisms from fungi to mammals (Nelson et al. 2010). Based on the range of responses in various insect species, a dozen photoperiodic time measurement models have been proposed (Vaz Nunes and Saunders 1999). Among them, two of the most influential models are

external coincidence and internal coincidence (Saunders 2021). In brief, the external coincidence model postulates the involvement of a single circadian clock that is entrainable to light and positions the photoinducible phase (φ_i) at the late scotophase (Pittendrigh and Minis 1964). φ_i is critical for assessing photoperiods. During summer, a longer light phase delays the phase of the clock, and thus φ_i falls in the photophase, which elicits a long-day response. During autumn, φ_i falls in scotophase, which induces a short-day response. The internal coincidence model postulates the involvement of two circadian oscillators: one is the dawn oscillator being entrainable to dawn or light-on, and the other is the dusk oscillator being entrainable to dusk or light-off. The phase relationships of these oscillators determine the response (Tyshchenko 1966).

The photoperiodic time measurement system is still highly conceptual, and its molecular mechanisms are largely unknown. In the species in which photoperiodic time measurement meets the external and internal coincidence models, molecular dissections of the processes occurring at φ_i and under the specific phase relationships, respectively, have long been awaited.

13.4 Circadian Clocks Involved in Photoperiodic Time Measurement

The circadian clock regulating circadian behavior is established by interlocked transcription-translation negative feedback loops consisting of circadian clock genes and their protein products. The circadian clock genes include *period* (*per*), *timeless* (*tim*), *cry-m*, *cycle* (*cyc*), *Clock* (*Clk*), *Par domain protein 1* (*Pdp1*), *vrille*, and *clockwork orange* to form several loops. In one loop, the CYCLE (CYC)/CLOCK (CLK) heterodimer promotes transcription of *per*, *tim*, *cry-m*, and other output genes, whereas the PER/TIM heterodimer suppresses CYC/CLK activity. Thus, *cyc* and *Clk* and their protein products are regarded as positive regulators, whereas *per*, *tim*, and *cry-m* and their protein products are negative regulators. For further details, see Chap. 4.

13.4.1 A Case of the Model Insect Drosophila melanogaster

Saunders et al. (1989) first used circadian clock mutants to ascertain the involvement of circadian clock genes in the photoperiodic response. Adult females of *D. melanogaster* developed their ovaries under long days, while they suppressed ovarian development under short days at a low temperature of 12 or 10 °C. Three null mutants of *per*, which show arrhythmicity in adult eclosion and adult locomotor activity, were capable of discriminating between long and short days, suggesting that *per* is not causally involved in photoperiodic time measurement.

The results of this landmark study are highly influential. However, we must bear in mind that the photoperiodic response of *D. melanogaster* is quite weak and observed only at a low temperature very close to the lower limit of their development. Furthermore, several studies revealed that temperature plays a major role in diapause induction and that photoperiod has no or little effect in this species (Emerson et al. 2009; Anduaga et al. 2018; Erickson et al. 2020). Although we have learned many from this species (Sandrelli et al. 2007; Tauber et al. 2007; Nagy et al. 2019; Abrieux et al. 2020; Meiselman et al. 2022), we must be very cautious about whether the information applies to the photoperiodic response in other insect species.

13.4.2 Cases of Species Other than Drosophila melanogaster

During the past decade, gene silencing and knockout techniques have been applied to ascertain the possible role of circadian clock genes in photoperiodism in various insect species with clear photoperiodic responses, other than *D. melanogaster*. RNAi targeted to clock or clock-related genes have been performed in ten species of six orders, i.e., *M. siamensis* and the band-legged ground cricket, *Dianemobius nigrofasciatus*, in Orthoptera (Sakamoto et al. 2009; Ueda et al. 2018; Goto and Nagata 2022); the bean bug, *Riptortus pedestris*, the brown-winged green bug, *Plautia stali*, and the linden bug, *Pyrrhocoris apterus*, in Hemiptera (Ikeno et al. 2010, 2011a, b, 2013; Bajgar et al. 2013; Omura et al. 2016; Urbanová et al. 2016; Kotwica-Rolinska et al. 2017; Tamai et al. 2019; Dong et al. 2021; Hasebe and Shiga 2021, 2022; Hasebe et al. 2022; Mano and Goto 2022); the jewel wasp, *Nasonia vitripennis*, in Hymenoptera (Mukai and Goto 2016; Dalla Benetta et al. 2019); the cabbage beetle, *Colaphellus bowringi*, in Coleoptera (Zhu et al. 2019); the Chinese oak silkmoth, *Antheraea pernyi*, in Lepidoptera (Mohamed et al. 2014); and the drosophilid fly *Chymomyza costata* and the Northern house mosquito, *Culex pipiens*, in Diptera (Pavelka et al. 2003; Meuti et al. 2015; Chang and Meuti 2020). Genome editing to disrupt clock gene function has been performed in three species in two orders, i.e., the commercial silkmoth, *Bombyx mori*, *D. plexippus*, and *P. apterus* (Iiams et al. 2019; Cui et al. 2021; Ikeda et al. 2021; Kotwica-Rolinska et al. 2022b; Tobita and Kiuchi 2022). These studies cover long-day species that develop or reproduce under long days (the most studied species) and short-day species that develop or reproduce under short days (*C. bowringi*). The studies also cover diapause in all developmental stages, i.e., embryo (*B. mori* and *D. nigrofasciatus*), larva (*C. costata* and *N. vitripennis*), nymph (*M. siamensis*), pupa (*A. pernyi*), and adult (*R. pedestris*, *P. stali*, *P. apterus*, *C. bowringi*, *D. plexippus*, and *C. pipiens*).

Riptortus pedestris shows clear photoperiodic responses; adults develop their reproductive organs and accumulate a small amount of lipids under long days, whereas they suppress their reproductive organ development and accumulate a large amount of lipids under short days (Numata and Hidaka 1982; Numata and

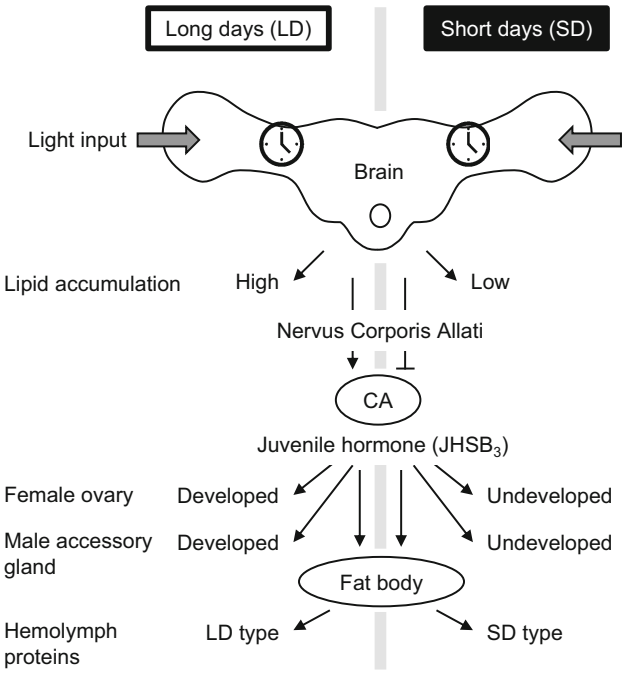


Fig. 13.1 The photoperiodic responses and the regulatory pathways in *Riptortus pedestris*. The responses are divided into juvenile hormone (JH)-independent (lipid accumulation) and JH-dependent (reproductive organ development and hemolymph protein expression) pathways. *CA* corpus allatum

Kobayashi 1989; Kobayashi and Numata 1993; Morita et al. 1999). The reproductive arrest is primarily caused by reduced activity of the corpus allatum (CA), which secretes juvenile hormone (JH) (Numata and Hidaka 1984; Morita and Numata 1997; Hirai et al. 1998; Ikeno et al. 2010; Dong et al. 2021; Mano and Goto 2022). On the other hand, photoperiodic lipid accumulation is independent of the CA (Morita et al. 1999). Thus, reproductive organ development and lipid accumulation are regulated by distinct endocrine effectors in this species (Fig. 13.1).

RNAi directed to the negative regulators in the circadian clock (*per* and *cry-m*) resulted in the development of the reproductive organs (female ovary and male accessory gland), even under diapause-inducing short days, whereas that directed to the positive regulators (*cyc* and *Clk*) resulted in suppression of the development even under diapause-averting long days in *R. pedestris* (Fig. 13.2a) (Ikeno et al. 2010, 2011a, 2013; Omura et al. 2016). The different phenotypes induced by silencing negative and positive regulators indicate that the clock arrested at specific phases activates distinct downstream cascades that control the photoperiodic response. This supports the idea that the circadian clock is involved in photoperiodic discrimination, i.e., photoperiodic time measurement (Goto 2022). Interestingly, the same responses are observed in lipid accumulation. RNAi directed to the negative

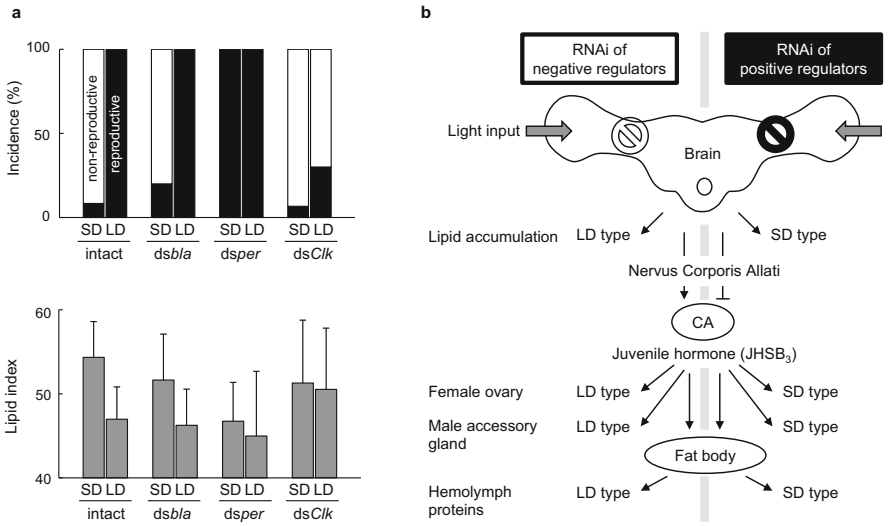


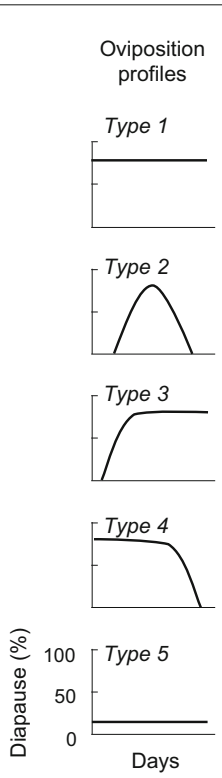
Fig. 13.2 Effects of RNAi targeted to *per* and *Clk* on ovarian development (**a**, upper panel) and lipid accumulation (**a**, lower panel; mean ± standard deviation) and phenotypes after RNAi targeted to the negative (*per* or *cry-m*) and positive (*cyc* or *Clk*) regulators of the circadian clock (**b**) in *Riptortus pedestris*. *bla*, *β-lactamase* (a control gene); *CA* corpus allatum, *ds* double-stranded RNA, *LD* long day, *SD* short day. Based on Omura et al. (2016)

regulator (*per*) resulted in the accumulation of a small amount of lipid even under short days, whereas that directed to the positive regulator (*Clk*) resulted in the accumulation of a large amount of lipid even under long days (Fig. 13.2a) (Omura et al. 2016). These results indicate that the process involving these clock genes resides in an upstream photoperiodic cascade and governs both photoperiodic responses (Fig. 13.2b). Distinct phenotypes induced by silencing negative and positive regulators were also observed in photoperiodic ovarian development in *D. plexippus* (Iiams et al. 2019).

Nasonia vitripennis shows a clear maternal effect. Females exposed to long days lay eggs that develop into adulthood without interruption, i.e., the nondiapause producer. In contrast, females maintained under short days lay eggs that are destined to enter larval diapause, i.e., the diapause producer. *per* RNAi disrupts photoperiodic induction of diapause, although *per* RNAi wasps are still able to become diapause producers in response to chilling (Mukai and Goto 2016). The results suggest that *per* is possibly involved in an upstream cascade regulating photoperiodism, such as photoperiodic time measurement, but not in a downstream cascade determining diapause-destined egg production. In *P. apterus*, *Clk* RNAi does not affect the reproduction of males under long days. However, it destroys the ability of diapause males to switch to the reproductive mode after transfer to long days (Urbanová et al. 2016). The results strongly support that *Clk* is involved in photoperiodic time measurement but not in diapause itself.

The involvement of the circadian clock or circadian clock genes independent of the clock (gene pleiotropy) in a downstream process is also proposed in some species. In *D. nigrofasciatus*, for example, RNAi directed to *Clk* revealed possible involvement of the gene not only in the photoperiodic time measurement but also in the diapause-egg production process (Goto and Nagata 2022). This species shows a clear maternal effect, in which females exposed to long days lay nondiapause eggs that develop into nymphs without interruption. In contrast, females maintained under short days lay eggs that are destined to enter diapause (Goto et al. 2008). When females are transferred from short days to long days, they gradually reduce and increase the numbers of diapause and nondiapause eggs laid, respectively, thereby resulting in a gradual reduction in the incidence of diapause (Kidokoro and Masaki 1978). This suggests that mother crickets monitor and assess photoperiod for several days or weeks and, on the basis of this assessment, determine the developmental trajectory of their offspring. Thus, if the circadian clock controls photoperiodic time measurement, it is likely that the dysfunction of the clock would be manifested by a gradual change in the incidence of diapause. In contrast, if the clock controls a downstream process of photoperiodic time measurement, it is assumed that the effects of dysfunction on the operation of the clock would be observed immediately.

To clarify this, females reared under short days were injected with double-stranded RNA (dsRNA) of a control gene *β-lactamase* (*bla*) or *Clk* and were continuously maintained under short days or transferred to long days (Goto and Nagata 2022). The observed oviposition profiles largely varied but could be categorized into five types (Fig. 13.3). All control crickets under short days deposited diapause eggs throughout the experimental period (*Type 1*), a typical short-day response. More than half of control crickets under long days gradually decreased diapause incidences (*Type 4*), a typical long-day response. The oviposition profiles of *Clk* RNAi crickets were distinct from those of control crickets. Under short days, most (9 out of 12) immediately deposited nondiapause eggs (*Type 2* and *Type 3*), and 6 out of 12 deposited those at the late stage of the experimental period (*Types 2* and *4*). Under long days, three out of nine *Clk* RNAi crickets were categorized as *Type 4*, a typical long-day response, whereas the remaining six were categorized as *Type 2*. The laying of nondiapause eggs by late-stage *Clk* RNAi females under short days appears to be similar to that of control crickets under long days. Collectively, these results indicate that *Clk* is involved in photoperiodic time measurement in *D. nigrofasciatus*. However, the immediate laying of nondiapause eggs by *Clk* RNAi females is unique. Such immediate effects cannot be explained in terms of the dysfunction of a circadian clock involved in photoperiodic time measurement. This immediate response might indicate the involvement of *Clk* in the production of diapause eggs. It is, however, uncertain why the immediate effect of producing nondiapause eggs did not persist in *Clk* RNAi crickets throughout the experimental period, as observed in the *Type 2* profile, although a plausible explanation is that with respect to the laying of diapause eggs, the RNAi effect persists only for a few days. Moreover, it has yet to be ascertained whether *Clk* regulates the production of diapause eggs as a component of the circadian clock or independently of the circadian clock (gene pleiotropy) (Goto and Nagata 2022).



Oviposition profiles	No. individuals			
	Short days		Long days	
	ds*bla*	ds*Clk*	ds*bla*	ds*Clk*
Type 1	5	1	3	0
Type 2	0	4	0	6
Type 3	0	5	0	0
Type 4	0	2	5	3
Type 5	0	0	1	0
Total no.	5	12	9	9

Fig. 13.3 Oviposition profiles and the number of females showing the profiles in *Dianemobius nigrofasciatus*. Females were injected with *β-lactamase* (*bla*) as a control gene or *Clock* (*Clk*) double-stranded (ds) RNA and reared under short- or long-day conditions. Based on Goto and Nagata (2022)

In *Drosophila triauraria*, crossing between a northern population with a clear photoperiodic response and southern strains with no photoperiodic ability revealed that the additive association of *tim* and *cry-d* alleles affected diapause incidence (Yamada and Yamamoto 2011). The lack of an interaction between these alleles, but their additive association, suggests that these genes are independently involved in the diapause program, i.e., noncircadian function. In the gut of *P. apterus*, *cry-m* and *Pdp1* play important noncircadian functions in diapause-/nondiapause-specific gene expression under the control of *Clk* and *cyc* as well as JH, which directly determines the diapause/nondiapause phenotype (Bajgar et al. 2013). The role of circadian clock genes in insect photoperiodism is further discussed by Goto (2022).

13.4.3 Circadian Output Signaling

Circadian clock output signal elements that regulate the photoperiodic response are still largely unknown, although the output elements regulating circadian behaviors have been extensively studied in *D. melanogaster* (King and Sehgal 2020). In *D. melanogaster*, many neuropeptides and neurotransmitters are detected in circadian clock neurons in the brain and are obvious candidate clock outputs, including pigment-dispersing factor (PDF), short neuropeptide F (sNPF), neuropeptide F (NPF), diuretic hormone 31 (DH31), ion transport peptide (ITP), neuropeptide-like precursor (NPLP1 or IPNamide), glycine, glutamate, and acetylcholine (Shafer et al. 2006; Johard et al. 2009; Hermann et al. 2012; Hermann-Luibl et al. 2014; Fujiwara et al. 2018; King and Sehgal 2020). Among them, PDF is one of the most important output factors in the clock network in *D. melanogaster*. RNAi directed to *pdf* induced a short-day response under long days in photoperiodic regulation of egg maturation and lipid accumulation in *C. pipiens* (Meuti et al. 2015). In *P. stali*, RNAi-mediated knockdown of *pdf* canceled oviposition arrest induced by the transfer from long- to short-day conditions and delayed oviposition onset after the change from short- to long-day conditions (Hasebe et al. 2022). Disruption of the photoperiodic responses by *pdf* RNAi indicates PDF as the important circadian clock output signal element. However, this is not the case in *R. pedestris*. The photoperiodic response is disrupted by surgical removal of the anterior proximal medulla region of the optic lobe where PER-immunoreactive clock cells reside in this species (Ikeno et al. 2014; Koide et al. 2021). However, PDF does not colocalize with PER, and RNAi directed to *pdf* does not affect the photoperiodic response in *R. pedestris* (Ikeno et al. 2014; Koide et al. 2021). These results indicate that PDF is not, but other neurotransmitters or neuropeptides expressed in the clock cells may be, involved in the photoperiodic response in this species. RNAi directed to *sNPF*, *NPF*, *Dh31*, *ITP*, *Nplp1*, and *choline acetyltransferase* (*ChAT*) also did not affect the photoperiodic response in *R. pedestris* (Des Marteaux et al. 2022). However, RNAi directed to *vesicular glutamate transporter* (*VGlut*), which determines the glutamate content of synaptic vesicles, weakly affected the photoperiodic response; *VGlut* RNAi females induced ovarian development even under diapause-inducing short days (Des Marteaux et al. 2022). Recently, Hasebe and Shiga (2022) demonstrated that extracellular glutamate dynamics in the brain are photoperiodically regulated by the clock gene and play an essential role in the photoperiodic control of reproduction via inhibitory pathways. These results suggest that glutamate is a promising candidate in the circadian output of *R. pedestris*.

Three mutants that lack PDF function were established in *P. apterus* by genome editing technology (Kotwica-Rolinska et al. 2022b). Females of the wild-type strain are reproductive under LD 17:7 h, while they enter reproductive diapause under LD 16:8 h. In contrast, females of these *pdf* null mutants failed to enter diapause under LD 16:8 h. These results indicate that PDF is the diapause-promoting factor. However, interestingly, females of these *pdf* null mutants successfully entered diapause when they had been reared under much shorter days, i.e., the shorter critical

day length, indicating not only that PDF is an important player but also that other crucial circadian clock-dependent factors are also involved in the photoperiodic response of this species (Kotwica-Rolinska et al. 2022b).

In diapausing *A. pernyi* pupae, the circadian clock cells with PER, CLK, and CYC in the brain are immunoreactive with antisera against melatonin and arylalkylamine *N*-acetyltransferase (NAT), the rate-limiting enzyme in melatonin synthesis. Gene expression of *nat* and melatonin amounts in the brain are clock-regulated. RNAi targeted to *per* upregulates the brain melatonin content and terminates diapause, and RNAi targeted to *nat* maintains diapause. The cells expressing the prothoracicotropic hormone (PTTH), which triggers ecdysteroidogenesis in the prothoracic gland to terminate diapause, are immunoreactive with antisera against the melatonin receptor. These results suggest that melatonin is an important mediator between the circadian clock and the endocrine effector. The plausible hypothesis is that higher melatonin content in the brain caused by upregulation of *nat* under short days suppresses the activity of PTTH cells (Mohamed et al. 2014).

In *D. melanogaster*, PDF and sNPF in clock neurons inhibit reproductive dormancy by maintaining insulin-producing cells (IPCs) in an active state (Nagy et al. 2019). In addition, a subset of clock neurons (DN3s) that express the neuropeptide allatostatin C (AstC) facilitates recovery from cold-induced reproductive dormancy. The stimulatory effect of AstC on egg production, independently of the insulin pathway, is mediated by cholinergic AstC receptor-2 neurons (Meiselman et al. 2022). Although it is still unknown whether the cold-induced dormancy in *D. melanogaster* is comparable to photoperiodic diapause in other insect species, AstC may also be a candidate for the circadian clock output that regulates the photoperiodic response.

13.5 Photoperiodic Counter

The photoperiodic counter registers the number of photoperiodic cycles during the photoperiod-sensitive period. According to the numbers counted until the end of the sensitive period, the counter system conveys the information to the endocrine effectors to elicit a physiological response. In a model of photoperiodic summation, insects accumulate a hypothetical "diapause titer" in the counter system according to the number of short days that individuals have experienced (Gibbs 1975). This putative substance can accumulate quantitatively in a photoperiod-dependent manner, and such a photoperiod-dependent accumulation of the putative substance is now incorporated into the model (Tagaya et al. 2010).

Although the molecular components of the photoperiodic counter have received little attention, some clues can be drawn from the cabbage armyworm, *Mamestra brassicae*, as well as *A. pernyi*. Dopamine accumulates under short days in the hemolymph and the brain of the prepupae and early pupae of *M. brassicae*. Furthermore, diapause was induced even under long days when the final instar larvae were fed with the dopamine precursor $_{L}$-dihydroxyphenylalanine ($_{L}$-DOPA) (Noguchi and

Hayakawa 1997). These results indicate that dopamine acts as a putative diapause-promoting substance. The same author found that *B. mori* female larvae fed $_{L}$-DOPA become diapause producers that deposit diapause eggs (Noguchi and Hayakawa 2001). Moreover, in *M. brassicae*, the *receptor for activated protein kinase C* (*Rack*) gene, which is expressed in several cells around the medial protocerebral neuropile, was upregulated in response to short days as well as dopamine treatment (Uryu et al. 2003). Rack may act as a diapause substance by binding to and stimulating the nuclear translocation of protein kinase C. However, successive studies have not been performed to verify its role.

In the brain and subesophageal ganglion complex of diapausing *A. pernyi* pupae, transcriptional downregulation of one of the serotonin (5-hydroxytryptamine, 5HT) receptors, $5HTR_B$, in response to the number of long-day exposure, was detected. $5HTR_B$ is expressed in PTTH cells, and RNAi targeted to $5HTR_B$ results in diapause termination (Wang et al. 2013). Furthermore, by the transition from short days to long days, the transcriptional upregulation of *nat* and the transcriptional downregulation of DOPA decarboxylase (DDC), the rate-limiting enzyme to produce dopamine, are accompanied in the brains of diapausing pupae. Melatonin and flupentixol, a dopamine receptor antagonist, terminate diapause, while dopamine and luzindole, a melatonin receptor antagonist, maintain diapause. These results suggest that dopamine and melatonin are the key molecules involved in the photoperiodic counter and could potentially function through mutual inhibition (Wang et al. 2015a). Thus, in *A. pernyi*, $5HTR_B$, melatonin, and dopamine are promising elements involved in the photoperiodic counter.

The possible significance of melatonin in photoperiodism is also proposed in the pea aphid *Acyrthosiphon pisum*. This species reproduces by viviparous parthenogenesis under long days, while reproducing sexually to enter embryonic diapause under short days, i.e., the holocyclic life cycle (see Chap. 15). Alongside the cycle, there are naturally occurring anholocyclic lineages, i.e., no photoperiodic response. The anholocyclic lineage does not respond to changes in photoperiod and reproduces parthenogenetically year-round. Melatonin levels are significantly higher in holocyclic aphids reared under short days than under long days, while no differences were found between anholocyclic aphids under the same conditions (Barberà et al. 2020).

13.6 Omics Approaches

The diapause program is characterized by three ecophysiological phases: prediapause, diapause, and postdiapause (Koštál 2006). During the prediapause phase, insects are sensitive to environmental cue(s) and decide whether to enter diapause. Thus, photoreception, photoperiodic time measurement, and photoperiodic counting are performed during this phase. During the diapause phase, which is operated by the endocrine effector, metabolism is reduced, and direct development is arrested. Finally, during the postdiapause phase, insects terminate diapause, and

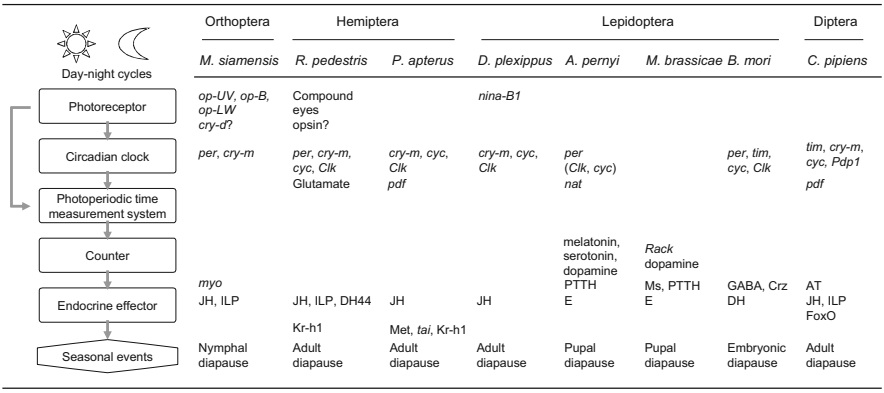
postdiapause direct development is resumed. Recently, numerous omics approaches have been adopted to clarify the mechanisms underlying photoperiodic diapause. Most of them approach mechanisms operating during the diapause phase. Of course, they are very informative, but we could perceive little about the central mechanisms in photoperiodism from the dataset, i.e., photoreception, photoperiodic time measurement, and photoperiodic counting. Studies on the prediapause phase, especially those focusing on events in the brain, are limited.

One example is the comparative transcriptomic analysis in *C. costata* (Poupardin et al. 2015; Koštál et al. 2017). This species discriminates photoperiod at the larval stage and enters diapause at the late third larval instar. The early third instar larvae destined to diapause upregulated the genes involved in the metabolism of lipids, amino acids, and organic acids and development of chitin-based cuticle, while they downregulated the genes involved in the development, cell division cycle, and DNA replication (Poupardin et al. 2015). Interestingly, a large variation in gene expression was also observed by transferring from long days to short days, which includes upregulation of genes involved in processing in the endoplasmic reticulum, the stress response mediated by heat-shock protein, metabolism of lipids and organic acids, and cuticle development and downregulation of genes involved in microtubular transport, spermatogenesis, mRNA processing, and the enzymatic complex of mitochondrial TCA cycles (Poupardin et al. 2015). In this species, however, the diapause induction phase (the decision for diapause has just been taken but the diapause phenotype is not yet expressed) and diapause initiation phase (the diapause phenotype starts to be expressed) appear to be overlapping, and thereby, it is still difficult to understand the significance of the processes detected from the context of the photoperiodic cascade.

The blow fly, *Protophormia terraenovae*, enters reproductive diapause in response to short days and a low temperature. The median region of the brain, where the neurons verified to be indispensable for the photoperiodic response reside, was collected at the very early adult stages under diapause-inducing and diapause-averting conditions. RNA-seq analysis revealed that 44.1% and 55.9% among 549 contigs are specifically upregulated under diapause-inducing and diapause-averting conditions, respectively (Hase et al. 2017). One of these genes is the angiotensin-converting enzyme (ACE), whose expression significantly increased 3 days after changing the photoperiod from long days to short days. ACE is supposed to have peptidase activities that inactivate the neuropeptide. Antisera against *P. terraenovae* ACE stained one pair of cells lateral to the esophageal foramen. How ACE is involved in photoperiodism and which photoperiodic process involves it are still unknown.

13.7 Concluding Remarks

We have accumulated information on the molecular mechanisms underlying photoperiodism, but the information is still fragmented (Fig. 13.4). Although multiple opsins play a role as photoreceptor molecules, the role of CRY-d in photoperiodic photoreception is still unclear. A circadian clock consisting of circadian clock genes is involved in photoperiodic time measurement. However, the clock also plays an important role in other processes. The molecular mechanisms underpinning photoperiodic time measurement are largely unknown. We know very little about the counter, but biogenic amines are possibly involved in the process. Although omics technologies are powerful for the clarification of numerous processes possibly involved in photoperiodism, they show only correlations. Functional analyses with the aid of RNAi or genome editing are needed to clarify how the detected genes and processes are involved in the photoperiodic response. Furthermore, we used a limited number of species to dissect the mechanisms, and therefore, we still do not know whether the molecular elements that we have clarified are indeed applicable to other insect species. Extensive studies with various insect species are very welcomed.



	Orthoptera	Hemiptera		Lepidoptera				Diptera
	M. siamensis	*R. pedestris*	*P. apterus*	*D. plexippus*	*A. pernyi*	*M. brassicae*	*B. mori*	*C. pipiens*
Photoreceptor	*op-UV, op-B, op-LW cry-d?*	Compound eyes opsin?		*nina-B1*				
Circadian clock	*per, cry-m*	*per, cry-m, cyc, Clk* Glutamate	*cry-m, cyc, Clk pdf*	*cry-m, cyc, Clk*	*per* (*Clk, cyc*) *nat*		*per, tim, cyc, Clk*	*tim, cry-m, cyc, Pdp1 pdf*
Counter					melatonin, serotonin, dopamine	*Rack* dopamine		
Endocrine effector	*myo* JH, ILP	JH, ILP, DH44 Kr-h1	JH Met, *tai*, Kr-h1	JH	PTTH E	Ms, PTTH E	GABA, Crz DH	AT JH, ILP FoxO
Seasonal events	Nymphal diapause	Adult diapause	Adult diapause	Adult diapause	Pupal diapause	Pupal diapause	Embryonic diapause	Adult diapause

Fig. 13.4 Physiological modules that constitute insect photoperiodism and possible molecular elements involved in these modules. *Modicogryllus siamensis* (Sakamoto et al. 2009; Tamaki et al. 2013; Ueda et al. 2018; Miki et al. 2020), *Riptortus pedestris* (Ikeno et al. 2010, 2011a, b, 2013; Omura et al. 2016; Dong et al. 2021; Hasebe and Shiga 2021, 2022; Des Marteaux et al. 2022; Mano and Goto 2022), *Pyrrhocoris apterus* (Hejnikova et al. 2016; Urbanová et al. 2016; Kotwica-Rolinska et al. 2017, 2022b; Hejníková et al. 2022), *Danaus plexippus* (Herman 1981; Iiams et al. 2019), *Antheraea pernyi* (Wang et al. 2013, 2015a, b; Mohamed et al. 2014), *Mamestra brassicae* (Noguchi and Hayakawa 1997; Uryu et al. 2003; Mizoguchi et al. 2013; Yamada et al. 2017), *Bombyx mori* (Yamashita 1996; Noguchi and Hayakawa 2001; Shiomi et al. 2015; Cui et al. 2021; Ikeda et al. 2021; Tsuchiya et al. 2021; Tobita and Kiuchi 2022), and *Culex pipiens* (Sim and Denlinger 2008, 2009, 2013; Kang et al. 2014; Meuti et al. 2015; Chang and Meuti 2020)

References

Abrieux A, Xue Y, Cai Y, Lewald KM, Nguyen HN, Zhang Y et al (2020) EYES ABSENT and TIMELESS integrate photoperiodic and temperature cues to regulate seasonal physiology in *Drosophila*. Proc Natl Acad Sci USA 117:15293–15304. https://doi.org/10.1073/pnas.2004262117

Anduaga AM, Nagy D, Costa R, Kyriacou CP (2018) Diapause in *Drosophila melanogaster*—photoperiodicity, cold tolerance and metabolites. J Insect Physiol 105:46–53. https://doi.org/10.1016/j.jinsphys.2018.01.003

Bajgar A, Jindra M, Dolezel D (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc Natl Acad Sci USA 110:4416–4421. https://doi.org/10.1073/pnas.1217060110

Barberà M, Escrivá L, Collantes-Alegre JM, Meca G, Rosato E, Martínez-Torres D (2020) Melatonin in the seasonal response of the aphid *Acyrthosiphon pisum*. Insect Sci 27:224–238. https://doi.org/10.1111/1744-7917.12652

Berndt A, Kottke T, Breitkreuz H, Dvorsky R, Hennig S, Alexander M et al (2007) A novel photoreaction mechanism for the circadian blue light photoreceptor *Drosophila* cryptochrome. J Biol Chem 282:13011–13021. https://doi.org/10.1074/jbc.M608872200

Bosse TC, Veerman A (1996) Involvement of vitamin A in the photoperiodic induction of diapause in the spider mite *Tetranychus urticae* is demonstrated by rearing an albino mutant on a semi-synthetic diet with and without β-carotene or vitamin A. Physiol Entomol 21:188–192. https://doi.org/10.1111/j.1365-3032.1996.tb00854.x

Bryon A, Kurlovs AH, Dermauw W, Greenhalgh R, Riga M, Grbic M et al (2017) Disruption of a horizontally transferred phytoene desaturase abolishes carotenoid accumulation and diapause in *Tetranychus urticae*. Proc Natl Acad Sci USA 114:E5871–E5880. https://doi.org/10.1073/pnas.1706865114

Bünning E (1936) Die endonome Tagesrhythmik als Grundlage der photoperiodischen Reaktion. Ber Dtsch Bot Ges 54:590–607

Chang V, Meuti ME (2020) Circadian transcription factors differentially regulate features of the adult overwintering diapause in the Northern house mosquito, *Culex pipiens*. Insect Biochem Mol Biol 121:103365. https://doi.org/10.1016/j.ibmb.2020.103365

Cui W-Z, Qiu J-F, Dai T-M, Chen Z, Li J-L, Liu K et al (2021) Circadian clock gene *period* contributes to diapause via GABAeric-diapause hormone pathway in *Bombyx mori*. Biology (Basel) 10:842. https://doi.org/10.3390/biology10090842

Dalla Benetta E, Beukeboom LW, Van De Zande L (2019) Adaptive differences in circadian clock gene expression patterns and photoperiodic diapause induction in *Nasonia vitripennis*. Am Nat 193:881–896. https://doi.org/10.1086/703159

Denlinger DL (2022) Insect diapause. Cambridge University Press, Cambridge

Des Marteaux L, Xi J, Mano G, Goto SG (2022) Circadian clock outputs regulating insect photoperiodism: a potential role for glutamate transporter. Biochem Biophys Res Commun 589:100–106. https://doi.org/10.1016/j.bbrc.2021.12.014

Dong L, Udaka H, Numata H, Ito C (2021) Regulation of *Krüppel homolog 1* expression by photoperiod in the bean bug, *Riptortus pedestris*. Physiol Entomol 46:82–93. https://doi.org/10.1111/phen.12347sp

Emerson KJ, Uyemura AM, McDaniel KL, Schmidt PS, Bradshaw WE, Holzapfel CM (2009) Environmental control of ovarian dormancy in natural populations of *Drosophila melanogaster*. J Comp Physiol A 195:825–829. https://doi.org/10.1007/s00359-009-0460-5

Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) Cry, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679. https://doi.org/10.1016/S0092-8674(00)81637-2

Emery P, Stanewsky R, Helfrich-Förster C, Emery-Le M, Hall JC, Rosbash M (2000) *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron 26:493–504. https://doi.org/10.1016/S0896-6273(00)81181-2

Erickson PA, Weller CA, Song DY, Bangerter AS, Schmidt P, Bergland AO (2020) Unique genetic signatures of local adaptation over space and time for diapause, an ecologically relevant complex trait, in *Drosophila melanogaster*. PLoS Genet 16:e1009110. https://doi.org/10.1371/JOURNAL.PGEN.1009110

Fujiwara Y, Hermann-Luibl C, Katsura M, Sekiguchi M, Ida T, Helfrich-Förster C et al (2018) The CCHamide1 neuropeptide expressed in the anterior dorsal neuron 1 conveys a circadian signal to the ventral lateral neurons in *Drosophila melanogaster*. Front Physiol 9:1276. https://doi.org/10.3389/FPHYS.2018.01276/BIBTEX

Gibbs D (1975) Reversal of pupal diapause in *Sarcophaga argyrostoma* by temperature shifts after puparium formation. J Insect Physiol 21:1179–1186. https://doi.org/10.1016/0022-1910(75)90085-2

Goto SG (2022) Photoperiodic time measurement, photoreception, and circadian clocks in insect photoperiodism. Appl Entomol Zool 57:193–212. https://doi.org/10.1007/s13355-022-00785-7

Goto SG, Nagata M (2022) The circadian clock gene (*Clock*) regulates photoperiodic time measurement and its downstream process in maternal induction of embryonic diapause in a cricket. Eur J Entomol 119:12–22. https://doi.org/10.14411/eje.2022.002

Goto SG, Doi K, Nakayama S, Numata H (2008) Maternal control of cold and desiccation tolerance in eggs of the band-legged ground cricket *Dianemobius nigrofasciatus* in relation to embryonic diapause. Entomol Res 38:17–23. https://doi.org/10.1111/j.1748-5967.2008.00140.x

Goto SG, Shiga S, Numata H (2010) Photoperiodism in insects: perception of light and the role of clock genes. In: Nelson RJ, Denlinger DL, Somers DE (eds) Photoperiodism: the biological calendar. Oxford University Press, Oxford, pp 258–286

Hase H, Koukai M, Hamanaka Y, Goto SG, Tachibana S-I, Shiga S (2017) Transcriptome analysis of the brain under diapause and nondiapause conditions in the blowfly *Protophormia terraenovae*. Physiol Entomol 42:282–289. https://doi.org/10.1111/phen.12205

Hasebe M, Shiga S (2021) Oviposition-promoting pars intercerebralis neurons show *period*-dependent photoperiodic changes in their firing activity in the bean bug. Proc Natl Acad Sci USA 118:e2018823118. https://doi.org/10.1073/pnas.2018823118

Hasebe M, Shiga S (2022) Clock gene-dependent glutamate dynamics in the bean bug brain regulate photoperiodic reproduction. PLoS Biol 20:e3001734. https://doi.org/10.1371/journal.pbio.3001734

Hasebe M, Kotaki T, Shiga S (2022) Pigment-dispersing factor is involved in photoperiodic control of reproduction in the brown-winged green bug, *Plautia stali*. J Insect Physiol 137:104359. https://doi.org/10.1016/j.jinsphys.2022.104359

Hejnikova M, Paroulek M, Hodkova M (2016) Decrease in *Methoprene tolerant* and *Taiman* expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden bug *Pyrrhocoris apterus*. J Insect Physiol 93–94:72–80. https://doi.org/10.1016/j.jinsphys.2016.08.009

Hejníková M, Nouzova M, Ramirez CE, Fernandez-Lima F, Noriega FG, Doležel D (2022) Sexual dimorphism of diapause regulation in the hemipteran bug *Pyrrhocoris apterus*. Insect Biochem Mol Biol 142:103721. https://doi.org/10.1016/j.ibmb.2022.103721

Herman WS (1981) Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus*. Biol Bull 160:89–106. https://doi.org/10.2307/1540903

Hermann C, Yoshii T, Dusik V, Helfrich-Förster C (2012) Neuropeptide F immunoreactive clock neurons modify evening locomotor activity and free-running period in *Drosophila melanogaster*. J Comp Neurol 520:970–987. https://doi.org/10.1002/cne.22742

Hermann-Luibl C, Yoshii T, Senthilan PR, Dircksen H, Helfrich-Förster C (2014) The ion transport peptide is a new functional clock neuropeptide in the fruit fly *Drosophila melanogaster*. J Neurosci 34:9522–9536. https://doi.org/10.1523/JNEUROSCI.0111-14.2014

Hirai M, Yuda M, Shinoda T, Chinzei Y (1998) Identification and cDNA cloning of novel juvenile hormone responsive genes from fat body of the bean bug, *Riptortus clavatus* by mRNA differential display. Insect Biochem Mol Biol 28:181–189. https://doi.org/10.1016/S0965-1748(97)00116-1

Iiams SE, Lugena AB, Zhang Y, Hayden AN, Merlin C (2019) Photoperiodic and clock regulation of the vitamin A pathway in the brain mediates seasonal responsiveness in the monarch butterfly. Proc Natl Acad Sci USA 116:25214–25221. https://doi.org/10.1073/pnas.1913915116

Ikeda K, Daimon T, Shiomi K, Udaka H, Numata H (2021) Involvement of the clock gene *period* in the photoperiodism of the silkmoth *Bombyx mori*. Zool Sci 38:523–530. https://doi.org/10.2108/zs210081

Ikeno T, Tanaka SI, Numata H, Goto SG (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol 8:116. https://doi.org/10.1186/1741-7007-8-116

Ikeno T, Numata H, Goto SG (2011a) Photoperiodic response requires *mammalian-type cryptochrome* in the bean bug *Riptortus pedestris*. Biochem Biophys Res Commun 410:394–397. https://doi.org/10.1016/j.bbrc.2011.05.142

Ikeno T, Numata H, Goto SG (2011b) Circadian clock genes *period* and *cycle* regulate photoperiodic diapause in the bean bug *Riptortus pedestris* males. J Insect Physiol 57:935–938. https://doi.org/10.1016/j.jinsphys.2011.04.006

Ikeno T, Ishikawa K, Numata H, Goto SG (2013) Circadian clock gene *Clock* is involved in the photoperiodic response of the bean bug *Riptortus pedestris*. Physiol Entomol 38:157–162. https://doi.org/10.1111/phen.12013

Ikeno T, Numata H, Goto SG, Shiga S (2014) Involvement of the brain region containing pigment-dispersing factor-immunoreactive neurons in the photoperiodic response of the bean bug, *Riptortus pedestris*. J Exp Biol 217:453–462. https://doi.org/10.1242/jeb.091801

Johard HAD, Yoshii T, Dircksen H, Cusumano P, Rouyer F, Helfrich-Förster C et al (2009) Peptidergic clock neurons in *Drosophila*: ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. J Comp Neurol 516:59–73. https://doi.org/10.1002/cne.22099

Kang DS, Denlinger DL, Sim C (2014) Suppression of allatotropin simulates reproductive diapause in the mosquito *Culex pipiens*. J Insect Physiol 64:48–53. https://doi.org/10.1016/j.jinsphys.2014.03.005

Kidokoro T, Masaki S (1978) Photoperiodic response in relation to variable voltinism in the ground cricket, *Pteronemobius fascipes* Walker (Orthoptera: Gryllidae). Jpn J Ecol 28:291–298. https://doi.org/10.18960/SEITAI.28.4_291

King AN, Sehgal A (2020) Molecular and circuit mechanisms mediating circadian clock output in the *Drosophila* brain. Eur J Neurosci 51:268–281. https://doi.org/10.1111/ejn.14092

Kobayashi S, Numata H (1993) Photoperiodic responses controlling the induction of adult diapause and the determination of seasonal form in the bean bug, *Riptortus clavatus*. Zool Sci 10:983–990

Koide R, Xi J, Hamanaka Y, Shiga S (2021) Mapping PERIOD-immunoreactive cells with neurons relevant to photoperiodic response in the bean bug *Riptortus pedestris*. Cell Tissue Res 385:571–583. https://doi.org/10.1007/s00441-021-03451-6

Koštál V (2006) Eco-physiological phases of insect diapause. J Insect Physiol 52:113–127. https://doi.org/10.1016/j.jinsphys.2005.09.008

Koštál V, Stetina T, Poupardin R, Korbelová J, Bruce AW (2017) Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. Proc Natl Acad Sci USA 114:8532–8537. https://doi.org/10.1073/pnas.1707281114

Kotwica-Rolinska J, Pivarciova L, Vaneckova H, Dolezel D (2017) The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage. Physiol Entomol 42:266–273. https://doi.org/10.1111/phen.12197

Kotwica-Rolinska J, Chodáková L, Smýkal V, Damulewicz M, Provazník J, Wu BC-H et al (2022a) Loss of timeless underlies an evolutionary transition within the circadian clock. Mol Biol Evol 39:msab346. https://doi.org/10.1093/molbev/msab346

Kotwica-Rolinska J, Damulewicz M, Chodakova L, Kristofova L, Dolezel D (2022b) Pigment dispersing factor is a circadian clock output and regulates photoperiodic response in the linden bug, *Pyrrhocoris apterus*. Front Physiol 13:884909. https://doi.org/10.3389/fphys.2022.884909

Kutaragi Y, Tokuoka A, Tomiyama Y, Nose M, Watanabe T, Bando T et al (2018) A novel photic entrainment mechanism for the circadian clock in an insect: involvement of *c-fos* and *cryptochrome*s. Zool Lett 4:26. https://doi.org/10.1186/s40851-018-0109-8

Mano G, Goto SG (2022) Photoperiod controls insulin and juvenile hormone signaling pathways via the circadian clock in the bean bug *Riptortus pedestris*. Appl Entomol Zool 57:363–377. https://doi.org/10.1007/s13355-022-00795-5

Meiselman MR, Alpert MH, Cui X, Shea J, Gregg I, Gallio M et al (2022) Recovery from cold-induced reproductive dormancy is regulated by temperature-dependent AstC signaling. Curr Biol 32:1362–1375. https://doi.org/10.1016/j.cub.2022.01.061

Meuti ME, Stone M, Ikeno T, Denlinger DL (2015) Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, *Culex pipiens*. J Exp Biol 218: 412–422. https://doi.org/10.1242/jeb.113233

Miki T, Shinohara T, Chafino S, Noji S, Tomioka K (2020) Photoperiod and temperature separately regulate nymphal development through JH and insulin/TOR signaling pathways in an insect. Proc Natl Acad Sci USA 117:5525–5531. https://doi.org/10.1073/pnas.1922747117

Mizoguchi A, Ohsumi S, Kobayashi K, Okamoto N, Yamada N, Tateishi K et al (2013) Prothoracicotropic hormone acts as a neuroendocrine switch between pupal diapause and adult development. PLoS One 8:e608824. https://doi.org/10.1371/journal.pone.0060824

Mohamed AAM, Wang Q, Bembenek J, Ichihara N, Hiragaki S, Suzuki T et al (2014) *N-acetyltransferase* (*nat*) is a critical conjunct of photoperiodism between the circadian system and endocrine axis in *Antheraea pernyi*. PLoS One 9:e92680. https://doi.org/10.1371/journal.pone.0092680

Morita A, Numata H (1997) Role of the neuroendocrine complex in the control of adult diapause in the bean bug, *Riptortus clavatus*. Arch Insect Biochem Physiol 35:347–355. https://doi.org/10.1002/(sici)1520-6327(199705)35:3%3C347::aid-arch8%3E3.0.co;2-q

Morita A, Soga K, Hoson T, Kamisaka S, Numata H (1999) Changes in mechanical properties of the cuticle and lipid accumulation in relation to adult diapause in the bean bug, *Riptortus clavatus*. J Insect Physiol 45:241–247. https://doi.org/10.1016/S0022-1910(98)00119-X

Mukai A, Goto SG (2016) The clock gene *period* is essential for the photoperiodic response in the jewel wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae). Appl Entomol Zool 51:185–194. https://doi.org/10.1007/s13355-015-0384-1

Nagy D, Cusumano P, Andreatta G, Anduaga AM, Hermann-Luibl C, Reinhard N et al (2019) Peptidergic signaling from clock neurons regulates reproductive dormancy in *Drosophila melanogaster*. PLoS Genet 15:e1008158. https://doi.org/10.1371/journal.pgen.1008158

Nelson RJ, Denlinger DL, Somers DE (2010) Photoperiodism: the biological calendar. Oxford University Press, Oxford

Noguchi H, Hayakawa Y (1997) Role of dopamine at the onset of pupal diapause in the cabbage armyworm *Mamestra brassicae*. FEBS Lett 413:157–161. https://doi.org/10.1016/S0014-5793(97)00848-X

Noguchi H, Hayakawa Y (2001) Dopamine is a key factor for the induction of egg diapause of the silkworm, *Bombyx mori*. Eur J Biochem 268:774–780. https://doi.org/10.1046/j.1432-1327.2001.01933.x

Numata H, Hidaka T (1982) Photoperiodic control of adult diapause in the bean bug, *Riptortus clavatus* Thunberg (Heteroptera: Coreidae) I. Reversible induction and termination of diapause. Appl Entomol Zool 17:530–538. https://doi.org/10.1303/aez.17.530

Numata H, Hidaka T (1984) Termination of adult diapause by a juvenile hormone analogue in the bean bug, *Riptortus clavatus*. Zool Sci 1:751–754

Numata H, Kobayashi S (1989) Morphological and behavioral character of adult diapause and its termination by a juvenile hormone analogue in *Riptortus clavatus*. In: Tonner M, Soldan T, Bennettova B (eds) Regulation of insect reproduction IV, Proceedings of a symposium held in Zinkovy, Sept 1987. Academia Publishing House of the Czech Academy of Sciences, Praha, pp 401–411

Omura S, Numata H, Goto SG (2016) Circadian clock regulates photoperiodic responses governed by distinct output pathways in the bean bug, *Riptortus pedestris*. Biol Rhythm Res 47:937–945. https://doi.org/10.1080/09291016.2016.1212515

Pavelka J, Shimada K, Kostal V (2003) TIMELESS: a link between fly's circadian and photoperiodic clocks? Eur J Entomol 100:255–265. https://doi.org/10.14411/eje.2003.041

Pittendrigh CS, Minis DH (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. Am Nat 98:261–294. https://doi.org/10.1086/282327

Poupardin R, Schöttner K, Korbelová J, Provazník J, Doležel D, Pavlinic D et al (2015) Early transcriptional events linked to induction of diapause revealed by RNAseq in larvae of drosophilid fly, *Chymomyza costata*. BMC Genomics 16:1–19. https://doi.org/10.1186/s12864-015-1907-4

Sakamoto T, Tomioka K (2007) Effects of unilateral compound-eye removal on the photoperiodic responses of nymphal development in the cricket *Modicogryllus siamensis*. Zool Sci 24:604–610. https://doi.org/10.2108/zsj.24.604

Sakamoto T, Uryu O, Tomioka K (2009) The clock gene *period* plays an essential role in photoperiodic control of nymphal development in the cricket *Modicogryllus siamensis*. J Biol Rhythm 24:379–390. https://doi.org/10.1177/0748730409341523

Sandrelli F, Tauber E, Pegoraro M, Mazzotta G, Cisotto P, Landskron J et al (2007) A molecular basis for natural selection at the *timeless* locus in *Drosophila melanogaster*. Science 316:1898–1900. https://doi.org/10.1126/science.1138426

Saunders D (2021) Insect photoperiodism: Bünning's hypothesis, the history and development of an idea. Eur J Entomol 118:1–13. https://doi.org/10.14411/EJE.2021.001

Saunders DS, Henrich VC, Gilbert LI (1989) Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. Proc Natl Acad Sci USA 86:3748–3752. https://doi.org/10.1073/pnas.86.10.3748

Senthilan PR, Grebler R, Reinhard N, Rieger D, Helfrich-Förster C (2019) Role of rhodopsins as circadian photoreceptors in the *Drosophila melanogaster*. Biology (Basel) 8:6. https://doi.org/10.3390/biology8010006

Shafer OT, Helfrich-Förster C, Renn SCP, Taghert PH (2006) Reevaluation of *Drosophila melanogaster*'s neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol 498:180–193. https://doi.org/10.1002/CNE.21021

Shiomi K, Takasu Y, Kunii M, Tsuchiya R, Mukaida M et al (2015) Disruption of diapause induction by TALEN-based gene mutagenesis in relation to a unique neuropeptide signaling pathway in *Bombyx*. Sci Rep 5:15566. https://doi.org/10.1038/srep15566

Sim C, Denlinger DL (2008) Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc Natl Acad Sci USA 105:6777–6781. https://doi.org/10.1073/pnas.0802067105

Sim C, Denlinger DL (2009) A shut-down in expression of an insulin-like peptide, ILP-1, halts ovarian maturation during the overwintering diapause of the mosquito *Culex pipiens*. Insect Mol Biol 18:325–332. https://doi.org/10.1111/j.1365-2583.2009.00872.x

Sim C, Denlinger DL (2013) Juvenile hormone III suppresses forkhead of transcription factor in the fat body and reduces fat accumulation in the diapausing mosquito, *Culex pipiens*. Insect Mol Biol 22:1–11. https://doi.org/10.1111/j.1365-2583.2012.01166.x

Song SH, Öztürk N, Denaro TR, Arat NÖ, Kao YT, Zhu H et al (2007) Formation and function of flavin anion radical in cryptochrome 1 blue-light photoreceptor of monarch butterfly. J Biol Chem 282:17608–17612. https://doi.org/10.1074/jbc.M702874200

Tagaya J, Numata H, Goto SG (2010) Sexual difference in the photoperiodic induction of pupal diapause in the flesh fly *Sarcophaga similis*. Entomol Sci 13:311–319. https://doi.org/10.1111/j.1479-8298.2010.00394.x

Tamai T, Shiga S, Goto SG (2019) Roles of the circadian clock and endocrine regulator in the photoperiodic response of the brown-winged green bug *Plautia stali*. Physiol Entomol 44:43–52. https://doi.org/10.1111/phen.12274

Tamaki S, Takemoto S, Uryu O, Kamae Y, Tomioka K (2013) Opsins are involved in nymphal photoperiodic responses in the cricket *Modicogryllus siamensis*. Physiol Entomol 38:163–172. https://doi.org/10.1111/phen.12015

Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C et al (2007) Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science 316: 1895–1898. https://doi.org/10.1126/science.1138412

Tobita H, Kiuchi T (2022) Knockouts of positive and negative elements of the circadian clock disrupt photoperiodic diapause induction in the silkworm, *Bombyx mori*. Insect Biochem Mol Biol 149:103842. https://doi.org/10.1016/j.ibmb.2022.103842

Tokuoka A, Itoh TQ, Hori S, Uryu O, Danbara Y, Nose M et al (2017) *Cryptochrome* genes form an oscillatory loop independent of the *per/tim* loop in the circadian clockwork of the cricket *Gryllus bimaculatus*. Zool Lett 3:1–14. https://doi.org/10.1186/s40851-017-0066-7

Tsuchiya R, Kaneshima A, Kobayashi M, Yamazaki M, Takasu Y, Sezutsu H et al (2021) Maternal GABAergic and GnRH/corazonin pathway modulates egg diapause phenotype of the silkworm *Bombyx mori*. Proc Natl Acad Sci USA 118:e2020028008. https://doi.org/10.1073/pnas.2020028118

Tyshchenko VP (1966) Two-oscillatory model of the physiological mechanism of insect photoperiodic reaction. Zh Obshch Biol 27:209–222. (in Russian)

Ueda H, Tamaki S, Miki T, Uryu O, Kamae Y, Nose M et al (2018) *cryptochrome* genes mediate photoperiodic responses in the cricket *Modicogryllus siamensis*. Physiol Entomol 43:285–294. https://doi.org/10.1111/phen.12258

Urbanová V, Bazalová O, Vaněčková H, Dolezel D (2016) Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. Insect Biochem Mol Biol 70:184–190. https://doi.org/10.1016/j.ibmb.2016.01.003

Uryu M, Ninomiya Y, Yokoi T, Tsuzuki S, Hayakawa Y (2003) Enhanced expression of genes in the brains of larvae of *Mamestra brassicae* (Lepidoptera: Noctuidae) exposed to short daylength or fed Dopa. Eur J Entomol 100:245–250

Van Der Kooi CJ, Stavenga DG, Arikawa K, Belušič G, Kelber A (2021) Evolution of insect color vision: from spectral sensitivity to visual ecology. Annu Rev Entomol 66:435–461. https://doi.org/10.1146/annurev-ento-061720-071644

Vaz Nunes M, Saunders D (1999) Photoperiodic time measurement in insects: a review of clock models. J Biol Rhythm 14:84–104. https://doi.org/10.1177/074873049901400202

Veerman A (1980) Functional involvement of carotenoids in photoperiodic induction of diapause in the spider mite, *Tetranychus urticae*. Physiol Entomol 5:291–300. https://doi.org/10.1111/j.1365-3032.1980.tb00237.x

Wang Q, Mohamed AAM, Takeda M (2013) Serotonin receptor B may lock the gate of PTTH release/synthesis in the Chinese silk moth, *Antheraea pernyi*; a diapause initiation/maintenance mechanism? PLoS One 8:1–13. https://doi.org/10.1371/journal.pone.0079381

Wang Q, Egi Y, Takeda M, Oishi K, Sakamoto K (2015a) Melatonin pathway transmits information to terminate pupal diapause in the Chinese oak silkmoth *Antheraea pernyi* and through reciprocated inhibition of dopamine pathway functions as a photoperiodic counter. Entomol Sci 18:74–84. https://doi.org/10.1111/ens.12083

Wang Q, Hanatani I, Takeda M, Oishi K, Sakamoto K (2015b) D2-like dopamine receptors mediate regulation of pupal diapause in Chinese oak silkmoth *Antheraea pernyi*. Entomol Sci 18:193–198. https://doi.org/10.1111/ens.12099

Yamada H, Yamamoto MT (2011) Association between circadian clock genes and diapause incidence in *Drosophila triauraria*. PLoS One 6:e27493. https://doi.org/10.1371/journal.pone.0027493

Yamada N, Kataoka H, Mizoguchi A (2017) Myosuppressin is involved in the regulation of pupal diapause in the cabbage army moth *Mamestra brassicae*. Sci Rep 7:1–9. https://doi.org/10.1038/srep41651

Yamashita O (1996) Diapause hormone of the silkworm, *Bombyx mori*: structure, gene expression and function. J Insect Physiol 42:669–679. https://doi.org/10.1016/0022-1910(96)00003-0

Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24:948–955. https://doi.org/10.1093/molbev/msm011

Zhu L, Tian Z, Guo S, Liu W, Zhu F, Wang X-P (2019) Circadian clock genes link photoperiodic signals to lipid accumulation during diapause preparation in the diapause-destined female cabbage beetles *Colaphellus bowringi*. Insect Biochem Mol Biol 104:1–10. https://doi.org/10.1016/j.ibmb.2018.11.001

Chapter 14
Neural Mechanism of Photoperiodism

Sakiko Shiga

Abstract In neural mechanisms underlying photoperiodism, photoperiodic information is received by photoreceptors and is processed in the photoperiodic clock and counter in the brain. The processed signals are then switched to the endocrine organs. Neuroanatomy, microsurgery, and electrophysiology, in combination with RNA interference, have revealed plausible photoperiodic neural circuitries that employ circadian clock cells and neurosecretory cells in the pars intercerebralis and pars lateralis. In the blow fly *Protophormia terraenovae* and the bean bug *Riptortus pedestris*, an anterior base of the medulla region containing clock protein PERIOD cells and neuropeptide pigment-dispersing factor cells is a potential site for photoperiodic mechanisms. In *P. terraenovae* PERIOD nuclear localization suggests that clock cells of s-LNv and DNm have different phase settings to environmental light-dark cycles. By comparing the clock phases of s-LNv and DNm, short and long days are distinguishable. In *R. pedestris*, pars intercerebralis neurons show a photoperiodic response in their firing activities, depending on the *period* expression. The neurotransmitter glutamate mediates short-day signals to pars intercerebralis neurons under *period* expression. In the proposed neural circuitry, cellular responses such as electrical activities, gene expression, and fiber projection patterns to short and long days should be revealed to understand the photoperiodic clock and counter mechanisms in future studies.

Keywords Circadian clock · Pars intercerebralis · Pars lateralis · Photoperiodic clock · Photoperiodic response · Pigment-dispersing factor

S. Shiga (✉)
Graduate School of Science, Osaka University, Toyonaka, Japan
e-mail: shigask@bio.sci.osaka-u.ac.jp

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14.1 Introduction

Organisms inhabiting the middle to high latitudes encounter seasonally occurring adverse conditions. Many insects predict the coming seasons through day (or night) length and acquire hardiness before the arrival of severe seasons (Tauber et al. 1986). Photoperiodic responses are critical for seasonal adaptation. To overcome severe environmental conditions, insects usually exhibit characteristic seasonal phenotypes, including diapause, at a fixed developmental stage (Denlinger 2002; Bradshaw and Holzapfel 2007). The photoperiodic mechanism that controls seasonal phenotypes involves several enigmatic questions in neurobiology. How is the length of the day or night measured? How does the brain translate the measured day length to "short" or "long" days? In insects, usually only 1-day information is not able to switch seasonal phenotypes, and a certain number of days are required, meaning that a mechanism to count or store the day-length information is necessary. Although measurement and storage/counting mechanisms are interesting issues, they have not been adequately addressed at the neural level.

Since Bünning (1936) first proposed that endogenous daily rhythmicity underlies photoperiodic responses during scarlet bean flowering, the circadian clock has been considered to play a crucial role in photoperiodic mechanisms in plants and animals. Subsequently, models such as external (Pittendrigh and Minis 1964) and internal (Tyshchenko 1966) coincidence have been developed. In these models, long- or short-day information is distinguished by the coincidence between a certain circadian clock phase and environmental light or by the coincidence of a special phase between two internal circadian clocks (Chap. 12). According to these ideas, cells and genes constituting the circadian clock are crucial elements in the photoperiodic mechanism. Understanding the molecular machinery involved in circadian clock functions during the last few decades has allowed us to examine the effects of the elimination or downregulation of clock gene expression on photoperiodic responses. In fact, it has been reported in many insect species that the expression of canonical circadian clock genes is a prerequisite for photoperiodic responses (Chap. 13). However, it is well known that clock genes are ubiquitously expressed in various tissues (Plautz et al. 1997; Giebultowicz 1999; Bajgar et al. 2013), and the neural circuitry in which clock genes operate in photoperiodic mechanisms remains mostly unsolved.

This chapter provides an overview of the photoperiodic mechanism with relevant organs and cells and introduces neural mechanisms focusing on dipteran and hemipteran species with future perspectives.

14.2 General Scheme for Photoperiodic Mechanisms

14.2.1 Photoreceptors

In photoperiodic responses, the light information of the photoperiod is received through photoreceptors (Fig. 14.1). Insects are sensitive to the photoperiod at fixed developmental stages depending on the species, and the photoreceptor organ is species- and stage-specific (Numata et al. 1997; Goto et al. 2010). In Calliphoridae of Diptera, both *Calliphora vicina* and *Protophormia terraenovae* are sensitive to photoperiod at the adult stage but use different photoreceptor organs. *C. vicina* uses extraretinal photoreceptors to control larval diapause in its progeny (Saunders and Cymborowski 1996), whereas *P. terraenovae* uses compound eyes (retinal photoreceptors) to control reproductive diapause (Shiga and Numata 1997). Even within the same species, different photoreceptor organs are used in the larval and adult

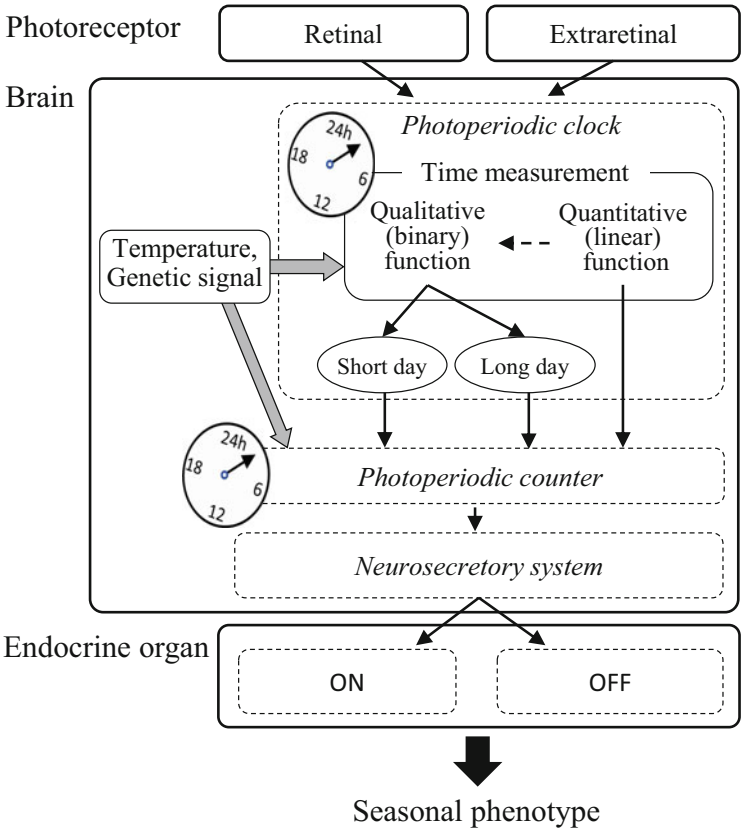

Fig. 14.1 Schematic representation of photoreceptor, brain components, and endocrine organ for photoperiodic response

stages of the carabid beetle *Leptocarabus kumagaii* (Shintani et al. 2009). There appear to be no uniform rules for the photoreceptor organs among the sensitive stages or insect orders. Although the photoreceptive organs and effective wavelengths for photoperiodism have been investigated, photoreceptor molecules and cells have rarely been examined. Only opsin-ultraviolet in the compound eyes is known to be necessary for the photoperiodic control of nymphal diapause in the cricket *Modicogryllus siamensis* (Tamaki et al. 2013). In particular, the identification of extraretinal photoreceptor cells for photoperiodic responses is awaited (Gao et al. 1999). The number of extraretinal photoreceptor cells must be small, and they may constitute a simple ascending path to the next neuron. Clarification of extraretinal photoreceptors in the brain may provide easy access to the center of the photoperiodic mechanisms.

14.2.2 Photoperiodic Clock and Counter

Light and dark information through the photoreceptor is conveyed to the neurons responsible for the "photoperiodic clock" and then to the "photoperiodic counter" circuits in the brain (Fig. 14.1). The photoperiodic clock and counter constitute the core photoperiodic mechanism (Saunders 2002, 2021; Takeda and Skopik 1997). The photoperiodic clock measures the day or night length (hereafter referred to as day length) of a day and determines short or long days. The photoperiodic counter stores successive cycles of short or long days during the sensitive period and counts the number of days up to an internal threshold to switch to the neurosecretory system (Fig. 14.1; Saunders 1971, 2002; Tyshchenko et al. 1972).

In a photoperiodic clock, there is a time measurement mechanism that quantitatively measures the day length (linear function) and qualitatively distinguishes short and long days (binary function). Quantitative phenotypic responses are known to control seasonal morphs such as body coloration and wing form, whose grades are produced in a linear function of day length (Numata and Kobayashi 1994; Shintani 2011; Hiroyoshi et al. 2019; Chap. 12). To express linear phenotypes, day-length information may be directly conveyed to the photoperiodic counter (Fig. 14.1). In qualitative time measurements that distinguish between short and long days, both external and internal coincidence models are applicable (Pittendrigh and Minis 1964; Tyshchenko 1966), although the latter can also explain quantitative time measurements (Tyshchenko 1966). In addition, there may be a coincidence-independent mechanism in which day-length information from a quantitative function is translated into short-day or long-day information through a qualitative binary function referring to an internal critical value. Internal and external coincidence models also need critical values such as "active phase" and "photoinducible phase" to distinguish short and long days (Chap. 12).

There is a variation in individual critical values to a certain degree in a population, and the day length at which the short-day effect (or long-day effect) is induced in 50% of the population is called "critical day length." The critical day length depends

on the species, and even in the same species, it depends on temperature and latitude (Danilevskii 1965; Yamaguchi and Goto 2019). Therefore, there must be some modulatory input of temperature and genetic information into the internal critical value setting for time measurement (Fig. 14.1).

Next, the number of short or long days was counted using the photoperiodic counter. Goryshin and Tyshchenko (1974) and Gibbs (1975) considered a mechanism for storing day-length information as discrete packets of information using a memory link (storage mechanism) and comparing the stored information to another internal threshold value of day numbers.

With regard to information storage, the brain mechanism of memory for learning may be comparable to that of the photoperiodic counter. It is well demonstrated in the honey bee *Apis mellifera* and the fruit fly *Drosophila melanogaster* that neural mechanisms underlying olfactory learning involve the mushroom bodies in the protocerebrum (Erber et al. 1987; Dubnau and Tully 1998; Waddel and Quinn 2001). In this regard, the ablation effects of mushroom bodies on the photoperiodic response and olfactory learning were compared in *P. terraenovae* (Ikeda et al. 2005). Feeding on hydroxyurea in a fixed early stage of the first instar larvae produced the brain without the mushroom body, as in *D. melanogaster* (De Belle and Heisenberg 1994). In females with ablated mushroom bodies, olfactory learning ability was disrupted, but the photoperiodic response remained the same as in intact flies (Ikeda et al. 2005). This suggests that the storage mechanisms in the photoperiodic counter and learning are distinct, and the former occurs in a region different from the mushroom body.

To measure the length of the light period per day (photoperiodic clock) and for counting packets or cycle numbers of a 24-h day (photoperiodic counter), it may be natural to consider that the circadian clock is used in reference to Bünning's idea (1936). Therefore, the identification of brain circadian clock cells expressing clock genes in the neural network is an important step in addressing the photoperiodic clock and counter mechanisms. When the number of counted days exceeds the internal day threshold in the photoperiodic counter, a proper neuroendocrine system must be driven to signal endocrine organs to release or suppress hormones that control seasonal phenotypes (Fig. 14.1).

14.2.3 Neurosecretory System

In the insect brain, the pars intercerebralis (PI) and pars lateralis (PL) contain neurosecretory cells (NSCs) innervating the endocrine organs of the prothoracic gland (PG), corpus cardiacum (CC), or corpus allatum (CA) (Raabe 1989; Shiga 2003). The neuronal control of endocrine organs posteriorly attached to the brain resembles that of the vertebrate hypothalamic-hypophyseal system (Veelaert et al. 1998). In fact, a *D. melanogaster* study showed that the PI and CC are developmentally equivalent to the mammalian hypothalamic-hypophysial axis (De Velasco et al. 2004). As secretion of ecdysteroids from the PG and that of juvenile hormones (JH,

sesquiterpenoid) from the CA is critical for controlling larval and pupal development and reproductive maturation, these endocrine organs are important for controlling diapause as a developmental arrest (Denlinger et al. 2012). Surgical ablation experiments suggested that PI and PL are important for switching between short-day and long-day effects in various species and that these regions appear to contain a neurosecretory system that controls the photoperiodic response (Fig. 14.1; Shiga 2003; Shiga and Numata 2007).

In this scheme, it is suggested that the neural circuitry for photoperiodic responses employs circadian clock cells, PI and PL NSCs, and output signals from the circuitry activate or suppress endocrine cells in endocrine organs such as the PG and CA. Examination of the cellular responses to different day lengths or cumulative numbers of days is critical for photoperiodic mechanisms. The electrical activity, gene expression, and plasticity of the fiber projection patterns of cells in the circuitry can be studied. The neural mechanisms of dipteran and hemipteran species have been studied.

14.3 Neural Circuitry and Its Functions Proposed in Different Species

14.3.1 Blow Fly Protophormia terraenovae

Adult blow flies, *P. terraenovae*, reproduce under long days and suppress reproductive activity to enter diapause under short days (Fig. 14.2a; Numata and Shiga 1995; Tanigawa et al. 1999; Shiga and Numata 2009). During reproductive diapause, JH production rates in the CA and ecdysteroid titers in the hemolymph/ovaries are significantly reduced (Shiga et al. 2003; Tanaka et al. 2013). As removal of the CA completely suppresses ovarian development under long days and transection of the nerve from the brain to the CA causes ovarian development under short days, JH from the CA is necessary for ovarian development, as reported in many insect species, and brain neurons must suppress JH synthesis in the CA under short days (Matsuo et al. 1997; Denlinger et al. 2012). Therefore, CA is a crucial endocrine organ for photoperiodic output.

14.3.1.1 Neurosecretory System

Ablation experiments have shown that PL is necessary for diapause, whereas PI is required for ovarian development (Shiga and Numata 2000). However, the removal of PL or PI did not affect JH production rates by CA (Shiga et al. 2003). This may mean that PL and PI neurons control ovarian development through humoral factors other than JH. However, certain types of PL and PI neurons have dense fiber arborization deep “inside” of the CA, suggesting that these neurons possibly control

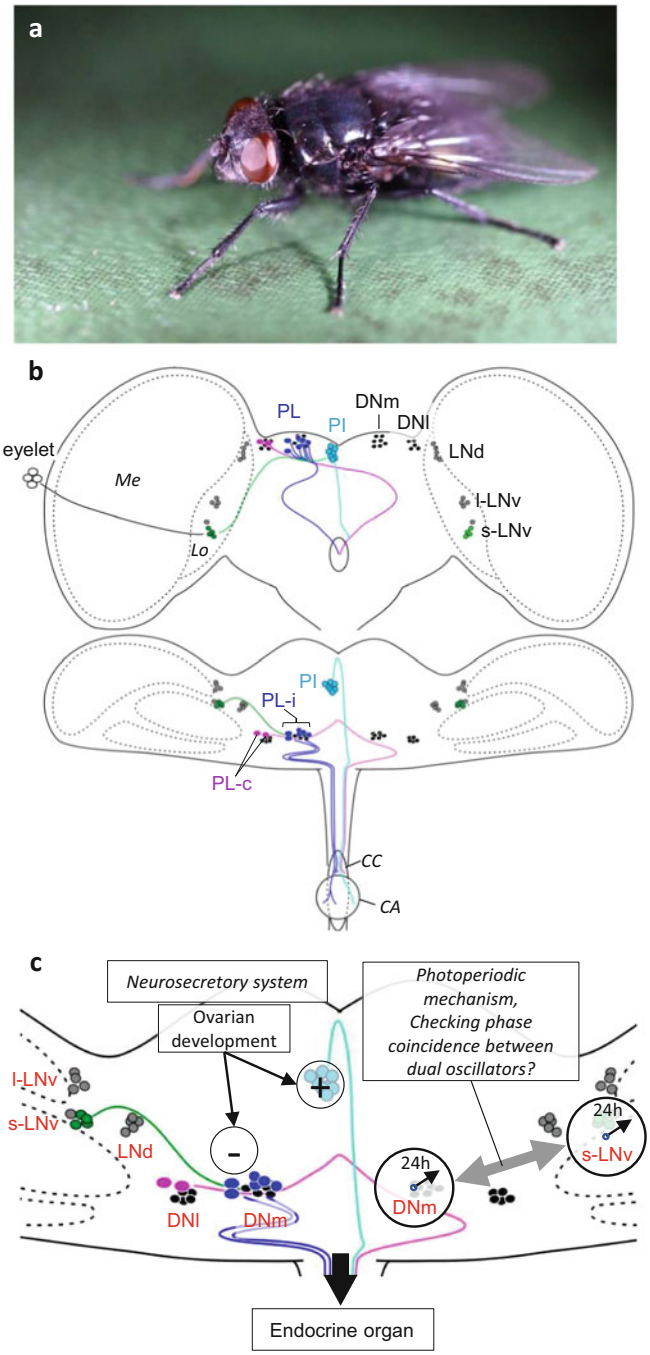

Fig. 14.2 Neuronal circuitry proposed in the blow fly *Protophormia terraenovae*. (**a**) an adult female, which develops ovaries under long day but suppresses ovarian development under short days. (**b**) frontal (upper) and dorsal view (lower) of the pars intercerebralis (PI, light blue), pars lateralis (PL) neurons (PL-i, dark blue; PL-c, magenta) innervating to the corpora cardiacum

JH production by endocrine cells in the CA as in the cockroach *Diploptera punctata* (Chiang et al. 2002; Stay et al. 2002; Hamanaka et al. 2004). There might be compensatory mechanisms for the control of JH production, causing no significant effects of PI or PL ablation on JH production in *P. terraenovae* (Shiga et al. 2003). In *D. melanogaster*, the *Drosophila* insulin-like peptide (Dilp) receptor and a type of dopamine receptor (Dop1R1) are expressed in CA (Ojima et al. 2018; Andreatta et al. 2018). Because reproductive suppression in *D. melanogaster* has both quiescence and diapause properties, it is called dormancy (Kurogi et al. 2021). Multiple controls of JH production appear to affect reproductive dormancy (Ojima et al. 2018; Andreatta et al. 2018), although pathways to carry Dilp and dopamine to the CA have not been identified, and their effects on JH production have not been shown yet. In *P. terraenovae*, ablation experiments and neuroanatomical studies suggest that PL and PI neurons play important roles in the neurosecretory system in the photoperiodic response. In the near future, neurons and molecules that directly control JH production in the CA should be identified in dipteran insects.

The PL and PI neurons of *P. terraenovae* are mostly peptidergic and heterogeneous in their neurochemistry (Hamanaka et al. 2007). The questions are which PL neurons are involved in the induction of reproductive diapause and which PI neurons are necessary for reproduction. There are two types of PL neurons in *P. terraenovae*. One type sends an axon to the complex of the CC and CA (CCCA) through the contralateral brain designated as PL-c (two neurons per hemisphere, Fig. 14.2b magenta); the other is called PL-i neurons (six to ten neurons per hemisphere), sending an ipsilateral axon to the CCCA (Fig. 14.2b dark blue; Shiga and Numata 2000). In the PI, many NSCs send contralateral axons to reach the CCCA (Fig. 14.2b light blue; Shiga and Numata 2000). Single-cell recording and staining revealed that among these neurons, one type of PL-i and one type of PI neurons send fiber branches inside the CA (Hamanaka et al. 2004). The electrophysiological properties of these neurons during daytime recordings did not differ between diapause-inducing (short day and low temperature) and diapause-averting (long day and high temperature) conditions. Instead, other PI and PL neurons without innervation into the CA showed a slight difference, although the physiological significance of this difference remains unclear (Hamanaka et al. 2004). It is likely that the neurons responsible for photoperiodic regulation of endocrine organs alter spiking activities depending on photoperiod, and thus further analysis, including more stable and long-term recording at different times of day, may lead to the identification of responsible neurons and neurophysiological bases underlying photoperiodic regulation of reproductive diapause. In particular, the identification of transmitters released from CA-innervating PL and PI neurons and their function in the CA is awaited.

Fig. 14.2 (continued) (CC) or corpus allatum (CA). PERIOD-immunoreactive cells (s-LNv, green; fifth s-LNv, l-LNv, LNd, gray; DN, black) are also shown. (**c**) possible functional roles in the circuit in dorsal view. Based on Shiga et al. (2000), Shiga and Numata (2000, 2009), Hamanaka et al. (2005), and Yasuyama et al. (2006)

14.3.1.2 Clock Cells and Their Importance in Photoperiodic Neural Circuitry

In *D. melanogaster*, circadian clock cells in the brain are classified into seven clusters: three lateral neurons (LN: LNd, s-LNv, and l-LNv), three dorsal neurons (DN: DN1, DN2, and DN3), and one lateral posterior neuron (LPN) (Helfrich-Förster 1995; Shafer et al. 2006; Chap. 5). In *P. terraenovae*, the circadian clock protein PERIOD (PER)-immunoreactive (-ir) cells are located in regions anatomically homologous to *D. melanogaster* and are grouped into five clusters: dorsal lateral neurons (LNd, $n = 5$), large ventral lateral neurons (l-LNv, $n = 4$), small ventral lateral neurons (s-LNv, $n = 5$), medial dorsal neurons (DNm, $n = 11$–14), and lateral dorsal neurons (DNl, $n = 4$–6) (Fig. 14.2b; Shiga and Numata 2009). The cell numbers and location of LNds, l-LNvs, and s-LNvs were comparable between *D. melanogaster* and *P. terraenovae*. The DNm located in the PL probably corresponds to DN1 and DN2 in *D. melanogaster*. In addition, DNl is possibly homologous to *Drosophila* DN3 according to anatomical location, although cell numbers in *P. terraenovae* ($n = 4$–6) are much lower than those in *D. melanogaster* ($n = \sim 40$) (Fig. 14.2; Shiga and Numata 2009). Cells corresponding to the LPN of *D. melanogaster* have not been found in *P. terraenovae*.

Surgical removal of a region containing s-LNvs causes arrhythmicity in locomotor activity and disruption of the photoperiodic response in *P. terraenovae*, suggesting that s-LNvs are the main oscillator cells for behavioral rhythms, as shown in *D. melanogaster* (Renn et al. 1999; Grima et al. 2004; Stoleru et al. 2004, 2005; Blanchardon et al. 2001), and are crucial for the photoperiodic response (Shiga and Numata 2009). Therefore, s-LNvs must be included in the photoperiodic circuitry, potentially as photoperiodic clock elements (Figs. 14.1 and 14.2).

In *P. terraenovae*, four s-LNvs are immunolabeled with a pigment-dispersing factor (PDF) antibody, and the fifth s-LNv is PDF negative. Neuroanatomy using back fills, immunohistochemistry, and electron microscopy revealed that PDF-positive s-LNvs provide synaptic input to some PL and PI neurons (Hamanaka et al. 2005; Yasuyama et al. 2015). However, PL and PI neurons receiving s-LNv synaptic input have not been identified at the single-cell level, and their projection patterns in the CCCA are not known. Immuno-electron microscopy showed that PDF immunoreactivity was not found in synaptic vesicles, but in dense-core vesicles, suggesting that fast-acting neurotransmitters from s-LNvs to PL neurons are different from PDF (Hamanaka et al. 2005). In *D. melanogaster*, s-LNvs contain short neuropeptide F and glycine, in addition to PDF (Johard et al. 2009; Frenkel et al. 2017). Because neuropeptides are usually found not in synaptic vesicles but in dense-core vesicles (Zupanic 1996), s-LNvs in *P. terraenovae* may use glycine as a synaptic transmitter. It is also important to know whether PL and PI neurons receiving synaptic input from s-LNv innervate the CA.

The role of PDF in photoperiodic responses remains controversial. *D. melanogaster* exhibits a shallow photoperiodic response only at low temperatures (Saunders and Gilbert 1990; Nagy et al. 2018). The null mutation of *pdf* did not have

a significant effect on the photoperiodic control of diapause under normally fed conditions (Nagy et al. 2019), whereas the *pdf* mutation diminished it under starved conditions (Ojima et al. 2018). However, because ovarian development in *D. melanogaster* is very sensitive to temperature, photoperiodism during ovarian dormancy may be due to thermoperiodic stimuli rather than photoperiodic stimuli (Anduaga et al. 2018).

In the bean bug *Riptortus pedestris* (Heteroptera), *pdf* knockdown did not cause significant effects on its photoperiodic response, but surgical ablation of PDF-ir cells disrupted the response, supporting the idea in *P. terraenovae* that PDF-ir neurons (s-LNvs) are important; however, transmitter molecules other than PDF are in charge of transmission in the photoperiodic circuitry (Ikeno et al. 2014; Shiga and Numata 2009). However, recent studies in another heteropteran species, the brown-winged green bug *Plautia stali*, showed that *pdf* expression is required to respond to photoperiod changes (from short to long days or vice versa), regulating ovarian development (Hasebe et al. 2022). This suggests that PDF may play a role in the photoperiodic mechanism, although the effects of *pdf* downregulation are barely visible under simple constant photoperiodic conditions.

14.3.1.3 Plausible Function of the Clock Neuron Network in Photoperiodic Time Measurement

In *D. melanogaster*, neural connections from s-LNvs to other clock neurons have been well documented. *Drosophila* DN1 is further classified into DN1a and DN1p based on anatomical, molecular, and developmental criteria (Shafer et al. 2006). Bidirectional communication between s-LNv and DN1a has been previously reported (Hamasaka et al. 2007; Collins et al. 2012; Shafer et al. 2006; Fujiwara et al. 2018). PDF-positive s-LNvs also provide synaptic input to DN1p, cryptochrome-positive LNd, and PDF-negative fifth s-LNv (Seluzicki et al. 2014; Gorostiza et al. 2014). Interestingly, s-LNvs appear to differentially contact specific subsets of clock cells throughout the day (Gorostiza et al. 2014). If *P. terraenovae* clock cells constitute a similar clock cell network, there may be communication between s-LNv and DNm (corresponding to *Drosophila* DN1a, p). In *P. terraenovae*, PDF antiserum labels cells in the PL (Nässel et al. 1993; Hamanaka et al. 2007). PER- and PDF-double immunohistochemistry showed that DNms only reside near the PDF-ir PL somata, and a few cells in the PL contain both PER and PDF (Shiga and Numata 2009). Anatomical proximity suggests that there is functional relevance between DNm and PL neurons and that DNms are also involved in photoperiodic neural circuitry.

Photoperiodic information might be processed in the network between the s-LNv and DNm before reaching the PL and PI neurons. In the possible interaction, s-LNv and DNm may consult each other regarding the clock phase to check whether phase coincidence occurs for the photoperiodic mechanism (Fig. 14.2c). Muguruma et al. (2010) compared the clock phases among different clock neurons using PER subcellular localization (Fig. 14.3a). The transcriptional and translational feedback

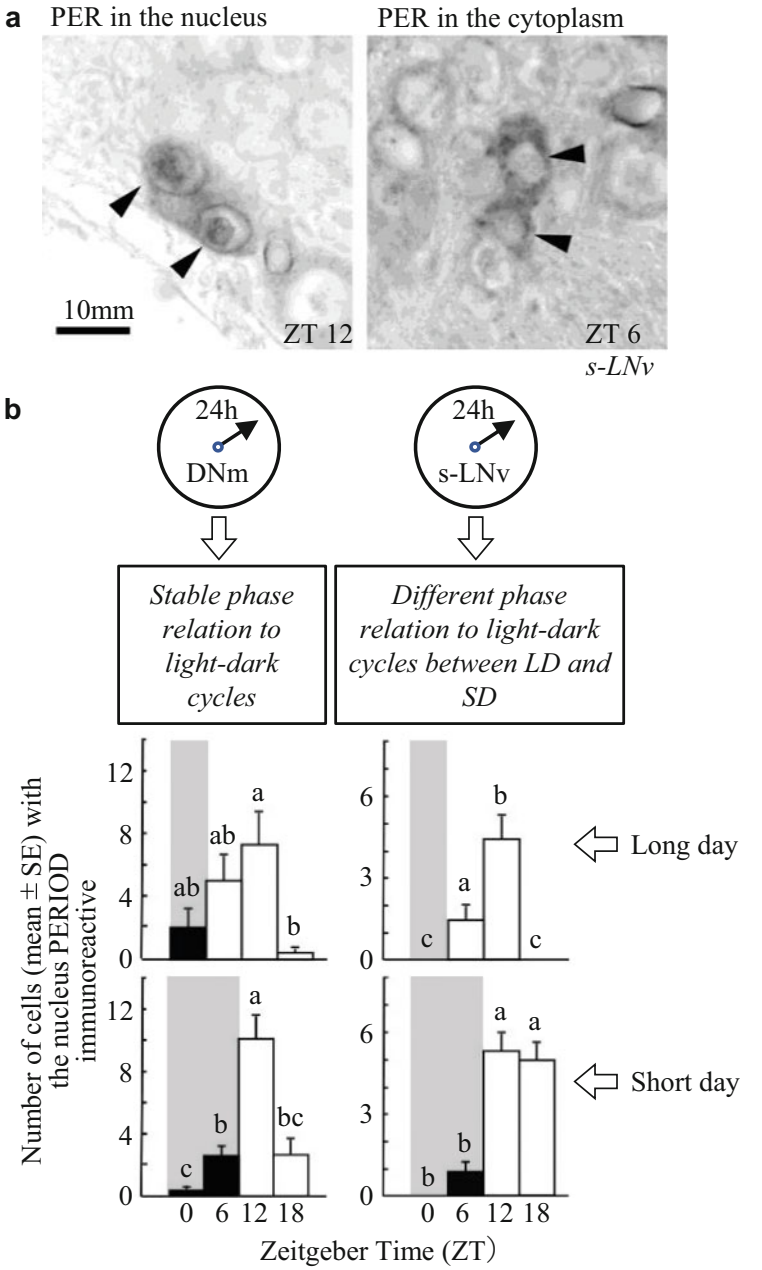

Fig. 14.3 Response of PERIOD-cellular localization in clock cells DNm and s-LNv to long and short days in *Protophormia terraenovae*. (**a**) PERIOD immunoreactivity in s-LNv at different zeitgeber time (ZT, here light-off at ZT = 0) under LD 12:12h. PERIOD nuclear staining is observed at ZT12, but not ZT6. (**b**) DNm and s-LNv respond to photoperiod in a different manner. Adapted from Muguruma et al. (2010), with permission from Springer

loop of clock genes, including *period* (*per*), is a core oscillatory mechanism in which nuclear localization of clock proteins indicates a certain clock phase (Chaps. 4 and 5). In DNm, PER nuclear localization was observed mostly from the beginning to the middle of the photophase, but little at the end of the photophase and the whole scotophase in both short and long days. DNm appears to oscillate in a stable phase relationship with light-dark cycles for both short and long days (Fig. 14.3b). In contrast, in s-LNv, PER nuclear localization was observed only in the middle of the daytime under long days, but in the whole daytime period in short days (Fig. 14.3b). During scotophase, PER nuclear localization rarely occurred during both photoperiods. Nuclear localization responses to light-dark cycles suggest that s-LNv employs a circadian entrainment mechanism different from that of DNm (Muguruma et al. 2010). Although a higher time resolution study is necessary, different timing of PER nuclear entry may indicate the occurrence of a different phase setting between DNm and s-LNv to light-dark cycles. By comparing the clock phases between s-LNv and DNm, the difference appears clearer under short-day conditions than under long-day conditions (Fig. 14.3b). Similar clock phases may provide a long-day decision, and different clock phases may provide a short-day decision to the neurosecretory system. In the flesh fly *Sarcophaga similis*, in which larvae respond to photoperiod to control pupal diapause, the number of PER-ir cells changes across a day, and the changing pattern under long and short days also differs between DN and LN in the larval brain (Yamamoto et al. 2017). Further research is needed to test the idea that clock phase comparison between DNm and s-LNv is involved in the qualitative binary decision of day length (Figs. 14.1 and 14.2c).

Different phase settings for environmental light-dark cycles between two oscillators remind us of the internal coincidence model by Tyshchenko (1966), in which two oscillators are entrained to dawn and dusk, respectively, and this alters the phase angle between the two oscillators under different day lengths, causing a long-day or short-day effect. Pittendrigh and Daan (1976) suggested that this dual oscillator model using "morning (M) clock" and "evening (E) clock" could also explain seasonal change of circadian activity behavior optimally adapting the animal's activity pattern to colder short and warmer long days. In *D. melanogaster*, the M and E oscillators are thought to track dawn and dusk, respectively, and each set of clock cells constituting the respective M and E oscillators has been described in different studies (Stoleru et al. 2007; Hermann-Luibl and Helfrich-Förster 2015). However, it has been reported that the clock cells responsible for the M and E oscillators change depending on the environmental conditions, and it is difficult to explain M and E oscillators at the cell level (Yoshii et al. 2012; Hermann-Luibl and Helfrich-Förster 2015). However, we might be able to apply the dual oscillator idea simply to clock cell clusters of s-LNvs and DNms to explain the mechanisms underlying day-length decisions (Fig. 14.2c).

14.3.2 Sternorrhyncha aphids, Megoura viciae, *and* Acyrthosiphon pisum

Marcovitch (1924) first reported animal photoperiodism in insects: The strawberry root aphid, *Aphis forbesi*, is responsive to day length for controlling reproductive morphs. Under long days, aphids produce virginoparae (parthenogenic progeny), but under short days, oviparae (sexual progeny). Later findings of the photoperiodic response were extended to other aphids, such as *Aphis chloris*, *Brevicoryne brassicae*, *Acyrthosiphon pisum*, and *Megoura viciae* (Wilson 1938; Bonnemaison 1951; Kenten 1955; Lees 1959).

14.3.2.1 Neurosecretory System and ILP in the PI

In the 1960s and the 1970s *M. viciae* was extensively studied for its photoperiodic mechanisms. In *M. viciae*, five groups of NSCs, Group I–V cells, were found in the brain (Fig. 14.4; Steel 1977). Radio-frequency microcautery and histological examination of the damaged region suggested that Group I cells and their lateral regions are important for the photoperiodic response (Fig. 14.4; Steel and Lees 1977).

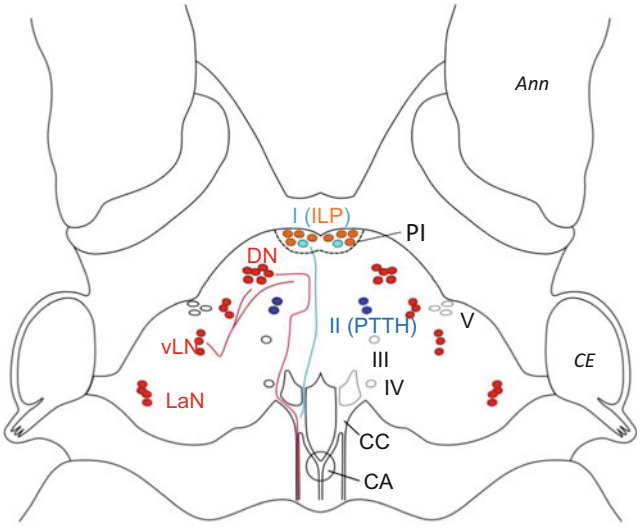

Fig. 14.4 Distribution of neurosecretory cells (blue and orange) and clock cells (red) in the pea aphid *Acyrthosiphon pisum*, in which reproductive morphs, virginoparae and oviparae, are photoperiodically controlled. Dorsal view. Among five neurosecretory cells I in the pars intercerebralis (PI), four cells expressing insulin-like peptide (ILP, orange). Two neurosecretory cells II expressing prothoracicotropic hormone (PTTH, blue). Ann, antennae; CE, compound eyes. For other explanation, see Fig. 14.2. Based on Barberà and Martínez-Torres (2017), Barberà et al. (2017, 2019, 2022), Colizzi et al. (2021), and Steel (1977)

Microcautery of Group I cells resulted in the production of oviparous progenies, even under long days, suggesting that Group I cells secrete virginopara-promoting substances for long days. Damage to the lateral region of Group I cells also cancelled the photoperiodic response. This operation did not destroy Group I cells, but caused depletion of neurosecretory materials inside these cells, suggesting that the lateral region may contain a photoperiodic mechanism to send information to Group I cells (Steel and Lees 1977). From the cell location in the medio-dorsal protocerebrum and staining with paraldehyde fuchsin (PAF), Group I cells appear to correspond to NSCs in the PI, in which many PAF-positive large NSCs have been identified in different insect species (Shiga 2003).

Since the genome sequence is available for *A. pisum* (The International Aphid Genomics Consortium 2010), orthologs of neuropeptide-coding genes were searched, and their expressing cells were mapped in the brain (Fig. 14.4). In *M. viciae*, there are five pairs of Group I cells in the PI (Steel 1977), and immunohistochemistry has revealed four pairs of insulin-like peptide 4 (ILP4)-immunoreactive (-ir) cells in the PI (Cuti et al. 2021). In *A. pisum*, immunohistochemistry and in situ hybridization confirmed four pairs in the PI containing peptide and mRNA of ILP4 (Cuti et al. 2021), while the other Group I cells may have different transmitters and fiber distributions in the ventral nerve cord (Steel 1977). ILP4-ir cells contralaterally extend their axons ventrally and posteriorly to the CC, through which ILP4-ir fibers run in the medial and lateral nerves to the peripheral tissues or organs in the abdomen. In the staining of the first-instar nymph, ILP4-ir fibers in the lateral nerve terminate near the germarium in the ovary (Cuti et al. 2021). Based on the cell location and fiber projection patterns in the brain, Group I cells and ILP4-ir cells are similar, except that Group I cells send fibers to the subesophageal zone and thoracic ganglia, whereas ILP4-ir fibers do not.

In *A. pisum ilp1*, *ilp4*, and *insulin receptor* (*ilr*) mRNA levels in the head are higher under long days than under short days at zeitgeber time (ZT, where ZT0 corresponds to "light on") 6 but not at ZT 18 (Barberà et al. 2019). Higher *ilp4* expression in the brain was also observed during long days in *A. pisum* (Cuti et al. 2021). Long fiber projections from brain cells to the germarium in nymphs support the idea that ILP4 in Group I cells may work as a virginopara-promoting substance to be secreted around the ovary. However, various physiological functions of ILP are known to promote JH synthesis, egg laying, and hemolymph glucose levels in different species (de Velasco et al. 2007; Hasebe and Shiga 2021a, b). A loss-of-function study is required to show the role of ILP in reproduction or photoperiodic response in aphids.

14.3.2.2 Mapping of Circadian Clock Cells with Neurosecretory Cells in the Brain

Clock cells expressing *per*, *timeless* (*tim*), and *cryptochrome* were mapped by in situ hybridization, and four types of cells were identified in the brain of *A. pisum*. These were named dorsal neurons (DNs), dorsal lateral neurons (dLN), ventral lateral

neurons (vLN), and neurons at the lamina (LaN) (Fig. 14.4; Barberà et al. 2017, 2022; Colizzi et al. 2021). DNs are located in the PL, which corresponds to a region lateral to Group I cells of the PI. In the PL, *per* and *tim* in situ hybridization labeled six to eight small DNs. These authors suggested the colocalization of *per* and *tim*. In addition, two other large DNs were found to express *tim*, but not *per*. Immunohistochemistry also showed six to eight PER-ir DNs, and double labeling showed that about half of them were also cryptochrome (CRY) immunoreactive (Colizzi et al. 2021). PER immunoreactivity was observed in the nucleus, whereas CRY immunoreactivity was detected in the cytoplasm. Interestingly, the CRY antibody labeled not only somata but also fibers in *A. pisum*. In the protocerebrum, CRY-positive fibers from the DN projected medially to the PI and to the CC and CA. CRY was also detected in the LN, and double staining showed that CRY and PER were colocalized in at least one LN. The CRY antibody stained a single LN, and its fibers dorsally projected to the PL. The LN fiber projection was similar to that of flies, *D. melanogaster*, and *P. terraenovae*. The ILP4 antibody stained lateral fibers from the PI cells and CRY antibody-stained medial fibers from the PL. This suggests communication between DN and Group I cells, as suggested by Steel and Lees (1977) for *M. viciae*.

In aphids, neuroanatomical studies of photoperiodic mechanisms have resumed after a long break from Lees and Steel (1977). Instead of the small body size, the size of single neurons is large enough to explore the mechanism, and stained fibers from neurosecretory cells are thick and have simple projections (Colizzi et al. 2021). The silencing of genes expressed in the identified neurons is waited to explore their functional aspects in photoperiodic mechanisms.

14.3.3 Heteropteran Bugs Riptortus pedestris, Pyrrhocoris apterus, *and* Plautia stali

Photoperiodic mechanisms have been extensively studied in several heteropteran species, including *R. pedestris* (Fig. 14.5a), the linden bug, *Pyrrhocoris apterus*, and *P. stali*, all of which respond to photoperiod to control reproductive diapause and reproduce under long days but enter reproductive diapause under short days (Hodek 1971; Numata and Hidaka 1982; Kotaki and Yagi 1989; Numata et al. 1993). Because RNA interference-mediated knockdown is effective, these species are good model insects for photoperiodic mechanisms using a molecular approach. In all species RNAi studies showed that clock genes are indispensable for the photoperiodic response (Ikeno et al. 2010; Kotwica-Rolinska et al. 2017; Tamai et al. 2019; Hasebe and Shiga 2021a; Hasebe et al. 2022; Chap. 13).

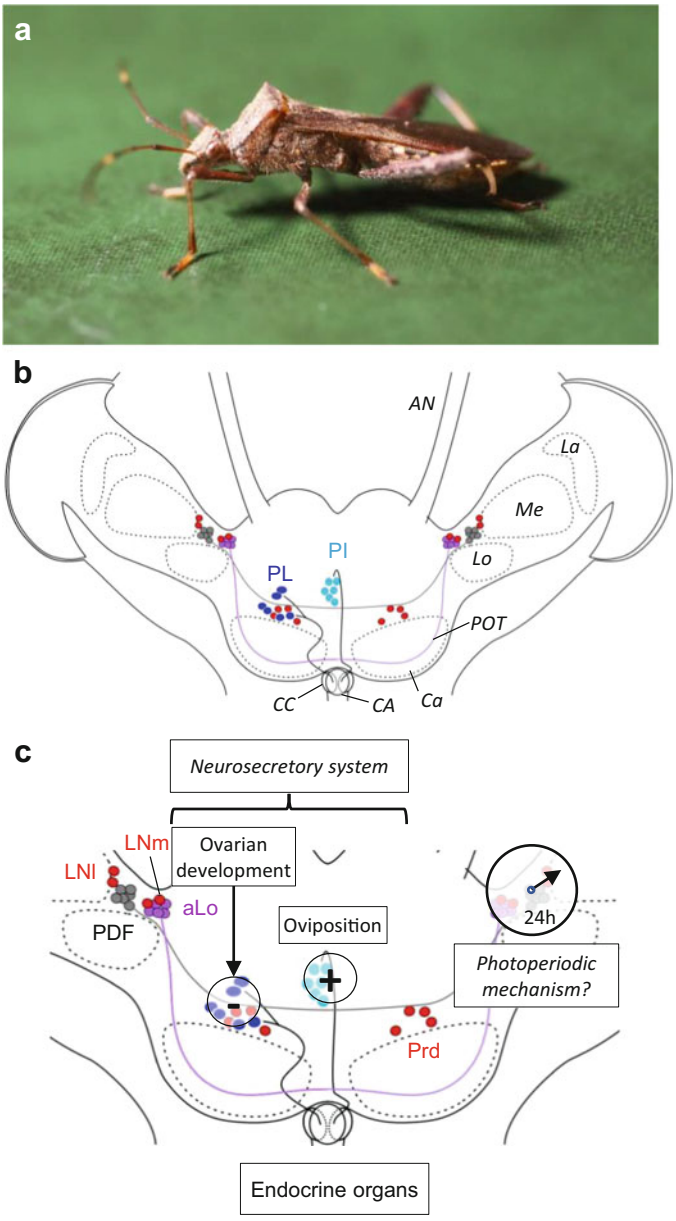

Fig. 14.5 Neuronal circuitry proposed in the bean bug *Riptortus pedestris*. (**a**) an adult female, which develops ovaries under long day but suppresses ovarian development under short days. (**b**) dorsal view of the pars intercerebralis (PI), pars lateralis (PL) neurons (pale and dark blue) innervating to the corpora cardiacum (CC) and corpus allatum (CA), PERIOD-immunoreactive cells (red), and pigment-dispersing factor (PDF)-immunoreactive cells (gray) in the brain. (**c**) possible functional roles in the circuit. Based on Shimokawa et al. (2008, 2014), Ikeno et al. (2014), and Koide et al. (2021)

14.3.3.1 Dual Neurosecretory Systems Controlling Independent Reproductive Activities in Photoperiodic Response of *R. pedestris*

Backfills from the CCCA stained large somata in the PI and PL of *R. pedestris* and *P. stali* (Fig. 14.5b; Shimokawa et al. 2008; Matsumoto et al. 2013). In *P. apterus*, surgical ablation showed the importance of the PI in photoperiodic suppression of reproductive activity, and later *per* and *Clock* gene expression was shown in some PI NSCs (Hodková 1976; Syrova et al. 2003). *P. apterus* continues egg laying under long days, but it completely stops under short days, and after PI ablation, both short-day and long-day females lay eggs, suggesting that PI suppresses reproductive activity under short days (Hodková 1976). In contrast, not the PI but PL have inhibitory roles in ovarian development as reproductive activities in *R. pedestris*, because PL removal causes ovarian development irrespective of photoperiod (Shimokawa et al. 2008). Removal of the PI in *R. pedestris* did not affect the photoperiodic control of ovarian development but reduced the number of deposited eggs under long days (Shimokawa et al. 2008, 2014). This suggests that ovarian development and egg laying are controlled by different neurosecretory systems: the PL controls the former and the PI controls the latter in *R. pedestris* (Fig. 14.5c). In *P. apterus*, it is noteworthy that after PI ablation, egg laying continues for shorter periods than in sham-operated females under long days (Hodková 1976). The PI NSCs are heterogeneous, and some cells may promote egg laying in *P. apterus*, similar to *R. pedestris*.

In *R. pedestris*, some PL neurons send varicose fibers in the CA (Shimokawa et al. 2008). This may indicate that PL neurons play a role in JH production in the CA, although there is no direct evidence to support this.

In *P. stali*, a clear photoperiodic response was observed in JH production rates (Matsumoto et al. 2013). In this species, myoinhibitory peptides (Plast-MIP) have been identified as allatostatins that inhibit JH production in CA (Matsumoto et al. 2017). Plast-MIP immunoreactivity and *plast-mip* mRNA were found in some PI NSCs (Hasegawa et al. 2020; Hasebe and Shiga 2021b). *Plast-mip* knockdown increased the number of reproductive females, even in short days (Tamai et al. 2019). These results suggest that Plast-MIP in some PI NSCs suppresses JH production in short days, causing reproductive diapause. The roles of PI NSCs appear to depend on heteropteran species: in *P apterus* and *P. stali*, PI cells have inhibitory roles, whereas in *R. pedestris*, they have stimulatory roles in reproductive activities (Hodková 1976; Shimokawa et al. 2008; Hasebe and Shiga 2021b).

Recent electrophysiological studies in *P. stali* and *R. pedestris* showed that PI NSCs exhibit photoperiodic responses in their firing activities, but their responses are opposite: PI NCSs are more active under short days in *P. stali*, whereas they are more active under long days in *R. pedestris* (Fig. 14.6a; Hasebe and Shiga 2021a, b). Although the difference was not dramatic in *P. stali*, the higher activity suggests the release of Plast-MIPs from PI under short days. Single-cell PCR additionally revealed *ilp* and *diuretic hormone 44* (*dh44*) expression in PI NSCs in both

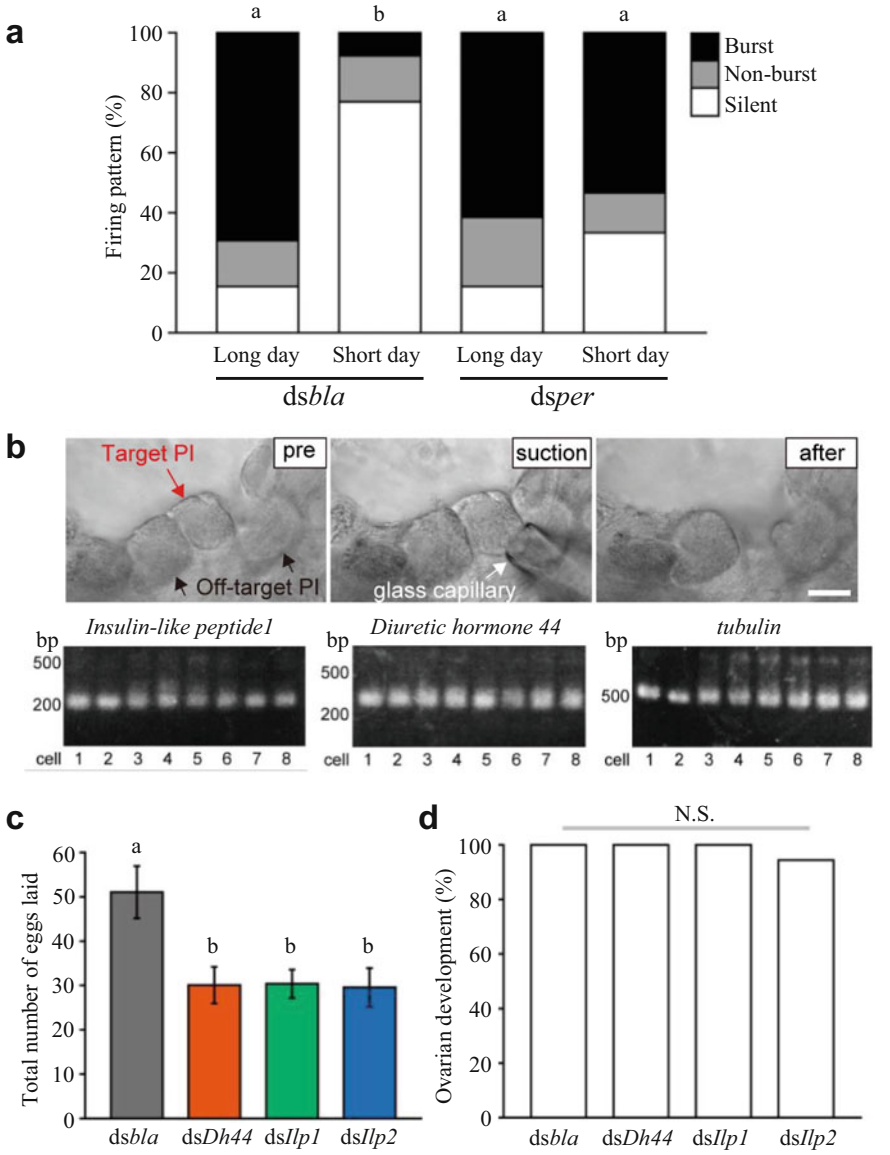

Fig. 14.6 *Period*-dependent photoperiodic response of the pars intercerebralis (PI) cells in *Riptortus pedestris*. (**a**) more PI cells have bursting activity under long days than short days. *period* knockdown cancels photoperiodic response in the PI cell activities. (**b**) peptide gene expression in single PI cells. Upper panels show collection process of a single PI cell. (**c**) peptide gene knockdown reduces numbers of deposited eggs, (**d**) but not ovarian development under long days [adapted from Hasebe and Shiga (2021a)]

P. stali and *R. pedestris* (Fig. 14.6b). In *R. pedestris*, knockdown of *ilp* and *dh44* reduced the number of deposited eggs under long days (Fig. 14.6c), suggesting that PI NSCs actively release these peptides to promote egg laying under long days by promoting firing activity. In contrast, *ilp* and *dh44* knockdown did not interrupt ovarian development in *R. pedestris*, suggesting that the peptides expressed in PI cells are not involved in ovarian development (Fig. 14.6d). The roles of *ilp* and *dh44* in *P. stali* PI cells have not been identified.

Interestingly, in *R. pedestris*, *per* RNAi disrupted the photoperiodic response in PI firing activity (Fig. 14.6a; Hasebe and Shiga 2021a). This suggests that the photoperiodic response of PI activity is under the control of a *per*-expression-dependent photoperiodic clock. *per* RNAi also prevents photoperiodic control of ovarian development (Ikeno et al. 2010), but PI neurons are not required for photoperiodic control of ovaries. Photoperiodic control of ovaries may be mediated by other NSCs, such as PL neurons. *R. pedestris* employs dual neurosecretory systems in which PI neurons control oviposition and PL neuron ovarian development, as suggested above (Fig. 14.5c). The PL NSC might also respond to photoperiod to change the electrical activity to control ovarian development. Although electrode access is rather difficult for PL NSCs, a combination of electrophysiology, single-cell PCR, and RNAi could also be applied to the PL to find important cells and molecules to control ovarian development dependent on photoperiod in the near future.

The next question is which molecules or neurons control the PI or PL NSCs in the photoperiodic response. Recently, Hasebe and Shiga (2022) revealed that glutamate directly controls the photoperiodic response of the PI in *R. pedestris*. L-Glutamate acts as an inhibitory signal to PI neurons via glutamate-gated chloride channels. Knockdown of glutamate-metabolizing enzyme genes and *glutamate-gated chloride channels* disrupted the cellular photoperiodic responses of PI neurons. Knockdown of these genes also attenuates the photoperiodic response to ovarian development. In fact, extracellular glutamate levels in the whole brain were significantly higher under short days than those under long days. The photoperiodic change in glutamate levels was abolished by knockdown of *per*. These results revealed that extracellular glutamate dynamics are photoperiodically regulated depending on the clock gene and play an essential role in the photoperiodic control of ovarian development and PI neuron-mediated oviposition (Fig. 14.7; Hasebe and Shiga 2022). Based on this finding, upstream mechanisms controlling glutamate dynamics in the brain may provide clues for approaching the photoperiodic clock-counter system in the brain.

14.3.3.2 Clock Cells and Relevant Neurons to Photoperiodic Responses

As *per* knockdown cancelled photoperiodic responses in brain extracellular glutamate levels, PI activities, ovarian development, and oviposition (Ikeno et al. 2010; Hasebe and Shiga 2021a, 2022), *per*-expressing cells in the brain must be involved in the photoperiodic clock-counter circuit. Immunohistochemistry mapped six groups of PER-ir cells in the brains of *R. pedestris* (Koide et al. 2021). Three of

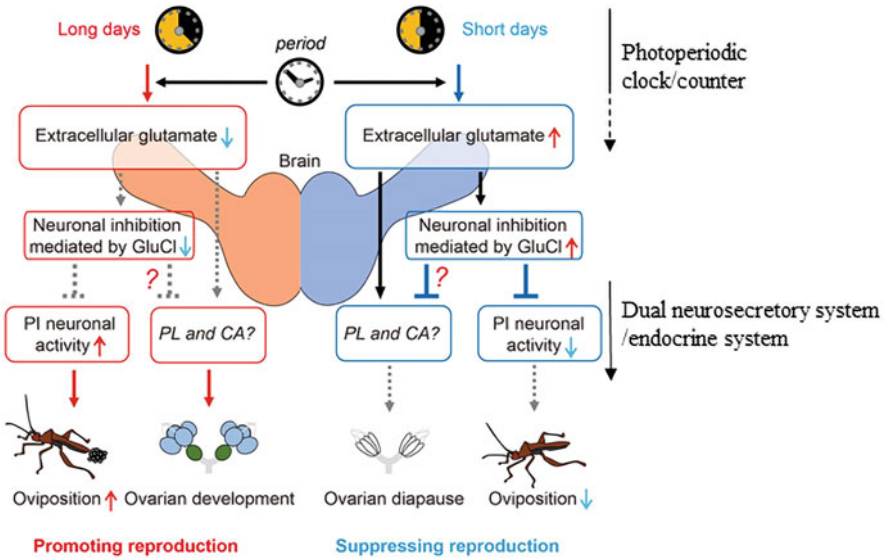

Fig. 14.7 Predicted hierarchical glutamatergic pathway for photoperiodic control of reproduction in *Riptortus pedestris*. Brain glutamate levels photoperiodically change depending on the clock gene *period*. The glutamatergic signal may regulate in parallel the dual neurosecretory system of the pars intercerebralis (PI) neurons for oviposition and the pars lateralis (PL) neurons for ovarian development (adapted from Hasebe and Shiga 2022). *GluCl* glutamate-gated chloride channel, *CA* corpus allatum

them were anatomically comparable to the fly clock cells (Fig. 14.5b, c). Lateral neuron lateral (LNl, cell number, $n = 2$) at the anterior base of the medulla probably corresponds to s-LNv in flies. LN medial (LNm, $n = 3$) located in the anterior region of the lobula may correspond to lLNv in flies. Dorsal protocerebrum neurons (Prd, $n = 4$–16) were found in the dorsal region of the protocerebrum. Prds probably correspond to DNm/DN1,2 in the flies.

Multiple labeling showed that LNl, LNm, PDF-ir somata, and somata of the anterior lobula (aLO) are distinct sets of cells located very close to each other (Fig. 14.5c; Koide et al. 2021). By microsurgery of the brain, aLO neurons connecting the bilateral optic lobes through the posterior optic tract (POT) and a small region containing PDF-ir cells at the base of the medulla were suggested to be indispensable for the photoperiodic control of ovarian development (Fig. 14.5c; Ikeno et al. 2014; Xi et al. 2017). The aLO neurons were labeled by tracing afferent pathways from the compound eye, the photoperiodic photoreceptor (Morita and Numata 1997), to the central brain, and the severance of the POT prevented the photoperiodic response (Xi et al. 2017). The aLO neurons send a long fiber to the contralateral aLO soma region (Fig. 14.5b). PDF-ir varicose fibers project onto dense aLO fibers in the basal medulla. Although the fiber distribution has not yet been identified, PER-ir LNm and LNl may be connected with PDF or aLO neurons in this area. Although ablation of a region containing PDF-ir somata disrupted the

photoperiodic response, *pdf* knockdown did not (Ikeno et al. 2014). This suggests that PDF-expressing neurons, but not PDF peptides, may be required for the photoperiodic response. An alternative interpretation could be that disruption of the photoperiodic response is caused by ablation of *per*-expressing LNl, which resides very close to PDF-ir cells (Ikeno et al. 2014: Koide et al. 2021).

Microsurgery, neuroanatomy, and RNAi studies have shown that the anterior basal medulla region in the optic lobe is a candidate area for the photoperiodic mechanism. This region is called the accessory medulla, into which most clock neurons send fibers and receive light inputs from photoreceptors directly or indirectly in *D. melanogaster* (Schlichting et al. 2016; Li et al. 2018). In *R. pedestris*, this region may also serve as a hub for receiving photic and clock information relevant to the photoperiodic clock (Koide et al. 2021).

When the location and projection of PL neurons, PER-ir neurons, and PDF-ir neurons are compared by single stained images, both PL somata and PER-ir Prd cells appear to be located in the PL, and PDF-ir fibers may reach there (Ikeno et al. 2014; Koide et al. 2021; Shimokawa et al. 2008). In addition, identification of glutamatergic neurons is necessary. Multiple staining of PDF-ir, PER-ir, PL, and glutamatergic neurons should explore the neural circuitry involved in how photoperiodic signals are processed to reach the PI and PL NSCs and alter their activities.

14.4 Future Perspectives

Neurosecretory mechanisms that control seasonal phenotypes have been studied in different insect orders (Shiga and Numata 2007). In contrast, the mechanisms underlying the photoperiodic clock and counter remain unsolved. However, recent progress in the anatomy and physiology of PI- and PL-NSCs in combination with RNAi in heteropteran species has encouraged us to develop further understanding of photoperiodic NSCs and access their upstream pathways reaching the photoperiodic clock and counter. Using knowledge of the *Drosophila* clock neuron network, flies are also a good model species for examining the photoperiodic clock. The application of single-cell PCR to *P. terraenovae* NSCs may lead to the identification of molecules and neurons controlling JH production in the CA mediating photoperiodic response. A few clock cells in the optic lobe may constitute a simple clock network in hemipteran species. Careful examination of the clock network is required for these species.

The importance of the anterior base of the medulla, including clock cells (s-LNv, LNl) and PDF cells in the photoperiodic response, is a common feature between *P. terraenovae* and *R. pedestris* (Figs. 14.2c and 14.5c). In future studies, it will be important to examine how these cells respond to different photoperiods. However, to obtain electrical recordings from clock cells, cell labeling using a knock-in technique is desirable for targeting these cells. Plasticity in fiber projections may be involved (Shiga 2013). In *D. melanogaster*, s-LNv fibers are well known to show plasticity in a circadian manner at dorsal projections in the protocerebrum, changing synaptic

partners (Gorostiza et al. 2014; Herrero et al. 2020; Fernandez et al. 2020). There might be photoperiodic plasticity in s-LNv flies, depending on the day length. In addition, plasticity may occur in the dendritic region of PI or PL neurons by increasing the number of short- or long-day receptions. This may be an approach for exploring the photoperiodic counting mechanism. With the help of gene engineering techniques, photoperiodic clock and counter mechanisms should be solved at the network level.

References

Andreatta G, Kyriacou CP, Flatt T, Costa R (2018) Aminergic signaling controls ovarian dormancy in *Drosophila*. Sci Rep 8:2030. https://doi.org/10.1038/s41598-018-20407-z

Anduaga AM, Nagy D, Costa R, Kyriacou CP (2018) Diapause in *Drosophila melanogaster*—photoperiodicity, cold tolerance and metabolites. J Insect Physiol 105:46–53. https://doi.org/10.1016/j.jinsphys.2018.01.003

Bajgar A, Jindra M, Dolezel D (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc Natl Acad Sci USA 110:4416–4421. https://doi.org/10.1073/pnas.1217060110

Barberà M, Martínez-Torres D (2017) Identification of the *prothoracicotropic hormone* (*Ptth*) coding gene and localization of its site of expression in the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 26:654–664. https://doi.org/10.1111/imb.12326

Barberà M, Collantes-Alegre JM, Martínez-Torres D (2017) Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrthosiphon pisum*. Insect Biochem Mol Biol 83:54–67. https://doi.org/10.1016/j.ibmb.2017.02.006

Barberà M, Cañas-Cañas R, Martínez-Torres D (2019) Insulin-like peptides involved in photoperiodism in the aphid *Acyrthosiphon pisum*. Insect Biochem Mol Biol 112:103185. https://doi.org/10.1016/j.ibmb.2019.103185

Barberà M, Collantes-Alegre JM, Martínez-Torres D (2022) Mapping and quantification of cryptochrome expression in the brain of the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 31:159–169. https://doi.org/10.1111/imb.12747

Blanchardon E, Grima B, Klarsfeld A, Chélot E, Hardin PE, Préat T et al (2001) Defining the role of *Drosophila* lateral neurons in the control of circadian rhythms in motor activity and eclosion by targeted genetic ablation and PERIOD protein overexpression. Eur J Neurosci 13:871–888. https://doi.org/10.1046/j.0953-816x.2000.01450.x

Bonnemaison L (1951) Contribution a l'etude des facteurs provoquant l'apparition des formes ailees et sexuees chez les Aphidinae. Ann Epiphyt 2:1–380

Bradshaw and Holzapfel (2007) Evolution of animal photoperiodism. Annu Rev Ecol Evol Syst 38:1–25. https://doi.org/10.1146/annurev.ecolsys.37.091305.110115

Bünning E (1936) Die endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion. Ber Dtsch Bot Ges 54:590–607

Chiang AS, Lin WY, Liu HP, Pszczolkowski MS, Fu T-F, Chiuet S-L et al (2002) Insect NMDA receptors mediate juvenile hormone biosynthesis. Proc Natl Acad Sci USA 99:37–42. https://doi.org/10.1073/pnas.012318899

Colizzi FS, Beer K, Cuti P, Deppisch P, Martínez-Torres D, Yoshii T et al (2021) Antibodies against the clock proteins Period and Cryptochrome reveal the neuronal organization of the circadian clock in the pea aphid. Front Physiol 12:705048. https://doi.org/10.3389/fphys.2021.705048

Collins B, Kane EA, Reeves DC, Akabas MH, Blau J (2012) Balance of activity between LN(v)s and glutamatergic dorsal clock neurons promotes robust circadian rhythms in *Drosophila*. Neuron 74:706–718. https://doi.org/10.1016/j.neuron.2012.02.034

Cuti P, Barberà M, Veenstra JA, Martínez-Torres D (2021) Progress in the characterization of insulin-like peptides in aphids: immunohistochemical mapping of ILP4. Insect Biochem Mol Biol 136:103623. https://doi.org/10.1016/j.ibmb.2021.103623

Danilevskii AS (1965) Photoperiodism and seasonal development of insects. Oliver and Boyd, Edinburgh

De Belle JS, Heisenberg M (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. Science 263:692–625. https://doi.org/10.1126/science.8303280

De Velasco B, Shen J, Go S, Hartenstein V (2004) Embryonic development of the *Drosophila* corpus cardiacum, a neuroendocrine gland with similarity to the vertebrate pituitary, is controlled by sine oculis and glass. Dev Biol 274:280–294. https://doi.org/10.1016/j.ydbio.2004.07.015

De Velasco B, Erclik T, Shy D, Sclafania J, Lipshitz H, McInnes R et al (2007) Specification and development of the pars intercerebralis and pars lateralis, neuroendocrine command centers in the *Drosophila* brain. Dev Biol 302:309–323. https://doi.org/10.1016/j.ydbio.2006.09.035

Denlinger DL (2002) Regulation of diapause. Annu Rev Entomol 47:93–122. https://doi.org/10.1146/annurev.ento.47.091201.145137

Denlinger DL, Yocum GD, Rinehart JP (2012) Hormonal control of diapause. In: Gilbert LI (ed) Insect endocrinology. Academic Press, London, pp 430–463

Dubnau J, Tully T (1998) Gene discovery in *Drosophila*: new insights for learning and memory. Annu Rev Neurosci 21:407–444. https://doi.org/10.1146/annurev.neuro.21.1.407

Erber J, Homberg U, Gronenberg W (1987) Functional roles of the mushroom bodies in insects. In: Gupta A (ed) Arthropod brain. Wiley-Interscience, New York, pp 485–511

Fernandez M, Pettibone HL, Bogart JT, Roell CJ, Davey CE, Pranevicius A et al (2020) Sites of circadian clock neuron plasticity mediate sensory integration and entrainment. Curr Biol 30:2225–2237.e5. https://doi.org/10.1016/j.cub.2020.04.025

Frenkel L, Muraro NI, González ANB, Marcora MS, Bernabó G, Hermann-Luibl C et al (2017) Organization of circadian behavior relies on glycinergic transmission. Cell Rep 19:72–85. https://doi.org/10.1016/j.celrep.2017.03.034

Fujiwara Y, Hermann-Luibl C, Katsura M, Sekiguchi M, Ida T, Helfrich-Förster C et al (2018) The CCHamide1 neuropeptide expressed in the anterior dorsal neuron 1 conveys a circadian signal to the ventral lateral neurons in *Drosophila melanogaster*. Front Physiol 9:1276. https://doi.org/10.3389/fphys.2018.01276

Gao N, von Schantz M, Foster RG, Hardie J (1999) The putative brain photoperiodic photoreceptors in the vetch aphid, *Megoura viciae*. J Insect Physiol 45:1011–1019. https://doi.org/10.1016/s0022-1910(99)00082-7

Gibbs D (1975) Reversal of pupal diapause in *Sarcophaga argyrostoma* by temperature shifts after puparium formation. J Insect Physiol 21:1179–1186. https://doi.org/10.1016/0022-1910(75)90085-2

Giebultowicz JM (1999) Insect circadian clocks: is it all in their heads? J Insect Physiol 45:791–800. https://doi.org/10.1016/S0022-1910(99)00055-4

Gorostiza EA, Depetris-Chauvin A, Frenkel L, Pírez N, Ceriani MF (2014) Circadian pacemaker neurons change synaptic contacts across the day. Curr Biol 24:2161–2167. https://doi.org/10.1016/j.cub.2014.07.063

Goryshin NI, Tyshchenko VP (1974) The place of the memory link in the mechanism of photoperiodic reaction in insects. Zh Obshch Biol 35:518–530. (in Russian)

Goto SG, Shiga S, Numata H (2010) Photoperiodism in insects: perception of light and the role of clock genes. In: Nelson RJ, Denlinger DL, Somers DE (eds) Photoperiodism: the biological calendar. Oxford University Press, Oxford, pp 258–286

Grima B, Chélot E, Xia R, Rouyer F (2004) Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature 431:869–873. https://doi.org/10.1038/nature02935

Hamanaka Y, Numata H, Shiga S (2004) Morphology and electrical properties of neurons projecting to the retrocerebral complex in the blow fly, *Protophormia terraenovae*. Cell Tissue Res 318:403–418. https://doi.org/10.1007/s00441-004-0935-1

Hamanaka Y, Yasuyama K, Numata H, Shiga S (2005) Synaptic connections between pigment-dispersing factor-immunoreactive neurons and neurons in the pars lateralis of the blow fly *Protophormia terraenovae*. J Comp Neurol 49:390–399. https://doi.org/10.1002/cne.20712

Hamanaka Y, Tanaka S, Numata H, Shiga S (2007) Peptide immunocytochemistry of neurons projecting to the retrocerebral complex in the blow fly, *Protophormia terraenovae*. Cell Tissue Res 329:581–593. https://doi.org/10.1007/s00441-007-0433-3

Hamasaka Y, Rieger D, Parmentier ML, Grau Y, Helfrich-Förster C, Nässel DR (2007) Glutamate and its metabotropic receptor in *Drosophila* clock neuron circuits. J Comp Neurol 505:32–45. https://doi.org/10.1002/cne.21471

Hasebe M, Shiga S (2021a) Oviposition-promoting pars intercerebralis neurons show period-dependent photoperiodic changes in their firing activity in the bean bug. Proc Natl Acad Sci USA 118:e2018823118. https://doi.org/10.1073/pnas.2018823118

Hasebe M, Shiga S (2021b) Photoperiodic response in the pars intercerebralis neurons, including Plast-MIP neurons, in the brown-winged green bug, *Plautia stali*. Zool Sci 38:317–325. https://doi.org/10.2108/zs210005

Hasebe M, Shiga S (2022) Clock gene-dependent glutamate dynamics in the bean bug brain regulate photoperiodic reproduction. PLoS Biol 20(9):e3001734. https://doi.org/10.1371/journal.pbio.3001734

Hasebe M, Kotaki T, Shiga S (2022) Pigment-dispersing factor is involved in photoperiodic control of reproduction in the brown-winged green bug, *Plautia stali*. J Insect Physiol 137:104359. https://doi.org/10.1016/j.jinsphys.2022.104359

Hasegawa T, Hasebe M, Shiga S (2020) Immunohistochemical and direct mass spectral analyses of *Plautia stali* myoinhibitory peptides in the cephalic ganglia of the brown-winged green bug Plautia stali. Zool Sci 37:42–49. https://doi.org/10.2108/zs190092

Helfrich-Förster C (1995) The period gene is expressed in CNS neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. Proc Natl Acad Sci USA 92:612–616. https://doi.org/10.1073/pnas.92.2.612

Hermann-Luibl C, Helfrich-Förster C (2015) Clock network in *Drosophila*. Curr Opin Insect Sci 7:65–70. https://doi.org/10.1016/j.cois.2014.11.003

Herrero A, Yoshii T, Ispizua JI (2020) Coupling neuropeptide levels to structural plasticity in *Drosophila* clock neurons. Curr Biol 30:3154–3166.e4. https://doi.org/10.1016/j.cub.2020.06.009

Hiroyoshi S, Takeda M, Mitsunaga T, Reddy GVP (2019) Quantitative response to photoperiod and weak coupling between seasonal morphs and diapause regulation in the Asian comma butterfly, *Polygonia c-aureum* (Lepidoptera: Nymphalidae). Eur J Entomol 116:123–132. https://doi.org/10.14411/eje.2019.014

Hodek I (1971) Sensitivity of larvae to photoperiods controlling the adult diapause of two insects. J Insect Physiol 50:373–381. https://doi.org/10.1016/j.jinsphys.2004.01.002

Hodková M (1976) Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. Nature 263:521–523. https://doi.org/10.1038/263521a0

Ikeda K, Numata H, Shiga S (2005) Roles of the mushroom bodies in olfactory learning and photoperiodism in the blow fly *Protophormia terraenovae*. J Insect Physiol 51:669–680. https://doi.org/10.1016/j.jinsphys.2005.05.001

Ikeno T, Tanaka SI, Numata H, Goto SG (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol 8:116. https://doi.org/10.1186/1741-7007-8-116

Ikeno T, Numata H, Goto SG, Shiga S (2014) Involvement of the brain region containing pigment-dispersing factor-immunoreactive neurons in the photoperiodic response of the bean bug, *Riptortus pedestris*. J Exp Biol 217:453–462. https://doi.org/10.1242/jeb.091801

Johard HA, Yoshii T, Dircksen H, Cusumano P, Rouyer F, Helfrich-Förster C et al (2009) Peptidergic clock neurons in *Drosophila*: ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. J Comp Neurol 516:59–73. https://doi.org/10.1002/cne.22099

Kenten J (1955) The effect of photoperiod and temperature on reproduction in *Acyrthosiphon pisum* (Harris) and on the forms produced. Bull Entomol Res 46:599–624. https://doi.org/10.1017/S0007485300039572

Koide R, Jili X, Hamanaka Y, Shiga S (2021) Mapping PERIOD-immunoreactive cells with neurons relevant to photoperiodic response in the bean bug *Riptortus pedestris*. Cell Tissue Res 385:571–583. https://doi.org/10.1007/s00441-021-03451-6

Kotaki T, Yagi S (1989) Hormonal control of adult diapause in the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). Appl Entomol Zool 24:42–51. https://doi.org/10.1303/aez.24.42

Kotwica-Rolinska J, Pivarciova L, Vaneckova H, Dolezel D (2017) The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage. Physiol Entomol 42:266–273. https://doi.org/10.1111/phen.12197

Kurogi Y, Mizuno Y, Imura Y, Niwa R (2021) Neuroendocrine regulation of reproductive dormancy in the fruit fly *Drosophila melanogaster*: a review of juvenile hormone-dependent regulation. Front Ecol Evol 9:715029. https://doi.org/10.3389/fevo.2021.715029

Lees AD (1959) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—I: the influence of these factors on apterous virginoparae and their progeny. J Insect Physiol 3:92–117. https://doi.org/10.1016/0022-1910(59)90024-1

Li MT, Cao LH, Xiao N, Tang M, Deng B, Yang T et al (2018) Hub-organized parallel circuits of central circadian pacemaker neurons for visual photoentrainment in *Drosophila*. Nat Commun 9:4247. https://doi.org/10.1038/s41467-018-06506-5

Marcovitch S (1924) The migration of the Aphididae and the appearance of sexual forms as affected by the relative length of daily exposure. J Agric Res 27:513–522

Matsumoto K, Numata H, Shiga S (2013) Role of the brain in photoperiodic regulation of juvenile hormone biosynthesis in the brown-winged green bug *Plautia stali*. J Insect Physiol 59:387–393. https://doi.org/10.1016/j.jinsphys.2013.01.007

Matsumoto K, Suetsugu Y, Tanaka Y, Kotaki T, Goto SG, Shinoda T et al (2017) Identification of allatostatins in the brown-winged green bug *Plautia stali*. J Insect Physiol 96:21–28. https://doi.org/10.1016/j.jinsphys.2016.10.005

Matsuo J, Nakayama S, Numata H (1997) Role of the corpus allatum in the control of adult diapause in the blow fly, *Protophormia terraenovae*. J Insect Physiol 43:211–216. https://doi.org/10.1016/s0022-1910(96)00103-5

Morita A, Numata H (1997) Distribution of photoperiodic receptors in the compound eyes of the bean bug, *Riptortus clavatus*. J Comp Physiol A 180:181–185. https://doi.org/10.1007/s003590050039

Muguruma F, Goto SG, Numata H, Shiga S (2010) Effect of photoperiod on clock gene expression and subcellular distribution of PERIOD in the circadian clock neurons of the blow fly *Protophormia terraenovae*. Cell Tissue Res 340:497–507. https://doi.org/10.1007/s00441-010-0966-8

Nagy D, Andreatta G, Bastianello S, Anduaga AM, Mazzotta G, Kyriacou CP et al (2018) A semi-natural approach for studying seasonal diapause in *Drosophila melanogaster* reveals robust photoperiodicity. J Biol Rhythm 33:117–125. https://doi.org/10.1177/0748730417754116

Nagy D, Cusumano P, Andreatta G, Anduaga AM, Hermann-Luibl C, Reinhard N et al (2019) Peptidergic signaling from clock neurons regulates reproductive dormancy in *Drosophila melanogaster*. PLoS Genet 15:e1008158. https://doi.org/10.1371/journal.pgen.1008158

Nässel DR, Shiga S, Mohrherr CJ, Rao KR (1993) Pigment-dispersing hormone-like peptide in the nervous system of the flies *Phormia* and *Drosophila*: immunocytochemistry and partial characterization. J Comp Neurol 331:183–198. https://doi.org/10.1002/cne.903310204

Numata H, Hidaka T (1982) Photoperiodic control of adult diapause in the bean bug, *Riptortus clavatus* Thunberg (Heteroptera: Coreidae). I. Reversible induction and termination of diapause. Appl Entomol Zool 17:530–538. https://doi.org/10.1303/aez.17.530

Numata H, Kobayashi S (1994) Threshold and quantitative photoperiodic responses exist in an insect. Experientia 50:969–971. https://doi.org/10.1007/BF01923489

Numata H, Shiga S (1995) Induction of adult diapause by photoperiod and temperature in *Protophormia terraenovae* (Diptera: Calliphoridae) in Japan. Environ Entomol 24:1633–1636. https://doi.org/10.1093/ee/24.6.1633

Numata H, Saulich AH, Volkovich TA (1993) Photoperiodic responses of the linden bug, *Pyrrhocoris apterus*, under conditions of constant temperature and under thermoperiodic conditions. Zool Sci 10:521–527

Numata H, Shiga S, Morita A (1997) Photoperiodic receptors in arthropods. Zool Sci 14:187–197

Ojima N, Hara Y, Ito H, Yamamoto D (2018) Genetic dissection of stress-induced reproductive arrest in *Drosophila melanogaster* females. PLoS Genet 14:e1007434. https://doi.org/10.1371/journal.pgen.1007434

Pittendrigh CS, Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents V. Pacemaker structure: a clock for all seasons. J Comp Physiol 106:333–355. https://doi.org/10.1007/BF01417860

Pittendrigh CS, Minis DH (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. Am Nat 98:261–294

Plautz JD, Kaneko M, Hall JC, Kay SA (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278:1632–1635. https://doi.org/10.1126/science.278.5343.1632

Raabe M (1989) Recent developments in insect neurohormones. Plenum Press, New York

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802. https://doi.org/10.1016/s0092-8674(00)81676-1

Saunders DS (1971) The temperature-compensated photoperiodic clock 'programming' development and pupal diapause in the flesh fly, *Sarcophaga argyrostoma*. J Insect Physiol 17:801–812. https://doi.org/10.1016/0022-1910(71)90098-9

Saunders DS (2002) Insect clocks, 3rd edn. Elsevier, Amsterdam

Saunders DS (2021) Insect photoperiodism: Bünning's hypothesis, the history and development of an idea. Eur J Entomol 118:1–13. https://doi.org/10.14411/eje.2021.001

Saunders DS, Cymborowski B (1996) Removal of optic lobes of adult blow flies (*Calliphora vicina*) leaves photoperiodic induction of larval diapause intact. J Insect Physiol 42:807–811. https://doi.org/10.1016/0022-1910(96)00007-8

Saunders DS, Gilbert LI (1990) Regulation of ovarian diapause in *Drosophila melanogaster* by photoperiod and moderately low temperature. J Insect Physiol 36:195–200. https://doi.org/10.1016/0022-1910(90)90122-V

Schlichting M, Menegazzi P, Lelito KR, Yao Z, Buh E, Benetta ED et al (2016) A neural network underlying circadian entrainment and photoperiodic adjustment of sleep and activity in *Drosophila*. J Neurosci 36:9084–9096. https://doi.org/10.1523/JNEUROSCI.0992-16.2016

Seluzicki A, Flourakis M, Kula-Eversole E, Zhang L, Kilman V, Allada R (2014) Dual PDF signaling pathways reset clocks via TIMELESS and acutely excite target neurons to control circadian behavior. PLoS Biol 12:e1001810. https://doi.org/10.1371/journal.pbio.1001810

Shafer OT, Helfrich-Förster C, Renn SCP, Taghert PH (2006) Reevaluation of *Drosophila melanogaster*'s neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol 498:180–193. https://doi.org/10.1002/cne.21021

Shiga S (2003) Anatomy and functions of brain neurosecretory cells in Diptera. Microsc Res Tech 62:114–131. https://doi.org/10.1002/jemt.10366

Shiga S (2013) Photoperiodic plasticity in circadian clock neurons in insects. Front Physiol 4:69. https://doi.org/10.3389/fphys.2013.00069

Shiga S, Numata H (1997) Induction of reproductive diapause via perception of photoperiod through the compound eyes in the adult blow fly, *Protophormia terraenovae*. J Comp Physiol A 181:35–40. https://doi.org/10.1007/s003590050090

Shiga S, Numata H (2000) The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae*. Naturwissenschaften 87:125–128. https://doi.org/10.1007/s001140050689

Shiga S, Numata H (2007) Neuroanatomical approaches to the study of insect photoperiodism. Photochem Photobiol 83:76–86. https://doi.org/10.1562/2006-03-31-IR-863

Shiga S, Numata H (2009) Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, *Protophormia terraenovae*. J Exp Biol 212:867–877. https://doi.org/10.1242/jeb.027003

Shiga S, Toyoda I, Numata H (2000) Neurons projecting to the retrocerebral complex of the adult blow fly, *Protophormia terraenovae*. Cell Tissue Res 299:427–439. https://doi.org/10.1007/s004419900110

Shiga S, Hamanaka Y, Tatsu Y, Okuda T, Numata H (2003) Juvenile hormone biosynthesis in diapause and nondiapause females of the adult blow fly *Protophormia terraenovae*. Zool Sci 20:1199–1206. https://doi.org/10.2108/zsj.20.1199

Shimokawa K, Numata H, Shiga S (2008) Neurons important for the photoperiodic control of diapause in the bean bug, *Riptortus pedestris*. J Comp Physiol A 194:751–762. https://doi.org/10.1007/s00359-008-0346-y

Shimokawa K, Numata H, Shiga S (2014) Pars intercerebralis promotes oviposition in the bean bug, *Riptortus pedestris* (Heteroptera: Alydidae). Appl Entomol Zool 49:525–528. https://doi.org/10.1007/s13355-014-0281-z

Shintani Y (2011) Quantitative short-day photoperiodic response in larval development and its adaptive significance in an adult-overwintering cerambycid beetle, *Phytoecia rufiventris*. J Insect Physiol 57:1053–1059. https://doi.org/10.1016/j.jinsphys.2011.05.005

Shintani Y, Shiga S, Numata H (2009) Different photoreceptor organs are used for photoperiodism in the larval and adult stages of the carabid beetle, *Leptocarabus kumagaii*. J Exp Biol 212:3651–3655. https://doi.org/10.1242/jeb.034033

Stay B, Zhang JR, Tobe SS (2002) Methyl farnesoate and juvenile hormone production in embryos of *Diploptera punctata* in relation to innervation of corpora allata and their sensitivity to allatostatin. Peptides 23:1981–1990. https://doi.org/10.1016/S0196-9781(02)00185-7

Steel CGH (1977) The neurosecretory system in the aphid *Megoura viciae*, with reference to unusual features associated with long distance transport of neurosecretion. Gen Comp Endocrinol 31:307–322. https://doi.org/10.1016/0016-6480(77)90095-8

Steel CGH, Lees AD (1977) The role of neurosecretion in the photoperiodic control of polymorphism in the aphid *Megoura viciae*. J Exp Biol 67:117–135. https://doi.org/10.1242/jeb.67.1.117

Stoleru D, Peng Y, Agosto J, Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. Nature 431:862–868. https://doi.org/10.1038/nature02926

Stoleru D, Peng Y, Nawathean P, Rosbash M (2005) A resetting signal between *Drosophila* pacemakers synchronizes morning and evening activity. Nature 438:238–242. https://doi.org/10.1038/nature04192

Stoleru D, Nawathean P, de la Paz Fernández M, Menet JS, Ceriani MF, Rosbash M (2007) The *Drosophila* circadian network is a seasonal timer. Cell 129:207–219. https://doi.org/10.1016/j.cell.2007.02.038

Syrova Z, Dolezel D, Sauman I, Hodková M (2003) Photoperiodic regulation of diapause in linden bugs: are period and Clock genes involved? Cell Mol Life Sci 60:2510–2515. https://doi.org/10.1007/s00018-003-3227-0

Takeda M, Skopik SD (1997) Photoperiodic time measurement and related physiological mechanisms in insects and mites. Annu Rev Entomol 42:323–349. https://doi.org/10.1146/annurev.ento.42.1.323

Tamai T, Shiga S, Goto SG (2019) Roles of the circadian clock and endocrine regulator in the photoperiodic response of the brown-winged green bug *Plautia stali*. Physiol Entomol 44:43–52. https://doi.org/10.1111/phen.12274

Tamaki S, Takemoto S, Uryu O, Kamae Y, Nose M, Shinohara T et al (2013) Opsins are involved in nymphal photoperiodic responses in the cricket *Modicogryllus siamensis*. Physiol Entomol 38:163–172. https://doi.org/10.1111/phen.12258

Tanaka A, Kuga Y, Tanaka Y, Goto SG, Numata H, Shiga S (2013) Effects of ablation of the pars intercerebralis on ecdysteroid quantities and yolk protein expression in the blowfly *Protophormia terraenovae*. Physiol Entomol 38:192–201. https://doi.org/10.1111/phen.12012

Tanigawa N, Shiga S, Numata H (1999) Role of the corpus allatum in the control of reproductive diapause in the male blow fly, *Protophormia terraenovae*. Zool Sci 16:639–644. https://doi.org/10.2108/zsj.16.639

Tauber MJ, Tauber CA, Masaki S (1986) Seasonal adaptations of insects. Oxford University Press, New York

The International Aphid Genomics Consortium (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol 8:e1000313. https://doi.org/10.1371/journal.pbio.1000313

Tyshchenko VP (1966) Two-oscillatory model of the physiological mechanism of insect photoperiodic reaction. Zh Obshch Biol 27:209–222. (in Russian)

Tyshchenko VP, Goryshin NI, Azarian AG (1972) Role of circadian processes in the photoperiodism of insects. Zh Obshch Biol 33:21–31. (in Russian)

Veelaert D, Schoofs L, De Loof A (1998) Peptidergic control of the corpus cardiacum-corpora allata complex of locusts. Int Rev Cytol 182:249–302. https://doi.org/10.1016/s0074-7696(08)62171-3

Waddel S, Quinn WG (2001) Flies, genes, and learning. Annu Rev Neurosci 24:1283–1309. https://doi.org/10.1146/annurev.neuro.24.1.1283

Wilson F (1938) Some experiments on the influence of environment upon the forms of *Aphis chloris* Koch (Aphididae). Trans R Entomol Soc Lond 87:165–180

Xi J, Toyoda I, Shiga S (2017) Afferent neural pathways from the photoperiodic receptor in the bean bug, *Riptortus pedestris*. Cell Tissue Res 368:469–485. https://doi.org/10.1007/s00441-016-2565-9

Yamaguchi K, Goto SG (2019) Distinct physiological mechanisms induce latitudinal and sexual differences in the photoperiodic induction of diapause in a fly. J Biol Rhythm 34:293–306. https://doi.org/10.1177/0748730419841931

Yamamoto M, Shiga S, Goto SG (2017) Distribution of PERIOD-immunoreactive neurons and temporal change of the immunoreactivity under long-day and short-day conditions in the larval brain of the flesh fly *Sarcophaga similis*. Chronobiol Int 34:819–825. https://doi.org/10.1080/07420528.2017.1310736

Yasuyama K, Okada Y, Hamanaka Y, Shiga S (2006) Synaptic connections between eyelet photoreceptors and pigment dispersing factor-immunoreactive neurons of the blowfly *Protophormia terraenovae*. J Comp Neurol 494:331–344. https://doi.org/10.1002/cne.20812

Yasuyama K, Hase H, Shiga S (2015) Neuroanatomy of pars intercerebralis neurons with special reference to their connections with neurons immunoreactive for pigment-dispersing factor in the blow fly *Protophormia terraenovae*. Cell Tissue Res 362:33–43. https://doi.org/10.1007/s00441-015-2192-x

Yoshii T, Rieger D, Helfrich-Förster C (2012) Two clocks in the brain: an update of the morning and evening oscillator model in *Drosophila*. Prog Brain Res 199:59–82. https://doi.org/10.1016/B978-0-444-59427-3.00027-7

Zupanic GK (1996) Peptidergic transmission: from morphological correlates to functional implications. Micron 27:35–91. https://doi.org/10.1016/0968-4328(95)00028-3

Chapter 15
Seasonal Timer in Aphids

Naoki Matsuda

Abstract Aphids seasonally switch their reproductive mode between viviparous parthenogenesis and oviparous bisexual reproduction, and this switch is driven by short days. The response to short days is suppressed by a transgenerational mechanism called a seasonal timer over several generations from the overwintered generation. The duration for which the seasonal timer functions is determined not by the number of generations but the absolute time from the overwintered generation. The duration of the seasonal timer decreases with increasing temperatures, while it is independent of photoperiod. The adaptive value of the seasonal timer is to prevent untimely production of oviparous sexual females and males in spring. The duration of the seasonal timer varies among and within species, and a weak seasonal timer of high-arctic species is considered to have adapted to the short warm season. RNA sequencing in the pea aphid, *Acyrthosiphon pisum*, revealed that epigenetic modifications play an important role in the switching of the two reproductive modes, but its involvement in the molecular mechanism underlying the seasonal timer remains unclear.

Keywords Epigenetics · Life cycle · Photoperiodism · Polyphenism · Transgenerational effect

15.1 Life Cycle and Photoperiodism of Aphids

In most species of aphids (Hemiptera: Aphididae), the typical annual life cycle consists of several parthenogenetic generations followed by one sexual generation (Moran 1992; Simon et al. 2002). Many aphid species with the typical life cycle overwinter as diapause eggs, from which females called “fundatrices” or “stem mothers” hatch in spring. These females and their descendant viviparous females (viviparae) reproduce parthenogenetically and viviparously until autumn. In autumn,

N. Matsuda (✉)
Graduate School of Agriculture, Kyoto University, Kyoto, Japan
e-mail: matsuda.naoki.3y@kyoto-u.ac.jp

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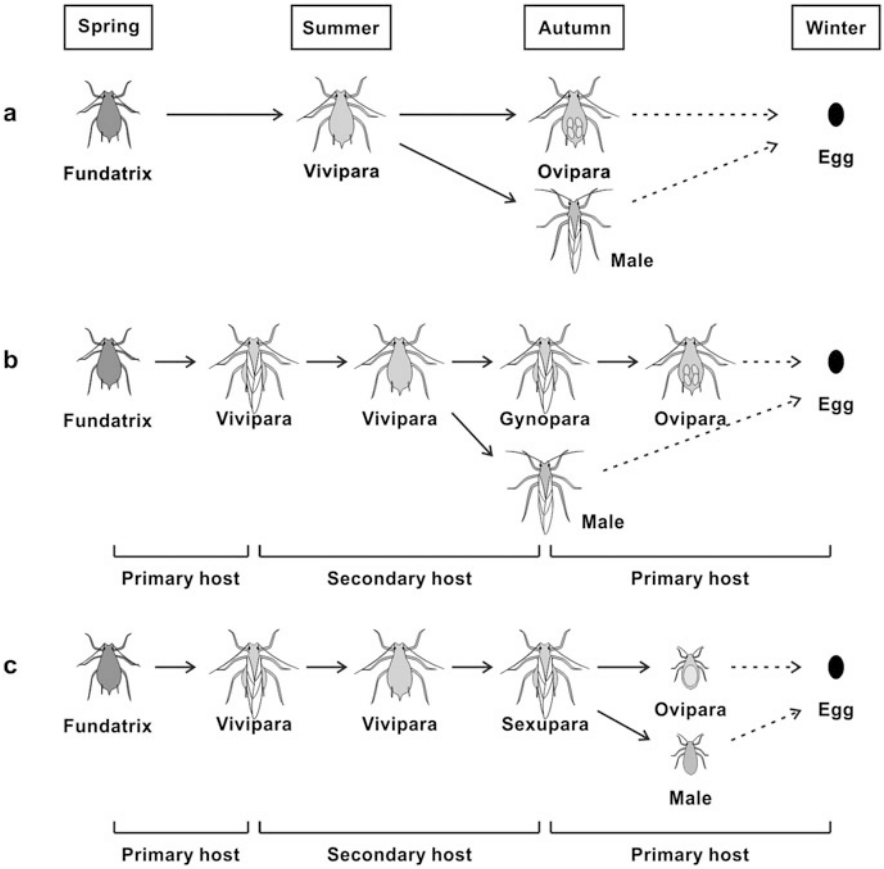

Fig. 15.1 Schematic representations of the seasonal life cycle of aphids. (**a**) Species without host alternation. (**b**) Species of Aphidinae with host alternation. (**c**) Species of the other subfamilies in Aphididae with host alternation. Solid and dotted arrows represent parthenogenesis and bisexual reproduction, respectively

oviparous sexual females (oviparae) and males appear, mate with each other, and lay overwintering eggs (Fig. 15.1a). In many aphid species, a short photoperiod is a major environmental cue for producing the sexual generation. Marcovitch (1923) discovered that the production of oviparae and males was successfully induced under short-day conditions of 7.5-h light and 16.5-h darkness (LD 7.5:16.5) in the strawberry root aphid, *Aphis forbesi*. This was the first photoperiodism reported in animals. This type of photoperiodism has since been demonstrated in various aphid species (Wilson 1938; Bonnemaison 1951; Kenten 1955; Lees 1959). In contrast, the production of diapause eggs is not necessary for overwintering at low latitudes, in which winter is not so severe. Therefore, some aphids have lost the ability to respond to short photoperiods, and the sexual generation has been eliminated from the life cycle (Moran 1992).

Some groups of aphids have a complex life cycle with seasonal alternation between two disjunct host plants (Fig. 15.1b). In host-alternating species of Aphidinae, a vivipara exposed to short days produces winged males and gynoparae, which are winged viviparous females specialized for the production of oviparae, instead of direct production of oviparae (Davidson 1929; Bonnemaison 1951). In contrast, in host-alternating species of the other subfamily, such as Eriosomatinae, autumnal winged viviparous females, called sexupara, parthenogenetically produce oviparae and males (Fig. 15.1c).

15.2 History of the Discovery of the Seasonal Timer

The photoperiodic switch from viviparous parthenogenesis to oviparous bisexual reproduction is suppressed over several generations from fundatrices due to a transgenerational "seasonal timer" in aphids. One year after the first report of photoperiodism in *Ap. forbesi* (Marcovitch 1923), Marcovitch (1924) found that the spring generation did not immediately yield sexual progenies even under short days. For example, when nymphs of *Ap. forbesi* that hatched from eggs in February under outdoor conditions in Tennessee were subjected 1 week later to short-day conditions of LD 7.5:16.5, oviparae were not observed until May. He also reported such retardation in the appearance of the sexual generation in *Aphis rumicis* and host-alternating species, including *Dysaphis sorbi* (formerly *Aphis sorbi*) and *Capitophorus hippophaes* (Marcovitch 1924). In the host-alternating species, the appearance of gynoparae was also retarded. Wilson (1938) reared fundatrices and their descendants of the aphid *Aphis chloris* under short-day conditions and obtained similar results. Although each generation was started with an early-born progeny of the previous one in his experiments, he estimated that the generation time would have been approximately doubled if a late-born progeny had been selected. He further pointed out that sexual females are produced over a limited period in nature, even though the individual ovipara may be preceded by a highly variable number of parthenogenetic generations. He therefore postulated the existence of a transgenerational "time factor," which inhibited the early appearance of the sexual generation and became weak as the post-fundatrix interval lengthened. This concept of a "time factor" was supported in the mealy cabbage aphid, *Brevicoryne brassicae*, by Bonnemaison (1951) and, more firmly, in the vetch aphid, *Megoura viciae*, by Lees (1960). They established two lineages of aphids, one consisting of individuals born early in each generation and the other of those born late (Fig. 15.2). These "early-born" and "late-born" lines were exposed to short-day conditions from the time of hatching, and the first sexual generation was born about the same date in both lineages when the early-born line had passed through the larger number of generations from the fundatrix than the late-born line had. Thus, Bonnemaison (1951) and Lees (1960) concluded that the number of days from hatching over several generations, rather than the number of generations from hatching, determined the duration for which the response to short days was suppressed, and therefore they called the

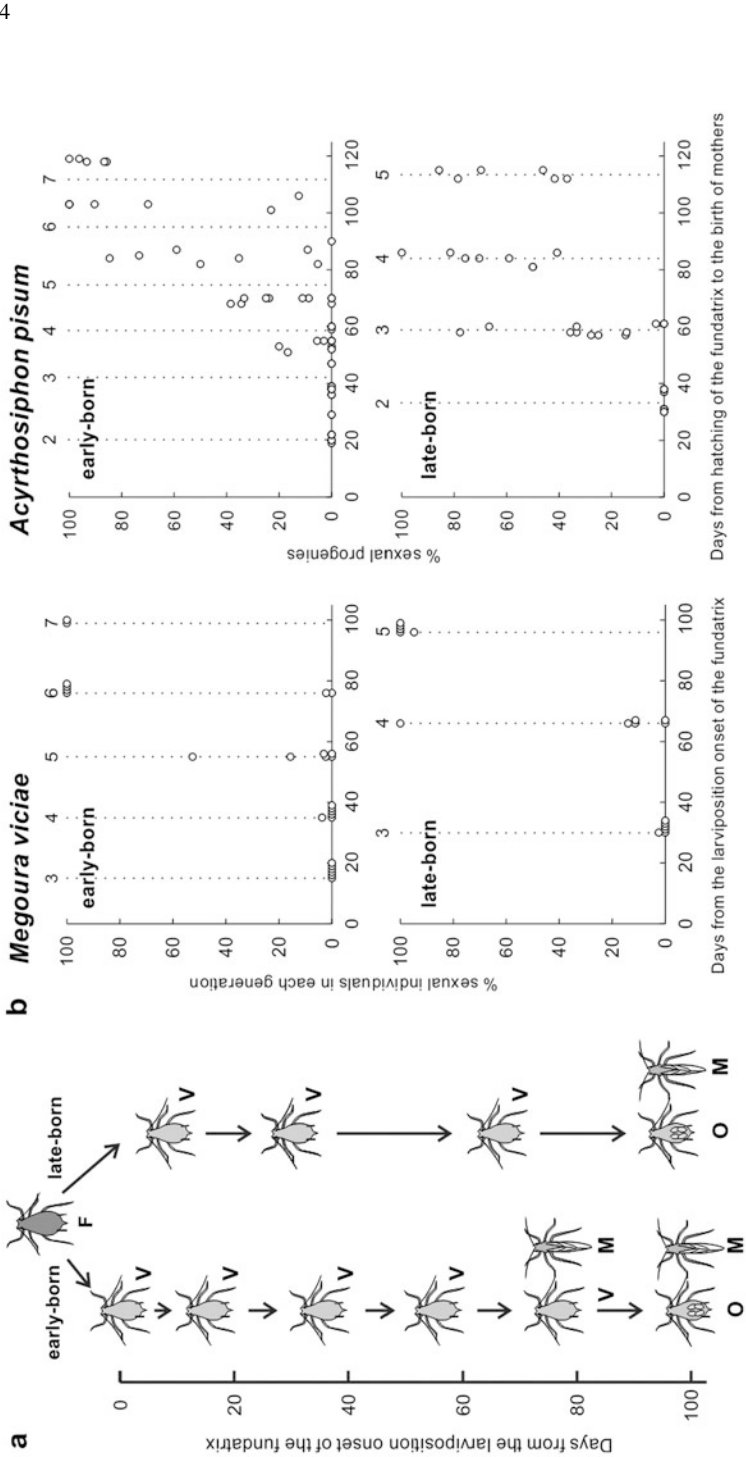

Fig. 15.2 The seasonal timer measures the number of days, not the number of generations, in *Megoura viciae* and *Acyrthosiphon pisum*. (**a**) A schematic representation of the method to establish early-born and late-born lineages. The number of days was based on the experiment in *M. viciae*. (**b**) Relationship between sexual morph production and the number of days from the larviposition onset of fundatrices. A vertical dotted line passes through the mean birth date for every generation, and the number of generations is shown above them. *F* fundatrix, *V* vivipara, *O* ovipara, *M* male. Based on Lees (1960) and adapted from Matsuda et al. (2017), with permission from Elsevier

mechanism responsible for this insensitiveness "a facteur fondatrice" and "an interval timer," respectively. Hereafter, I call this mechanism a seasonal timer to discriminate it from other mechanisms that measure specific time intervals and thus are called an interval timer (e.g., MacKay 1977; Nagao and Shimozawa 1987). Later, the seasonal timer measuring the absolute time from hatching was also supported in the small raspberry aphid, *Aphis rubicola* (Brodel and Schaefers 1979), and in the pea aphid, *Acyrthosiphon pisum* (Matsuda et al. 2017), by comparing early- and late-born lineages (Fig. 15.2). To date, the existence of the seasonal timer has been reported in more than 20 species across four subfamilies of Aphididae, including both species with and without host alternation, although it was not verified in most of them that the absolute time from hatching is important rather than the number of generations (Table 15.1).

15.3 Physiological Characteristics of the Seasonal Timer

Some researchers have examined whether rearing conditions such as temperature and photoperiod affect the duration of the seasonal timer. Lees (1960) reared post-fundatrix lineages of *M. viciae* under short days at three different temperatures, i.e., 20, 15, and 11 °C, and demonstrated that the higher the rearing temperature was, the shorter the duration of the seasonal timer (Fig. 15.3a; Table 15.1), unlike the free-running period of circadian clocks (Pittendrigh 1954). Moreover, Lees (1960) showed further that post-fundatrix lineages of *M. viciae* kept under long days for several generations switched to ovipara producers just after transfer to short days, indicating that the seasonal timer expires not only under short days but also under long days. Later, Brodel and Schaefers (1979) demonstrated that the duration of the seasonal timer is determined independently of the photoperiod in *Ap. rubicola* by comparing the number of days from hatching to the birth of ovipara producers between two lineages reared under different photoperiodic conditions: one was kept continuously under short days, and the other was reared under long days and transferred to short days in the next generation. As a result, initial exposure of lineages to long days did not expand the number of days required to regain the ability to produce sexual progenies, indicating that the photoperiod has no effect on the duration of the seasonal timer in *Ap. rubicola*. Our recent study in *Ac. pisum* also supported both the temperature dependence and photoperiod independence of the seasonal timer (Matsuda et al. 2017). The duration of the seasonal timer was longer at 15 °C than at 20 °C in post-fundatrix lineages of *Ac. pisum* (Fig. 15.3a; Table 15.1), while it was independent of whether the lineages had experienced long or short days.

Expiration of the seasonal timer may occur gradually or abruptly (Fig. 15.3a). The proportion of sexual progenies gradually increased as time passed after hatching in most species with the seasonal timer, such as *Ac. pisum* (Matsuda et al. 2017), *Ap. chloris* (Wilson 1938), and *Ap. rubicola* (Brodel and Schaefers 1979). This gradual transition from viviparae production to sexual morph production suggests that the

Table 15.1 List of aphid species and durations of the seasonal timer suppressing appearance of each morph

				Seasonal timer duration			
Subfamily	Species	Host alternation	Temp.	Day	Gen no.	Morph	References
Aphidinae	*Acyrthosiphon brevicorne*	No	20 °C	–	2	Male, ovipara	Strathdee and Bale (1996)
	Acyrthosiphon pisum	No	20 °C	33[a]	3	Ovipara	Bonnemaison (1972)
				35[a]	3	Male	
			15 °C	90.3[b]	–	Male, ovipara	Matsuda et al. (2017)
			20 °C	65.3–69.7[b]	–	Male, ovipara	
	Acyrthosiphon svalbardicum	No	20 °C	–	2	Male, ovipara	Strathdee et al. (1993)
	Aphis chloris	No	21 °C	–	7	Ovipara	Wilson (1938)
	Aphis forbesi	No	Outdoor	80[c]	–	Ovipara	Marcovitch (1924)
	Aphis grossulariae	Yes	16 °C	–	4	Male	Harrington (1981)
				–	6	Ovipara	
	Aphis rubicola	No	21 °C	43.5–46.5[b]	–	Ovipara	Brodel and Schaefers (1979)
	Aphis rumicis	No	Outdoor	65[a]	–	Male	Marcovitch (1924)
	Brevicoryne brassicae	No	Unclear	–	7	Gynopara, male	Bonnemaison (1951)
	Capitophorus hippophaes	Yes	Outdoor	44[a]	–	Gynopara, male	Marcovitch (1924)
	Cryptomyzus galeopsidis	Yes	16 °C	–	4	Ovipara	Harrington (1981)
				–	5	Male	
	Dysaphis plantaginea	Yes	20 °C	44[a]	–	Gynopara	Bonnemaison (1972)
				58[a]	–	Male	
	Dysaphis sorbi	Yes	Outdoor	46[a]	–	Gynopara	Marcovitch (1924)
	Hyperomyzus lactucae	Yes	16 °C	–	4	Male, ovipara	Harrington (1981)
	Megoura viciae	No	11 °C	114[a]	5–6	Ovipara	Lees (1960)

			15 °C	35–76[a]	4–5	Male	
				55–76[a]	5–6	Ovipara	
			20 °C	20–32[a]	4–5	Male	
				32–44[a]	5–6	Ovipara	
	Myzus persicae	Yes	Unclear	42[a]	–	Gynopara	Bonnemaison (1951)
				85[a]	–	Male	
			17 °C	–	3	Gynopara	Margaritopoulos et al. (2002)
				–	5	Male	
	Nasonovia ribisnigri	Yes	16 °C	–	4	Ovipara	Harrington (1981)
	Phorodon humuli	No	18 °C	–	4	Gynopara	Campbell and Tregidga (2006)
	Rhopalosiphum padi	Yes	15 °C	74–110[c]	–	Male	Lushai et al. (1996)
				68–128[c]	–	Ovipara	
	Sitobion avenae	No	20 °C	<6 months–3 years[d]	–	Ovipara	Dedryver et al. (2012)
Drepanosiphinae	*Drepanosiphum platanoides*	No	10 °C	–	2	Ovipara	Dixon (1971)
				–	4	Male	
	Eucallipterus tiliae	No	15 °C	–	2	Ovipara	Dixon (1972)
				–	3	Male	
Eriosomatinae	*Pemphigus bursarius*	Yes	18 °C	–	4	Sexupara	Judge (1968)
Lachninae	*Cinara todocola*	No	15°C	–	3	Male, ovipara	Yamaguchi (1976)

[a]The number of days from adult emergence of fundatrices to birth of the first sexual morphs
[b]The number of days from hatching of fundatrices to birth of viviparae which produced 50% sexual progenies
[c]The number of days from hatching of fundatrices to birth of the first sexual morphs
[d]The number of days from hatching of fundatrices to birth of viviparae in which the proportion of sexual progenies reached the plateau

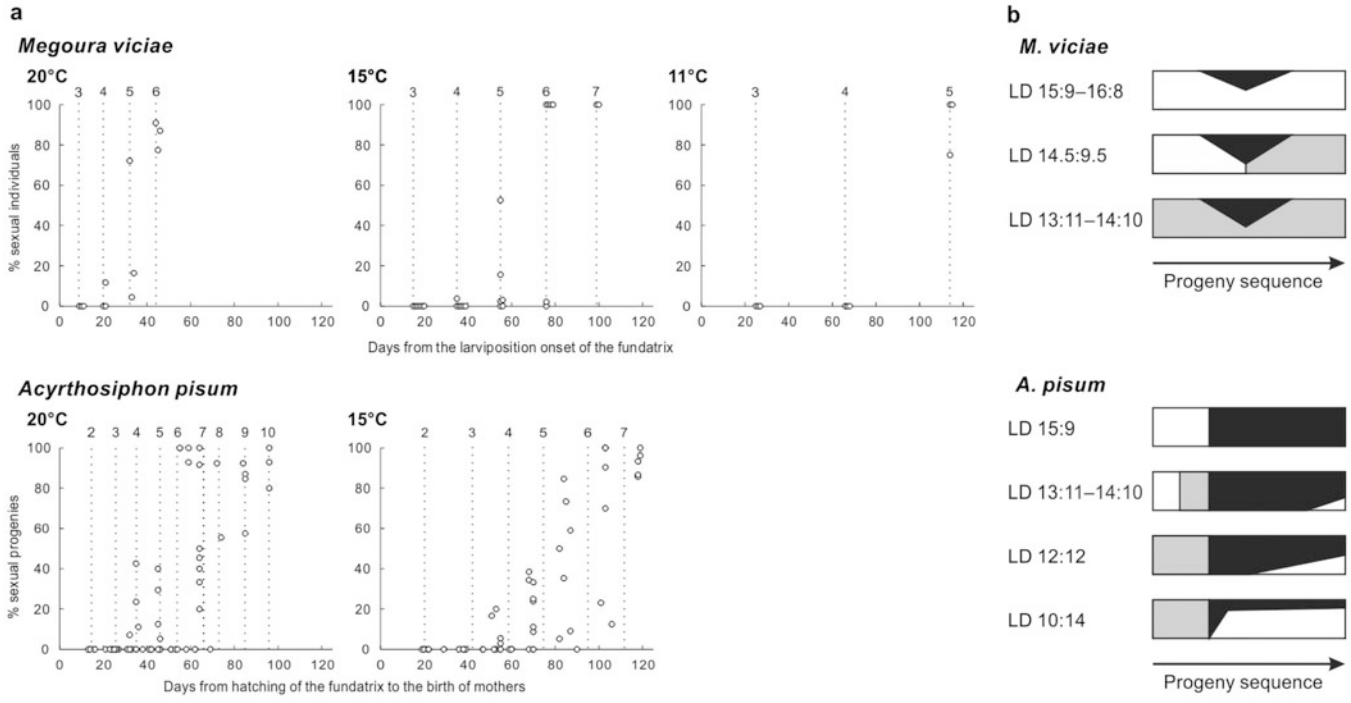

Fig. 15.3 (**a**) Effects of rearing temperatures on the duration of the seasonal timer in *Megoura viciae* and *Acyrthosiphon pisum*. *M. viciae* were reared under 12-h light and 12-h darkness, and *Ac. pisum* were reared under 10-h light and 14-h darkness. Although early-born and late-born lineages were reared in parallel for both species, only the results in the early-born lineages were shown. A vertical dotted line passes through the mean birth date for every generation, and the number of generations is shown above them. Based on Lees (1960) and adapted from Matsuda et al. (2017), with permission from Elsevier. (**b**) Schematic representation of reproductive sequences of *M. viciae* and *A. pisum* under different photoperiods. White, gray, and black bars indicate production of viviparae, oviparae, and males, respectively. Based on Lees (1959) and MacKay (1987)

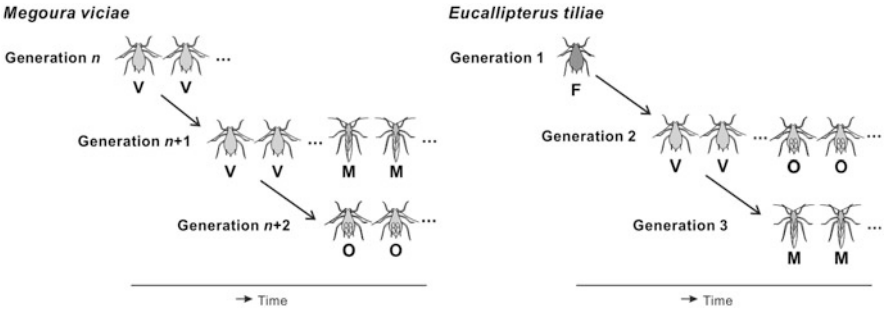

Fig. 15.4 Schematic representations of the reproductive patterns in three successive generations of *Megoura viciae* and *Eucallipterus tiliae*. The timing of the appearance of oviparae and males is estimated from the duration of the seasonal timer for each morph and the reproductive sequence under short days in the two species. See text for details. *F* fundatrix, *V* vivipara, *M* ovipara, *M* male. Based on Lees (1960) and Dixon (1972)

function of the seasonal timer is weakened in a quantitative manner in these species. In contrast, the proportion of sexual progenies switched from 0% in one generation to almost 100% in the next generation in *M. viciae* (Lees 1960). Although it is unclear why such gradual and abrupt switching patterns are observed in different species, it is interesting that the relationship between the photophase length and the morph determination shows similar gradual and abrupt patterns in *Ac. pisum* and *M. viciae*, respectively (Fig. 15.3b). In *Ac. pisum*, photoperiodic morph determination is a graded response, in which the proportion of sexual progenies varies quantitatively with photophase length and seldom reaches 100% (MacKay 1987; Erlykova 2003). In contrast, in *M. viciae*, photoperiodic morph determination is also a quantitative but more drastic response, in which the proportion of oviparae among the female offspring is 0% under LD 15:9 and almost 100% under LD 14:10 (Lees 1959).

Although both males and oviparae are induced by short days, the duration of the seasonal timer is different between them in many species (Table 15.1). The production of oviparae (or gynoparae in host-alternating species) is inhibited longer than that of males in some species, e.g., *M. viciae* (Lees 1960) and *Aphis grossulariae* (Harrington 1981), and shorter in other species, e.g., *Drepanosiphum platanoides* (Dixon 1971) and *Eucallipterus tiliae* (Dixon 1972). These results indicate that the production of males and oviparae might be regulated by two seasonal timers with different durations. Such a difference in the durations might be compensated by the reproductive sequence specific to each aphid species (Fig. 15.4). In *M. viciae*, males are born predominantly in the middle of the progeny sequence, while oviparae are born in the early and late phases (Lees 1959). Because the seasonal timer of this species is longer for oviparae than for males by approximately 20 days (Lees 1960), males born in one generation and oviparae born in the early phase of the next generation would mature synchronously. In *E. tiliae*, in contrast, males in each generation appear predominantly in the first half, while oviparae appear in the second half of the progeny sequence, and the first oviparae and males appear in

the second and third generations from hatching, respectively (Dixon 1972). This results in the first males and first oviparae maturing together.

15.4 Ecological and Evolutionary Aspects of the Seasonal Timer

Wilson (1938) suggested that fundatrices and their progenies would be exposed to short daylengths in early spring, and the adaptive significance of the seasonal timer would be to inhibit the early appearance of the sexual generation. The early appearance of wild males before summer has been reported in several aphid species, including *B. brassicae* and the bird cherry aphid, *Rhopalosiphum padi* (Taylor et al. 1998). Such early males are likely descendants of viviparae that have overwintered without resetting the seasonal timer and induced by short days in spring. These males would have fewer chances of mating because there would be few, if any, oviparae in the same period. Moreover, the untimely production of sexual morphs in spring would result in reproductive disadvantages due to the cost of sex and a slow developmental rate of sexually produced eggs. While it has been considered for many years that the seasonal timer suppresses untimely production of sexual morphs (Lees 1960; Brodel and Schaefers 1979; Lushai et al. 1996; Margaritopoulos et al. 2002; Campbell and Tregidga 2006), we showed for the first time experimentally that sexual morph production of *Ac. pisum* can be induced under daylengths in spring and that this untimely response is averted due to the function of the seasonal timer (Matsuda et al. 2020a). We reared viviparae of a strain originating from Sapporo (43.0 °N) and fundatrices from inbreeding of the strain simultaneously from the first instar under natural daylengths and temperatures in Sapporo (Fig. 15.5a). Contrary to our expectation, however, both viviparae of the original strain and fundatrices produced only viviparae (Fig. 15.5b). These results indicate that the seasonal timer is not necessary for this *Ac. pisum* strain to avoid producing sexual progenies, at least at normal temperatures in its original habitat. Then, we conducted the same experiment in Kyoto (35.0 °N). As a result, hatching occurred earlier by a month in Kyoto than in Sapporo (Fig. 15.5a), and most of the viviparae of the original strain produced oviparae, males, or both in addition to viviparae, whereas all the fundatrices produced only viviparae (Fig. 15.5b). Because the viviparae of the original strain and the inbred fundatrices had a similar genetic background, whether sexual morphs were produced should depend on the state of the seasonal timer. Although it is unlikely that populations of *Ac. pisum* in Kyoto overwinter as diapause eggs (Kanbe and Akimoto 2009), populations in Sapporo might face a warm spring similar to the experimental conditions in Kyoto due to yearly variation in temperatures. Therefore, we concluded that the seasonal timer is a safeguarding mechanism against the untimely appearance of sexual morphs induced in a warm spring. It is possible that the seasonal timer is maintained as an adaptation

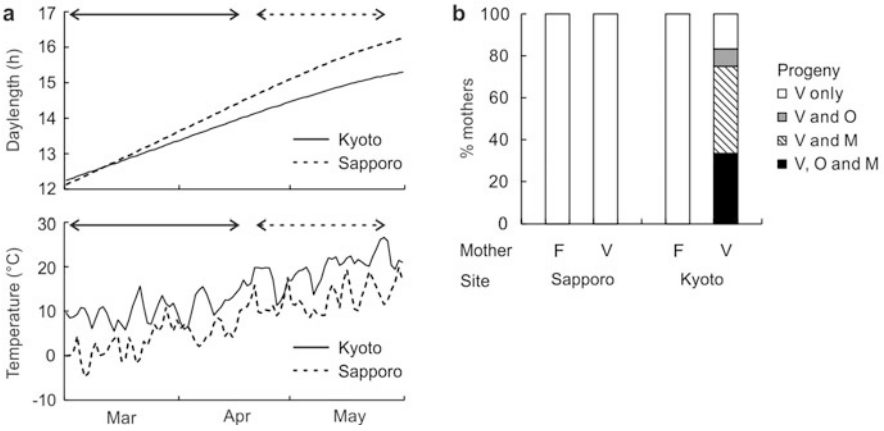

Fig. 15.5 Photoperiodic response in viviparae of the Sapporo strain and its inbred fundatrices of *Acyrthosiphon pisum* in spring. (**a**) Seasonal changes in natural daylength, including civil twilight and daily mean air temperature. Solid and dashed arrows indicate the period when fundatrices and viviparae were undergoing nymphal development in Kyoto and Sapporo, respectively. (**b**) Morph determination of progenies in the tested mothers (N = 10–12). Abbreviations aphid morphs: *F* fundatrix, *V* vivipara, *O* ovipara, *M* male. Adapted from Matsuda et al. (2020a), with permission from Wiley

to an occasionally warm spring in populations of *Ac. pisum* in Sapporo (Matsuda et al. 2020a).

The duration of the seasonal timer varies among aphid species (Table 15.1). The seasonal timer operates for 30–100 days or three to seven generations from hatching of fundatrices in most species with the seasonal timer. In such species, sexual morph production is most strongly suppressed in fundatrices, and they produce only viviparae. However, in *Acyrthosiphon svalbardicum* and *Acyrthosiphon brevicorne*, which are distributed in the high-Arctic region, fundatrices directly produce a small proportion of sexual progenies (Strathdee et al. 1993; Strathdee and Bale 1996). The percentage of sexual progenies increases in the next generation up to 100%, indicating that these two species have a weak and short seasonal timer that has adapted to the short summer available for parthenogenetic reproduction in the high Arctic region (Table 15.1). In contrast, Dedryver et al. (2012) reported a very long seasonal timer that operates for a few years in the grain aphid, *Sitobion avenae*. Although the authors tested the ability to produce sexual progenies only once a year, it took 1–4 years until sexual progenies were produced in some post-fundatrix lineages. If winters are sometimes, but not always, mild enough to ensure a high probability of winter survival as viviparae, strains with such a long seasonal timer might avoid disappearance in the first autumn and increase the frequency of their genes in *Si. avenae* populations through parthenogenesis for longer than 1 year. On the other hand, expiration of the long seasonal timer might provide the minimum required opportunities for bisexual reproduction, eliminating deleterious mutations that have

accumulated through parthenogenesis (Simon et al. 2002). It is unclear whether such a long seasonal timer exists in other aphid species.

The duration of the seasonal timer can also be different within a species. Lushai et al. (1996) showed intraspecific variations in the duration of the seasonal timer in *R. padi*. There was a difference of up to 21 days in the mean duration of the seasonal timer among strains collected from latitudinally different sites in the UK. In this case, the length of the warm season may not be the main factor of the geographic variation in the seasonal timer duration because the Roslin strains, which were collected from the northernmost site, had the longest seasonal timer. Such variation in the seasonal timer duration was also observed in crosses obtained in the laboratory. Interestingly, the durations of the seasonal timer for the field-collected strains were generally shorter than those for the laboratory crosses. A larger variation in the duration of the seasonal timer was observed in *Si. avenae*. Dedryver et al. (2012) reported in this species that the seasonal timer duration varied from less than 6 months to 3 years, and crosses with longer egg diapause durations tended to have longer seasonal timers. Aphid egg mortality in nature correlates with the time spent at the egg stage (Leather et al. 1993), and not winter severity but predators are a major mortality factor for aphid eggs (Leather 1992). Therefore, the authors hypothesized that eggs with the longest seasonal timer might be eliminated by predation before hatching in nature and that the longer seasonal timer of the laboratory crosses of *R. padi* might be explained by the same reason (Dedryver et al. 2012).

Similar to aphids, a few other insects have been reported to have a mechanism suppressing photoperiodic responses over several generations after diapause. In the parasitoid wasps *Trichogramma principium* and *Trichogramma telengai*, prepupal diapause is induced by low temperatures and short photoperiods experienced by the maternal generation. The ability to enter diapause was lost during one or more post-diapause generations and restored as generations pass, although it is unclear whether a mechanism responsible for this phenomenon measures the absolute time or the number of generations from the previously experienced diapause (Reznik and Samartsev 2015). This seasonal timer of *T. principium* was recently reported to be dependent on rearing temperatures: the rate of restoration of the ability to enter diapause linearly increased with mean temperature, and the number of generations at which 50% of individuals entered diapause was similar among different temperature conditions (Reznik and Voinovich 2021), similar to the results in *M. viciae* (Lees 1960). These findings suggest that the seasonal timer widely exists in insects, although it remains to be discovered.

15.5 Possible Mechanism of the Seasonal Timer

It remains largely unknown how the seasonal timer suppresses the sexual morph production and how it is transferred to subsequent generations. A high level of juvenile hormone (JH) generally induces the production of viviparae instead of sexual morphs in aphids (Mittler et al. 1979; Hardie 1981; Hardie and Lees 1985;

Ishikawa et al. 2012). Thus, one possibility is that the seasonal timer could result from a high level of JH in the ovarioles of fundatrices, which is diluted through generations until it falls below a critical threshold (Tagu et al. 2005). However, it is unlikely that such a molecule operating the seasonal timer could resist so many dilutions due to cell divisions through several generations and nymphal stages (Lees 1960; Tagu et al. 2005).

Another possibility is that the seasonal timer might be operated by epigenetic modifications such as DNA methylation and histone acetylation, which regulate gene expression and are passed on across one or more generations (Ho and Burggren 2010). Epigenetic modifications have been shown to be involved in photoperiodic induction of diapause in several insect species. In the jewel wasp, *Nasonia vitripennis*, genome-wide DNA methylation profiles were different between females reared under diapause-inducing short days and females reared under diapause-averting long days, and RNA interference or pharmacological inhibition of DNA methyltransferases caused females to produce diapause progenies regardless of photoperiod (Pegoraro et al. 2016). In the flesh fly *Sarcophaga bullata*, which enters diapause as pupae under short days, histone deacetylase genes were differentially expressed in larvae between long and short days (Reynolds et al. 2016). In this species, individuals produced by females that have experienced pupal diapause are unable to enter diapause regardless of photoperiod (Henrich and Denlinger 1982). Such a maternal effect is similar to the aphid seasonal timer in that the mechanism is activated by diapause and suppresses the sensitivity to photoperiod in the next generation. Interestingly, expression levels of the histone deacetylase genes in larvae also depended on the diapause history of their mothers (Reynolds et al. 2016). These examples in other insects suggest the possibility that epigenetic modifications are likely to also be involved in the aphid seasonal timer.

Although the sequencing of the whole genome of *Ac. pisum*, the first sequencing in hemimetabolous insects (The International Aphid Genomics Consortium 2010), facilitates molecular approaches to aphid photoperiodism (Le Trionnaire et al. 2013), the molecular mechanism underlying the seasonal timer remains largely unknown. Recently, we performed RNA-sequence-based transcriptome analyses in the head of *Ac. pisum* to identify genes differentially expressed in relation to the seasonal timer and photoperiods (Matsuda et al. 2020b). Viviparae with an expired seasonal timer under short-day conditions, which produce sexual progenies, showed higher expression of 773 genes than viviparae with an expired seasonal timer under long-day conditions and of 751 genes than viviparae with an operative seasonal timer under short-day conditions, both of which produce no sexual progenies. These 773 and 751 genes included 612 common genes, which are considered to be involved in sexual morph production. Gene Ontology (GO) analysis of these 612 genes showed that histone modification, gene silencing by RNA, and protein sumoylation are significantly enriched in aphids producing sexual progenies. These results suggested that not only epigenetic modifications but also posttranscriptional and posttranslational modifications might play a role in the maternal switching of the two reproductive modes. If some genes are differentially expressed depending on the state of the seasonal timer under both photoperiodic conditions, these genes are considered

to be involved in the expiration of the seasonal timer. However, such an expression pattern was observed in only 36 genes: 9 genes were upregulated in viviparae with an operative seasonal timer, while 27 genes were conversely regulated, and no GO terms were significantly enriched. Therefore, there remains no evidence for the involvement of epigenetic modifications in the seasonal timer.

15.6 Future Perspectives

It is expected that the molecular mechanisms underlying how the seasonal timer measures a specific length of time can be addressed with advanced technologies, including genomic tools and genome editing. To date, the genomes of more than 20 species have been sequenced in Aphididae (Shigenobu and Yorimoto 2022), facilitating comparative genomic approaches into the seasonal timer. The sequencing of multiple genomes of aphids with different lengths of the seasonal timer might identify key loci responsible for the intra- and interspecific variation in the duration of the seasonal timer. Moreover, genome-wide analysis of chromatin accessibility or DNA methylation is now available in *Ac. pisum* (Richard et al. 2017; Mathers et al. 2019), and these technologies might be effective in identifying genomic regions that are differentially epigenetically regulated depending on the elapsed time from hatching of fundatrices. For functional analysis of candidate genes responsible for regulation of the seasonal timer, in addition to gene knockdown by RNA interference (Mutti et al. 2006), the CRISPR/Cas9 genome editing technique has been improved in *Ac. pisum* (Le Trionnaire et al. 2019). Future works adopting these approaches may pioneer the new research field of transgenerational biological timer.

References

Bonnemaison L (1951) Contribution á l'étude des facteurs provoquant l'apparition des formes ailées et sexuées chez les Aphidinae. Ann Epiphyt 2:1–380

Bonnemaison L (1972) Ètude du "facteur-fondatrice" chez *Dysaphis plantaginea* Pass. et *Acyrthosiphon pisum* Harris (Homoptères, Aphididae). Ann Entomol Fr 8:607–627

Brodel CF, Schaefers GA (1979) An "interval timer" for the production of oviparae in *Aphis rubicola* (Homoptera: Aphididae). Entomol Exp Appl 25:1–8. https://doi.org/10.1111/j.1570-7458.1979.tb02841.x

Campbell CAM, Tregidga EL (2006) A transgenerational interval timer inhibits unseasonal sexual morph production in damson-hop aphid, *Phorodon humuli*. Physiol Entomol 31:394–397. https://doi.org/10.1111/j.1365-3032.2006.00531.x

Davidson J (1929) On the occurrence of the partheno-genetic and sexual forms in *Aphis lumicis* L., with special reference to the influence of environmental factors. Ann Appl Biol 1:104–134. https://doi.org/10.1111/j.1744-7348.1929.tb07124.x

Dedryver C-A, Le Gallic J-F, Mahéo F, Parisey N, Tagu D (2012) Delayed setting of the photoperiodic response in recombinant clones of the aphid species *Sitobion avenae*. Ecol Entomol 37:293–299. https://doi.org/10.1111/j.1365-2311.2012.01365.x

Dixon AFG (1971) The "interval timer" and photoperiod in the determination of parthenogenetic and sexual morphs in the aphid, *Drepanosiphum platanoides*. J Insect Physiol 17:251–260. https://doi.org/10.1016/0022-1910(71)90209-5

Dixon AFG (1972) The "interval timer", photoperiod and temperature in the seasonal development of parthenogenetic and sexual morphs in the lime aphid, *Eucallipterus tiliae* L. Oecologia 9: 301–310. https://doi.org/10.1007/BF00345334

Erlykova N (2003) Inter- and intraclonal variability in the photoperiodic response and fecundity in the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae). Eur J Entomol 100:31–37. https://doi.org/10.14411/eje.2003.006

Hardie J (1981) Juvenile hormone and photoperiodically controlled polymorphism in *Aphis fabae*: prenatal effects on presumptive oviparae. J Insect Physiol 27:347–355. https://doi.org/10.1016/0022-1910(81)90059-7

Hardie J, Lees AD (1985) The induction of normal and teratoid viviparae by a juvenile hormone and kinoprene in two species of aphids. Physiol Entomol 10:65–74. https://doi.org/10.1111/j.1365-3032.1985.tb00020.x

Harrington R (1981) Comparative studies of sexual morph production by aphids: with special reference to photoperiodism. PhD thesis, Imperial College London

Henrich VC, Denlinger DL (1982) A maternal effect that eliminates pupal diapause in progeny of the flesh fly, *Sarcophaga bullata*. J Insect Physiol 28:881–884. https://doi.org/10.1016/0022-1910(82)90102-0

Ho DH, Burggren WW (2010) Epigenetics and transgenerational transfer: a physiological perspective. J Exp Biol 213:3–16. https://doi.org/10.1242/jeb.019752

Ishikawa A, Ogawa K, Gotoh H, Walsh TK, Tagu D, Brisson JA et al (2012) Juvenile hormone titre and related gene expression during the change of reproductive modes in the pea aphid. Insect Mol Biol 21:49–60. https://doi.org/10.1111/j.1365-2583.2011.01111.x

Judge FD (1968) Polymorphism in a Subterranean aphid, *Pemphigus bursarius*. I. Factors affecting the development of sexuparae. Ann Entomol Soc Am 61:819–827. https://doi.org/10.1093/aesa/61.4.819

Kanbe T, Akimoto S (2009) Allelic and genotypic diversity in long-term asexual populations of the pea aphid, *Acyrthosiphon pisum* in comparison with sexual populations. Mol Ecol 18:801–816. https://doi.org/10.1111/j.1365-294X.2008.04077.x

Kenten J (1955) The effect of photoperiod and temperature on reproduction in *Acyrthosiphon pisum* (Harris) and on the forms produced. Bull Entomol Res 46:599–624. https://doi.org/10.1017/S0007485300039572

Le Trionnaire G, Wucher V, Tagu D (2013) Genome expression control during the photoperiodic response of aphids. Physiol Entomol 38:117–125. https://doi.org/10.1111/phen.12021

Le Trionnaire G, Tanguy S, Hudaverdian S, Gleonnec F, Richard G, Cayrol B et al (2019) An integrated protocol for targeted mutagenesis with CRISPR-Cas9 system in the pea aphid. Insect Biochem Mol Biol 110:34–44. https://doi.org/10.1016/j.ibmb.2019.04.016

Leather SR (1992) Aspects of aphid overwintering (Homoptera: Aphidinea: Aphididae). Entomol Gen 17:101–113. https://doi.org/10.1127/entom.gen/17/1992/101

Leather SR, Walters KFA, Bale JS (1993) The ecology of insect overwintering. Cambridge University Press, Cambridge. https://doi.org/10.1017/CBO9780511525834

Lees AD (1959) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—I. The influence of these factors on apterous virginoparae and their progeny. J Insect Physiol 3:92–117. https://doi.org/10.1016/0022-1910(59)90024-1

Lees AD (1960) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—II. The operation of the "interval timer" in young clones. J Insect Physiol 4:154–175. https://doi.org/10.1016/0022-1910(60)90078-0

Lushai G, Hardie J, Harrison RG (1996) Inhibition of sexual morph production in the bird cherry aphid, *Rhopalosiphum padi*. Entomol Exp Appl 81:117–119. https://doi.org/10.1111/j.1570-7458.1996.tb02022.x

MacKay PA (1977) Alata-production by an aphid: the "interval timer" concept and maternal age effects. J Insect Physiol 23:889–893. https://doi.org/10.1016/0022-1910(77)90016-6

MacKay PA (1987) Production of sexual and asexual morphs and changes in reproductive sequence associated with photoperiod in the pea aphid, *Acyrthosiphon pisum* (Harris). Can J Zool 65:2602–2606. https://doi.org/10.1139/z87-394

Marcovitch S (1923) Plant lice and light exposure. Science 58:537–538. https://doi.org/10.1126/science.58.1513.537.b

Marcovitch S (1924) The migration of the Aphididae and the appearance of the sexual forms as affected by the relative length of daily light exposure. J Agric Res 27:513–522

Margaritopoulos JT, Tsitsipis JA, Prophetou-Athanasiadou DA (2002) An interval timer controls the production of sexual morphs in *Myzus persicae* (Homoptera: Aphididae). Physiol Entomol 27:251–255. https://doi.org/10.1046/j.1365-3032.2002.00287.x

Mathers TC, Mugford ST, Percival-Alwyn L, Chen Y, Kaithakottil G, Swarbreck D et al (2019) Sex-specific changes in the aphid DNA methylation landscape. Mol Ecol 28:4228–4241. https://doi.org/10.1111/mec.15216

Matsuda N, Kanbe T, Akimoto S, Numata H (2017) Transgenerational seasonal timer for suppression of sexual morph production in the pea aphid, *Acyrthosiphon pisum*. J Insect Physiol 101:1–6. https://doi.org/10.1016/j.jinsphys.2017.06.008

Matsuda N, Kanbe T, Endo J, Akimoto S, Numata H (2020a) Suppression of autumnal sexual morph production in spring by a seasonal timer in an aphid. Physiol Entomol 45:103–109. https://doi.org/10.1111/phen.12322

Matsuda N, Numata H, Udaka H (2020b) Transcriptomic changes in the pea aphid, *Acyrthosiphon pisum*: effects of the seasonal timer and photoperiod. Comp Biochem Physiol D Genomics Proteomics 36:100740. https://doi.org/10.1016/j.cbd.2020.100740

Mittler TE, Eisenbach J, Searle JB, Matsuka M, Narrar SG (1979) Inhibition by kinoprene of photoperiod-induced male production by apterous and alate viviparae of the aphid *Myzus persicae*. J Insect Physiol 25:219–226. https://doi.org/10.1016/0022-1910(79)90046-5

Moran N (1992) The evolution of aphid life cycles. Annu Rev Entomol 37:321–348. https://doi.org/10.1146/annurev.en.37.010192.001541

Mutti NS, Park Y, Reese JC, Reeck GR (2006) RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. J Insect Sci 6:38. https://doi.org/10.1673/031.006.3801

Nagao T, Shimozawa T (1987) A fixed time-interval between two behavioural elements in the mating behaviour of male crickets, *Gryllus bimaculatus*. Anim Behav 35:122–130. https://doi.org/10.1016/S0003-3472(87)80217-8

Pegoraro M, Bafna A, Davies NJ, Shuker DM, Tauber E (2016) DNA methylation changes induced by long and short photoperiods in *Nasonia*. Genome Res 26:203–210. https://doi.org/10.1101/gr.196204.115

Pittendrigh CS (1954) On temperature independence in the clock system controlling emergence time in *Drosophila*. Proc Natl Acad Sci USA 40:1018–1029. https://doi.org/10.1073/pnas.40.10.1018

Reynolds JA, Bautista-Jimenez R, Denlinger DL (2016) Changes in histone acetylation as potential mediators of pupal diapause in the flesh fly, *Sarcophaga bullata*. Insect Biochem Mol Biol 76:29–37. https://doi.org/10.1016/j.ibmb.2016.06.012

Reznik SY, Samartsev KG (2015) Multigenerational maternal inhibition of prepupal diapause in two *Trichogramma* species (Hymenoptera: Trichogrammatidae). J Insect Physiol 81:14–20. https://doi.org/10.1016/j.jinsphys.2015.06.012

Reznik SY, Voinovich ND (2021) Influence of temperature on the restoration of the ability of *Trichogramma principium* to undergo diapause. J Insect Physiol 132:104271. https://doi.org/10.1016/j.jinsphys.2021.104271

Richard G, Legeai F, Prunier-Leterme N, Bretaudeau A, Tagu D, Jaquiéry J, Le Trionnaire G (2017) Dosage compensation and sex-specific epigenetic landscape of the X chromosome in the pea aphid. Epigenetics Chromatin 10:30. https://doi.org/10.1186/s13072-017-0137-1

Shigenobu S, Yorimoto S (2022) Aphid hologenomics: current status and future challenges. Curr Opin Insect Sci 50:100882. https://doi.org/10.1016/j.cois.2022.100882

Simon JC, Rispe C, Sunnucks P (2002) Ecology and evolution of sex in aphids. Trends Ecol Evol 17:34–39. https://doi.org/10.1016/S0169-5347(01)02331-X

Strathdee AT, Bale JS (1996) Life-cycle and morph production in the Arctic aphid *Acyrthosiphon brevicorne*. Polar Biol 16:293–300. https://doi.org/10.1007/s003000050056

Strathdee AT, Bale JS, Block WC, Webb NR, Hodkinson ID, Coulson SJ (1993) Extreme adaptive life-cycle in a high arctic aphid, *Acyrthosiphon svalbardicum*. Ecol Entomol 18:254–258. https://doi.org/10.1111/j.1365-2311.1993.tb01098.x

Tagu D, Sabater-Muñoz B, Simon JC (2005) Deciphering reproductive polyphenism in aphids. Invertebr Reprod Dev 48:71–80. https://doi.org/10.1080/07924259.2005.9652172

Taylor MS, Harrington R, Clark SJ (1998) Unseasonal male aphids. In: Nieto Nafría JM, Dixon AFG (eds) Aphids in natural and managed ecosystems. Universidad de León, León, pp 287–293

The International Aphid Genomics Consortium (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol 8:e1000313. https://doi.org/10.1371/journal.pbio.1000313

Wilson F (1938) Some experiments on the influence of environment upon the forms of *Aphis chloris* Koch (Aphididae). Trans R Entomol Soc Lond 87:165–180. https://doi.org/10.1111/j.1365-2311.1938.tb02685.x

Yamaguchi H (1976) Biological studies on the Todo-fir aphid *Cinara todocola* Inouye, with special reference to its population dynamics and morph determination. Bull Govern Forest Exp Stn 283: 1–102

Chapter 16
Time-Compensated Celestial Navigation

James Frederick Cheeseman

Abstract Animals and in particular insects, despite their diminutive size, have a remarkable ability to use the environmental information around them to perform complex navigational tasks to forage for food and to return home. Some of the most prominent of these environmental cues are those observed in the sky, first among which is the sun. The purpose of this chapter is to describe the time-compensated celestial navigation in insects which relies on an accurate continually consulted circadian clock. It draws heavily on the research on two species, the honey bee *Apis mellifera* and the monarch butterfly *Danaus plexippus*, which have been used extensively to study complementary aspects of navigation behavior.

Keywords Circadian · Migration · Orientation · Sun compass

16.1 Introduction

For an animal to show true navigation, it needs not only to know in which direction to move but also to have a concept of where it is in its geographic environment even in unfamiliar territory. For the purposes of this chapter, this definition of navigation is broadened to include orientation more generally. Many insects achieve way-finding through visual cues including using landmarks either in isolation or through panorama matching. These are relatively simple way-finding mechanisms which are robust while the animal is in visual range of them.

The strongest celestial cue which provides direction is light from the sun either directly during the day or reflected by the moon at night. It provides an instantaneous compass direction and allows the animal to calculate a bearing.

Even when the sun is occluded from view by cloud, a partial view of the sky is enough to see and orient by the polarized pattern of light known as the e-vector

J. F. Cheeseman (✉)
Department of Anaesthesiology, School of Medicine, The University of Auckland, Auckland, New Zealand
e-mail: j.cheeseman@auckland.ac.nz

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(Rossel et al. 1978), and knowledge of this e-vector is sufficient to obtain compass information by several insect models including the honey bee *Apis mellifera* (Evangelista et al. 2014), the fruit fly *Drosophila melanogaster* (Warren et al. 2019), and the monarch butterfly *Danaus plexippus* (Reppert et al. 2004).

The challenge is that celestial cues, viewed by those on earth, move across the sky. During the day the sun rises in the east and tracks across the southern part of the sky (in the northern hemisphere) before setting in the west. In the southern hemisphere, the sun's path tracks to the north. The night sky is of course quite different. The moon is the most prominent cue but is not always visible and even when full provides a fraction of the daytime light from the sun. At night at least in the northern hemisphere, the sky appears to rotate around the northern pole star. Mammals and birds can use stars for orientation, but there is little evidence that insects can decipher individual stars and might rather use the generalized pattern of the Milky Way such as in the example of the dung beetle *Scarabaeus satyrus* (Dacke et al. 2013).

For short-distance homing in many insects, the movement of the celestial cues may not be a problem because the timeframe of the excursions is so short. However, for insects which fly and forage over several kilometers over several hours, this poses a significant challenge as the sun or moon will have moved in the sky over time.

The travel of the sun across the sky every day means that in order to use it as a compass, animals require an accurate circadian clock to interpret the directional information. This ability to consult a continuously updated biological clock gives rise to the concept of time-compensated sun-compass orientation first described in insects by von Frisch (1954) in honey bees.

16.2 Honey Bee Flight Using the Sun as a Compass

16.2.1 Navigating with a Clock

Karl von Frisch (1954) explained how the flights of honey bees are made with reference to the sun's position and that they communicate this information through the famous waggle. Bees can be trained to and from a sugar water feeder simulating a flower's nectar with relative ease. Depending on the quality of the "nectar," recruits are quickly added from the hive. The number of recruits can be titrated by increasing or decreasing the concentration of sugar water. Trained foragers fly quickly and directly between the hive and the feeder in a "vector flight" (Menzel et al. 1998) and once satiated return to the hive reversing this vector. The direction of this vector is made with specific reference to the sun. This ingrained behavior provides a system that can be manipulated experimentally in a number of ways to test the role of the sun and other navigational cues and has been the fundamental research tool in the study of honey bee behavior for several decades.

The experimental proof of time-compensated sun-compass orientation in bees has been described by Lindauer (1961) and others since in several elegant stages. First

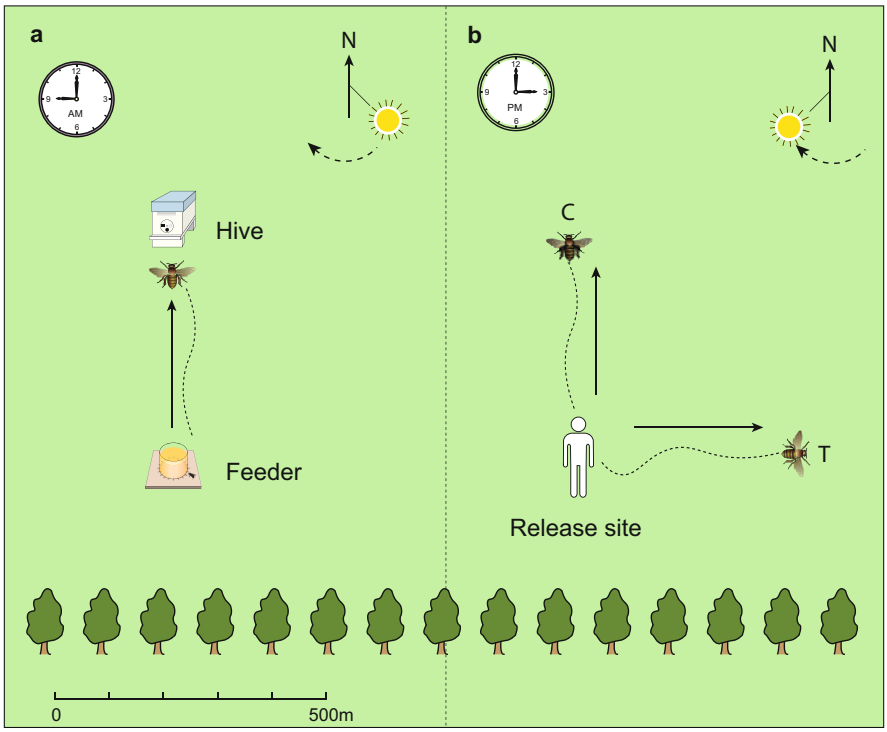

Fig. 16.1 Diagrammatic representation of sun-compass orientation in honey bee flights. (**a**) and (**b**) are contiguous in space but separated in time by 6 h in time. In (**a**) the bees are trained to a feeder and return to the hive by a vector flight north with reference to the sun. In (**b**) two examples are shown. Control bees (C) that have been caught at the feeder and displaced by several hundred meters are released 6 h later and, with functional clocks, compensate for the movement of the sun in the sky and fly their vector flight north to where the hive should be. Anesthetized bees (T) in which the clock has been phase shifted make an error in their vector flight direction proportional to the phase shift of the clock

when bees are trained to a feeder, for example, to the south of the hive, and that feeder and bees are displaced several hundred meters to the east from its original position, the bees still fly north when released on their homeward flight (Fig. 16.1). In this instance the bees have made an error by heading in the northerly direction that will not return them home. It is typically not until they reach the end of the return vector flight that they perform search patterns and rely on a hierarchy of other navigational information such as landmarks to find their hive (Menzel et al. 1998). However it is not just the case that the bees fly "blindly" with respect to sun compass and if prominent landmarks are sufficiently strong, the bees will override the incorrect sun-compass information navigating instead with regard to familiar or generalized landmarks (Cheeseman et al. 2014).

Once it has been established that the bees use the sun to calculate their vector flights, a second experiment can be performed to test whether they can compensate

for the movement of the sun. In this instance bees trained to the original feeder are caught as they take off full of nectar for the flight home. These bees are kept in the dark, without reference to the sun, for several hours before being released and observed to fly directly and quickly home on their correctly attuned homeward vector flight direction (north in the above example). Importantly the bees fly in this northerly direction regardless of whether the release site has been displaced indicating they have compensated for the movement of the sun (Lindauer 1961).

A third experiment can be performed in which the same system of hive and feeder can be employed with the catch and release method. Once trained to feeder, the entire hive can be moved overnight to a completely novel site outside the experience of the bees. In this way one might discount the possible influence of landmarks or other cues that might influence the behavior. Multiple feeders, this time without sugar or scent, can be arranged around the hive, and the number of bee visits is observed at each feeder. Invariably the feeder in the compass direction relative to the hive in its original site is the one most visited by the foragers and also at the right time.

Together these experiments indicate that bees have an innate time sense (named Zeitgedächtnis) which is driven by a circadian clock. "Flower constancy" is a strong driver of this behavior as individual species of flower show maximum secretion of nectar over limited periods each day with bees visiting each species at the appropriate time such as illustrated in Linnaeus' "Horologium florae (Flower clock)" (Linnaeus 1751). So accurate is the bees' time sense that it is possible to train foragers to between at least five (von Frisch 1954) and as many as nine (Bloch 2010) separate periods during a single day. The resolution of the clock is such that it is accurate to within minutes.

The remarkable ability for the forager bees to communicate the position of food (distance and direction) with respect to the sun through the waggle dance is further evidence of the time-compensated behavior. Even in poor weather when bees are confined to the hive by rain, foragers will continue to dance adjusting their angle on the comb relative to the sun's position. If the quality of the food source has been sufficiently good, dancing may continue in the so-called marathon dances which go into the night indicating the position of the food relative to the sun on the other side of the world where the bees have never seen it.

The circadian clock of the honey bee has been very well described and is based on the familiar positive and negative feedback loops of the known clock genes which regulate their own transcription. Interestingly the bee clock seems to be more similar in its mechanism to the mammalian clock in some respects than to other insect models such as *Drosophila* (Rubin et al. 2006; Yuan et al. 2007; Bloch 2010). *cryptochrome* (*cry*) and *period* (*per*) mRNA levels in the brain undergo strong oscillations, and *cycle* protein oscillates in antiphase to *per* (Bloch 2010) in diurnal light cycles as well in the constant conditions of the hive.

It is relatively straightforward to phase shift individuals (Ludin et al. 2012) and whole colonies of bees (Moore and Rankin 1993) in the laboratory with light cycles and temperature cycles, and these experiments can reliably phase shift times of food anticipatory behavior. Similar experiments have been conducted in the hoverfly

(Massy et al. 2021). A phase response curve derived from the response of locomotor activity to 1-h light pulses administered to individual bees indicates phase delays in the evening (between CT9[1] and CT18) (Ludin et al. 2012). Smaller phase advances were observed late in the subjective night and into the early morning (between CT18 and CT3).

16.2.2 Shifting the Circadian Clock with Anesthesia and Its Effect on the Time Compensation of the Sun Compass

It transpires that the circadian clock can be phase shifted with anesthesia. Exposing honey bees to the general anesthetic isoflurane during the day causes a phase delay in their foraging behavior. This has been demonstrated in several experiments (Cheeseman et al. 2012) and provides a useful tool to interrogate the bees' navigation systems.

Bees fitted with radiofrequency identification tags can be time-trained to a feeder over several days, and these foragers continue to return to that feeder at the same time of day for several days after the feeder is emptied of food. Anesthesia of the whole colony for 6 h during the day delays the flights of foragers on subsequent days by at least 3 h (Cheeseman et al. 2012). Importantly this delay continues for several days despite the colony's being kept in the open with exposure to the sun. Only after the third day do the flights of the foragers return to their preanesthesia schedule. This suggests strongly that time perception has been altered by anesthesia and therefore could affect time compensation of the sun compass. To test this the catch and release experimental paradigm can once again be employed to investigate the effect of the anesthesia on the bee's navigation. The advancement of technology to track individual bees in flight with harmonic radar (Riley et al. 1996; Menzel et al. 2005) has greatly increased the ability to investigate flight tracks. To return to the method in the earlier example (Fig. 16.1a), bees can be trained from a hive to a feeder to the south. As they alight the feeder full of nectar, they perform the fast vector flight north toward home. This behavior can be exploited to test the effect of anesthesia on time compensation too. If they are caught just as they take off from the feeder and anaesthetized in the dark, we can test the hypothesis that the anesthesia does in fact alter time perception. One might reasonably predict if anesthesia stops the perception of the passage of time the bees may make an error in their time compensation, equal to the time anaesthetized, and miscalculate the correction to make in the time the sun has moved. Given the sun moves on average 15° per hour, a 6-h anesthetic would therefore result in a 90° error in flight direction (Fig. 16.1b (T)). The bees do indeed make an error consistent with a period of time being lost; however it is not an error of the magnitude equal to that under anesthesia. After

[1] Circadian time. CT0 and CT12 correspond to onset and offset of activity, respectively.

6 h the average error of bees returning home was approximately 60°, the implication being that anesthesia has phase shifted rather than stopped the clock.

The comprehensive proof that anesthesia is acting on the clock itself comes from analysis of the expression of the clock genes themselves. In a further series of experiments, whole colonies of bees were kept in constant dim light in the laboratory and the activity rhythms of the colony measured for at least a week. The whole colonies were anaesthetized for 6 h during the day or at night and activity monitored for a further week. During this behavioral experiment, individually marked foragers of known age were collected and assayed for mRNA levels of *per*, *cry*, and *Clock* every 6 h over a time course of 72 h (approximately 36 h before and 36 h after anesthesia). Anesthesia during the day strongly phase delayed the expression patterns of *per* and *cry* and did not change the expression of *Clock*. This was entirely consistent with a delay in the colony's overall activity of the same magnitude. However when the experiment was repeated in a control experiment at night, no such phase delay occurred in either activity behavior or clock gene expression. This is not just a diurnal effect but a circadian one, and the subtlety of the effects of anesthesia on the bee clock has since been further described in an anesthesia phase response curve (Ludin et al. 2016) in which both advances and delays are observed on activity rhythms over the circadian cycle.

The importance of these findings is that the mechanism of navigation (time-compensated sun-compass orientation) is, perhaps unsurprisingly, inextricably linked to the circadian clock. However exactly how they are connected and where this occurs in the brain in the bee is not yet known. To investigate this further, we turn to another insect model the monarch butterfly.

16.3 Long-Distance Migration Using a Time-Compensated Sun Compass in the Monarch Butterfly

16.3.1 The Remarkable Journey

Perhaps the most impressive performance of insect navigation described is the migration of the monarch butterfly in which millions make their way from North America to their overwintering grounds in Mexico (Reppert and de Roode 2018). There are several reasons for this migration: to escape the freezing temperatures and the lack of their primary food source, milkweed plants, and also to escape from a protozoan parasite *Ophryocystis elektroscirrha* (Reppert and de Roode 2018).

The migration is unique because it typically takes between three and five generations of butterflies to complete the approximately 4500-km round journey (Miller et al. 2012; Tyler Flockhart et al. 2013, 2017). Furthermore the familial descendants return to the same location (Reppert and de Roode 2018). A significant change in physiology occurs between generations. In the late summer, the generation about to set out on the southern migration enters reproductive diapause to survive the journey

and winter before becoming reproductively active again in the spring. Several generations are then required to populate the northern range over the spring and summer (Reppert and de Roode 2018).

The time-compensated sun compass is once again critical to this behavior of the monarch butterfly and relies on a functional circadian clock for successful navigation (Froy et al. 2003). The study of the mechanisms at the behavioral, physiological, neural, and genetic levels has been championed by Steven Reppert and his laboratory, some highlights of which are summarized here.

16.3.2 Sensing the Position of the Sun

The monarch's compound eye retina is attuned to sensing the sun's azimuth, and the dorsal rim of the eye is sensitive to the angle of polarization (the e-vector) (Reppert et al. 2004; Labhart et al. 2009). This dorsal rim area is a specialized detector of linearly polarized light and is common to *Drosophila* (Warren et al. 2019), desert locust *Schistocerca gregaria* (Homberg 2004), the honey bee, ants (Lebhardt and Ronacher 2014), and others (Labhart et al. 2009). Both signals, the sun's azimuth and e-vector information, are sent from the eye to the butterfly's central complex in the brain which is believed to integrate them to determine the sun's position. It is this central complex which is most likely the core structure of sun-compass orientation in the monarch (Reppert and de Roode 2018), and this is consistent with findings in other insects including *Drosophila* (Strauss 2002), the dung beetles *Scarabaeus lamarcki* and *S. satyrus* (el Jundi et al. 2018), and the desert locust (Homberg 2004) in which the central complex is implicated in integration of information from celestial cues for movement and navigation.

16.3.3 Testing the Time Compensation of the Sun Compass

The strength of the autumn migratory behavior can be exploited experimentally by flying harnessed monarchs in a flight simulator. This has provided a powerful paradigm to test the sun-compass mechanism. In the simulator individuals with a view of the sun orient southwest and happily fly for many days. Clock shifting experiments using artificial light cycles reliably change the subsequent flight direction of the phase-shifted individuals so that a phase shift of 6 h results in a 90° change in direction (Merlin et al. 2009).

16.3.4 Location of the Mechanism for Time-Compensated Sun-Compass Orientation

Using the same flight simulator, protocol allowed the discovery that the clock mechanism for the monarch's sun-compass system is located in the antennae. Each antenna has a circadian clock which is directly photosensitive and can be entrained by light. Stopping the light signal by painting black or removing both antennae from an individual means it cannot perform time-compensated sun-compass orientation. Removing the paint from antennae rescues this ability (Guerra et al. 2012). Only one antenna is required for correct orientation behavior. If one is damaged or removed, the other is sufficient for correct orientation. However conflicting information from each intact antenna disorients the individual if either antenna is covered in paint blocking the light (Guerra et al. 2012).

These peripheral, antennal clocks are able to operate independently or at least are not strongly coupled together. Furthermore the clocks in the antennae appear to act somewhat independently of the central clock in the brain. The blocking of the light to the antennae results in free-running of the antennal clocks, but the central brain clocks remain entrained.

Examination of the expression of key core clock genes *per* and *timeless* (*tim*) in the antennae shows strong oscillations in light-dark cycles. In the example above when an antenna is painted black stopping light transmission, both *per* and *tim* expressions are highly dampened (Guerra et al. 2012). This is consistent with the general observation that peripheral clocks are not as strongly persistent in the absence of entrainment (Hardin 1994; Yildirim et al. 2022).

In contrast the central brain clock, generally predicted to be in the paired pars lateralis in Lepidoptera (Brady et al. 2021), is strongly endogenous. The implication is that it is not the central clock, in the monarch at least, that is important for providing the timing mechanism to synchronize the sun-compass information but rather the peripheral antennal clocks. What is fascinating is that there seem to be several systems working requiring clock input. The central clock in the brain determines the circadian phase of the animal and presumably the diurnal cycle, but the antennal clocks provide the key to time-compensate the sun-compass information.

16.4 Time-Compensated Moon Compass

So far the examples of time-compensated celestial navigation discussed have all been with reference to the sun compass. The hypothesis of a time-compensated moon compass has been discussed, if only rarely since 1960 (Papi 1960). One can imagine a similar time-compensated system using a lunar clock of period close to 24.8 h that could function a similar way as the sun compass. However there are several additional challenges an animal faces in being able to use this. The moon

does not always rise at the same time, and the intensity changes with the lunar cycle and also when covered by cloud. Nevertheless behavioral experiments in at least one species of amphipod sand hopper, *Talitrus saltator*, have been performed which indicate clock shifting the lunar clock results in change in orientation (Ugolini et al. 1999). However, at this point at least, there does not appear to be an example of an insect which uses time compensation to translate positional information from the moon to correct its course. Furthermore, unlike the circadian clock system which is very well described, we know very little of the circalunar clock in terms of mechanism, so there is much to learn in this field.

The moon is not the only nighttime celestial cue. Both the dung beetle (Dacke et al. 2013) and the large yellow underwing moth, *Noctua pronuba* (Sotthibandhu and Baker 1979), for example, can orient by the stars as well. Analysis of the tethered flight behavior of the moth showed no evidence that the night sky cues were time-compensated (Sotthibandhu and Baker 1979; Dreyer et al. 2018a). A final example of an insect which undergoes long-distance migration similar to the monarch but entirely at night is the bogong moth, *Agrotis infusa*, of Australia. This example would be ideal if it were shown to use a moon- or star-compensated compass and this has been investigated. However, the recent evidence suggests that it employs a terrestrial-based compass in the form of magnetic sense to make the 1000-m migration rather than nighttime celestial cues (Dreyer et al. 2018b).

16.5 Conclusions

Time-compensated celestial navigation has been observed in many insects. It is complex system with many components. Not only does the animal need a circadian, or perhaps circa-lunar, clock but also some way integrating the spatial and clock-based information to determine orientation and/or position. Several aspects of circadian clock-based information are used including but not exclusively (1) a clock to tell absolute time, (2) a clock to tell the organism when to feed, and (3) a clock to interpret the sun-compass information.

Much of what we know about the system so far comes from the work on two remarkable insects, the honey bee and the monarch butterfly. There are clear similarities and differences in these model insects. How insects perceive light directly from the sun and polarized light through the dorsal rim of the compound eye is well conserved across several insect orders. However, the antennal clocks of the monarch have not yet been described in other species like the bee.

The immediate future direction for study into the time compensation of the celestial compass is through the comparative anatomy of the brain and sensory systems in insects. This has begun and there is a lot to learn.

At this point in time, there does not appear to be evidence of the ability of insects to compensate for the movement of the celestial components of the night sky. It is, however, tempting to think there could be species such as moths which do fly large

distances at night that might employ orientation behavior based on the night sky in a time-compensated way.

References

Bloch G (2010) The social clock of the honeybee. J Biol Rhythm 25:307–317. https://doi.org/10.1177/0748730410380149

Brady D, Saviane A, Cappellozza S, Sandrelli F (2021) The circadian clock in Lepidoptera. Front Physiol 12:776826. https://doi.org/10.3389/fphys.2021.776826

Cheeseman JF, Winnebeck EC, Millar CD, Kirkland LS, Sleigh J, Goodwin M et al (2012) General anesthesia alters time perception by phase shifting the circadian clock. Proc Natl Acad Sci USA 109:7061–7066. https://doi.org/10.1073/pnas.1201734109

Cheeseman JF, Millar CD, Greggers U, Lehmann K, Pawley MDM, Gallistel CR et al (2014) Way-finding in displaced clock-shifted bees proves bees use a cognitive map. Proc Natl Acad Sci USA 111:8949–8954. https://doi.org/10.1073/pnas.1408039111

Dacke M, Baird E, Byrne M, Scholtz CH, Warrant EJ (2013) Dung beetles use the milky way for orientation. Curr Biol 23:298–300. https://doi.org/10.1016/J.CUB.2012.12.034

Dreyer D, el Jundi B, Kishkinev D, Suchentrunk C, Campostrini L, Frost BJ et al (2018a) Evidence for a southward autumn migration of nocturnal noctuid moths in central Europe. J Exp Biol 221 (24):jeb179218. https://doi.org/10.1242/JEB.179218

Dreyer D, Frost B, Mouritsen H, Günther A, Green K, Whitehouse M et al (2018b) The earth's magnetic field and visual landmarks steer migratory flight behavior in the nocturnal Australian bogong moth. Curr Biol 28:2160–2166.e5. https://doi.org/10.1016/j.cub.2018.05.030

el Jundi B, Warrant EJ, Pfeiffer K, Dacke M (2018) Neuroarchitecture of the dung beetle central complex. J Comp Neurol 526:2612–2630. https://doi.org/10.1002/CNE.24520

Evangelista C, Kraft P, Dacke M, Labhart T, Srinivasan MV (2014) Honeybee navigation: critically examining the role of the polarization compass. Philos Trans R Soc B Biol Sci 369:20130037. https://doi.org/10.1098/rstb.2013.0037

Froy O, Gotter AL, Casselman AL, Reppert SM (2003) Illuminating the circadian clock in monarch butterfly migration. Science 300:1303–1305. https://doi.org/10.1126/science.1084874

Guerra PA, Merlin C, Gegear RJ, Reppert SM (2012) Discordant timing between antennae disrupts sun compass orientation in migratory monarch butterflies. Nat Commun 3:958. https://doi.org/10.1038/ncomms1965

Hardin PE (1994) Analysis of period mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. Mol Cell Biol 14:7211–7218. https://doi.org/10.1128/MCB.14.11.7211-7218.1994

Homberg U (2004) In search of the sky compass in the insect brain. Naturwissenschaften 91:199–208. https://doi.org/10.1007/s00114-004-0525-9

Labhart T, Baumann F, Bernard GD (2009) Specialized ommatidia of the polarization-sensitive dorsal rim area in the eye of monarch butterflies have non-functional reflecting tapeta. Cell Tissue Res 338:391–400. https://doi.org/10.1007/s00441-009-0886-7

Lebhardt F, Ronacher B (2014) Interactions of the polarization and the sun compass in path integration of desert ants. J Comp Physiol A 200:711–720. https://doi.org/10.1007/s00359-013-0871-1

Lindauer M (1961) Time-compensated sun orientation in bees. Cold Springs Harb Symp Quant Biol 25:371–377. https://doi.org/10.1101/SQB.1960.025.01.039

Linnaeus C (1751) Philosophia botanica. apud Godofr. Kiesewetter, Stockholm

Ludin NM, Rescan M, Cheeseman JF, Millar CD, Warman GR (2012) A honey bee (*Apis mellifera*) light phase response curve. Chronobiol Int 29:523–526. https://doi.org/10.3109/07420528.2012.667468

Ludin NM, Cheeseman JF, Merry AF, Millar CD, Warman GR (2016) The effects of the general anaesthetic isoflurane on the honey bee (*Apis mellifera*) circadian clock. Chronobiol Int 33:128–133. https://doi.org/10.3109/07420528.2015.1113987

Massy R, Hawkes WLS, Doyle T, Troscianko J, Menz MHM, Roberts NW et al (2021) Hoverflies use a time-compensated sun compass to orientate during autumn migration. Proc R Soc B 288: 20211805. https://doi.org/10.1098/rspb.2021.1805

Menzel R, Geiger K, Joerges J, Müller U, Chittka L (1998) Bees travel novel homeward routes by integrating separately acquired vector memories. Anim Behav 55:139–152. https://doi.org/10.1006/anbe.1997.0574

Menzel R, Greggers U, Smith A, Berger S, Brandt R, Brunke S et al (2005) Honey bees navigate according to a map-like spatial memory. Proc Natl Acad Sci USA 102:3040–3045. https://doi.org/10.1073/pnas.0408550102

Merlin C, Gegear RJ, Reppert SM (2009) Antennal circadian clocks coordinate sun compass orientation in migratory monarch butterflies. Science 325:1700–1704. https://doi.org/10.1126/science.1176221

Miller NG, Wassenaar LI, Hobson KA, Norris D (2012) Migratory connectivity of the monarch butterfly (*Danaus plexippus*): patterns of spring re-colonization in eastern North America. PLoS One 7:e31891. https://doi.org/10.1371/JOURNAL.PONE.0031891

Moore D, Rankin MA (1993) Light and temperature entrainment of a locomotor rhythm in honeybees. Physiol Entomol 18:271–278. https://doi.org/10.1111/j.1365-3032.1993.tb00599.x

Papi F (1960) Orientation by night: the moon. Cold Spring Harb Symp Quant Biol 25:475–480. https://doi.org/10.1101/SQB.1960.025.01.049

Reppert SM, de Roode JC (2018) Demystifying monarch butterfly migration. Curr Biol 28:R1009–R1022. https://doi.org/10.1016/j.cub.2018.02.067

Reppert SM, Zhu H, White RH (2004) Polarized light helps monarch butterflies navigate. Curr Biol 14:155–158. https://doi.org/10.1016/J.CUB.2003.12.034

Riley JR, Smith AD, Reynolds DR, Edwards AS, Osborne JL, Williams IH et al (1996) Tracking bees with harmonic radar. Nature 379:29–30. https://doi.org/10.1038/379029b0

Rossel S, Wehner R, Lindauer M (1978) E-vector orientation in bees. J Comp Physiol 125:1–12. https://doi.org/10.1007/BF00656826

Rubin EB, Shemesh Y, Cohen M, Elgavish S, Robertson HM, Bloch G (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. Genome Res 16:1352–1365. http://www.genome.org/cgi/doi/10.1101/gr.5094806

Sotthibandhu S, Baker RR (1979) Celestial orientation by the large yellow underwing moth, *Noctua pronuba* L. Anim Behav 27:786–800. https://doi.org/10.1016/0003-3472(79)90015-0

Strauss R (2002) The central complex and the genetic dissection of locomotor behaviour. Curr Opin Neurobiol 12:633–638. https://doi.org/10.1016/S0959-4388(02)00385-9

Tyler Flockhart DT, Wassenaar LI, Martin TG, Hobson KA, Wunder MB, Norris DR (2013) Tracking multi-generational colonization of the breeding grounds by monarch butterflies in eastern North America. Proc R Soc B 280:20131087. https://doi.org/10.1098/RSPB.2013.1087

Tyler Flockhart DT, Fitz-gerald B, Brower LP, Derbyshire R, Altizer S et al (2017) Migration distance as a selective episode for wing morphology in a migratory insect. Mov Ecol 5:7. https://doi.org/10.1186/S40462-017-0098-9

Ugolini A, Melis C, Innocenti R, Tiribilli B, Castellini C (1999) Moon and sun compasses in sandhoppers rely on two separate chronometric mechanisms. Proc R Soc B 266:749–752. https://doi.org/10.1098/rspb.1999.0700

von Frisch K (1954) The dancing bees: an account of the life and senses of the honey bee. Methuen, London

Warren TL, Giraldo YM, Dickinson MH (2019) Celestial navigation in *Drosophila*. J Exp Biol 222 (Suppl. 1):jeb186148. https://doi.org/10.1242/jeb.186148

Yildirim E, Curtis R, Hwangbo D-S (2022) Roles of peripheral clocks: lessons from the fly. FEBS Lett 596:263–293. https://doi.org/10.1002/1873-3468.14251

Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24:948–955. https://doi.org/10.1093/molbev/msm011

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