Luis María Vaschetto *Editor*

Epigenetics, Development, Ecology and Evolution

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A mi viejo, José Benjamín Vaschetto
(1944–2021)

Preface

In the last decades, it has been suggested that epigenetic mechanisms might play fundamental roles, both in evolution and development. In multicellular animals, epigenetic pathways acting during development may represent the underlying basis of phenotypic variation for important traits observed in natural populations. It is especially important when considering that epigenetic variation exhibits an extraordinarily high potential and sensitivity to environmental conditions. This book provides a renewed piece for understanding the evolution of metazoans at the highest levels (including the evolution of human traits) from an epigenetic perspective. The book is aimed at showing some of the latest progresses in evolutionary epigenetics, and the intersection of this emerging fascinating field with developmental biology. For that purpose, I have compiled the works of scientists who are nowadays contributing to our understanding on developmental epigenetics and evolutionary origins of epigenetic regulation associated with ecology features and adaptive phenotypes.

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Alta Gracia, Córdoba, Argentina — Luis María Vaschetto

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Chapter 1
An Introduction to Epigenetics, Development, Ecology, and Evolution

Luis María Vaschetto

Contents

Abstract Epigenetics refers to the study of heritable modifications in gene expression without changes in the DNA sequence. Epigenetics pathways, which include DNA methylation, histone marks, and ncRNA pathways, work in a synergistic way in order to modulate gene expression and promote cell differentiation. In higher organisms, epigenetic mechanisms may represent the underlying cause of phenotypic variation and diversification depending on the environmental (ecological) conditions. *Epigenetics, Development, Ecology, and Evolution* is a reading piece that aims to collect some of the latest progress in evolutionary epigenetics, and its intersection with ecology and developmental biology.

Keywords Epigenetics · Developmental biology · Ecology · Evolution

1.1 Introduction

The term 'epigenetics' can be defined as the study of the heritable changes in gene expression that occurs without modifications in the nucleotide (DNA) sequence (Jablonka and Lamb 2005; Vaschetto 2015). The epigenetic-mediated changes in transcriptional activity include DNA methylation, histone modifications (e.g., acetylation, methylation), and non-coding regulatory RNA pathways (e.g., miRNAs, lncRNAs, piwiRNAs) (Skinner 2014, 2015). These epigenetic mechanisms work together in order to shape target gene expression, and they are found to be mutually

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reinforced through intricate feedback processes (Holmquist and Ashley 2006; McEachern 2011). The complex epigenetic networks play a pivotal role during development of multicellular organisms and often represent an underlying cause of phenotypic plasticity in natural populations.

In the second chapter of this book, Jeremias et al. (2022) provide a comprehensive reading piece in order to explain why and how epigenetic regulation represents a point of intersection among development, adaptive strategies, and microevolutionary change. This section is an analytical piece focused on the critical role of the epigenetic machinery during cellular differentiation and maintenance of phenotypically adaptive profiles. Moreover, the authors also discuss the mechanisms through which the organism's epigenome can promote adaptive strategies in offspring via transgenerational inheritance effects and the influence that environmentally induced epigenetic modifications exhibit on these processes in different animal groups (i.e., reptiles, amphibians, and birds).

In the next section, Dr. Vogt (2022) provides an interesting review to understand in deep the epigenetic mechanisms capable of shaping phenotypic evolution and cellular differentiation during animal development. The author elegantly explains the epigenetically mediated phenotypic effects in development and evolution of natural populations, while simultaneously describing the ecological significance of epigenetic evolutionary changes in species morphology over time. Dr. Vogt also exemplifies the ways in which epigenetically mediated variation of gene expression could drive adaptive responses to challenging environmental conditions, building an imaginary bridge that will allow readers to understand the crosstalk between environmental factors and differential gene expression during development.

In the fourth chapter, Drs. Thorson and Skinner (2022) describe in detail the associations between environmentally induced epigenetic transgenerational inheritance mechanisms and adaptive phenotypes. Here, the authors reveal how epigenetic regulation and related non-genetic inheritance mechanisms can be successfully integrated into the modern evolutionary synthesis, the most recent paradigm in evolutionary biology. In this review, Drs. Thorson and Skinner demonstrate why and how environmentally induced epigenetic mechanisms can be considered 'Rosetta Stones' capable of connecting developmental pathways from an evolutionary perspective.

In the fifth chapter, Dr. Bautista (2022) describes transgenerational epigenetic programming mechanisms that show potential to influence Darwinian fitness and adaptability to different environmental conditions, thus providing a renewed framework for the concept of 'survival of the fittest'. For that purpose, the author clearly explains the connections between core principles of evolution, going through concepts such as epigenetics, transgenerational inheritance, and developmental programming. Moreover, this chapter also provides useful insights into the environmental relevance of epigenetic modulation. This review is an excellent contribution to unravel the hidden potential of epigenetic phenomena that are capable of accelerating the occurrence of genome fixation and inducing evolutionary change.

In the next section, Dr. Guerrero-Bosagna et al. (2022) describe epigenetic mechanisms driving the evolution of developmental pathways in birds. Research

in birds has greatly contributed to our understanding of epigenetics and its roles from both the Waddingtonian perspective and at the molecular biology level. It is an excellent review that compiles the most relevant studies in epigenetics of birds, ranging from the ideas of preformation and epigenesis to molecular epigenetic mechanisms underlying gene regulation. The authors also describe the ways in which environmental conditions may act as a link between genetic and epigenetic pathways, buffering their effects on adaptive phenotypes. The environmental factors examined in this Chapter include hypoxia, temperature, and environmental pollutants.

In the seventh chapter, Ingelson-Filpula et al. (2022) describe epigenetic pathways associated with extreme stress responses. The authors explore the association between epigenetic mechanisms and adaptive responses to different types of extreme stress conditions. The adaptive strategies analyzed in this section include freeze tolerance, torpor/hibernation, hypoxia, anoxia, estivation, and dehydration. Both short-term and long-term responses to extreme factors are achievable thanks to the physiological phenomenon of 'metabolic rate depression', an interesting process of metabolic reorganization that has recently been associated with epigenetic pathways and post-transcriptional gene regulation.

In the eighth chapter, Drs. Ragsdale and Foley (2022) provide a comprehensive overview of the scope of epigenetics in human behavior and culture. For that purpose, the authors describe how adaptation to local ecologies might represent the first step toward a process known as 'gene-culture co-evolution'. In this chapter, the authors propose the mechanisms by which the adaptation of cultural heritage to local ecologies could be influenced by epigenetic-cultural coadaptation and co-evolution. Here, epigenetic mechanisms are employed to evidence how complex phenotypic traits (e.g., behavior and personality) might result in being adaptive. Finally, Ragsdale and Foley examine epigenetic changes associated with diet capable of influencing cognitive development and behavior.

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Chapter 2
Epigenetic Regulation: The Cross-Talk among Development, Adaptive Strategies, and Microevolutionary Change

Guilherme Jeremias, Fernando J. M. Gonçalves, Jana Asselman, and Joana L. Pereira

Contents

Abstract In this chapter, we aim to demonstrate that epigenetic regulation is a central, cohesive and comprehensive process that underpins, reflects in and brings together developmental aspects, phenotypic responses and inheritance that may constrain evolutionary pathways. In particular, evidence on the critical role of epigenetics in developmental biology was compiled, which demonstrates that, despite the stability of such processes, epigenetic mechanisms remain highly responsive to environmental cues, especially during the early stages of life. By exploring how epigenetic changes during development can have persistent effects, often impinging heredity and evolution, epigenetic inheritance is highlighted as the core of the cross-talk between early life, adulthood and transgenerational effects on phenotypes. Accordingly, we then discuss the role of epigenetic changes in shaping species' adaptive strategies, as well as the role of epigenetic inheritance in defining actual adaptation, therefore highlighting the importance of epigenetic mechanisms

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and inheritance for evolutionary processes. Moreover, critical data gaps were pointed out throughout the different subjects addressed, thereby opening interesting avenues for epigenetic research on development, ecology, ecotoxicology and evolution.

Keywords Epigenetic Inheritance · Developmental Epigenetics · Epigenetic Adaptation · Evolutionary Epigenetics

2.1 Introduction

Epigenetic mechanisms concern potentially heritable modifications in gene activity and expression, excluding those related to changes in the DNA sequence itself (Bird 2002, 2007). Three main epigenetic mechanisms are usually considered: (i) DNA methylation—involves the transference of a methyl group mostly to the fifth position of a cytosine (although other DNA bases can also be methylated); (ii) post-translational histone modifications—wrapped around the DNA, histones are a structural part of nucleosomes; and (iii) non-coding RNAs, that form complex regulatory networks of the genome by being coupled to epigenetic machinery through regulatory loops (Iorio et al. 2010; Peschansky and Wahlestedt 2014).

Epigenetic changes can arise sporadically or be induced by the environment during the life span of the most different organisms—see, e.g. Baccarelli and Bollati (2009), Bollati and Baccarelli (2010) and Cortessis et al. (2012) for a detailed view on the interplay between environmental cues and epigenetic responses. Epigenetic marks/patterns can be induced and then immediately reversed once exposure ceases, but these molecular marks may also persist throughout the life time of the affected organism (Bird 2007; Mirbahai and Chipman 2014; Jeremias et al. 2020). It can be also extended to the non-exposed progeny when the epigenetic mark/pattern is transmitted to following generations, picturing this exciting field of research on epigenetic inheritance (Skinner 2011a, b; Jeremias et al. 2018b)—see Box 2.1

Epigenetic changes can translate into phenotype variations through the epigenetic regulation of gene expression. Precisely, some of the most notorious epigenetic studies focused on the origin and importance of epigenetically mediated phenotypes, and the role of environmental clues in their establishment. Some of these hallmark studies pictured that (i) monozygotic twins are epigenetically indistinguishable in young ages, while older twins showed remarkable differences in their DNA methylation and histone acetylation and that these epigenetic differences accumulating throughout life translate into different gene expression portraits and phenotype outcomes (Fraga et al. 2005); (ii) DNA methylation changes in the glucocorticoid receptor of the hippocampus of genetically identical rat pups are established according to low or high nurturing behaviour by rat mothers, and such phenomenon largely influences the personality and behaviour of the pups in adulthood (Weaver et al. 2004); (iii) the realization that fertile queens and sterile worker honey bees

develop from genetically identical larvae, and the differential feeding of these larvae accounts for an epigenetic global reprogramming that underpins profound shifts in development, thereby regulating the contrasting reproductive and behavioural statuses of these females (Kucharski et al. 2008). Indeed, different phenotypes can originate from identical genotypes through epigenetic changes, supporting claims that epigenetic mechanisms play a key role in controlling phenotypic plasticity and phenotype determination (Barros and Offenbacher 2009; Duncan et al. 2014; Vogt 2015; Burggren 2016; Banta and Richards 2018). Furthermore, evidences exist on the occurrence of a very well-defined chain of events, in which an environmental exposure can determine specific epigenetic changes that, in turn, translate into different cell/organismal phenotype outcomes (Meaney 2010; Skinner 2011a; Burggren 2016). Furthermore, these processes are widespread in nature—for reviews on the topic, see, e.g. Feinberg (2007), Burggren (2015) and Norouzitallab et al. (2019). Still, it is also fair to recognize that a better understanding of the connection among epigenetic mechanisms, changes in gene expression and higher-level effects, including those on cells and at the individual level, is urgently required (EFSA 2016; Jeremias et al. 2020).

Overall, the message to retain at this stage is that epigenetically determined phenotypes can shape the life of organisms, not only by mediating short-term responses to environmental factors, but also by influencing responses to environmental cues over large temporal scales (Jablonka and Lamb 2007; Jeremias et al. 2018b). Despite the existence of data gaps in the scientific landscape (see Box 2.1), epigenetic mechanisms and epigenetic inheritance not only regulate phenotypic variation and determination through development, but also shape adaptive and microevolutionary responses of individual- and population-level traits (see Fig. 2.1; Jablonka and Lamb 2007; Jeremias et al. 2018b). Accordingly, the traditional view of evolution, based on the assumptions that phenotypes arise from the expression of genetic variants that are selected by DNA-based forms of inheritance, has been challenged by epigenetics (Skinner 2011a; Burggren 2016; Jeremias et al. 2018b).

Box 2.1 Brief Notes on Three Key Knowledge Gaps Impairing the Interpretation of Epigenetic Data within the Interplay of Developmental, Adaptive, and Evolutionary Frameworks

- *Unbalanced epigenetics knowledge range among different biological groups.*

Epigenetic research has focused mostly on mammal species, and while there is still much to be discovered in this context, it is known that DNA methylation patterns are well conserved across mammalian species, with methylation mainly targeting cytosine residues within a CpG context (Head 2014; Mendizabal et al. 2014). In addition, mammals generally have heavily methylated genomes, except for those cytosines within the CpG islands in the

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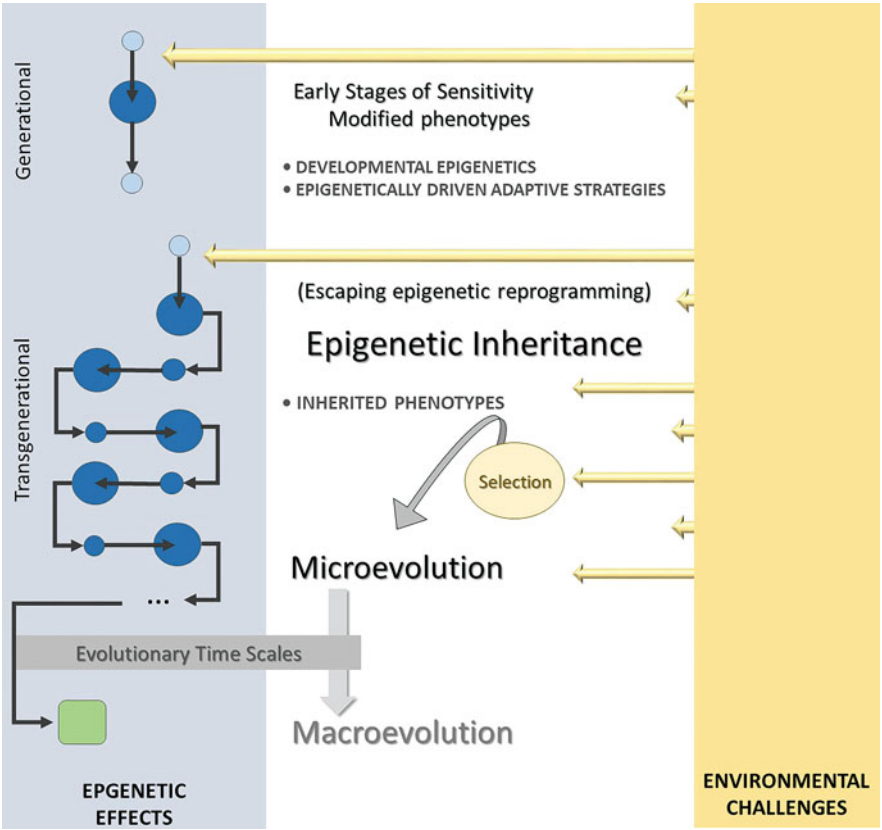

Fig. 2.1 Conceptual diagram representing the role of epigenetic regulation as a central and cohesive process in the cross-talk between development, adaptive strategies and evolution. The effects of environmental cues (note yellow arrows dynamics) on early stages of development are represented (symbols changing size), as well as their immediate effects on phenotypes (symbols changing colour). The possibility of epigenetic marks escaping reprogramming is displayed, in addition to the corresponding persistence of previously modified phenotypes by means of epigenetic inheritance. Lastly, the possible selection of epigenetic inherited phenotypes and resulting arise of microevolutionary patterns are represented, as well as macroevolutionary effects over evolutionary time scales (note the change in shape and colour of the symbol)

Box 2.1 (continued)
promoters of genes, as methylation in these regions is associated with the repression of transcription (Zemach et al. 2010; Head 2014). The scarcity of knowledge concerning the role of epigenetic mechanisms in developmental, adaptive and evolutionary processes of vertebrate groups other than mammals (e.g. birds, amphibians and reptiles), as well as invertebrate species in general,

(continued)

Box 2.1 (continued)
is problematic (Frésard et al. 2013; Holt 2017; Jeremias et al. 2018b). This is because the diversity within these less studied groups of animals and of the corresponding habitats and environmental contexts is large, which renders any attempts to postulate general trends for epigenetic patterns mostly speculative.

- *Short availability of genomic knowledge.*

The availability of accurate genomic information is critical for a feasible understanding of epigenetic mechanisms and patterns. Although the shortage of this kind of information is applicable to a large range of non-mammalian vertebrates, the scenario is more dramatic for invertebrate species. Until recently, there was a severe lack of invertebrate genomes available, which limited the study of epigenetic phenomena to only a few invertebrate species (Rivière 2014; Jeremias et al. 2020). Nevertheless, from the limited information available, it is possible to extract that DNA methylation levels in invertebrates are extremely variable, with some species exhibiting no DNA methylation while others are methylated to a level that is comparable to some vertebrates (Head 2014; Olson and Roberts 2014). Remarkably, even in similar groups of invertebrate organisms, variation can be extreme; for instance, DNA methylation in insect species is known to vary immensely, regarding both the quantity and genome location (Head 2014; Yan et al. 2015). Also, invertebrates seem to present a lower number of epigenetic marks; specifically, invertebrates' DNA methylation has been shown to be sparse and mainly targeted to gene bodies, contrary to the widespread methylation of intergenic regions in vertebrates (Suzuki et al. 2007; Feng et al. 2010; Zemach et al. 2010; Sarda et al. 2012). The meaning of intragenic DNA methylation in invertebrates is largely unknown, if actually existing in relevant levels. Interestingly, gene body methylation has been correlated with higher gene transcription in both vertebrates and invertebrates, thus suggesting an important role of such epigenetic marks as regulators of gene expression in both groups (Suzuki et al. 2007; Sarda et al. 2012; Kvist et al. 2018).

- *Unclear definition of directly induced and inherited epigenetic marks.*

The term epigenetic inheritance has been poorly used in the context of epigenetic research (Heard and Martienssen 2014; Skinner 2014). This is because epigenetic inheritance only occurs in cases where epigenetic marks are inherited through the germline (Heard and Martienssen 2014; Nilsson et al. 2018). Therefore, the existence of epigenetic inheritance can only be confirmed in cases where future generations (F1, F2, F3, etc.) are not exposed to environmental cues directly challenging the parental generation (F0), which often challenge also germ lines of future generations (Heard and Martienssen 2014). Appropriately distinguishing epigenetic marks found following direct

(continued)

Box 2.1 (continued)
exposure to environmental cues from those that were actually inherited by the progeny is critical to accurately separate the role of epigenetics in driving immediate adaptive strategies (configuring phenotypic plasticity) or a negative phenotypic effect, and the role of epigenetics as a driver of adaptation, hence (co-) paving evolutionary paths. This distinction reflects in an accurate definition of what can be classified as a transgenerational persistence of epigenetic marks, which is inherently different from their intergenerational and multigenerational persistence. The maintenance of an epigenetically induced phenotype in generations that were never exposed to the driving environmental challenge (note that in the case of female mammals and many other organisms the exposure of germ cells in developing embryos cannot be ruled out when parent adults are exposed; thus, F1 and F2 are likely exposed along with F0) reflects transgenerational persistence of epigenetic marks, thus true inheritance of an environmentally driven change (e.g. Hanson and Skinner 2016). Whenever direct exposure of a given descent generation cannot be ruled out (e.g. in F1 and F2 in mammals, when the female parent is considered) that may induce a given effect, one can only assume that a multigenerational or an intergenerational effect is being observed through successive generations. The touting of this non-transgenerational effect as multigenerational is as common as its touting as intergenerational in the epigenetics literature, which no particular reference to the differences between these two terms (compare, e.g. Hanson and Skinner 2016 with Tuscher and Day 2019). Although the apparent interchangeability of these terms applied in several fields, including epigenetics, they do not mean exactly the same as argued by, e.g., Villar (2007). Intergenerational implies interaction between/among generations, meaning that intergenerational effects encompass changes in the overall outcome from one generation to another (e.g. an already methylated gene produces a phenotype that is better prepared to withstand a given environmental condition that induced methylation also in the parental generation soma). Multigenerational is a broader term that refers simply to the sharing of a given trait among generations, e.g. the observation of methylation marks in a given gene across successive generations. Under this more systematic definition of the terms, a transgenerational epigenetic effect will always be a multigenerational effect but the opposite is not necessarily true, while not all multigenerational effects are intergenerational.

The major role of epigenetics in defining phenotypes as a response to diverse and inherently distinct environmental cues, both in the short and in the long term, is intertwined by its major fundamental role in shaping organisms' development. This also configures epigenetic inheritance as a central and cohesive process that drives the function of the epigenome in development, adaptive and evolutionary aspects. Such a complex framework is synthetized in Fig. 2.1, which serves as a clarifying

conceptual guide through epigenetics research, and concomitantly as a roadmap for the present chapter. Aiming at clarifying direct and indirect roles of epigenetic mechanisms in biological development, adaptation and evolution, we first explore the key role of epigenetics in developmental processes of both vertebrate and invertebrate species, specifically focusing on both the stability and environmental responsiveness of epigenetic mechanisms during critical developmental stages (Sect. 2.2), as well as on the possibility of epigenetic marks escaping epigenetic reprogramming, thus opening the possibilities for epigenetic inheritance (Sect. 2.3). Consequently, we then discuss the implications of epigenetics for evolution by focusing on the related role of epigenetic mechanisms and phenotypes, specifically the processes by which epigenetic phenomena and inheritance shape adaptive strategies and the overall adaptive capacity of organisms (Sect. 2.4). In order to better frame these questions, critical data gaps are highlighted in each of the different sections of this chapter.

2.2 Developmental Epigenetics

The term "epigenetics" was firstly introduced by the embryologist Conrad Waddington in 1942, as a part of the concept of the "epigenetic landscape" (Waddington 1942). As Waddington latter discussed, this concept aimed to represent the stable pathways during embryonic development in which genes and their products brought phenotypes into being (Waddington 1968; Jablonka and Lamb 2002; Noble 2015). According to Waddington's reasoning, development was "canalized", "*and this canalization or buffering was the outcome of natural selection for genes whose actions and interactions make the valleys in his epigenetic landscape deep and steep sided*"—see Jablonka and Lamb (2002) for an historical perspective on the definition of epigenetics. Although the definition of epigenetics has largely been extended from its origin, this shows that epigenetic research has been coupled to development since the first days of the field (Jablonka and Lamb 2002; Holliday 2006). Throughout the years, epigenetic mechanisms have been increasingly positioned as key components of developmental biology, which in turn stands as a very broad discipline, comprising all the aspects of ageing, embryology, growth and regeneration (Jablonka and Lamb 2002; Felsenfeld 2014).

The integration of epigenetic concepts into developmental biology demonstrated how genetics and epigenetics act in concert to shape development processes (Bernstein et al. 2007; Cavalli and Heard 2019). The most remarkable example is the transformation of a single fertilized egg into the hundreds of specialized cell types that compose a multicellular organism, since all cells of the body contain the same DNA. Indeed, the key driving process governing cellular identities is the epigenetically mediated change in the expression of a defined set of cell lineage genes (Álvarez-Errico et al. 2015; Atlasi and Stunnenberg 2017; Cavalli and Heard 2019). Yet, this process is everything but a simple task as it is dependent on the coordinated deployment of hundreds of transcription factors that, with precision,

need to bind to multiple DNA sequences (Álvarez-Errico et al. 2015; Cavalli and Heard 2019). Once established, unique cellular expression patterns need to be maintained throughout the lifespan of organisms, which constitutes a separated process yet connected to the previous phase, ensuring the establishment of unique cellular gene expression patterns (Cavalli and Heard 2019). This cellular memory is achieved through the involvement of a plethora of non-DNA sequence chromatin cofactors, which support the maintenance of chromatin states through cell division, and thereby for extended periods of time (Álvarez-Errico et al. 2015; Cavalli and Heard 2019). Accordingly, epigenetic marks and patterns need to survive DNA replication and mitosis, and indeed, the mitotic transmission of epigenetic marks has been demonstrated in both animals and plants—see Cavalli and Heard (2019), Oomen and Dekker (2017) and Probst et al. (2009) for mechanistic insights on these processes.

Interestingly, different epigenetic marks are replicated at different stages of mitosis, as well as distinct components of cellular machinery are used to ensure the replication of epigenetic patterns. For example, the replication of DNA methylation patterns in newly synthesized strands occurs shortly after the replication fork passes (Wigler et al. 1981; Masai and Foiani 2018); non-coding RNAs ensure the stability of cell replication and non-coding RNA islands seem to be transmitted through mitosis (Akhade et al. 2017; Stojic et al. 2020); and histone patterns have also been shown to be faithfully inherited despite chromatin disassembly ahead of the replication fork (Annunziato 2015; Masai and Foiani 2018; Hugues et al. 2020). Overall, this shows that cell replication is not only about the creation of new DNA strands but also comprises the selective maintenance of epigenetic patterns through cell division (Probst et al. 2009; Cavalli and Heard 2019). Moreover, it seems that repressive epigenetic marks are not necessary for pre-implantation embryonic development and naïve cellular pluripotency, as both phases are usually associated with global DNA demethylation (Atlasi and Stunnenberg 2017; Takahashi et al. 2018). On the other hand, high levels of DNA methylation were observed in post-implantation processes, showing that the exit from naïve pluripotency and embryonic stem cell differentiation is accompanied by progressive restriction of chromatin accessibility (Bao et al. 2009; Atlasi and Stunnenberg 2017; Xu and Xie 2018). Therefore, epigenetic bivalency plays a prominent role in many aspects of cell functioning and embryonic development (Bernstein et al. 2006; Cuddapah et al. 2010; Bernhart et al. 2016; Xu and Xie 2018). Also, although epigenetic memory contributes to the stability of gene expression and cellular function during developmental stages, the epigenetic machinery of cells remains flexible, thereby allowing cells to respond to external stimuli, including direct environmental exposure, cellular communication and signalling (Wilson et al. 2009; Zaidi et al. 2011). Accordingly, the three epigenetic mechanisms play a key part in cellular plasticity and cell language, thus supporting the view that the epigenome is a highly dynamic entity that is constantly reshaped and under the influence of environmental stimuli during development (Carrell and Hammoud 2009; Wilson et al. 2009; Skinner 2011a; Steffen and Ringrose 2014).

Most epigenetic studies focused on developmental aspects targeted exclusively vertebrate species, with particular emphasis on humans and other mammals. Some of the most noteworthy examples in this context include the role of DNA methylation in the development of the mammalian immune system and regulation of neuronal cell fate decisions, as well as the importance of the three epigenetic mechanisms for X chromosome inactivation and genomic imprinting in mammals—see, e.g. Kiefer (2007), Li (2014) and Xu and Xie (2018) for reviews on the key role of epigenetics during the development of vertebrates. Nevertheless, DNA methylation is by far the best well-studied epigenetic mechanism in this context, despite there are a growing number of studies demonstrating the involvement of histones changes and non-coding RNAs in developmental processes, thus highlighting the potential for their involvement in many more aspects of development (Beermann et al. 2016; Li et al. 2018; Zhao et al. 2019).

Interestingly, the transition from invertebrate to invertebrate–vertebrate assemblages in the history of life was a major evolutionary event marked by major changes in development, including spectacular morphological and physiological innovations—the development of a spinal column and new organization of the nervous system, among other major features—supporting the existence of more complex forms of life (Keller et al. 2016b; Xu et al. 2019). As differences in epigenetic inheritance and reprogramming events are known to exist between vertebrates and invertebrates, it is suggested that these differences shaped the morphological complexity and evolutionary novelties that determined the referred evolutionary transition (Keller et al. 2016b; Xu et al. 2019). Despite the scarcity of studies, some promising results support the view that invertebrates' development is also under epigenetic control, at least in some species. These include observations that major changes in epigenetic status occur during different developmental stages and that developmental, cellular communication and adhesion genes are enriched for epigenetic changes in the oyster (*Crassostrea gigas*) and marbled crayfish (*Procambarus virginalis*)—e.g. Riviere et al. (2017), Song et al. (2017) and Vogt (2017). This suggests that there is a huge potential for the clarification of the epigenome dynamics during developmental processes, as well as on the interplay between environment and epigenetic responses during the early life stages of invertebrates. Accordingly, critical progress on these issues can now be made, by taking advantage on the increasing availability of vertebrate and invertebrate genomes, as well as by the establishment of non-mammalian organisms (e.g. fish, amphibians and reptiles) and invertebrate species (e.g. crustaceans and insects) as epigenetic model organisms—see, e.g. Bonasio (2015), Holt (2017), Jeremias et al. (2018b) and Jeremias et al. (2020) for a comprehensive discussion on some of the most useful organisms and epigenetic techniques for future studies in this context.

2.2.1 *Roles of Epigenetic Mechanisms in Early Windows of Development*

The epigenetic machinery plays an important role in cellular differentiation and maintenance of expression profiles, while also allowing cells to regulate their expression profiles in response to external stimuli (Wilson et al. 2009; Zaidi et al. 2011). However, it has been widely reported that the epigenome is much more sensitive to environmental cues during early stages of development rather than in adulthood or later stages in life (Dolinoy et al. 2011; Skinner 2011b). Many experimental studies have confirmed that a vast array of environmental stressors can determine specific epigenetic changes resulting from in utero and early life exposures, and such phenomena seem to be widespread in nature, concerning both invertebrates and vertebrates (Gicquel et al. 2008; Dolinoy et al. 2011; Norouzitallab et al. 2014). As an example of these processes in invertebrates, Norouzitallab et al. (2014) showed that when a population of the aquatic invertebrate *Artemia* was exposed during early life stages to heat stress, there was increased tolerance to the stressor, which seemed to be determined by alterations in the levels of global DNA methylation and histone H3 and H4 acetylation levels. Important, this phenotypic trait, i.e. increased tolerance to heat stress, was transmitted to three successive generations (Norouzitallab et al. 2014).

Focusing on vertebrates, some promising studies demonstrated that epigenetic mechanisms may mediate development and/or regeneration processes in birds, amphibians, fishes and reptiles and that environmentally induced epigenetic changes during the development of such organisms can translate into long-term effects (Yakushiji et al. 2009; Holt 2017; Best et al. 2018). In particular, several fish species (with the best studied being the model zebrafish, *Danio rerio*) have provided a better understanding on both the role of epigenetic mechanisms in the development of vertebrates, and the phenotypic changes resulting from those environmentally induced epigenetic changes that occur during early life stages (Labbé et al. 2017; Best et al. 2018; Lakstygal et al. 2018; Seritrakul and Gross 2019). Also, a wide range of epigenetic responses has been documented during gametogenesis, development and differentiation of fishes, and the manipulation of gametes and early-stage embryos can determine specific epigenetic changes that translate into lifelong phenotypes (Leung et al. 2016; Metzger and Schulte 2016; Labbé et al. 2017; Best et al. 2018).

Although much fewer epigenetic studies can be found for reptiles, amphibians and birds, several developmental processes in these organisms seem also to be under the influence of environmentally induced epigenetic changes (Frésard et al. 2013; Sun et al. 2014; Piferrer et al. 2019). For example, it is known that epigenetic regulation is an important mechanism controlling sex determination in several reptiles and fishes and that such epigenetically mediated processes are also present in invertebrates and other vertebrates (Piferrer 2013; Kuroki and Tachibana 2018; Piferrer et al. 2019; Singh et al. 2020). The effects of teratogenesis (prenatal toxicity) in these organisms are also, at least in part, epigenetically mediated, and such

molecular processes account for malformations and other abnormalities observed during early stages of life and/or adulthood (Mudbhary and Sadler 2011; Martín-Del-Campo et al. 2019). Another paradigmatic example in this context regards the development of amphibians, in which the environments experienced during early life critically define the character and timing of development, often having profound effects on phenotypic traits later in life (Denver 2009; Sarma et al. 2020).

As detailed above, it is increasingly evident epigenetic changes can induce shifts in developmental trajectories that can shape phenotypic responses and the adaptive potential of certain vertebrate groups and some invertebrate species (Denver 2009; Jeremias et al. 2018b; Petitjean et al. 2019; Sarma et al. 2020). On the other hand, much more studies have explored the role of epigenetic mechanisms in mammalian development. For instance, it has been reported that the male F1–F4 offspring of pregnant female rats exposed (Sprague Dawley and Fisher strains) to vinclozolin (an endocrine disruptor) during the time of sex determination presented higher levels of spermatogenic defects, and prostate, kidney and immune system disease, as well as higher rates of tumour development compared to offspring from non-exposed females (Skinner and Anway 2005; Anway et al. 2006; Bollati and Baccarelli 2010). Similar results were observed when studying both in utero and neonatal exposure of rats to the toxicant bisphenol A: exposed rats presented higher body weight, deficits in reproduction function and increased rates of prostate and breast cancer development (Dolinoy et al. 2007; Bollati and Baccarelli 2010). Accordingly, there are also evidences that the extent of DNA methylation at each allele is stochastic in the yellow agouti mice, as well as dependent upon maternal nutrition and environmental exposure, specifically during early development (Dolinoy and Jirtle 2008; Dolinoy et al. 2011). Interestingly, epigenetic changes occurring during early life have also been documented in humans, and such processes have been touted to have huge implications for epidemiological and medical studies (Hong and Wang 2014; Jeremias et al. 2020). In fact, adverse intrauterine environments, such as the shortage or excess of nutrients, are associated with increased risks for many complex diseases later in human life, namely increased lifelong risks for obesity, metabolic, cardiovascular and malignant diseases (Lehnen et al. 2013; Lee 2015). Similarly, severe effects of adverse conditions in early life, such as child abuse, have been documented on the development of the human brain, leading to increased vulnerability to mood disorders later in life (Murgatroyd and Spengler 2011). Despite these valuable insights, much work remains to be done towards better characterizing the environmental susceptibility of epigenetic mechanisms, especially histones modifications and non-coding RNAs, in the different developmental stages, as well as towards a better understanding of the effects of early life exposures on organismal sensitivities in later stages of life and in future generations (Wu et al. 2015; Hanna et al. 2018; Jeremias et al. 2020).

Despite more work is yet to be done, one can safely argue that even some of the most complex phenotypic traits can be affected by epigenetic changes during the early stages of development and that such epigenetic changes and resulting phenotypes can become permanent throughout the life of the organisms, thereby influencing phenotypic plasticity ranges and ultimately shaping the adaptive strategies of

organisms (Dolinoy et al. 2011; Guerrero-Bosagna and Skinner 2012; Jeremias et al. 2018b; Nilsson et al. 2018). Furthermore, it has been shown that peri-conceptional and intrauterine exposures occurring in regions resistant to epigenetic reprogramming can be inherited, showing that not all epigenetic marks are completely erased and reapplied, i.e. epigenetically reprogrammed (Dolinoy et al. 2011; Wu et al. 2015).

2.3 Epigenetic Inheritance: Escaping Reprogramming

Epigenetic reprogramming consists on the removal of epigenetic marks from germ cells and embryos, and such processes play a key role in the success of sexual reproduction and development (Li 2002; Sasaki and Matsui 2008). There are two major waves of genome-wide epigenetic reprogramming during mammal development: the first occurs during primordial germ-cell formation, while the second focuses on the zygote and occurs shortly after fertilization (Sasaki and Matsui 2008; Xu and Xie 2018). Interestingly, despite male and female gametes contribute with the same amount of DNA to the zygote, these germ cells are epigenetically distinct and different processes of epigenetic reprogramming have been described (Head 2014; Xu et al. 2019). For example, the extent and distribution of DNA methylation in sperm and oocytes were found to be distinct in several species, thus supporting the need of specific epigenetic reprogramming so that germ cells achieve equivalent epigenetic states before fertilization, thereby assuring compatibility for totipotency and development thereafter (Hackett and Surani 2013; Head 2014; Xu et al. 2019). In addition, the processes of epigenetic reprogramming seem to differ across species and may even be dramatically different in related groups of organisms (Head 2014; Xu et al. 2019). For instance, the epigenetic marks of the zebrafish sperm are inherited without reprogramming, while the mother's germ cells are largely reprogrammed in order to match the parental epigenetic state (Jiang et al. 2013; Head 2014); in contrast, both germ cells of mammals undergo extensive epigenetic reprogramming, such as genome-wide demethylation (Sasaki and Matsui 2008; Carrell and Hammoud 2009). Furthermore, while epigenetic reprogramming processes are relatively well characterized in mammals and some other vertebrate species, they remain largely unexplored in invertebrate species. One remarkable exception is the study of Xu et al. (2019), who performed a complete gene ontology analysis on the methylome of gametes and early embryos of several vertebrate and invertebrate species. Their results show that the potential regulation of reprogramming of promoter DNA methylation was very limited in invertebrate species, while in vertebrates the enrichment of such epigenetic marks for metabolic, developmental and reproductive pathways was observed, indicating that DNA methylation reprogramming is associated with embryonic development and reproduction processes in vertebrates (Xu et al. 2019). Nevertheless, the authors also noticed that the parental methylomes of some of the invertebrate species were identical and stable during embryogenesis. In contrast, in invertebrate species

more closely related to vertebrates, such as echinoderms and invertebrate chordates, the reprogramming of parental methylomes was present, ultimately becoming more evident during vertebrate evolution (Xu et al. 2019). These findings confirm previous claims that epigenetic reprogramming was dramatically shaped during animal evolution, especially after the evolutionary transitions from invertebrates to vertebrates, and then to mammals (Keller et al. 2016b; Xu et al. 2019).

While some epigenetic marks can be reversed once exposure to the driving stimulus is ceased, others can sometimes be maintained through the lifetime of organisms or even be transmitted across different generations. The potential for epigenetic inheritance is limited in several groups of organisms, such as mammals, because efficient epigenetic reprogramming processes are in place (Morgan et al. 2005; Kovalchuk 2012) and considering that the erasing of epigenetic marks during development stands out as a natural barrier for the establishment of epigenetic inheritance (Bollati and Baccarelli 2010; Kovalchuk 2012; Heard and Martienssen 2014). However, there is a growing number of studies demonstrating long-term effects resulting from short-term exposure to environmental chemicals and other external factors by the means of epigenetic inheritance (Feil and Fraga 2012; Ladd-Acosta 2015; Schmidl et al. 2018). Peri-conceptional and intrauterine exposures occurring in regions resistant to epigenetic reprogramming can be inherited, confirming previous claims that not all epigenetic marks were completely erased and reapplied, i.e. epigenetically reprogrammed (Dolinoy et al. 2011; Wu et al. 2015). In agreement, epigenetic inheritance has been widely documented in several model species from diverse research fields, including human tissues and cells, different mammal species, plants, crustaceans and fishes (Jeremias et al. 2018a, Trijau et al. 2018; Perez and Lehner 2019).

There is a growing awareness that many examples of transgenerational epigenetic effects reported in the literature are rather the result of direct environmental exposure and thus should more accurately be described as multigenerational epigenetic effects (Skinner 2011a; Heard and Martienssen 2014). This is because when F0 male organisms are exposed to environmental cues, their gametes (that will originate the F1 generation) are also exposed to that environmental factor; thus, the first truly unexposed generation, where epigenetic inheritance can be detected, is the F2 generation (Skinner 2008; Heard and Martienssen 2014). The detection of true epigenetic inheritance is even more challenging in females because of the potential of pregnancy interfering with the detection of transgenerational effects (Kovalchuk 2012; Heard and Martienssen 2014). More precisely, in the case of an environmentally exposed pregnant female (F0), there is the potential for the exposure of the progeny (future F1) and the germ line of the progeny (future F2) (Skinner 2008; Kovalchuk 2012). In such cases, true epigenetic inheritance can only be isolated in the F3 generation and only when deliberate exposure occurs during F1, F2 and F3 lifespan (Kovalchuk 2012). Although monitoring successive non-exposed generations is of primary importance for the detection of true epigenetic inheritance, defining a non-exposed generation can sometimes be challenging, especially in cases where organisms show external fertilization and/or internal embryo development (Bell and Stein 2017; Shaw et al. 2017). In fact, much work remains to be done

towards a more detailed understanding of the mechanistic aspects of reversibility and inheritance of epigenetic marks, as well as towards confirming the prevalence of epigenetic inheritance processes in natural populations (Grossniklaus et al. 2013; Shaw et al. 2017; Jeremias et al. 2020). Furthermore, most studies exploring transgenerational effects have focused exclusively on DNA methylation, which is by far the best studied epigenetic mechanism; yet, the potential role of histone modifications and non-coding RNAs in epigenetic inheritance has been widely postulated, and sometimes demonstrated in experimental studies; thus, this issue is very important and a promising avenue to be explored in future studies (Skvortsova et al. 2018; Perez and Lehner 2019).

As previously discussed, the epigenomes of germ cells and early embryos are particularly susceptible to environmental cues (Laufer et al. 2017; Alvarado-Cruz et al. 2018). This highlights that epigenetic mechanisms allow for the stable regulation of gene expression and phenotypes at early ages, in ways that can be propagated over multiple cell divisions while also remaining flexible enough to respond to environmental stimuli (Bock 2009; Guerrero-Bosagna and Skinner 2012). This view has prompted the development of epigenetic biomarkers, which are promising tools to predict later-life health outcomes, especially from early life and in utero exposures (Bock 2009; Ladd-Acosta 2015; Ladd-Acosta et al. 2016; Leygo et al. 2017; Jeremias et al. 2020). Furthermore, epigenetic inheritance can be seen as an inheritent and robust biological mechanism by which cells remember previous environmental exposures. Therefore, epigenetic biomarkers can potentially also inform retrospectively on organismal lifetime exposure and parental exposures that are carried through the germ line (Mirbahai and Chipman 2014; Nilsson et al. 2018; Jeremias et al. 2020).

There are a growing number of studies demonstrating long-term effects resulting from exposure to environmental chemicals and other external factors by means of epigenetic inheritance (Feil and Fraga 2012; Ladd-Acosta 2015; Schmidl et al. 2018). Importantly, while the inheritance of epigenetic marks established during developmental stages can determine transgenerational disease, abnormal physiology and other negative effects, such processes also offer a window of opportunity enabling organisms to dynamically fit for differentiation and other developmental transitions. This could ultimately allow species to better adapt to their environments through both short- and long-term responses (Gicquel et al. 2008; Skinner 2016; Nilsson et al. 2018). More precisely, epigenetic inheritance can play an important evolutionary role by enabling the selection of fitter phenotypes, thus supporting better strategies to cope with environmental perturbation over evolutionary time scales (Jablonka and Raz 2009; Jeremias et al. 2018b; Nilsson et al. 2018).

2.4 Evolutionary and Adaptive Implications of Epigenetics

2.4.1 Extending the Theory of Evolution?

The surge of epigenetics as a distinct discipline of Biology has been accompanied by a raise in debatable, but important questions concerning both theoretical and practical aspects of evolution. More precisely, epigenetic phenomena may pose challenges to the widely accepted gene-centred neo-Darwinian version of Darwinism, which postulates that evolution acts mainly via natural selection of phenotypes originating from genetic mutations and other forms of genetic variability (Jablonka and Lamb 2002; Laland et al. 2015). Accordingly, this theory states that the evolutionary process is based on the progressive accumulation of fixated genetic (then phenotypic) differences through time, which shape the evolution of species (Laland et al. 2014, 2015). However, such molecular underlying basis of evolutionary processes does not accurately support some cases of rapid adaptation commonly reported in natural populations (Jablonka and Lamb 2007; Avise and Ayala 2009). In this regard, epigenetic research provides us the notion that both epigenetic marks and phenotypes can arise without genetic variability and that non-DNA variations can be transmitted across generations, i.e. inherited (see Sect. 2.3), both ideas being disruptive concepts for traditional evolutionary thinking (Jablonka and Lamb 1999; Manjrekar 2017; Stajic and Jansen 2021).

Indeed, nowadays it may seem obsolete to have a definition of evolution limited to changing DNA sequences and allele frequencies over time. Many scientists and evolutionary thinkers have been suggesting that the evolutionary process would be more completely described by incorporating non-genetic molecular processes and by furthering the scope of inheritance beyond DNA sequence-based inheritance (Mendelian genetics), thereby accounting for epigenetic inheritance (Jablonka and Lamb 2002; Laland et al. 2014; Manjrekar 2017; Banta and Richards 2018). As an example, the so-called "Extended Evolutionary Synthesis" is a theory that suggests an increase in the boundaries of the Modern Synthesis, by bringing together missing themes of developmental bias, phenotypic plasticity, niche construction and extragenomic inheritance, thereby representing the effort of bringing epigenetic concepts into evolutionary frameworks (Jablonka and Lamb 2007; Pigliucci and Muller 2010; Laland et al. 2014). Importantly, such a proposed theory does not refuse the concepts comprised in the Modern Synthesis, but rather combines them with epigenetic phenomena and others—see, e.g. Jablonka and Lamb (2007) and Pigliucci and Muller (2010) for a more comprehensive view on this issue. It is also important to remark that definitive conclusions regarding the evolutionary role of epigenetic mechanisms and inheritance require far more supporting evidence, especially evidence collected outside highly controlled laboratory settings—see, e.g. Laland et al. 2014 for an introduction on the need or not to revisit the processes considered fundamental for evolution.

2.4.2 *Epigenetic Phenotypes and Transgenerational Inheritance: Shaping Adaptive Strategies and Microevolution Patterns*

The life of all organisms is marked by the constant interaction with their surrounding environment. Throughout their lives, organisms can be exposed to a wide range of environmental cues, ranging from small fluctuations in external conditions to major environmental challenges (Bernhardt et al. 2020). At a certain threshold, an environmental perturbation will induce a battery of phenotypic responses by involving changes in functional traits (e.g. physiological and behavioural aspects), with the goal of allowing individuals to better cope with environmental challenges (Fusco and Minelli 2010; Wong and Candolin 2015; Bernhardt et al. 2020). In this regard, it has been shown that even temporary and low-level exposures to chemicals can affect the epigenome, which is generally described as being highly responsive to a wide range of external stimuli, especially at early stages of development (Skinner 2011b; Feil and Fraga 2012). Therefore, because individuals within natural populations typically experience environmental cues at the same time and to the same extent, there is possibility that the same epigenetic changes (and their corresponding phenotypes) may occur in different individuals of a given population (Feil and Fraga 2012; Burggren 2016).

While there is a lack of studies focusing on the occurrence of epigenetically mediated phenotypes in populations under natural conditions, some exceptions exist and seemingly confirm the rationale that epigenetic phenotypes are involved in the determination of different life-history traits in populations subjected to different evolutionary constrains (Richards 2008, 2011; Guillette et al. 2016; Angers et al. 2020). Moreover, similar aspects can be found between epigenetically and genetically determined phenotypes, with the most important being that both types can be "perceived" in the same way by natural selection, since they can be advantageous, disadvantageous or neutral (Burggren 2016; Banta and Richards 2018). Epigenetically mediated phenotypes likely arise faster, and perhaps even more broadly, than genetically determined phenotypes in response to an environmental perturbation (Jablonka and Lamb 2002; Burggren 2016). This contrast is determined by the underlying differences in the genetic and the epigenetic machinery, since epigenetic mechanisms are reversible and generally more dynamic than DNA sequences. It is worth remarking at this stage that components of the epigenetic machinery are themselves coded within the genome, which adds another dimension to the appraisal of the interplay between genetics and epigenetics in constraining phenotype outcomes and evolutionary effects—e.g. genetic mutations in components of the epigenetic machinery can have important downstream phenotype consequences, namely the class of diseases known as Mendelian disorders of epigenetic machinery (Bjornsson 2015; Griffiths 2017). Still, it is likely that epigenetic mechanisms act as faster sources of variation towards adaptation (Jablonka and Lamb 1999; Vogt 2017; Banta and Richards 2018). Particularly in cases of complex environmental conditions, microevolutionary processes towards local adaptation may be the key

mechanisms on which individuals and populations rely to prevent fitness loss (Peñuelas et al. 2013; Fasola et al. 2015).

The genetic machinery of cells is very stable, and it is this stability that settles the basis for life. The majority of environmental factors and exposures do not modify DNA sequences, and most natural genetic variations and many new experimentally induced mutations are not inherited; thus, there is no induction of evolutionary effects (Jablonka and Lamb 2002; Skinner et al. 2010; Skinner 2011a). On the other hand, the epigenetic information acquired in response to the environment can potentially be inherited; thus, epigenetic phenotypes can also impact the overall fitness of organisms over multiple generations (Jablonka and Lamb 1999; Burggren 2016; Jeremias et al. 2018b). Thereby, in the cases where epigenetically mediated phenotypes confer evolutionary advantages, organisms and populations can increase their adaptive capacity over evolutionary time scales at a much faster pace than in cases where adaptation is driven by genetic mutations (Jablonka and Lamb 2002; Burggren 2016).

Epigenetic variation is a theme of primary importance for understanding the existence of phenotypic diversity in natural populations and under laboratory settings (Vogt 2015, 2017; Baerwald et al. 2016). Many sources of epigenetic variation exist, including both stochastic and environmentally induced epigenetic changes. Firstly, methylation in the CpG context is an important determinant of proximal natural genetic variation, with methylated cytosines presenting higher rates of base mutation than unmethylated ones (Qu et al. 2012; Glastad et al. 2016). Interestingly, the analysis of methylation data across deep phylogenies revealed that such marks were largely conserved, while genomic regions showing DNA methylation divergence also exist, being mainly enriched for developmental and tissue specialization (Hernando-Herraez et al. 2013; Mendizabal et al. 2014). In particular, methylation divergence has been associated with gene expression and functional divergence under the influence of evolutionary constrains, thus making a point for the important evolutionary role of DNA methylation (Keller and Yi 2014; Mendizabal et al. 2014). Secondly, non-coding RNAs have no consistent conservation levels: some non-coding RNAs experienced sequence conservation, and others experienced rapid sequence evolution. Regardless of these patterns, non-coding RNAs potentially have important functional roles, suggesting that different non-coding RNAs respond differentially to evolutionary constraints (Pang et al. 2006; Mercer et al. 2009). In agreement, it has been suggested that non-coding RNAs are major sources of epigenetic variability and that such mechanisms may be at the centre of adaptation to environmental challenges (Repoila et al. 2003; Pang et al. 2006). Thirdly, histones are deeply associated with numerous genomic features, such as transposable elements and transcribed genes, and their importance as sources of epigenetic variability at specific loci has been increasingly demonstrated (Richards 2008; Duncan et al. 2014). More evidence in this context highlights the importance of epigenetic drifts, stochastic epimutations and epigenetic polymorphisms as sources of epigenetic variability, indicating that these mechanisms can sometimes act together to promote a better response to environmental perturbation (Keller et al. 2016a; Leung et al. 2016; Vogt 2017).

While further study is required to clarify the connection between different epigenetic processes and resulting epigenetic variability, it is clear that phenotypic variation arises from a combination of genomic composition, environmental input and epigenetic variability (either stochastic or environmentally induced). Thus, once epigenetically mediated phenotypic variation can be targeted by natural selection towards increasing fitness, the involvement of epigenetic mechanisms in the evolutionary process can be argued (Leung et al. 2016; Cavalli and Heard 2019). Because of this, it seems that evolutionary processes would be more accurately described by adding the concept of alleles with the concept of epialleles, thereby comprising the variation in gene expression and phenotypes provided by both genetic and epigenetic machinery (Finnegan 2002; Kakutani 2002; Banta and Richards 2018). This epialleles represent genomic regions at which epigenetic states vary between individuals, e.g. organisms of a given population or genetically identical organisms, such as twins (Finer et al. 2010; Dominguez-Salas et al. 2014). Different epigenetic modifications can contribute and act together towards this variability, possibly enlarging the boundaries of plasticity with which a given genotype is translated, and therefore influencing phenotypes (Zilberman et al. 2007; Finer et al. 2010). Interestingly, the widespread occurrence and transgenerational stability of epialleles outside laboratory settings is becoming increasingly recognized, with the best well-documented natural epialleles being spontaneous variants emerging in plant populations and agricultural fields, although epialleles transgenerationally inherited also exist in humans and other animals (Bertozzi and Ferguson-Smith 2020; Li et al. 2020). As Finer et al. (2010) highlighted, perhaps the most remarkable example in this context is the hypermethylated epiallele MLH1—a mismatch gene that is involved in the DNA repair of key genes associated with non-polyposis colon cancer heredity—that was originally identified by Suter et al. (2004) while studying individuals that lacked candidate gene mutations for the disease despite presenting a personal or family history of this cancer. However, descriptions of natural epialleles and their transgenerational inheritance are scarce and limited to very specific contexts; thus, their importance for evolutionary processes is very promising but largely unexplored (Finer et al. 2010; Weigel and Colot 2012).

Epigenetic variation is an important potential source of novel selectable traits, which are both common and enough to lead to evolutionary effects even over large temporal scales (Becker and Weigel 2012; Jeremias et al. 2018b). For many years, studies on the heritability of epigenetic variation were restricted to epidemiological studies and empirical observations based on experimental animal models. This is no longer the case, with more and more studies reporting that the inheritance of epigenetic marks is a stable, common and widespread mechanism in nature (Skinner 2011a; Guerrero-Bosagna and Skinner 2012). By this means, and for instance, a given epigenetically determined phenotype arising in response to a pollutant in an individual or group of individuals in a population can be inherited through successive generations, and consequently, advantageous phenotypes could be sustained by the force of microevolutionary mechanisms over large temporal scales (Jablonka and Lamb 2002; Jablonka and Raz 2009; Burggren 2016). Interestingly, this process allows for the explanation of some evolutionary cases that could not be explained by

DNA-based variability and inheritance, again showing that epigenetic inheritance extends the common view of Darwin's evolutionary theory (Jablonka and Lamb 1999; Pigliucci and Muller 2010). Indeed, while phenotypic adaptation driven by genetic change can positively adjust the fitness of populations, the selection of fitter phenotypes can be costly as it can reflect in a reduction of intra-population genetic variability or a trade-off with decreased tolerance to new stressors (Ribeiro and Lopes 2013; Merilä and Hendry 2014; Fasola et al. 2015). Consequently, epigenetic inheritance may better support the stabilization of phenotypes, as well as provide higher phenotypic variance at equilibrium due to different epigenetic states (Kilvitis et al. 2014; Kronholm and Collins 2016; Banta and Richards 2018).

Overall, epigenetic mechanisms seem to support both short- and long-term responses to environmental change. In fact, in cases of environmental fluctuations, the occurrence of environmentally induced epigenetic changes can determine changes in gene expression that allow for the broadening of physiological tolerance ranges (Kilvitis et al. 2014; Jeremias et al. 2018b). These epigenetic (and gene expression) modifications can be maintained throughout life because of the mitotic stability of epigenetic machinery, but there is the potential for germline persistence of epigenetic changes, especially if these occur during critical windows of development or if environmental pressures are sustained over the long term (Skinner 2011a, b; Jeremias et al. 2018b). By these means, epigenetically mediated phenotypes can allow organisms to increase their fitness, and because such traits can be selected, organisms can fine-tune their responses over evolutionary time scales (Artemov et al. 2017; Jeremias et al. 2018b). In agreement with this view, we previously compiled evidence on the role of epigenetic mechanisms in the adaptation of freshwater species to climate change (Jeremias et al. 2018b). In such ecosystems, adaptive strategies relying on phenotypic plasticity or genetic changes assume particular relevance in the response to environmental challenges because avoidance and escape are limited (Heino et al. 2009; Bush and Hoskins 2017; Jeremias et al. 2018b). Nevertheless, due to the fast pace of environmental transformation provoked by climate change, it seems that microevolution is the key process driving species resilience in the long term (Merilä and Hendry 2014; Merilä and Hoffmann 2016). In this particular context, epigenetic mechanisms seem critical to prevent extinction events by increasing adaptive capacities through the widening of plasticity ranges, but also because of microevolutionary adaptation mediated by epigenetically inherited phenotypes (Rey et al. 2016; Jeremias et al. 2018b). While this example highlights the evolutionary importance of epigenetics, several studies have been demonstrating that epigenetic differences can determine different genomic compositions, thereby showing that epigenetic inheritance may actually play a relevant role in both microevolutionary and macroevolutionary responses (Varriale and Bernardi 2006; Varriale 2014; Yi and Goodisman 2021). Future research will certainly contribute to further establish epigenetic inheritance as an issue of primary importance for clarifying theoretical and practical aspects of evolution.

2.5 Conclusion

Great advances have been made in the past few years towards demonstrating the importance of epigenetics for different systems and aspects of Biology. In this regard, the interplay between epigenetic and genetic machinery during development has been demonstrated. However, unlike genetic machinery, epigenetic mechanisms are highly responsive to environmental factors, and critical windows of susceptibility exist during early stages of development. Despite the existence of epigenetic reprogramming processes, epigenetic marks and patterns can persist in the germline. Therefore, epigenetically mediated phenotypes can be inherited. While this inheritance can translate into deleterious effects affecting successive generations, it can also facilitate the development of adaptive strategies and contribute to increase the overall fitness of individuals, populations and species. Thus, epigenetic mechanisms can play an important role not only in short-term responses, but also over large temporal scales in evolutionary patterns when epigenetic inheritance is involved.

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Chapter 3
Epigenetics and Phenotypic Plasticity in Animals

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Contents

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Abstract Phenotypic plasticity sensu lato, the generation of different phenotypes from the same genome, is caused by developmental programmes, developmental stochasticity and environmental impacts. These triggers can evoke changes of DNA methylation and histone modification marks on the chromatin and of non-coding RNA pathways that regulate DNA expression, leading finally to the production of different phenotypes from the same DNA sequence. The power of epigenetic mechanisms in shaping of phenotypes is most impressively demonstrated by the structurally and functionally different cell types in the body of multicellular animals and the phenotypically very different life stages of holometabolous insects that are produced from the single DNA of the zygote. However, epigenetic mechanisms can also help generating substantial phenotypic variation in populations, as revealed by experiments with clonal animals. This phenotypic variation is caused by bed-hedging developmental stochasticity and directional environmental induction, which usually act together but in different weighing, depending on the environment. The generation of epigenetically mediated phenotypic plasticity is obviously effective in all animal populations, but is particularly important for clonal and genetically impoverished populations helping them to survive when the environmental conditions change. It also helps invasive groups, sessile taxa and populations in extreme habitats to adapt to their particularly challenging environments. Epigenetic mechanisms are evolutionarily relevant as well. They were shown to trigger trait alteration in early domestication and consolidate speciation by contributing to reproductive isolation, chromatin remodelling and alteration of gene expression. Some epigenetically mediated phenotypes can be inherited to the next generations, particularly if they provide advantages in changing or new environments. Under long-lasting favourable conditions, they may be genetically integrated, starting new evolutionary trajectories. Because epigenetic changes can either be the consequence of genetic changes or trigger genetic changes, depending on context, they can be both followers and leaders in animal evolution.

Keywords Development · Domestication · Environmental adaptation · Epigenetic variation · Evolution · Phenotypic plasticity · Speciation

3.1 Introduction

How genotypes map to phenotypes is one of the most fundamental questions in biology. Previous research has focussed on the contribution of genetic and environmental variation to shaping of the phenotype (nature versus nurture debate) (Moore 2001), but studies with clonal organisms have revealed a third mechanism that can influence phenotypic outcomes in the absence of genetic or environmental heterogeneity, namely developmental stochasticity (Gärtner 1990; Vogt 2015a). This third component adds a flavour of indeterminism to the genetic and environmental determinism of the phenotype. There is increasing evidence that both stochastic developmental and environmentally induced phenotypic variation are mediated by epigenetic mechanisms (Leung et al. 2016; Vogt 2021).

This chapter examines the relationship between epigenetics and phenotypic plasticity sensu lato in animals and the relevance of epigenetically mediated phenotypic variation for development, ecology and evolution. Molecular biologists usually restrict the term epigenetics to stable, mitotically and sometimes meiotically inheritable alterations of gene expression that do not alter the DNA sequence (Gibney and Nolan 2010). These changes in gene expression are caused by epigenetic mechanisms like DNA methylation, histone modifications and non-coding RNA pathways that are responsive to environmental cues (Jaenisch and Bird 2003; Lennartsson and Ekwall 2009; Moutinho and Esteller 2017). Developmental and evolutionary biologists working with multicellular organisms additionally consider higher-level epigenetic processes that can lead to the variable expression of phenotypic traits from the same genome, e.g. chemical and mechanical cell-to-cell interactions, self-organization of tissues and morphogenic diffusion–reaction systems. These higher-level epigenetic mechanisms are not considered in this chapter. Readers interested in this field are referred to Nijhout (1990), Kelsh et al. (2008), Hallgrímsson and Hall (2011) and Landge et al. (2020).

The term phenotypic plasticity is here used in a common and broader sense covering all the phenotypic variation that can be generated from the same genome including cell-type heterogeneity in an animal's body, fluctuating asymmetry between body sides, polyphenism (morphologically and behaviourally distinct life stages and castes of insects) and non-genetic phenotypic diversity in populations (Fig. 3.1). A narrower definition of phenotypic plasticity often used in literature (here called phenotypic plasticity sensu stricto) is the production of different phenotypes from the same genotype by influences of the external environment (DeWitt and Scheiner 2004; Fusco and Minelli 2010). Phenotypes can be morphological, physiological, biochemical, behavioural and life history related (growth, reproduction and life span).

The chapter starts with a description of the molecular epigenetic mechanisms that can produce different phenotypes from the same DNA sequence and outlines how these epigenetic mechanisms interact with the genome and the environment. The following sections deal with different phenomena of phenotypic plasticity sensu lato including the production of structurally and functionally different cell types from the

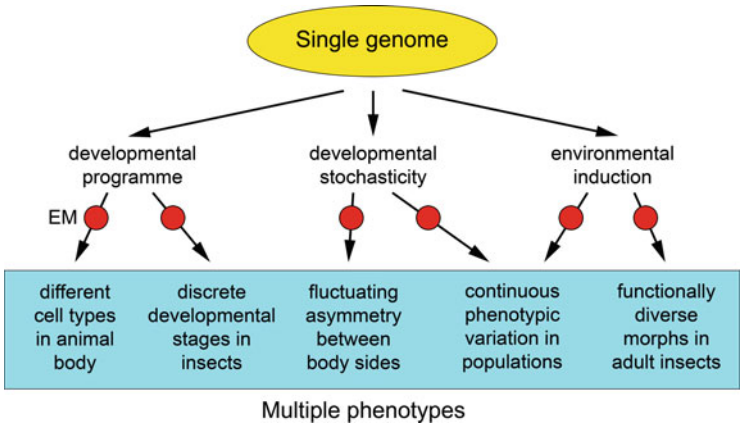

Fig. 3.1 Scheme showing the different manifestations of phenotypic plasticity sensu lato. Multiple phenotypes can be produced from the same DNA sequence by developmental programmes, developmental stochasticity (e.g. random epimutations) and environmental induction. These routes to phenotypic diversity are mediated by epigenetic mechanisms (EM, dots) (original illustration by author)

single genome of the zygote, the differences between body sides in bilaterally symmetrical animals, the generation of discrete alternative phenotypes from the same genome by developmental programmes or environmental cues and the generation of a continuum of phenotypes from the same genome in populations by developmental stochasticity and environmental induction. Thereafter, the role of epigenetically mediated phenotypic variation (non-genetic phenotypic variation) in ecology and evolution is discussed. The models used for explanation and illustration cover asexually reproducing and highly inbred animals, invaders, sessile taxa, troglobionts, domesticated animals and polyploid species. Whenever possible, I correlate phenotypic variation with the variation of particular epigenetic states. The chapter ends with a discussion on genome–epigenome–phenotype relationships and future research needs in the field of epigenetics and phenotypic plasticity.

In contrast to other work on the relationship of epigenetics and phenotypic plasticity, I here focus on the production of different phenotypes from a single genome (DNA sequence and identical copies). This approach allows recognition of the role of epigenetics in shaping of phenotypes more precisely than in sexually reproducing, genetically diverse experimental systems, where the influences of DNA sequence variation and epigenetic variation on phenotype are difficult to disentangle. By focussing on asexually reproducing and otherwise clonal animals, epigenotypes are identified as the first step to producing phenotypic diversity from a single genome.

3.2 Relationships between Genome, Environment, Epigenetic Mechanisms and Phenotype

There is no doubt that a single DNA sequence can produce more than one biochemical, morphological, behavioural or life history phenotype. These phenotypes arise by different expression of the DNA as the result of complex interactions between the genome and environmental factors. Gene expression is regulated at the level of transcription, translation and further downstream processes. Transcription is highly complicated and includes transcription factors, enhancers, silencers and numerous further supporting and regulating proteins and non-coding RNAs. Epigenetic mechanisms such as DNA methylation, histone modifications and non-coding RNAs modify the accessibility of the DNA and chromatin for sequence reading and interpreting molecules and help in processing of the transcripts. Epigenetic marks on the DNA and chromatin can change stochastically and in response to environmental cues. Alternative splicing and mRNA editing can also generate different phenotypes from a single gene. Through these processes, different information can be read out of the same DNA sequence and be used for the production of different phenotypes.

3.2.1 Epigenetic Mechanisms Involved in the Production of Phenotypic Plasticity

The best investigated epigenetic mechanisms that can trigger phenotypic plasticity are DNA methylation, histone modifications and non-coding RNA pathways (ncRNAs) (Fig. 3.2) (Jaenisch and Bird 2003; Lennartsson and Ekwall 2009; Moutinho and Esteller 2017). Phenotypic variation unrelated to variation of the DNA sequence can additionally be caused by less well-known chemical modifications of the mRNA and RNA editing (Coutinho Carneiro and Lyko 2020; Zhao et al. 2020). An example is the deamination of adenosine to inosine by the ADAR (adenosine deaminase acting on RNA) enzyme family (Fig. 3.2), which can lead to codon change and diversification of the proteome and phenome (Eisenberg and Levanon 2018).

DNA methylation occurs in most animals but has been evolutionarily lost in some species including the genetics models *Caenorhabditis elegans* (nematode) and *Drosophila melanogaster* (fruit fly) (Raddatz et al. 2013; Provataris et al. 2018; Vogt 2022a). There is no consistent correlation between the global DNA methylation level and evolutionary level or genome size as previously assumed (Vogt 2022a). The methylation marks are mostly on the cytosines of CpG dinucleotides (Fig. 3.2) and occur in promoters, gene bodies and repeats (Jaenisch and Bird 2003; Jones 2012; Schübeler 2015). Methylation of promoters, transposons and repeats usually results in transcriptional repression (Schübeler 2015). Gene body methylation modulates gene expression and seems to reduce transcriptional noise (Neri et al.

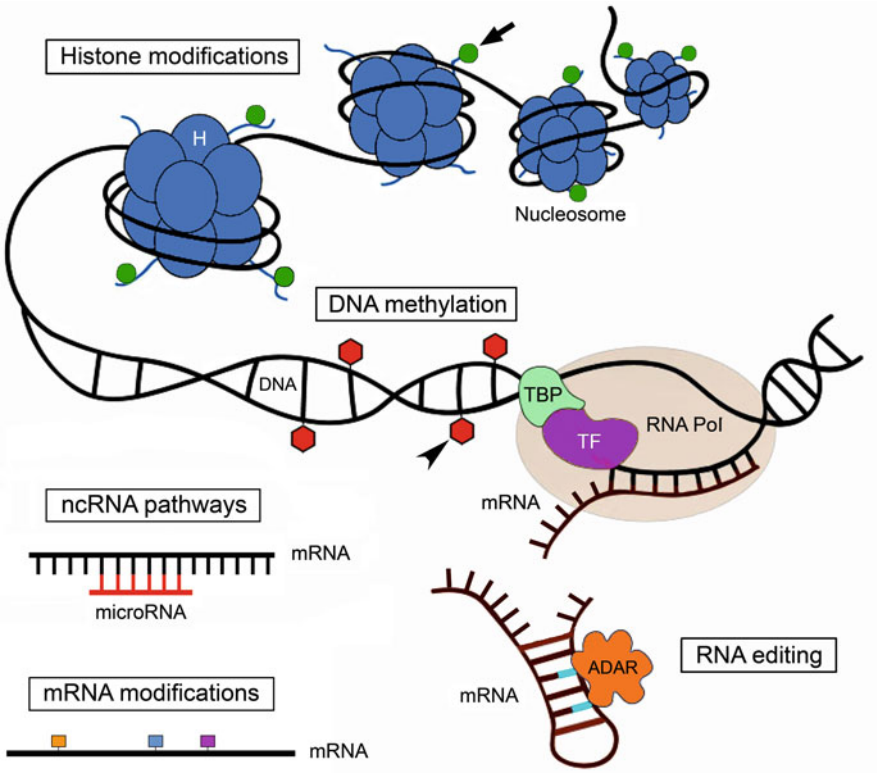

Fig. 3.2 Molecular epigenetic mechanisms involved in the generation of multiple phenotypes from a single DNA sequence. *Histone modifications:* The amino-terminal tails of the histones (H) that constitute the nucleosomes can be reversibly acetylated, methylated, phosphorylated and ubiquitinated (circles, arrow). These modifications affect chromatin structure and fine-tune the accessibility of the transcription machinery to the DNA. *DNA methylation:* DNA methylation refers to the addition of a methyl group to cytosines (hexagons, arrowhead) in CpG dinucleotides. Depending on the site of methylation in promoters, gene bodies or repeats, this mechanism can switch genes on and off, fine-tune their expression and repress transposons. *Non-coding RNA pathways exemplified by microRNAs:* miRNAs can help in regulating gene expression, e.g. by complementary base-pairing to mRNA leading finally to mRNA degradation. *mRNA modifications and mRNA editing:* Chemical base modifications of the mRNA (squares) can affect splicing, modify the speed of translation and induce codon change. Editing of mRNA by ADAR changes adenosine to inosine (light bars), which pairs with cytosine instead of thymidine diversifying the transcriptomic profile by codon change. *TBP* TATA box binding protein; *TF* transcription factor; *RNA Pol* RNA polymerase II complex (based on Coutinho Carneiro and Lyko 2020, Creative Commons Attribution License, http://creativecommons.org/licenses/by/4.0/)

2017). The DNA methylation marks are established by DNA methyltransferases (DNMTs) and erased by ten-eleven translocation enzymes (TETs) (Law and Jacobsen 2010; Wu and Zhang 2017; Lyko 2018).

The histones in the nucleosomes greatly influence DNA transcription by either shielding the DNA or allowing binding of transcription factors to the DNA. The

N-terminal tails of the histones carry post-translational modifications like methylation, acetylation, phosphorylation or ubiquitination (Fig. 3.2), which affect the chromatin structure. Histone acetylation often stimulates gene expression, whereas histone methylation often represses gene expression, depending on their location (Bannister and Kouzarides 2011; Allis and Jenuwein 2016). The chemical modifications on the histones are dynamically regulated by enzymes (Marmorstein and Trievel 2009; Morgan and Shilatifard 2020).

Small to long ncRNAs are further regulators of gene expression and contribute to the production of phenotypic variation (Frias-Laserre and Villagra 2017; Long et al. 2017). For example, microRNAs (miRNAs) can inhibit translation or cause mRNA degradation (Fig. 3.2) (Moutinho and Esteller 2017; O'Brien et al. 2018). Small interfering RNAs (siRNAs) can regulate gene transcription through transposable element silencing and the interaction with DNA methylation and histone modifications (Holoch and Moazed 2015). Long ncRNAs (lncRNAs) are involved in transcriptional regulation, dosage compensation and genomic imprinting (Li et al. 2019).

Polycomb group (PcG) and trithorax group (TrxG) proteins contribute significantly to the mitotic and meiotic inheritance of epigenetically mediated phenotypic variability by sustaining silent and active gene expression states, respectively (Steffen and Ringrose 2014). This epigenetic memory maintains gene expression states through cell generations or the germline without a change in DNA sequence and in the absence of the initiating signals. PcG and TrxG proteins are important for the long-term stability of gene expression and increase the robustness of gene regulatory networks, e.g. in different tissues. Ciabrelli et al. (2017) demonstrated experimentally how nuclear organization and PcG proteins can contribute to epigenetically inheritable phenotypic plasticity. They established stable and isogenic *Drosophila* lines that carried alternative epialleles defined by differential levels of polycomb-dependent histone modifications (H3K27me3). After being established, epialleles were dominantly transmitted to naive flies where they induced paramutations. These epilines could be reset to the naive state by disruption of chromatin interactions.

Alternative splicing of the mRNA is another means to generate phenotypic plasticity from the same DNA sequence. At first glance, alternative splicing seems to be a purely genetic mechanism, but epigenetic mechanisms can be crucially involved in this process as discussed in Zhang et al. (2020). For example, CpG methylation and histone modifications can mark an alternative exon, and these marks are then recognized by an adaptor protein that recruits splicing factors to promote the retention of the alternative exon. Another possibility is the regulation of the activity of splicing factors by ncRNAs (Zhang et al. 2020).

Most experiments on the role of epigenetics in mediating phenotypic plasticity focussed on single epigenetic mechanism like DNA methylation or histone modifications. However, in the real world, the different epigenetic mechanisms usually crosstalk and act together. An example is given by Loaeza-Loaeza et al. (2020) for a repressive epigenetic landscape.

3.2.2 *Epigenetically Mediated Variation of Gene Expression in Response to Environmental Signals*

Many studies have linked environmental exposures in plants and animals to alterations of gene expression and epigenetic modifications (Cavalli and Heard 2019). Expression changes in individual genes can modify gene networks resulting in phenotypic changes. The DNA contains all information that is necessary to produce different variants of a given phenotype: the genes encoding the proteins that finally make up the phenotype, the genes for the proteins that perceive and transmit environmental signals to the DNA, the enzymes and nucleic acids of the epigenetic mechanisms that are involved in regulation of the chromatin state and gene expression, and the CpG dinucleotides as main targets of the DNA methylation machinery. The environmental cues determine which of the possible phenotypes encoded in the DNA are to be produced, and the epigenome is a crucial interface between the genome and environment.

Understanding the genome–environment interaction requires answering of the following questions: How are the environmental signals conveyed to the nucleus of the target cells? Which molecules of the chromatin remodelling and gene expression machinery are sensitive to environmental signals? Who are the readers, writers and erasers of the epigenetic marks that are changed in response to environmental signals? Which molecules can read DNA sequences to target the readers, writers and erasers of epigenetic marks to the correct place?

Environmental cues can have direct effects on the target cells, e.g. fatty acids from the food, but mostly they are perceived by sense organs, translated into neurohormonal signals and conveyed to the target cells. The hormonal signals then elicit cellular signals that finally regulate target molecules in the nucleus involved in chromatin remodelling, gene expression and processing of the transcripts. Serotonin is a good example of a signal-transmitting hormone. In migratory locusts, which display density-dependent stationary and migratory phases (polyphenism), it regulates expression of density-responsive genes and the involved epigenetic mechanisms (Ernst et al. 2015; Foquet et al. 2021).

Many of the proteins and enzymes involved in chromatin architecture and gene expression are apparently sensitive to environmental and metabolic agents and can serve as mediators between environment and genome (Turner 2009). The demethylation enzyme TET1, which can interact with transcription factors and histone-modifying enzymes to regulate gene expression, is a good example. Zhu et al. (2020) listed a wide range of environmental factors that upregulate or downregulate TET1 in mammals including some food ingredients, ethanol, air pollution and radiation. Another environment-sensitive protein is the polycomb protein PRC2 that is involved in temperature-controlled sex determination in red-eared slider turtle, *Trachemys scripta elegans* (Ge et al. 2018). The temperature-sensitivity of polycomb proteins is supported by Voigt and Kost (2021) who found that genes regulated by the polycomb group in *Drosophila melanogaster* vary in their transcriptional output in response to changes in temperature. Other examples of

environment-sensitive proteins are transcription factors of the TCP family in plants that mediate environmental signals into growth responses (Danisman 2016).

The writers and erasers of the epigenetic code include the DNMTs and TETs and the histone-modifying enzymes (Wu and Zhang 2017; Lyko 2018; Morgan and Shilatifard 2020). Ravichandran et al. (2018) reviewed how DNMTs and TETs are recruited to specific genomic loci and how they interact with the chromatin to methylate and demethylate the DNA. DNMTs bind specifically to CpG sites but prefer specific flanking sequences over others. Proteins of the methyl-CpG-binding domain family (MBDs) are primary candidates for the readout of DNA methylation as they recruit methylases, histone deacetylases and other chromatin remodellers to methylated DNA associated with gene repression (Du et al. 2015). Most MBDs bind to methylated CpGs, but some MBD proteins also bind unmethylated DNA in active regulatory regions. Histone acetylation marks are mainly written by histone acetyltransferases (HATs) and read by bromodomain-containing proteins (BrDs) (Marmorstein and Zhou 2014).

Aside of chromatin and chromatin-modifying enzymes, transcription factors (TFs) are key components in the complex network through which the genome interacts with the environment (Thorne et al. 2009). Each animal possesses hundreds of TFs that bind to specific DNA sequences. Modification of the histones influences packaging and accessibility of the promoter DNA and can help guide TFs to their specific binding sites. The enzymes that put such modifications in place are dependent on metabolic components (e.g. acetyl CoA, S-adenosyl methionine) and susceptible to inhibition or activation by environmental factors. An example of the crosstalk of TFs with DNA sequences, DNA methylation marks and histone binding proteins is given by Huang et al. (2018).

Previously, it was thought that TFs bind only to unmethylated promoter regions of genes, whereas methylation of the binding sites prohibits transcription (Wang et al. 2018). However, many TFs bind to both methylated and unmethylated DNA suggesting that DNA methylation alters the binding specificity and intensity. Kribelbauer et al. (2020) demonstrated how the effect of CpG methylation on DNA groove geometry can influence DNA binding by TFs. Apparently, epigenetic modifications affect TF binding in a highly context-specific manner, with a direction and effect size that depend critically on their position within the TF binding site and the amino acid sequence of the TF.

Figure 3.3 illustrates how the interaction of the environment with the DNA, TFs, and readers and writers of epigenetic marks could yield different variants of a phenotypic trait from the same DNA sequence.

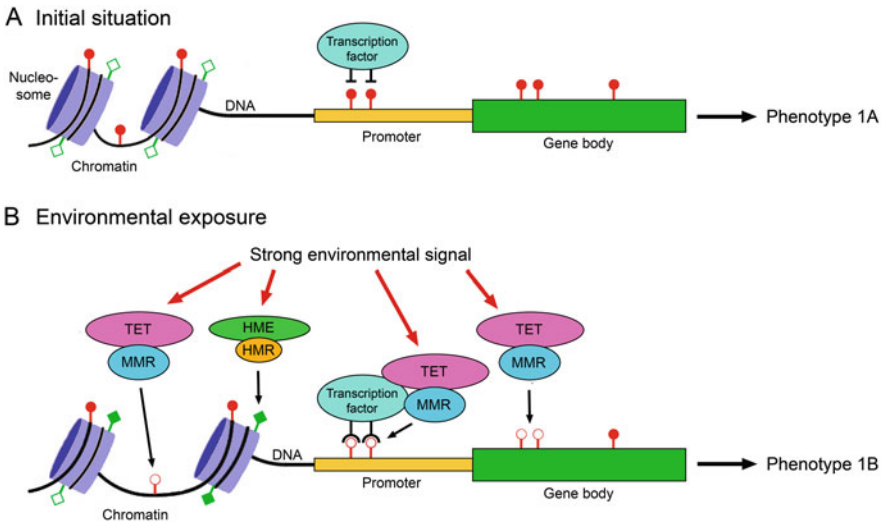

Fig. 3.3 Simplified scheme on the interaction of genome, epigenetic mechanisms and environment in producing phenotypic plasticity. (**a**) Section of chromatin of unexposed specimen marked by unacetylated histone tails (open rectangles) and DNA marked by methylated CpGs (filled circles). These epigenetic modifications cause compaction of the chromatin, reduced binding of the transcription factor to the promoter region and low gene transcription resulting in variant A of phenotype 1. (**b**) Strong environmental signals in a new environment cause acetylation of the histone tails (filled rectangles) and demethylation of some CpGs of the DNA (open circles) resulting in opening of the chromatin and higher binding of the transcription factor. These events lead to enhancement of gene transcription and the production of variant B of phenotype 1. *TET* environment-sensitive DNA demethylating ten-eleven translocation enzyme; *MMR* methylation mark reader targeting TET to specific methylation marks; *HME* environment-sensitive histone-modifying enzyme; *HMR* histone modification reader targeting HME to specific moieties of the histones (**a** and **b** original illustrations by author)

3.3 Association of Epigenetic and Phenotypic Changes during Embryonic Development

Development in animals is either direct or indirect. Direct developers show rather continuous phenotypic alterations from the zygote to the adult, whereas indirect developers have larval stages with different morphologies, behaviours and ecologies interspersed between the embryonic and adult stages. Examples of direct developers are mammals and examples of indirect developers are holometabolous insects. In this section, I will exemplify the relationship of epigenetics and developmental phenotypes in direct developers using mouse (*Mus musculus*) and marbled crayfish (*Procambarus virginalis*) as representatives of vertebrates and arthropods, respectively. Indirect developers that change phenotypes abruptly during development will be addressed in Sect. 3.4.3.

Mouse and marbled crayfish differ considerably in several aspects of embryonic development. Mouse embryos develop in the uterus of their mother and are not directly exposed to the external environmental conditions of the mother,

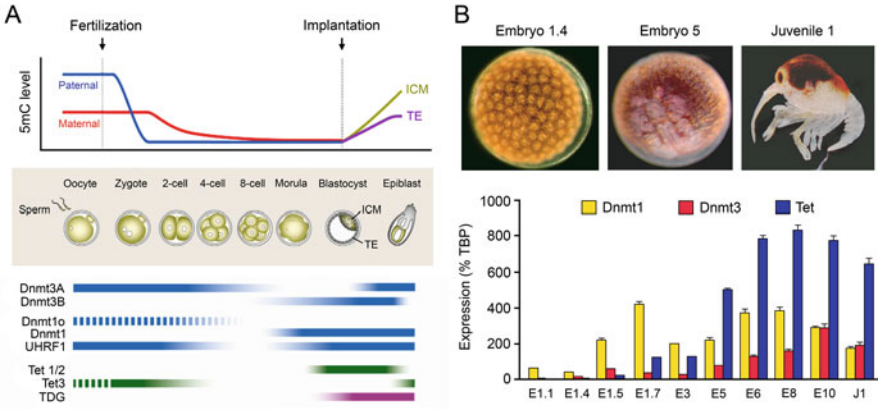

Fig. 3.4 Association of phenotypes and DNA methylation in embryonic development of mouse, *Mus musculus*, and marbled crayfish, *Procambarus virginalis*. (**a**) Dynamic changes in global cytosine methylation and expression of the DNA methylation and demethylation enzymes in early development of mouse. DNMT1o (oocyte-derived variant of DNMT1), DNMT1, UHRF1, DNMT3A and DNMT3B are methylation enzymes, and TET 1–3 and TDG are demethylation enzymes. The paternal 5-methylcytosine is more rapidly reduced after fertilization than the maternal 5mC. In the blastocyst stage, DNA methylation reaches a minimum. After implantation, the DNA methylation pattern is re-established, particularly in the inner cell mass (ICM) that develops into the mouse. The trophoectoderm (TE) becomes part of the placenta. (**b**) Phenotypic changes and dynamics of DNA methylation and demethylation enzymes during embryonic development of the parthenogenetic marbled crayfish. mRNA expression levels are given relative to TBP expression. The DNMT1, DNMT3 and TET genes show very low expression until the 128 nucleus stage (embryo 1.4) and quite different expression dynamics thereafter. Embryo 5 corresponds to about 50% of the duration of embryonic development, in which tissues are not yet discernable, and juvenile 1 is the hatching stage. Bars indicate standard deviation from three measurements (**a** based on Wu and Zhang 2014, with kind permission from Elsevier; **b** left and middle photograph from Vogt 2018b, with kind permission from Springer Nature; right photograph from Vogt and Tolley 2004, with kind permission from Wiley; graph based on Gatzmann et al. 2018, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/)

e.g. temperature. However, stress experienced by the mother can indirectly influence development of the embryo. Moreover, only a part of the blastocyst, namely the inner cell mass (Fig. 3.4a), develops into the mouse, while the outer trophectoderm cells contribute to formation of the placenta that transfers nutrients from the mother to the embryo. In the obligatory parthenogenetic marbled crayfish that develop from unfertilized eggs, dozens to hundreds of eggs are glued externally to the pleopods and brooded until juvenile stage 3, the first feeding stage. The embryo develops inside the egg shell (Fig. 3.4b), which is the only barrier to the external environment. Thus, they directly experience the same environmental conditions as their mother. Moreover, all embryonic cells are used to generate the crayfish body, and the nutrients required for development of the embryo come exclusively from the yolk that is deposited in the egg during oogenesis.

Global DNA methylation (5-methylcytosine per total cytosine) in the 2.6 Gb genome of adult mice is ~5%, depending on sex, tissue and condition (Nohara et al. 2011). Mice have one DNMT1, two DNMT3 and three TETs and some associated proteins like UHRF1 (ubiquitin-like plant homeodomain and RING finger domain 1) and TDG (thymine DNA glycosylase) that help in methylation and demethylation of the DNA, respectively. Dahlet et al. (2020) investigated the roles of DNA methylation enzymes by measuring the effects of genetic inactivation of DNMT1, DNMT3A and DNMT3B on the methylome and transcriptome. They found a strict division of function between DNMT1 that is responsible for maintenance methylation and DNMT3A and DNMT3B that serve for methylation acquisition in development. By analyzing severely hypomethylated embryos, they revealed that DNA methylation is used for repression of a panel of genes including imprinted genes, germline genes and lineage-committed genes. DNA methylation also suppressed multiple retrotransposons and illegitimate transcripts from cryptic promoters in transposons and gene bodies.

Embryonic development of mouse from the zygote to birth lasts 18–21 days. The DNA methylation marks are globally erased and re-established a first time in the zygote and the following pre-implantation stages (Fig. 3.4a) and a second time in the primordial germ cells (PGCs) (Seisenberger et al. 2012; Wu and Zhang 2014). The expression of the methylation and demethylation enzymes varies considerably during early embryonic development. After fertilization, DNA methylation of the sperm pronucleus in the zygote is actively reduced by TET3 (Fig. 3.4a). The DNA methylation marks in the maternal genome are passively lost over subsequent cell divisions because the oocyte-derived DNMT1o is largely excluded from the nucleus, and therefore, maintenance methylation is inefficient. The global DNA methylation level reaches a minimum around the blastocyst stage (32–140 cells) at day 4 of development. After implantation, DNA marks are re-established by DNMT3A and DNMT3B, particularly in the inner cell mass that develops into the mouse (Fig. 3.4a). Removal of the DNA methylation marks in the PGCs occurs at days 12–14 of embryonic development. They are re-established during further development of the PGCs to either sperm or oocytes restricting developmental potency.

The duration of embryonic development in parthenogenetic marbled crayfish is quite similar to mouse. Development from the unfertilized egg to hatching of the first juvenile stage lasts 17–26 days, depending on water temperature (Vogt et al. 2004; Seitz et al. 2005; Grimmer 2015). Based on morphological criteria, it was subdivided into 10 stages (Alwes and Scholtz 2006). Stage 1 that lasts from the spawned egg to the beginning of gastrulation was further divided into 8 substages named 1.1–1.8 (Grimmer 2015). Global DNA methylation of the 3.7 Gb genome is about 2.4% in adults (Vogt et al. 2015), corresponding to about 50% of the mouse value. The DNA is already well methylated from embryonic stage 5, the earliest stage where we could reliably determine global DNA methylation by mass spectrometry. The 5mC/total C ratio was 2.78% in this stage, which corresponds to about 50% of the duration of embryonic development, and 2.65% in embryonic stage 10. In 154-day-old juveniles and adults of about 2 years the values were 2.58% and 2.41%, respectively, suggesting that the global DNA methylation level declines slightly with age in this

indeterminately growing species. In contrast, in determinately growing mouse and humans, DNA methylation increases considerably with age (Fraga et al. 2005; Stubbs et al. 2017), e.g. from 2.5% in a 3-year-old child to 4.5% in a 50-year-old human.

Marbled crayfish have single copies of DNMT1, DNMT3 and TET (Gatzmann et al. 2018). The mRNA levels for these enzymes were generally low until the 124 nucleus stage (embryo 1.4 in Fig. 3.4b) (Gatzmann et al. 2018), in which cell membranes between the nuclei are still lacking. These are only established in the 256-cell stage. DNMT1 was strongly upregulated in embryonic stage 1.5 (256–512-cell stage), while DNMT3 expression increased continuously from this stage until stage 10. TET mRNA levels increased strongly during mid-embryogenesis and remained high until hatching (Fig. 3.4b) (Gatzmann et al. 2018). The expression levels and dynamics of the methylation and demethylation enzymes suggest that the DNA methylation pattern is intensely remodelled in the second half of embryonic development, the time in which the tissues and appendages are formed.

Histone modifications are also involved in regulation of embryonic development. Sarmento et al. (2004) studied changes in global levels of histone modifications in mouse during oocyte maturation and pre-implantation development using immunofluorescence and confocal microscopy. They revealed two strikingly distinct categories of histone modifications. The first category contained stable modifications including histone H3 lysine 9 methylation, histone H3 lysine 4 methylation and histone H4/H2A serine 1 phosphorylation. The second group contained dynamic and reversible marks including hyperacetylated histone H4, histone H3 arginine 17 methylation and histone H4 arginine 3 methylation.

Research with knock-out mice revealed that ncRNAs are further important regulators of animal embryogenesis (Beermann et al. 2016). For example, mice lacking miRNAs showed a depletion of multipotent stem cells and died at the eighth day of embryonic development. Pauli et al. (2011) emphasized that ncRNAs are involved in maintenance of pluripotency, patterning of body axes, specification and differentiation of cell types, and organogenesis. They control embryonic gene expression by several means, ranging from miRNA-induced degradation of mRNAs to lncRNA-mediated modification of chromatin. Isakova et al. (2020) investigated the role of ncRNAs in the development of 11 tissues in mouse and revealed that ~30% of the total ncRNA transcriptome is tissue-specific.

Li et al. (2019) reviewed the regulatory functions of ncRNAs in insect development including miRNAs, piwi-interacting RNAs (piRNAs), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs). piRNAs mainly silence transposable elements in the germline at the transcriptional and post-transcriptional levels (Senti and Brennecke 2010). The piRNAs in the cytoplasm of the oocyte can be considered as a maternal pool of the piRNA-induced silencing complex (piRISC) genome defence system that is inherited transgenerationally. miRNAs can silence target genes in insect cells through translation inhibition or mRNA decay by interfering with translation factors associated with the 5′-cap and 3′-tail structures of mRNA (Jonas and Izaurralde 2015). lncRNAs are involved in insect development, insecticide resistance and anti-viral defence (Wang et al. 2017a). In fruit fly

Drosophila melanogaster, lncRNAs were significantly upregulated in late embryonic and larval stages (Chen et al. 2016a).

3.4 Production of Discrete Phenotypes from the Same Genome with the Help of Epigenetic Mechanisms

Epigenetic mechanisms can either produce a limited number of discrete, alternative phenotypes or a continuous range of phenotypes from the same genome. Examples of the former alternative are presented in this section for the structurally and functionally different cell types in an animal's body, male and female phenotypes, morphologically and behaviourally different life stages of holometabolous insects, different castes of social insects and predator-induced defence structures in water fleas.

3.4.1 Different Cell Types in an Animal's Body

The numerous cell types in the body of multicellular animals all originate from a single cell, the zygote. Therefore, they contain the same DNA sequence, although they are morphologically and functionally highly diverse. An example of the ultrastructural and functional differences between isogenic cells is given in Fig. 3.5a-d for the shrimp *Penaeus monodon*. Measurement of the DNA methylation and demethylation enzymes DNMT1, DNMT3 and TET in a related decapod crustacean, the crayfish *Procambarus virginalis*, revealed differences between tissues and organs (Fig. 3.5e) (Gatzmann et al. 2018). DNMT1 showed the smallest difference between tissues, whereas DNMT3 was more variable, representing the most tissue-specific enzyme of the methylation machinery. TET expression was high in the haemocytes, brain, hepatopancreas and abdominal musculature, moderate in the heart and very low in the ovary.

In mouse and humans, DNA methylation has been identified as an important effector of tissue specificity (Lokk et al. 2014; Greenberg and Bourc'his 2019). Lokk et al. (2014) subjected 17 somatic tissues from four humans to functional genome analysis and identified a great number of tissue-specific, differently methylated regions (DMRs). Many of the genes carrying these DMRs had tissue-specific functions. Blake et al. (2020) performed a multi-tissue comparative study of gene expression and DNA methylation in primates using livers, kidneys, hearts and lungs from humans, chimpanzees and rhesus macaques. They found a high degree of conservation in gene expression levels when considering the same tissue across species. They also measured significant differences in DNA methylation between tissues (Fig. 3.5f) and identified tissue-specific DMRs. Zhang and Zhang (2011) reported that histone modification profiles also vary between human tissues and cells

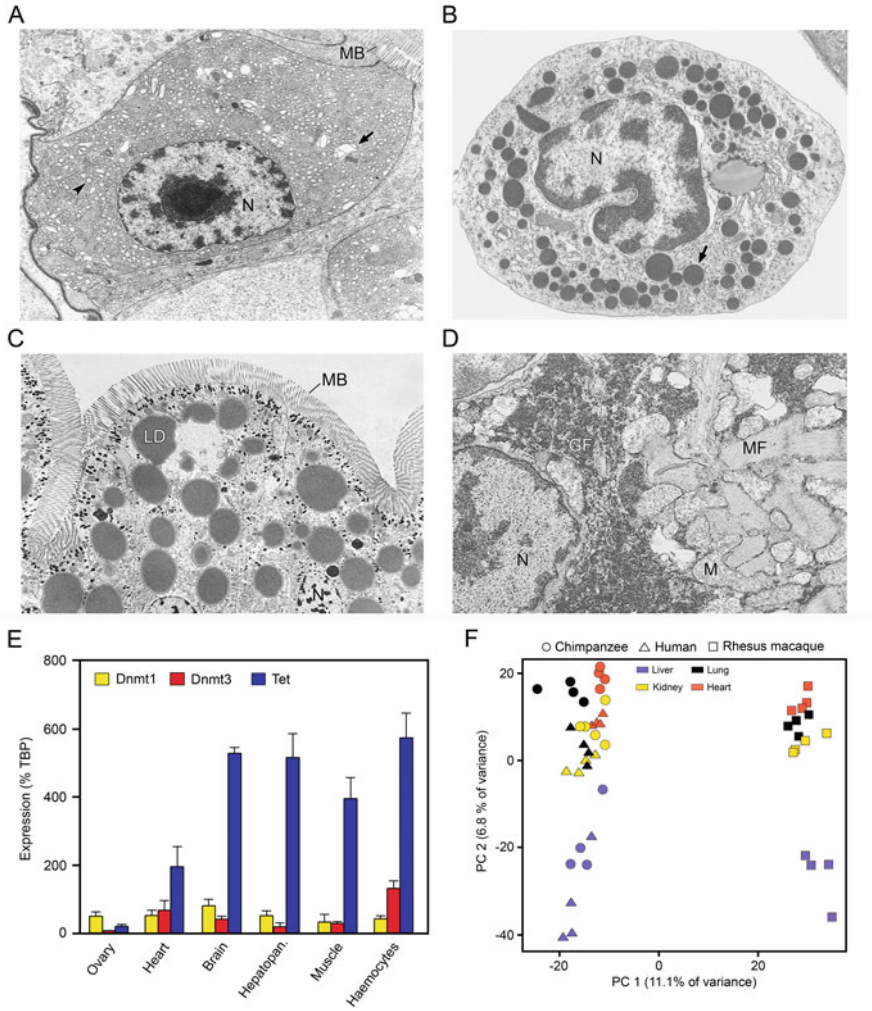

Fig. 3.5 Phenotypic and epigenetic differences between genetically identical cell types and organs. (**a**) Digestive enzyme synthesizing hepatopancreas cell of shrimp *Penaeus monodon* characterized by plenty of rough endoplasmic reticulum (arrowhead) and large Golgi bodies (arrow). N, nucleus; MB, microvillous border. (**b**) Haemocyte of shrimp characterized by numerous granules (arrow) containing components of the immune defence system. (**c**) Nutrient absorbing midgut cell of shrimp characterized by well-developed microvillous border and large lipid droplets (LD). (**d**) Contractile heart muscle cell of shrimp characterized by myofibrils (MF), glycogen fields (GF) and numerous mitochondria (M). (**e**) Different expression of methylation and demethylation enzymes in organs of crayfish *Procambarus virginalis*. mRNA expression levels are given relative to TBP expression. Bars indicate means ± SD from three measurements. (**f**) Principal components analysis (PCA) of average methylation levels in 47 tissue samples from 4 humans, *Homo sapiens*, 4 chimpanzees, *Pan troglodytes*, and 4 rhesus macaques, *Macaca mulatta*, showing conservation of tissue-specific DNA methylation in species (**a-c** from Vogt 2019b with kind permission from Wiley, **d** original from author; **e** based on Gatzmann et al. 2018, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/; **f** based on Blake et al. 2020, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/)

and considered them causative for cell-type-specific expression of protein-coding genes and miRNA genes.

3.4.2 Male and Female Phenotypes

Most animal species are sexually reproducing having phenotypically different males and females. Usually, both sexes are genetically different because they have different sex chromosomes or sex genes that pre-decide sexual fate at fertilization (genetic sex determination). However, in water fleas that reproduce by cyclic parthenogenesis, the regular alternation between sexual and asexual reproduction, sex is determined by environmental factors (environmental sex determination) (Vogt 2020a). In this case, males and females are genetically identical but phenotypically different.

Males and females of the water flea *Daphnia pulex* display large differences in morphology, metabolism, behaviour and lifespan despite genetic identity. In order to achieve a better understanding of the epigenetic factors that underlie the phenotypic differences between sexes, Kvist et al. (2020) investigated gene expression, DNA methylation and histone modifications in males and females raised in identical laboratory settings (Fig. 3.6a). The authors revealed that gene expression levels were positively correlated with DNA methylation and histone H3 trimethylation at lysine 4 (H3K4me3) in promoter regions. Conversely, gene expression was negatively correlated with elevated histone H3 trimethylation at lysine 27 (H3K27me3) distributed across the entire gene length. Epigenetic modifications that globally promote elevated gene expression were predominant in males, while epigenetic modifications that globally reduce gene expression were more frequent in females. These data demonstrate that there are vast epigenetic differences between males and females in *Daphnia pulex* despite genetic identity, which supposedly underpin the prominent morphological and life history differences between sexes.

In decapod crustaceans, the primary determinants of sex are genetic factors. The development of sexually dimorphic phenotypes is regulated by the insulin-like androgenic gland hormone (IAG) from the androgenic gland and the crustacean female sex hormone (CFSH) from the X-organ sinus gland system in the eyestalk ganglion (Toyota et al. 2021). The *IAG* gene is switched on in males and turned off in females, and the *CFSH* gene is switched on in females and turned off in males. Jiang et al. (2020) investigated the relationship between expression and silencing of the *CFSH* gene and DNA methylation in the mud crab *Scylla paramamosain*, which has a ZZ/ZW sex-determining system with the female being the heterogametic sex. The authors found gender-specific expression patterns as expected and variation of expression during vitellogenesis (Fig. 3.6b).

To explore the role of DNA methylation in *CFSH* expression in detail, the 5′-flanking region of the gene was cloned and a CpG island containing 12 CpG sites was identified by MethPrimer (Fig. 3.6c). Bisulphite sequencing and methylated DNA immunoprecipitation showed that CpG island methylation of the *CFSH* gene was significantly lower in the eyestalk ganglion of early vitellogenic females,

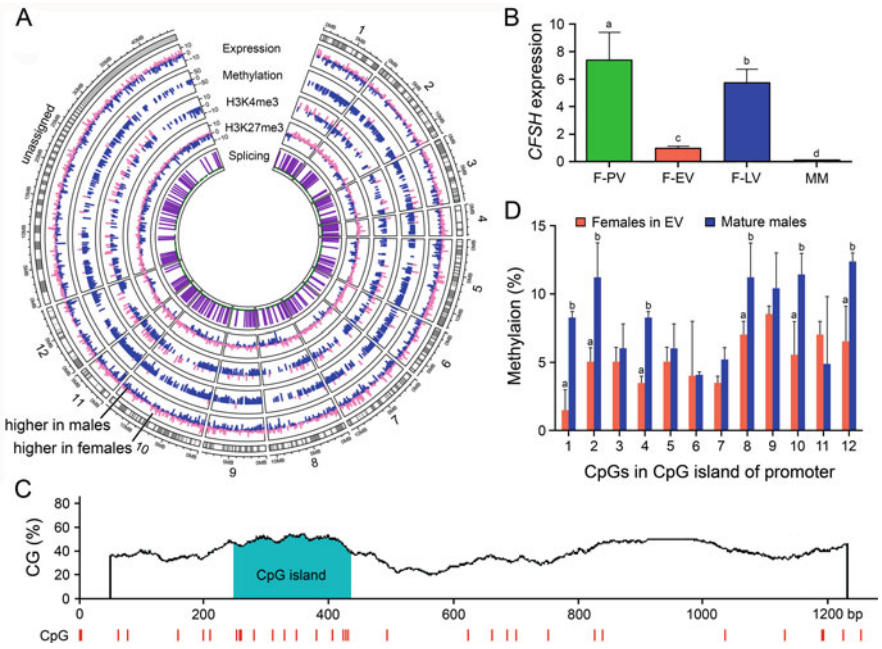

Fig. 3.6 Involvement of epigenetic mechanisms in the expression of sex-specific phenotypes in crustaceans. (**a**) Circos plot showing differences in multiple omics datasets between sexes in genetically identical *Daphnia pulex*, distributed across the genome. Inner bars in circles indicate higher values in males and outer bars indicate higher values in females. Numbers indicate scaffold assignment to chromosomes. There are marked differences between males and females in gene expression, DNA methylation, histone modifications and gene splicing. (**b**) Expression of sex-determining crustacean female sex hormone (CFSH) in the eyestalk ganglion of pre-vitellogenic (F-PV), early vitellogenic (F-EV) and late vitellogenic females (F-LV) and mature males (MM) of mud crab *Scylla paramamosain*, showing variation during vitellogenesis and very low value in males. Data are means ± SE ($n = 6$); different letters indicate statistical significance at $P < 0.05$. (**c**) CpG island in 5′-flanking sequence of *CFSH* gene including 12 CpGs (vertical bars). (**d**) Comparison of CpG methylation in *CFSH* promoter region between eyestalk ganglia of early vitellogenic females and mature males, showing significantly higher values in males. $n = 3$ per group; $P < 0.05$ (**a** based on Kvist et al. 2020, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/; **b-d** based on Jiang et al. 2020, Creative Commons Attribution License CC BY, https://creativecommons.org/licenses/by/4.0/)

the female stage with the lowest *CFSH* expression level, than in the eyestalk ganglion of males (Fig. 3.6d). CpG island methylation of the *CFSH* gene was also significantly lower in the hormone-producing eyestalk ganglion than in the musculature of females. These findings suggest that higher CpG promoter methylation suppresses *CFSH* expression and contributes to *CFSH* regulation in a gender and tissue-specific manner. Further analysis revealed that promoter methylation inhibited *CFSH* expression by blocking the binding of transcription factor Sp1 to the DNA.

3.4.3 Discrete Life Stages of Holometabolous Insects

Holometabolous insects produce morphologically, functionally and behaviourally very distinct life stages from the same genome, namely the larva, pupa and adult or imago (Fig. 3.7a). These diverse life stages allow insects to partition life history to feeding and growth (larva), quiescence and metamorphosis (pupa), and reproduction and dispersal (adult) (Simpson et al. 2011). Their expression is controlled by a hormone-mediated developmental programme (Rolff et al. 2019). The involvement of epigenetic mechanisms in generation of these different morphs from the same genome is only sparsely investigated.

Jones et al. (2018) studied genome-wide DNA methylation at single-nucleotide resolution in the larvae and adults of cotton bollworm moth, *Helicoverpa armigera*, a globally invasive pest of agriculture. They found that about 0.9% of the CpG sites were methylated and the methylation pattern was almost identical in the larvae and adults. In contrast, Cardoso-Júnior et al. (2017) observed intense DNA methylation and demethylation events in larvae and pupae of the stingless bee *Melipona scutellaris* using an ELISA-based methodology to quantify global DNA methylation (Fig. 3.7b). Using western blot assays, they also found significant differences in histone methylation and phosphorylation between newly emerged queens and workers.

3.4.4 Different Castes of Social Insects

In adults of many insects, environmental cues can induce different alternative phenotypes from the same genome. This morphological and behavioural polyphenism helps to optimally exploit resources (seasonal morphs), to cope with temporally heterogeneous environments (dispersal morphs) and to partition labour (castes of eusocial insects) (Simpson et al. 2011). Polyphenism is mediated by neurochemical and hormonal pathways that are apparently regulated with the help of environment-sensitive epigenetic mechanisms (Simpson et al. 2011; Glastad et al. 2018; Yang and Pospisilik 2019; Villagra and Frías-Lasserre 2020). Good examples are seasonal morphs in aphids, density-dependent phenotypes in locusts, and diet-mediated queens and workers in honeybees.

Migratory locusts change reversibly between solitarious and gregarious phases that differ dramatically in appearance, physiology and behaviour (Burrows et al. 2011; Ayali 2019). For example, in the desert locust, *Schistocerca gregaria*, the solitary and stationary phase is green and the gregarious and migratory phase is brown. Changes of different phase traits require different periods of time: some behavioural changes take just a few hours, colour change takes a lifetime, and alteration of the muscles and skeleton takes several generations. The establishment of gregarious behaviour is mainly caused by a substantial increase in serotonin, which is probably regulated by environment-sensitive and transgenerationally

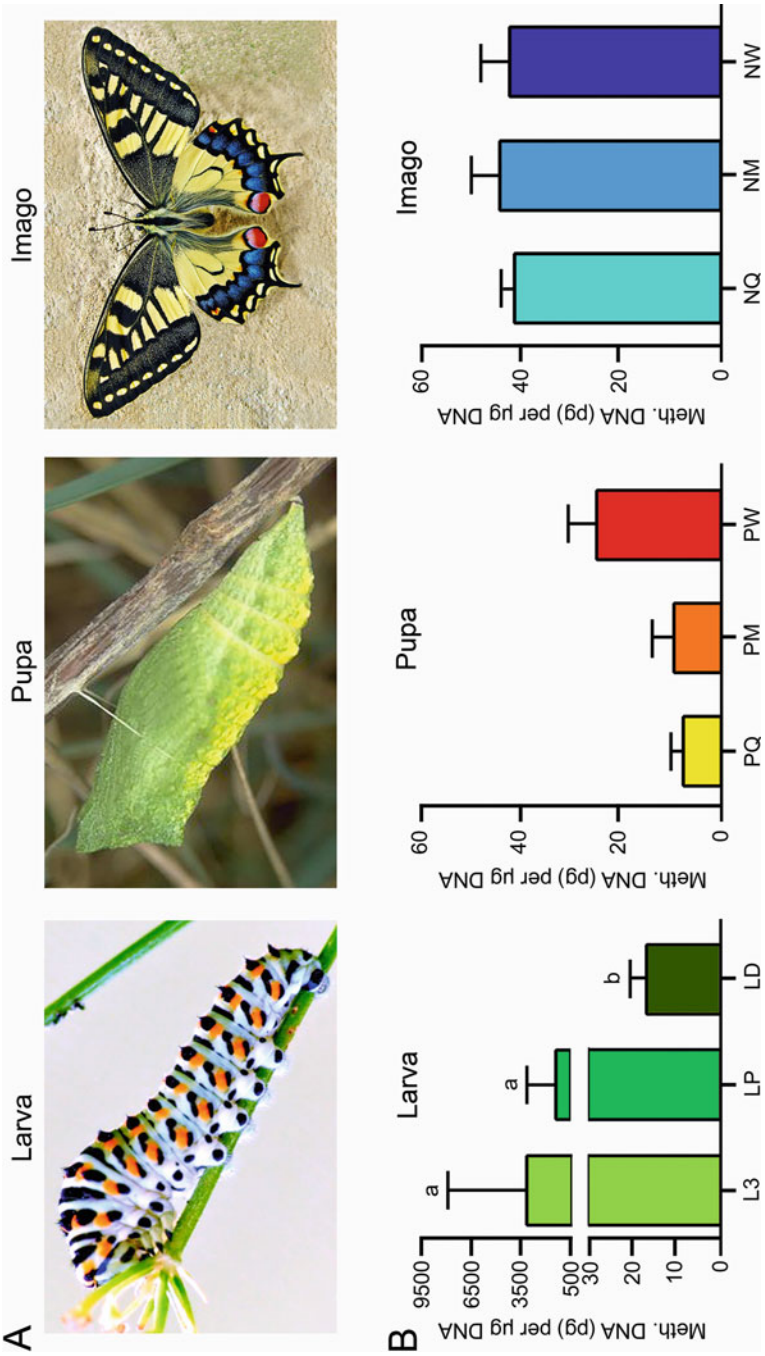

Fig. 3.7 Phenotypic and epigenetic differences between genetically identical life stages of holometabolous insects. (**a**) Phenotypic differences between feeding and growing larva, metamorphosing pupa and reproducing and dispersing imago of butterfly *Papilio machaon*. (**b**) Quantification of methylated cytosines in the DNA of larvae, pupae and newly emerged adults in stingless bee *Melipona scutellaris* by ELISA. There is a dramatic reduction of DNA methylation during larval development and an increase between pupa and hatching adults. The graphs show means ± SE ($n = 3$). Different letters indicate statistical significance at

inherited epigenetic signatures (Ernst et al. 2015). Falckenhayn et al. (2013) analysed the methylome of desert locust using whole-genome bisulphite sequencing (WGBS). They revealed a total cytosine methylation level of 1.3%, confinement of the methylation marks to CpGs and exons and methylation of a significant fraction of transposons. Genic sequences were densely methylated in a pronounced bimodal pattern suggesting a role for DNA methylation in the regulation of locust gene expression.

Ernst et al. (2015) emphasized that DNA methylation, histone modifications and ncRNAs are all involved in phase transition of locusts, but the database is still small. For example, about 90 genes are differentially methylated in gregarious versus solitarious *Locusta migratoria* (Wang et al. 2014), and the brains of gregarious *Schistocerca gregaria* contain more phosphorylated histone H3 when compared to solitarious specimens (Ernst et al. 2015). Wei et al. (2009) investigated the involvement of small ncRNAs in *Locusta migratoria* phase transition and found that gregarious animals had higher expression of RNAs with lengths below 22 nucleotides, whereas the solitary phase had higher expression of RNAs with lengths above 22 nucleotides. Gregarious locusts had considerably higher levels of miRNAs, but solitary locusts had higher levels of endo-siRNAs and piRNA-like small RNAs. Moreover, miRNA-133 has been shown to inhibit aggregation by controlling dopamine synthesis in locusts (Yang et al. 2014).

The honeybee *Apis mellifera* produces morphologically, behaviourally and reproductively different queens and workers from the same genome by differential feeding of the larvae. Presumptive queens are fed with royal jelly and presumptive workers with pollen. Both morphs are diploid, but the workers are considerably smaller and sterile. Queens (Fig. 3.8a) produce the entire offspring and regulate life in the hive by pheromones. Workers (Fig. 3.8a) act as foragers or nurses. Longevity is about 2 years in queens but only 3–6 weeks in workers.

Several papers demonstrate key roles for DNA methylation and chromatin modifications in inducing the queen and worker phenotypes in honeybee. For example, Lyko et al. (2010) reported that the DNA of the brain of queens and workers differ in methylation of more than 550 genes, including genes involved in metabolism, RNA synthesis, nucleic acids binding, signal transduction, brain development and neural functions. An example is shown in Fig. 3.8b for the *syd* gene that encodes the catalytic component of the chromatin structure-remodelling complex. Herb et al. (2012) found substantial differences in DNA methylation between nurse and forager subcastes of workers. Reverting foragers back to nurses re-established methylation signatures for a majority of genes.

Foret et al. (2012) sequenced the larval and adult methylomes in both queens and workers. They found that the number of differentially methylated genes (DMGs) in

Fig. 3.7 (continued) $P < 0.05$. *L3*, larva of third instar; *LP* pre-defecating larva; *LD* defecating larva; *PQ* pink-eyed queen pupa; *PM* pink-eyed male pupa; *PW* pink-eyed worker pupa; *NQ* newly emerged queen; *NM* newly emerged male; *NW* newly emerged worker (**a** from Vogt 2021, with kind permission from Springer Nature; **b** based on Cardoso-Júnior et al. 2017, Creative Commons Attribution License CC BY, https://creativecommons.org/licenses/by/4.0/)

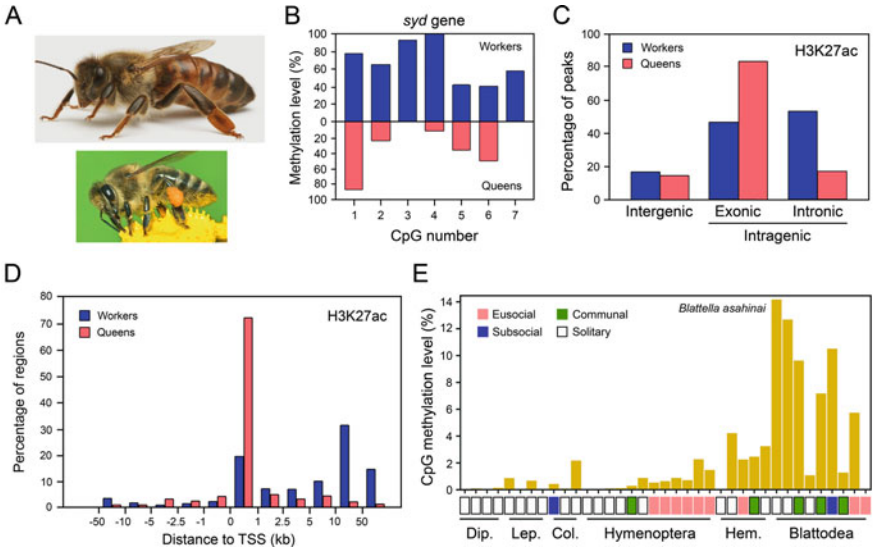

Fig. 3.8 Involvement of epigenetic mechanisms in polyphenism and sociality of insects. (**a**) Dimorphism of reproducing queen (upper panel) and foraging worker (lower panel) in honeybee, *Apis mellifera*. (**b**) Different methylation of CpGs in *syd* gene of workers and queens. (**c**) Different enrichment of unique H3K27ac ChIP-seq regions in intragenic and intergenic sites of 96 h workers and 96 h queens. (**d**) Different location of unique intronic H3K27ac ChIP-seq regions relative to the nearest transcription start site (TSS) in 96 h queens and 96 h workers. In queens, an enrichment of H3K27ac is almost exclusively observed close to the TSS, but in workers it is located more downstream. (**e**) Extensive variation of DNA methylation in insects revealed by WGBS. The genomic level of DNA methylation ranges from 0% in Diptera (Dip.) to 14% in cockroach *Blattella asahinai*. Overall, methylation levels are highest in the relatively basal Blattodea. There is no obvious correlation between DNA methylation level and social behaviour. Col., Coleoptera; Hem., Hemiptera; Lep., Lepidoptera (**a** photograph queen from Alex Wild, with kind permission; photograph worker from https://commons.wikimedia.org/wiki/File:Apis_mellifera_Western_honey_bee.jpg by Andreas Trepte, Creative Commons Attribution CC BY-SA 2.5, https://creativecommons.org/licenses/by-sa/2.5/deed.en; **b** based on Lyko et al. 2010, Creative Commons Attribution License, http://creativecommons.org/licenses/by/4.0; **c** and **d** based on Wojciechowski et al. 2018, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/; **e** based on Bewick et al. 2017, with kind permission from Oxford University Press)

the larval head is significantly increased relative to the adult brain (2399 versus 560) with more than 80% of DMGs being hypermethylated in worker larvae. Several highly conserved metabolic and signalling pathways were enriched in methylated genes including genes involved in the production of juvenile hormone and insulin, two hormones shown to regulate caste determination.

Wojciechowski et al. (2018) produced the first genome-wide maps of chromatin structure in honeybee at a key larval stage in which developmental canalization into queen or worker was virtually irreversible. Using ChIP-seq, which combines chromatin immunoprecipitation with DNA sequencing to identify the binding sites of DNA-associated proteins, they found extensive genome-wide differences in histone

modifications (H3K4me3, H3K27ac and H3K36me3), many of which correlated with caste-specific transcription. The authors identified H3K27ac as a key chromatin modification with a pronounced caste-specific distribution. This modification was found in exons, introns and intergenic regions (Fig. 3.8c). An increase in enrichment of H3K27ac in 96 h queens was almost exclusively located within 0–1 kbp downstream of the transcription start sites, whereas in 96 h workers H3K27ac enrichment was mostly located outside this region (Fig. 3.8d).

Apparently, there is no simple cause–effect relationship between individual epigenetic mechanisms and social behaviour in the species-rich and highly diverse insects. In the primitively eusocial wasp *Polistes dominula*, only seven genes are methylated and DNMT3 is absent (Standage et al. 2016), calling into question the general importance of DNA methylation in social behaviour. Bewick et al. (2017) came to the same conclusion by WGBS of 41 species from several insect orders and investigation of the bimodality of $CpG_{o/e}$ values in 123 social and asocial insect species (Fig. 3.8e).

3.4.5 Cyclomorphosis in Water Fleas

Small water fleas develop anti-predatory morphs when exposed to predator cues, which is called cyclomorphosis (Pijanowska 1990). These morphs are characterized by significantly increased helmets and elongation of the terminal spine (Fig. 3.9a). The defence structures can be inherited to the following generations even in the absence of the initial stimulus (Agrawal et al. 1999). Laforsch and Tollrian (2004) performed induction experiments with *Daphnia pulex* and *Daphnia cucullata* using chemical cues from the predators *Chaoborus flavicans* (insect larva), *Leptodora kindtii* (cladoceran) and *Cyclops* sp. (copepod). They revealed significantly longer helmets and tail spines in all size classes of the exposed daphnids (Fig. 3.9a). The level of protection against predation differed between size classes (Fig. 3.9b) and between predator cues.

Augusto et al. (2021) developed an ATAC-seq assay (assay for transposase-accessible chromatin using sequencing) for *Daphnia pulex* to link the development of defence structures in response to predator cues to alterations of the chromatin structure. ATAC-seq is a relatively new technique for assaying chromatin accessibility genome-wide. Augusto and colleagues found that the appearance of anti-predatory morphs was paralleled by profound reorganization of the chromatin (Fig. 3.9c, d), suggesting that both are functionally linked. Chromatin remodelling usually involves epigenetic mechanisms (Becker and Workman 2013), particularly histone modifications. Therefore, epigenetic mechanisms are assumed to be centrally involved in cyclomorphosis but respective proofs are still lacking.

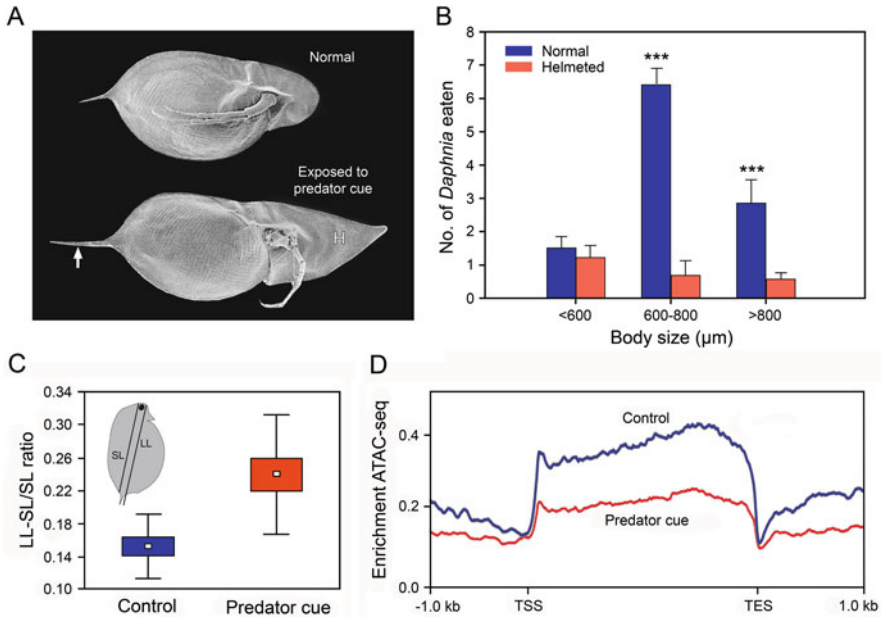

Fig. 3.9 Alteration of phenotype and chromatin structure during cyclomorphosis of water fleas. (**a**) Scanning electron micrographs of normal and predator-induced *Daphnia cucullata*. The predator-induced specimen has a larger helmet (H) and an elongate tail spine (arrow). (**b**) Number of normal and helmeted morphs of *Daphnia cucullata* in three size classes eaten by the predatory cladoceran *Leptodora kindtii*. Shown are means ± SE of 10 trials per condition, asterisks indicate highly significant difference at $P < 0.001$. (**c**) Morphometric difference between predator fish-exposed and unexposed *Daphnia pulex*. Boxes indicate means ± SE and bars indicate SD, $P < 0.001$. LL, long length; SL, short length. (**d**) Metagene ATAC-seq profiles of predator-fish exposed and unexposed *Daphnia pulex* populations. Predator-exposed groups had on average fewer reads over genes than control samples indicating induction of major changes in chromatin structure by the predator cues. TES, transcription end site; TSS, transcription start site (**a** and **b** based on Laforsch and Tollrian 2004, with kind permission from Wiley; **c** and **d** based on Augusto et al. 2021, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/)

3.5 Production of a Continuum of Phenotypes from the Same Genome with the Help of Epigenetic Mechanisms

Laboratory and field studies with asexually reproducing animals revealed that a DNA sequence and its identical copies can produce a continuous range of phenotypes by two different mechanisms, namely developmental stochasticity and environmental induction (Vogt et al. 2008; Vogt 2015a, 2017, 2020b; Leung et al. 2016; Angers et al. 2020). These proportions of non-genetic phenotypic variation, which are called SDPV (stochastic developmental phenotypic variation) and EIPV (environmentally induced phenotypic variation) in the following, are both mediated by

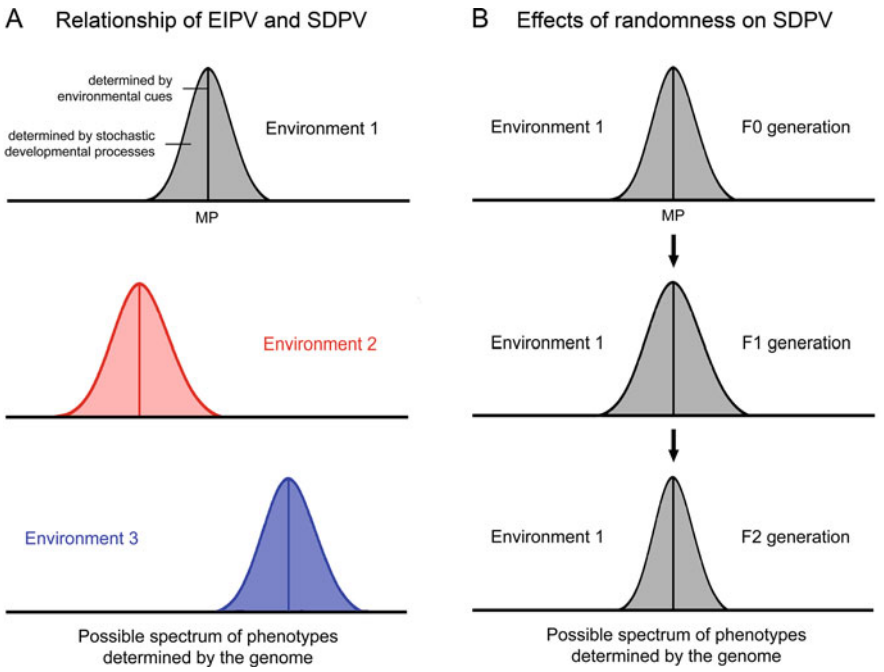

Fig. 3.10 Schematic illustration of the relationship of EIPV and SDPV on the example of clonal populations reared in highly standardized laboratory environments. In a given environment, genetic variation and environmental variation that could generate phenotypic variation are zero or close to zero. (**a**) Cues of the external environment determine the position of the mean or target phenotype (MP) in the spectrum of possible phenotypes, which is determined by the genome of the test organism. Different environmental cues induce different MPs, which together constitute EIPV (norm of reaction). In each environment, the MP is surrounded by a range of phenotypes that is due to SDPV. (**b**) In a given environment, the MP holds its position on the scale of genetically possible phenotypes throughout subsequent generations (F0–F2), but the range of SDPV around it may vary between generations due to the stochastic nature of SDPV (**a** and **b** based on Vogt 2017, Creative Commons Attribution NonCommercial License, http://creativecommons.org/licenses/by-nc/4.0)

epigenetic mechanisms but differ in quality and function as will be discussed below. They can be distinguished in the laboratory by raising genetically identical populations in either the same or different environments. Figure 3.10 illustrates the difference and interdependence between SDPV and EIPV on the example of clonal populations kept in uniform and highly controlled laboratory settings, in which the contributions of genetic variation and non-shared environmental variation to phenotypic variation are close to zero.

3.5.1 Stochastic Developmental Phenotypic Variation (SDPV) and Relationship to Epigenetics

Batchmates of highly inbred, artificially cloned, polyembryonic and apomictic parthenogenetic animals raised individually or communally in the same narrowly controlled laboratory setting were shown to regularly develop considerable phenotypic variation in numerous traits (Gärtner 1990; Vogt et al. 2008; Vogt 2015a, b, 2020b). Since the contribution of genetic variation and environmental variation to the observed phenotypic variation is considered very small in these conditions, the most likely explanation for the phenomenon is the production of phenotypic variation by developmental stochasticity.

3.5.1.1 Properties and Extent of SDPV in Animals

SDPV, sometimes called "developmental noise" or the "third component", is ubiquitous in animals. Since it also occurs in bacteria, protists, fungi and plants, it is considered a general biological principle generating phenotypic diversity from the same genome (reviewed in Vogt 2015a). SDPV is obviously produced by random alterations of epigenetic marks on the DNA and chromatin (epimutations) and higher-order probabilistic processes such as reaction–diffusion systems during patterning.

SDPV in animals is best demonstrated and quantified by laboratory experiments in uniform settings using offspring (clutchmates) from single, asexually reproducing females. In such experiments, there is still a considerable range of phenotypes observed around the mean (Fig. 3.10a) reflecting SDPV (Vogt 2017). The mean or target phenotype in such Gaussian curves is determined by the interaction of the genome and the prevailing environmental conditions. The phenotypes around the mean are the result of SDPV. The random a priori production of diverse epigenotypes and related phenotypes from the same genome without knowing the future conditions is a risk-spreading or bed-hedging strategy that enhances the chance of survival when the environmental conditions change (Vogt 2015a, 2017, 2020b; Leung et al. 2016; Angers et al. 2020).

Examples of the extent of SDPV in animals are given in Table 3.1 and Figs. 3.11 and 3.12 for various traits in highly inbred, polyembryonic, apomictic parthenogenetic and artificially cloned animals. As a rule of thumb, SDPV is relatively small in morphological traits, higher in biochemical and life history traits and particularly high in behavioural traits. Interestingly, spotted coloration is extremely variable identifying each clonemate individually, despite genetic identity (Vogt 2015a).

Table 3.1 Extent of SDPV in genetically identical and communally reared animals groups

Species	Trait	Range/mean[a]	CV (%)	Reference
Rattus norvegicus (rat) I, $n = 18$	Mandible length	26.8 mm	1.49	Flamme (1977)
	Heart weight	0.87 g	10.34	
	Liver weight	11.24 g	11.12	
	Body weight	333 g	12.91	
	Serum protein	67.3 g/l	9.66	
	GOT	43.3 U/l	36.72	
Dasypus novemcinctus (armadillo) P, $n = 4$	No. of scutes in BR	526–531	0.39	Storrs and Williams (1968)
	Brain weight	5.23–5.86% bw	5.52	
	Body weight	52.61–60.30 kg	5.72	
	Heart weight	0.45–0.64% bw	14.65	
	Spleen weight	0.13–0.24% bw	29.99	
	Glutamate in brain	12.24–20.57 rl	21.06	
	Alanine in brain	3.33–12.29 rl	55.80	
	Adrenaline in AG	0.05–1.60 µg/g	102.41	
Sus scrofa domestica (pig) C, $n = 5$	Weight at 27 wk	81.6–102.1 kg	9.25	Archer et al. (2003)
	Blood calcium	3.6–4.3 g/dl	0.93	
	Serum protein	7.0–7.7 g/dl	3.73	
	Blood albumin	10.7–10.9 mg/dl	7.25	
	Blood glucose	70–88 mg/dl	9.20	
	Blood urinary nitrogen	8.9–11.6 mg/dl	14.04	
	Triiodothyronine	43.41–54.63 ng/dl	20.54	
	Cortisol	3.2–6.7 µg/dl	28.98	
Capra aegagrus hircus (goat) C, $n = 5$	Weight at 52 wk PW	43.8 kg	15.34	Landry et al. (2005)
	Thyroxine	4.3 µg/dl	27.91	
	Insulin-like GF I	177.9 ng/ml	44.74	
	Insulin	17.7 µIU/ml	66.67	
	Growth hormone	3.4 ng/ml	135.29	
Oncorhynchus masou macrostomus (fish) C, $n = 22$	Standard length	8.0 cm	5.00	Iguchi et al. (2001)
	Body weight	8.2 g	12.20	
	Benthic feeding	31.28 freq/12 min	96.23	
	Horizontal movement	7.64 grids/min	112.43	
	Hiding	1.08 freq/12 min	215.74	
Procambarus virginalis (crayfish) AP, $n = 8$	Carapace length at 152 d	1.6–2.0 cm	9.55	Vogt et al. (2008)
	Total length at 152 d	3.4–4.4 cm	10.26	
	Life span of reproducers	437–910 d	21.31	
	Body weight at 152 d	0.99–2.40 g	30.91	
	Reproduction cycles	1–5	49.52	
	First spawning	157–531 d	52.46	
	No. of offspring at 430 d	0–219	90.68	

All groups were reared in captivity in highly standardized environments. [a]means are given when data on ranges were not available. *AG* adrenal gland; *AP* apomictic parthenogenesis; *BR* banded region; *bw* body weight; *C* artificial cloning; *CV* coefficient of variation; *freq* frequency; *GF* growth factor; *GOT* glutamic-oxaloacetic transaminase; *I* inbreeding; *P* polyembryony; *PW* post weaning; *rl* relative level

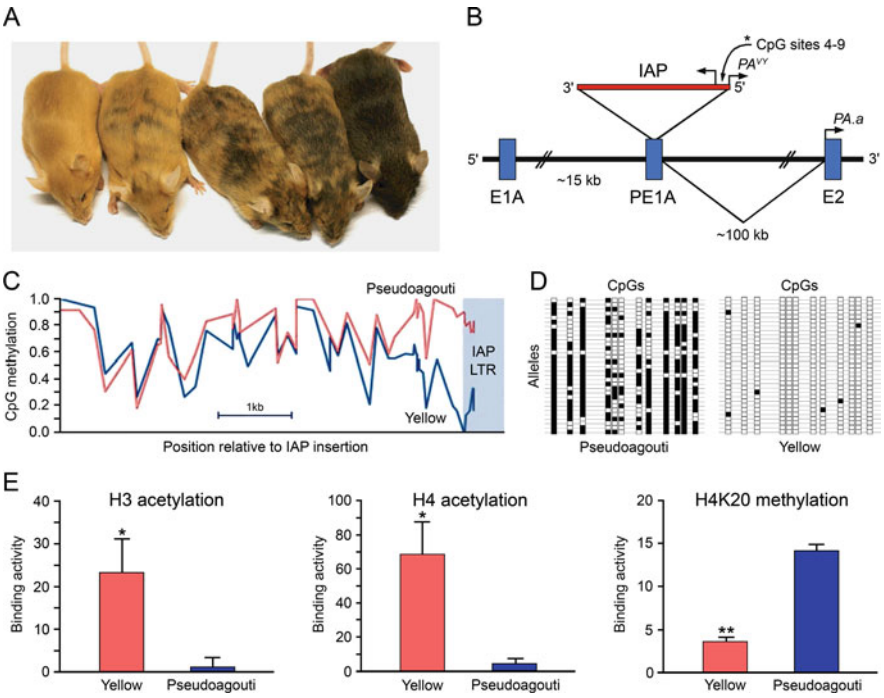

Fig. 3.11 Association of SDPV of coloration and epigenetic signatures in genetically identical littermates of A^{vy}/a laboratory mouse (*Mus muculus*). (**a**) Littermates showing colour variation from pure yellow (left) through yellow/brown speckled to brown (pseudoagouti) (right). (**b**) Scheme of viable yellow agouti gene A^{vy}. The gene contains a contra-oriented IAP insertion within pseudoexon 1A (PE1A). A cryptic promoter (arrow labelled PA^{VY}) drives constitutive ectopic *Agouti* expression. Transcription of the *Agouti* gene normally initiates from a specific promoter (arrow labelled PA, a) in exon 2 (E2). * indicates 5'LTR of the A^{vy} IAP region with CpGs. E1, exon 1. (**c**) Comparison of average DNA methylation levels of CpGs in yellow and pseudoagouti mice inside and outside the IAP insertion. Ectopic *agouti* transcripts originate from the LTR element. (**d**) Representative bisulphite sequencing profiles of individual alleles from yellow and pseudoagouti mice. Each row represents a single allele and each column a CpG within the IAP LTR and adjacent downstream region of pseudoexon 1 (white: unmethylated; black: methylated). (**e**) Chromatin precipitation data for acetylated histones H3 and H4 and methylated histones in the 5'LTR of the IAP showing enrichment of H3 diacetylation in yellow versus pseudoagouti A^{vy}/*a* mice ($n = 6$ per group; $P = 0.09$). The same holds for H4 diacetylation ($n = 3$ per group; $P = 0.08$). In contrast, H4K20 trimethylation is enriched in pseudoagouti mice ($n = 6$ per group; $P = 0.01$. Binding activity was calculated as per cent of pre-immunoprecipitated input DNA (**a** and **d** from Cropley et al. 2010, Creative Commons Attribution License, http://creativecommons.org/licenses/by/4.0/; **b** and **e** based on Dolinoy et al. 2010, Creative Commons Attribution-NonCommercial 3.0, http://creativecommons.org/licenses/by-nc/3.0; **c** based on Oey et al. 2015, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/)

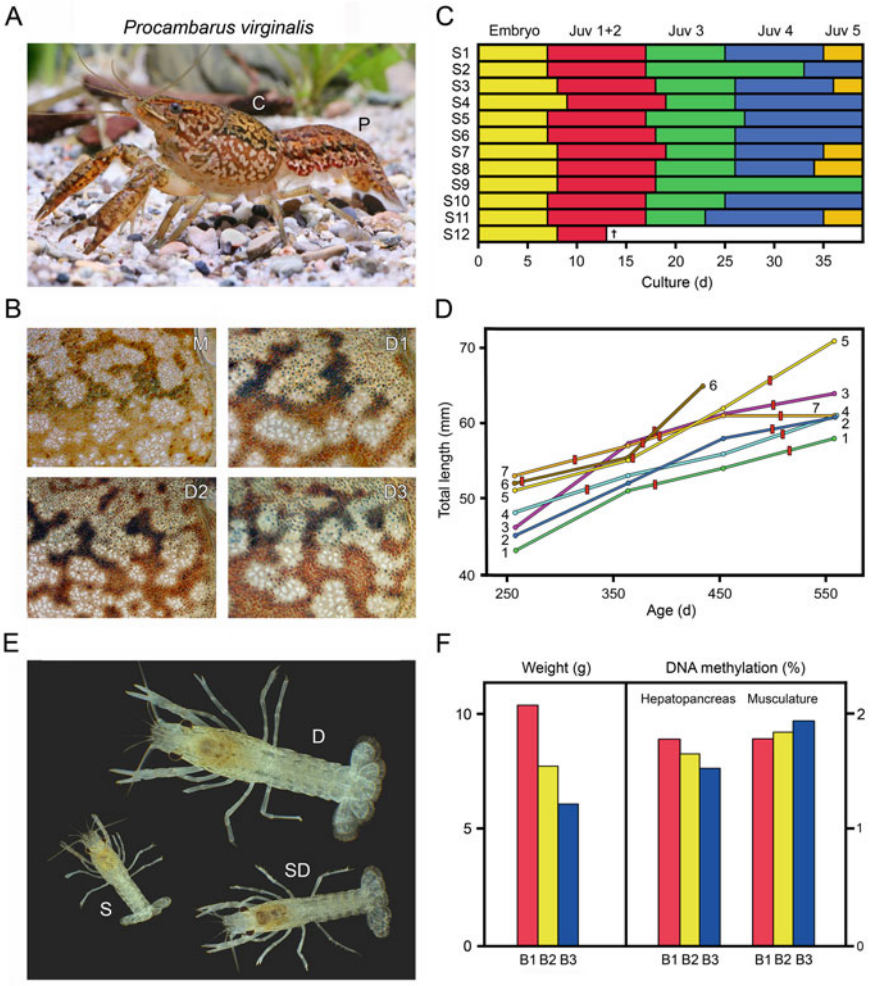

Fig. 3.12 SDPV of morphological, behavioural and life history traits in isogenic and identically raised clutchmates of parthenogenetic marbled crayfish, *Procambarus virginalis*. (**a**) Adult marbled crayfish showing eponymous coloration. C, carapace; P, pleon. (**b**) Colour pattern of posteriolateral carapace areas of a mother (M) and three adult daughters (D1–D3) from the same clutch. Note striking differences in marmoration among all individuals despite genetic identity, communal rearing and identical feeding. (**c**) Development of clutchmates (S1–12) raised individually in a 12-well microplate from late embryogenesis to juvenile stage 5 (Juv 5). Development is rather uniform in embryos and non-feeding juvenile stages 1 and 2 but becomes heterogeneous after onset of feeding in stage 3. (**d**) Variation in growth and reproduction among communally reared clutchmates (1–7). Vertical bars indicate time of oviposition. Note repeated group position changes of individuals over time with respect to growth. (**e**) Establishment of social hierarchy and growth differences in clutchmates kept for 34 days under social stress conditions. The experiment was started with five size-matched siblings of indifferent agonistic behaviour and ended with one dominant (D), one subdominant (SD) and three subordinates (S) of remarkably different size, although food was available in excess and not monopolized. (**f**) Variation of body weight and global DNA methylation (determined by capillary electrophoresis) in the hepatopancreas and abdominal musculature of three 626-day-old, communally reared clutchmates (B1–B3), showing

3.5.1.2 Stochastic Developmental Coat Colour Variation in Inbred A^{vy} Mice

Colour variation in A^{vy} agouti mice is a classical example of epigenetic metastability, in which a variable and partially heritable phenotype correlates with the epigenetic state of a gene (Blewitt et al. 2006). Metastable epialleles are variably expressed in genetically identical individuals due to epigenetic modifications established during early development. DNA methylation within metastable epialleles is principally stochastic due to probabilistic reprogramming of epigenetic marks during embryogenesis. However, maternal nutrition and environment can modify these methylation patterns and the resulting phenotypes, too (Dolinoy et al. 2010).

The A^{vy} mutation arose spontaneously in C3H/HeJ mice in 1962 and was detected because of the unusual yellow coat of its carrier (Jirtle 2014). Animals with this mutation were backcrossed with C57BL/6 J mice, followed by more than 200 generations of sibling mating. This has resulted in the generation of heterozygous A^{vy}/a mice with a genetically invariant background. Littermates range in colour from yellow through mottled (yellow and brown patches) to pseudoagouti (brown) (Fig. 3.11a). The mutation that causes this colour variation is a contra-oriented, intracisternal A particle (IAP) retrotransposon that has integrated upstream of the *agouti* gene. Expression at this locus is controlled by the 5′ long terminal repeat (LTR) of the retrotransposon that includes a promoter (Fig. 3.11b).

The phenotypic state of A^{vy} agouti mice correlates with CpG methylation of the promoter within the IAP LTR (Cropley et al. 2010). This region contains 6 CpG sites, which are variably methylated in isogenic A^{vy}/a offspring (Fig. 3.11c, d). The methylation state of the locus in an individual is conserved across tissue types suggesting establishment early in embryonic development. When unmethylated and active, this promoter drives constitutive transcription of *agouti* and results in a yellow coat. Yellow mice also become obese and are more prone to developing diabetes and cancer than the pseudoagouti mice. Methylated promoters are inactive and lead to pseudoagouti mice. When the activity of the allele differs between cells, the outcome is a mottled mouse with patches of yellow and pseudoagouti fur.

Utilizing chromatin immunoprecipitation followed by qPCR, Dolinoy et al. (2010) also observed variable histone patterns in the LTR of the A^{vy} epiallele. Yellow mice displayed enrichment of H3 and H4 diacetylation. Pseudoagouti mice, in which A^{vy} hypermethylation silences ectopic expression, exhibit enrichment of H4K20 trimethylation. No differences were observed for H3K4 trimethylation, a modification often enriched in the promoter of active genes. These results suggest that DNA methylation acts in concert with histone modifications to affect inter-individual variation of metastable epiallele expression.

Fig. 3.12 (continued) methylation differences between individuals and tissues (**a** from Vogt et al. 2015; Creative Commons Attribution License CC BY 3.0, http://creativecommons.org/licenses/by/3.0; **b-f** from Vogt et al. 2008, with permission from The Company of Biologists)

Yellow mothers produce more yellow offspring than agouti mothers indicating that this phenotype is epigenetically inherited following maternal but not paternal transmission of the relevant epigenetic marks (Blewitt et al. 2006). DNA methylation at the A^{vy} allele is not reprogrammed during primordial germ cell development. However, during pre-implantation development, the paternal allele is rapidly demethylated immediately following fertilization, whereas the maternal allele is not. At the blastocyst stage, the maternal allele is completely demethylated as well suggesting that DNA methylation is not the epigenetic mark that transmits the phenotype to the next generation. Histone modifications or ncRNAs are alternative candidates.

Oey et al. (2015) addressed the question how much of the phenotypic variability among A^{vy} littermates is driven by genetic differences and how much by differences of the epigenome by using whole-genome sequencing (WGS) and whole-genome bisulphite sequencing (WGBS). Unlike monozygotic twins, littermates in inbred mouse colonies arise from independent gametes, providing opportunities for genetic differences that result from germline mutations. WGS was carried out for one yellow and one pseudoagouti mouse, and the genomes were searched for variants against the C57BL/6 J reference genome. Genome-wide, a total of 985 single-nucleotide variants (SNVs) differed between the two mice. The majority of these SNVs were located in intergenic or intronic regions. Only 11 of the variants were located inside exons, and of these, seven were predicted to result in amino acid changes. No differences between the two mice were seen in the *Agouti* gene region. WGBS revealed 356 inter-individual differentially methylated regions (iiDMRs), 55 of which overlapped with endogenous retroviral elements (ERVs). The majority of ERV iiDMRs were metastable epialleles. Their methylation level correlated inversely with the mRNA level from neighbouring genes. Most other variable DMRs were tissue-specific. These results demonstrate that the phenotypic variation between the different A^{vy} phenotypes is purely epigenetically based and that there are apparently several loci involved in production of the spectrum of phenotypic differences.

3.5.1.3 Stochastic Developmental Trait Variation in Clutchmates of Parthenogenetic Crayfish

The marbled crayfish, *Procambarus virginalis* (Fig. 3.12a), is an apomictic parthenogenetic all-female species that produces offspring genetically identical to the mother and among each other (Vogt 2020c). Although detected only in 1995 in the German aquarium trade (Scholtz et al. 2003), it is meanwhile one of the best studied crayfish species (references in Vogt 2018a, b, 2020c). Marbled crayfish has a maximum total length (tip of carapace to end of pleon, Fig. 3.21a) of ca. 13 cm and is an autotriploid descendant of the sexually reproducing slough crayfish, *Procambarus fallax*, that is native to Florida and southern Georgia (Martin et al. 2010, 2015; Vogt et al. 2015). It is now viewed as a separate asexual species (Vogt et al. 2015; Lyko 2017).

Marbled crayfish has neither been found in the native range of the parent species nor in historical museum collections giving rise to the hypothesis that it is an evolutionarily very young species that even might have originated in captivity (Vogt et al. 2015; Vogt 2019a). Legrand et al. (2021) have recently estimated the origin of marbled crayfish from the yearly mutation rate and mutation accumulation over time and dated the most recent common ancestor to a time window between the years 1946 and 1996, confirming its young evolutionary age.

Marbled crayfish are directly developing and mostly reproduce twice a year (Vogt 2008, 2015b). They produce clutches of ~50–650 offspring, depending on female size (Vogt 2020c), providing an extraordinary source of genetically identical clutchmates for experimentation. The eggs and first three post-hatching juvenile stages are carried underneath the maternal pleon and brooded on the pleopods. The maximum age of marbled crayfish recorded was 4.5 years (Vogt 2010). They can be raised throughout life in very simple laboratory settings, the early life stages even in microplates. All life stages can be fed with the same pellet food (e.g. Tetra Wafer Mix) (Vogt 2008, 2020c). A higher degree of genetic and experimental standardization is hardly conceivable in animals.

Laboratory experiments with individually and communally raised isogenic clutchmates of marbled crayfish revealed SDPV of all traits investigated (Vogt et al. 2008). The lowest degree of SDPV was observed for morphological traits like body length and carapace length (Table 3.1) or numbers of olfactory and gustatory sense organs on the antennae and pereiopods, respectively (Vogt et al. 2008). The highest degree of variability was revealed for the marbled coloration pattern, which identifies each specimen unambiguously, despite genetic identity. This marbled pattern differs markedly between mother and offspring and between clutchmates (Fig. 3.12b) and is not inherited (Vogt et al. 2008).

Considerable SDPV levels were also recorded for life history traits like speed of development, growth, reproduction and longevity (Vogt et al. 2008). When clutchmates were individually raised in a 12-well microplate through the late embryonic and early juvenile stages, development was rather uniform in the lecithotrophic embryos and juvenile stages 1 and 2. However, starting from juvenile 3, the first feeding stage, the speed of development became increasingly diverse (Fig. 3.12c). Adult clutchmates communally reared for more than 550 days varied markedly in growth, number of reproductions and time points of spawning (Fig. 3.12d). Interestingly, individuals repeatedly changed their relative position within the group when considering one of these parameters. For example, regarding total length, specimen S5 was number 5 at day 258, number 1 at day 365 and number 2 at day 558. Life span of marbled crayfish in my laboratory population that had reached adulthood varied between 312 and1610 days.

Behavioural traits varied even more among genetically identical clutchmates. For example, when stage-6 juveniles with neutral agonistic behaviour were placed in a group of five in a culture vessel without solid shelters, a social hierarchy was gradually established in the following 34 days. Behavioural divergence started in juvenile stage 7, the first life stage with sclerotized chelae suitable for fighting. At the end of the experiment, the group consisted of 1 dominant, 1 subdominant and

3 subordinates (Vogt et al. 2008). During establishment of the social hierarchy, the dominant developed increasingly offensive behaviours, while its counterparts developed increasingly defensive and avoiding behaviours. Interestingly, growth of the dominant speeded up compared to the subdominant and subordinates (Fig. 3.12e) although all specimens had unlimited access to the food and fed regularly as revealed by the externally visible filling of the intestine. These differences in behaviour and growth probably developed from very small random behavioural differences via self-reinforcing circuitries including behaviour, metabolism and neuroendocrine feedback.

The mutation rate of marbled crayfish is 3.51×10^{-8} per nucleotide and year, and most of the mutations by far are in non-coding regions (Legrand et al. 2021). Therefore, the high phenotypic variation among identically raised clutchmates cannot be explained by random genetic mutations or by genetic recombination, which does not exist in the asexually reproducing crayfish. Due to the simplicity and uniformity of the experimental settings, it is also very unlikely that the observed phenotypic variation is the result of unshared environmental experiences, leaving epigenetically caused developmental stochasticity as the most plausible explanation.

The genome of marbled crayfish is well methylated, and therefore, variation of methylation marks may be among the factors underlying SDPV. Mass spectrometry and capillary electrophoresis with a laser-induced fluorescence detector revealed global DNA methylation levels of 2.4% and 1.8%, respectively (Fig. 3.12f) (Vogt et al. 2008, 2015). Feinberg and Irizarry (2010) demonstrated that the epigenetic marks on the DNA and chromatin can change randomly, indeed, resulting in epigenetic and sometimes phenotypic variation. Such spontaneous epimutations are reversible and, unlike genetic mutations, do not affect the DNA sequence. In the model plant *Arabidopsis thaliana,* epimutations are about five orders of magnitude more frequent than genetic mutations (10^{-4} versus 10^{-9} per base pair and generation) (Van der Graaf et al. 2015). Therefore, epimutations have the potential to generate phenotypic variation much more rapidly when compared to genetic mutations.

Measurement of global DNA methylation in communally raised clutchmates revealed differences between specimens in both the juvenile and adult life stages (Vogt et al. 2008). There were also differences between different tissues in the same individual as demonstrated for the hepatopancreas, the main metabolic organ of crayfish (Vogt 2019b), and the abdominal musculature (Fig. 3.12f). These epigenetic differences were not yet mechanistically linked to variation of the observed morphological, life history and behavioural traits.

3.5.1.4 Fluctuating Asymmetry

Fluctuating asymmetry (FA), the deviation of morphological structures from perfect symmetry in bilaterally symmetric animals (Graham et al. 2010), is a special aspect of the stochastic production of phenotypic variation from the same genome. It can easily be determined in the laboratory and the wild, including sexually reproducing

A

B

Stage	CL	Aesthetascs		Corrugated setae					
		A1-r	A1-l	P1-r	P1-l	P2-r	P2-l	P3-r	P3-l
Juv 6	?	9	10	35	33	?	23	?	25
Juv 7	4.9	12	12	47	42	27	28	30	29
Juv 8	5.3	15	14	?	?	?	?	?	?
Juv 9	6.1	17	16	54	53	39	38	37	36
Juv 10	7.6	18	20	58	55	41	40	40	39
Juv 11	9.0	24	25	72	67	50	48	48	48
Juv 12	10.1	?	?	76	73	?	?	?	?
Juv 13	11.9	37	41	91	86	?	70	69	71
Juv 14	12.5	39	41	92	91	70	71	71	72

Fig. 3.13 Fluctuating asymmetry (FA) between left and right body sides in marbled crayfish. (**a**) Dorsal view on carapace showing differences in marmoration pattern between left (l) and right (r) body side. (**b**) Alteration of carapace length (CL), number of olfactory aesthetascs on right and left first antenna (A1-r and A1-l), and number of gustatory corrugated setae on right and left pereiopods 1–3 (P1-r to P3-l) in a single crayfish through nine juvenile stages, measured from the exuviae. Development of the three traits is not narrowly correlated, and FA of the sense organs can fluctuate between body sides over time (arrows) (**a** and **b** from Vogt et al. 2008; with permission from The Company of Biologists)

and genetically diverse populations. However, FA can be caused not only by stochastic epigenetic differences but also by genetic disturbances and environmental stress (Parsons 1992). An example of FA of body coloration is shown in Fig. 3.13a for marbled crayfish. FA of different traits is semi-independent as shown in Fig. 3.13b for the olfactory aesthetascs on the first antenna and the gustatory corrugated setae on the chelae of pereiopods 1–3 in marbled crayfish. In a given group of laboratory-reared marbled crayfish, FA of a particular trait was always considerably smaller than SDPV of the same trait (Vogt et al. 2008), which was apparently due to repeated attempts in lifetime to correct asymmetry towards symmetry (Fig. 3.13b).

Epigenetic mechanisms are prime candidates for the symmetry modifying factors, as they are able to change gene expression in cells stochastically and in response to environmental cues without changing the DNA sequence. Studies on the relationship between FA and epigenetic mechanisms are not yet available for animals. However, knockdown of DNMT1 in zebrafish *Danio rerio* and frog *Xenopus laevis* revealed that DNA methylation is essential for the establishment of the asymmetric body plan in vertebrate embryos, in which some organs like heart, liver and pancreas occur only in singular and are lateralized (Wang et al. 2017b).

3.5.2 *Environmentally Induced Phenotypic Variation (EIPV) and Relationship to Epigenetics*

Most researchers working on non-genetic variation in populations did not distinguish between the stochastic developmental and environmentally induced proportions of phenotypic variation and treated them together under the term "phenotypic plasticity". To avoid confusion in meaning, I have used the term EIPV in this chapter for the proportion of phenotypic variation that is exclusively caused by cues of the external environment.

3.5.2.1 Properties and Extent of EIPV in Animals

EIPV is ubiquitous in all living organisms including bacteria, protists, fungi, plants and animals as documented by the extensive literature on phenotypic plasticity (e.g. Schlichting 1986; Pigliucci 2001; DeWitt and Scheiner 2004; Justice et al. 2008; Slepecky and Starmer 2009; Fox et al. 2019). It is another general biological principle aside of SDPV that can generate multiple phenotypes from the same genome via epigenetic mechanisms. Environmental cues can reach the target cells either directly (e.g. some chemicals and nutrients) or indirectly via sense organs and neurohormonal signals (e.g. light, predator odours). The environmental signals are able to modify the epigenetic signatures on the DNA and chromatin via environment-sensitive enzymes and proteins, leading to changes in gene expression (Fig. 3.3).

The extent of EIPV that can be produced from the same DNA sequence is best determined in laboratory experiments by splitting a clonal population into multiple groups and exposing these groups to different environmental conditions. The variation within each group is due to SDPV, whereas the variation between different groups is due to EIPV (Fig. 3.10). The extent of EIPV or "norm of reaction" of the tested genome results from the sum of all means revealed in the experiment. When the experimental condition in a given group is changed, the mean phenotype is shifted to another position on the scale of genetically possible phenotypes (Fig. 3.10a), and therefore, EIPV can be viewed as being directional.

Not all environmental cues are capable of producing EIPV. Many environmental signals result only in a short-term physiological response, and others result in no response at all. Starvation, strong predator pressure, harsh environmental conditions and toxicants are probably the most potent elicitors of EIPV (Skinner 2014, 2015; Guillette Jr et al. 2016; Strader et al. 2020).

3.5.2.2 pH-Induced Trait Alterations in Corals

Liew et al. (2018) investigated the association of environmentally induced variations of DNA methylation and phenotypic traits in laboratory-raised, genetically identical

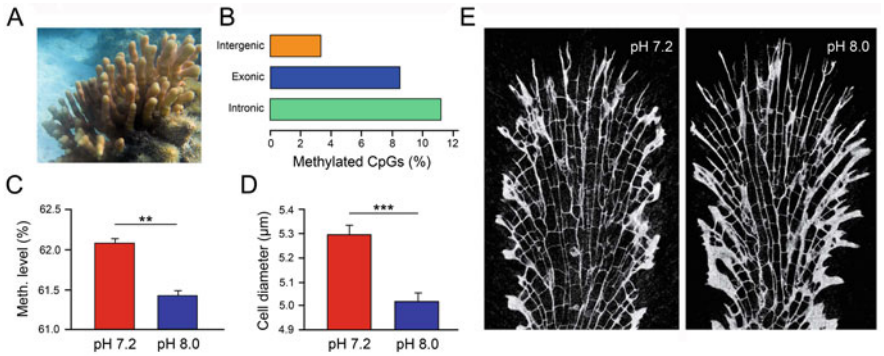

Fig. 3.14 Epigenetic and phenotypic variation in coral *Stylophora pistillata* grown in the laboratory from the same genet and exposed to different pH conditions. (**a**) *Stylophora pistillata*. (**b**) Genic distribution of DNA methylation showing that introns are more intensely methylated than exons or intergenic regions. (**c**) Effect of pH on DNA methylation. Mean methylation levels were significantly higher at stressful pH 7.2 ($P < 0.01$). (**d**) Effect of pH on cell size. Cells were significantly larger at pH 7.2 ($P < 0.001$). (**e**) Representative longitudinal sections of skeletons showing higher porosity at pH 7.2 (**a** from https://en.wikipedia.org/wiki/Stylophora_pistillata, Creative Commons Attribution-ShareAlike 4.0 International, https://creativecommons.org/licenses/by-sa/4.0/; **b-e** based on Liew et al. 2018; Commons Attribution NonCommercial License 4.0 (CC BY-NC), https://creativecommons.org/licenses/by-nc/4.0/)

genets of the coral *Stylophora pistillata* (Fig. 3.14a). They found that the introns have proportionally more methylated cytosines (11.3%) than the exons (8.6%) and intergenic regions (3.3%) (Fig. 3.14b). Exposure of the coral genets to long-term pH stress (pH 7.2) significantly increased mean methylation levels (Fig. 3.14c) when compared to the control (pH 8.0). Widespread methylation changes were observed in genes regulating cell cycle and body size. Enhanced DNA methylation at stressful pH was phenotypically accompanied by an increase in cell size (Fig. 3.14d) and polyp size resulting in more porous skeletons (Fig. 3.14e). The paper demonstrates that environmental cues can concomitantly trigger changes of epigenetic marks on the DNA and phenotypic traits, suggesting a causal relationship between the two.

3.5.2.3 Stress Response in Offspring of Differently Caring Rat Mothers

The influence of maternal care on the long-term behaviour of the offspring in laboratory rat, *Rattus norvegicus*, is one of the best investigated examples of environment–epigenome–phenotype relationships in animals (Jutapakdeegul et al. 2003; Szyf et al. 2005; Champagne 2008; Champagne and Curley 2009; McGowan et al. 2011). Differences in licking and grooming and arched-back nursing of pups by mothers over the first week after birth have pronounced effects on the stress response and social and reproductive behaviour of the offspring. Variation in maternal care occurs in a wide range and is inherited to subsequent generations. It is associated with epigenetic variation at the glucocorticoid receptor (GR) gene in the

hippocampus of the brain, which encodes the GR receptor. This receptor binds glucocorticoid hormones, which are important mediators of the stress response. The epigenetic signatures at the *GR* gene can be modified by cross-fostering between low and high-caring mothers, suggesting that maternal care variation is based on epigenetic mechanisms rather than genetic variation.

The epigenetic and phenotypic consequences of maternal care for the offspring were studied in two rat dam groups that showed twofold–threefold differences in the frequency of licking/grooming (low versus high LG) (Champagne and Curley 2009). High LG resulted in demethylation and acetylation of parts of the chromatin, particularly at the promoter region of the *GR* gene resulting in chromatin expansion, facilitated binding of the transcription factor NGFI-A to the DNA and enhanced transcription of the gene (Fig. 3.15a). Low LG resulted in methylation and deacetylation of the chromatin and promoter region of the *GR* gene resulting in chromatin compaction, blocking of NGFI-A binding and virtual silencing of the gene (Fig. 3.15a). The adult offspring of high LG females were more exploratory in a novel environment, having reduced plasma adrenocorticotropin and corticosterone in response to stress and elevated hippocampal GR mRNA compared to the offspring of low LG dams. Hippocampal GR regulates the hypothalamic–pituitary–adrenal (HPA) axis response to stress through a negative feedback relationship with higher levels of GR-mRNA associated with attenuated stress responsitivity.

Tactile stimulation of the pups in the form of maternal care or stroking with a paintbrush enhanced hippocampal GR expression via increases in NGFI-A, which is dependent on serotonergic activation of cAMP-coupled 5-HT7 receptors. The effects on GR expression of tactile stimulation could be mimicked by administration of a cAMP analogue and blocked by a 5-HT7 receptor antagonist. Central infusion of the adult offspring with the histone deacetylase inhibitor trichostatin A removed the previously defined differences in histone acetylation, DNA methylation, NGFI-A binding, glucocorticoid receptor expression and HPA response to stress, suggesting a causal relationship between maternal care, the epigenomic state, glucocorticoid receptor expression and stress response in the offspring.

The maternal care model of rats also revealed that the different intensities of maternal care can be inherited to the next generation (Champagne 2008; Champagne and Curley 2009) (Fig. 3.15b, c). The offspring of high LG dams exhibit high levels of maternal LG towards their own offspring, whereas the offspring of low LG dams are themselves low in LG. These effects are mediated by differential methylation at multiple regions within the promoter of the oestrogen receptor ERα in the medial pre-optic area (MPOA) of the hypothalamus including a binding site for signal transducer and activator of transcription protein Stat-5. The high levels of ERα promoter methylation observed in the female offspring of low LG dams result in less Stat-5 levels indicating that differential methylation of ERα has functional consequences for the binding of factors that normally enhance gene expression.

McGowan et al. (2011) doubted that epigenetic changes at single gene promoters are sufficient to account for the complex behavioural and physiological characteristics associated with different maternal care, which emerge in infancy and are sustained into adulthood. They investigated this question in depth by using high-

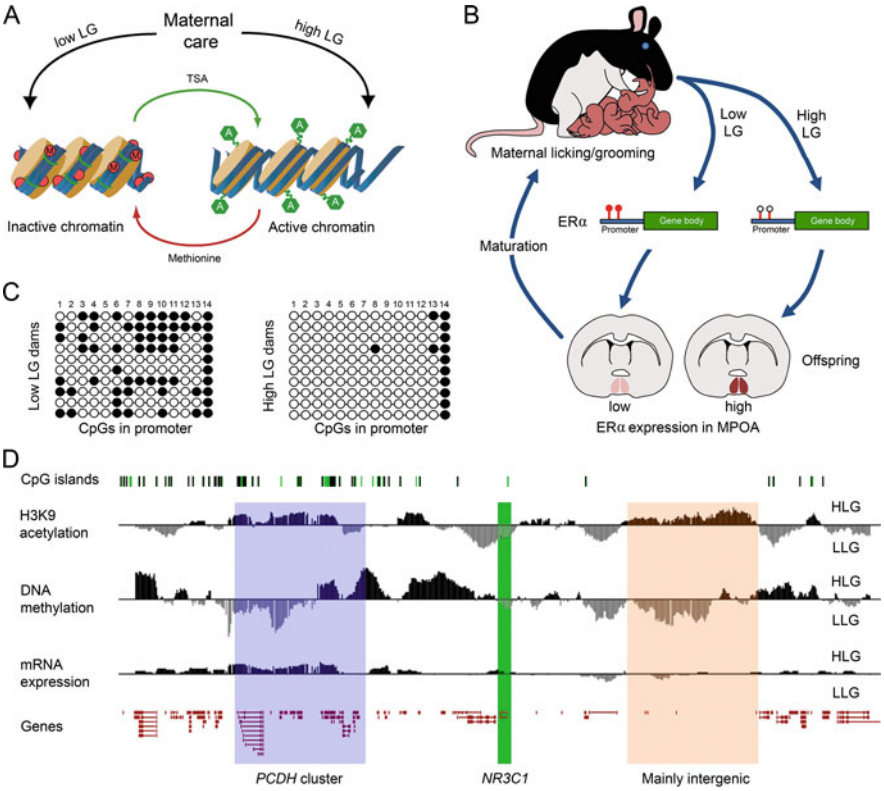

Fig. 3.15 Influence of maternal care on epigenetic signatures, gene expression and behaviour in offspring of laboratory rat, *Rattus norvegicus*. (**a**) Differences in chromatin structure triggered by different levels of maternal care. Low maternal licking/grooming (LG) leads to increased methylation (M) and deacetylation in the promoter regions of the glucocorticoid receptor gene in the hippocampus and the oestrogen receptor α gene (*ERα*) in the medial pre-optic area (MPOA) of the hypothalamus. These marks trigger chromatin compaction and gene silencing. High level of licking/grooming leads to histone acetylation (**A**) and reduction of methylation in the promoter regions of these genes resulting in chromatin expansion and gene activation. These environmentally induced epigenetic modifications can be partially reversed through administration of the histone deacetylase inhibitor trichostatin A (TSA) and the methyl donor methionine. (**b**) Epigenetic transmission of maternal care intensity from mother to offspring. Differences in licking/grooming by the mother lead to methylation differences in the promoter of *ERα* resulting in expression differences in the MPOA. (**c**) Bead-on-string illustration of methylation patterns in promoter region of ERα in MPOA, showing striking differences between offspring of HLG and LLG dams. Black circles indicate presence of 5-methylcytosine. Columns represent potential sites of differential CpG methylation within promoter sequence. (**d**) Differences of epigenetic signatures and gene expression between differently cared adult offspring across ~7 Mb of chromosome 18. Tracks show location of CpG islands and genes, and differences in H3K9 acetylation, DNA methylation and gene expression between offspring of HLG and LLG dams. Highlighted regions show sites of glucocorticoid receptor *NR3C1* gene, *protocadherin* gene cluster and large intergenic region (**a** based on Champagne and Curley 2009, with kind permission from Elsevier; **b** and **c** based on Champagne 2008, with kind permission from Elsevier; **d** based on McGowan et al. 2011, Creative Commons Attribution License, https://creativecommons.org/licenses/by/4.0/)

density oligonucleotide arrays to determine the state of DNA methylation, histone acetylation and gene expression in a ~ 7 Mb region of chromosome 18 from the hippocampus containing the *NR3C1* gene (Fig. 3.15d). The authors found that the adult offspring of high compared to low maternal care mothers showed epigenetic changes in promoters, exons and gene ends across many genes associated with higher transcriptional activity. Other genes in this region remained unchanged. Interestingly, the chromosomal region containing the protocadherin-a, protocadherin-b and protocadherin-c (Pcdh) gene families involved in synaptogenesis showed the highest differential response to maternal care. The results suggest that the epigenetic response to maternal care involves not only single candidate gene promoters but is patterned and coordinated in clusters across broad genomic areas.

The maternal care example in rat illustrates that a temporary environmental stimulus experienced in an early life period can change behavioural and physiological traits via alteration of the epigenome. These epigenetic and phenotypic changes are sustained into adulthood and inherited to subsequent generations, but they are potentially reversible as shown by cross-fostering experiments and pharmacological interventions. The example also shows that such complex trait alterations include multiple epigenetic mechanisms and affect multiple loci of the genome.

3.5.3 *Different Functions of Epigenetically Mediated SDPV and EIPV in Populations*

If SDPV is a bet-hedging strategy and EIPV a strategy that promotes adaptation to the prevailing environment, then both strategies should be differently selected in predictable and unpredictable environments. Leung et al. (2016) investigated this issue in natural populations of the gynogenetic fish *Chrosomus eos-neogaeus*. This all-female species occurs in North America in 14 clonal lineages originating from different hybridization events between the redbelly dace, *Chrosomus eos*, and the fine-scale dace, *Chrosomus neogaeus* (Angers and Schlosser 2007). Dating of hybridization events suggested an origin <50,000 years ago. Each hybrid lineage apparently originated from a single zygote and is genetically uniform with the exception of random mutations that accumulated over time.

The investigation of DNA methylation in *Chrosomus eos-neogaeus* lineages using MSAP (methylation-sensitive amplification polymorphism) revealed relative epigenetic similarity of individuals in a given lake but significant differences between lakes (Massicotte and Angers 2012). Analysis of DNA methylation in lineages from predictable and unpredictable environments (lakes versus intermittent headwater streams) in southern Quebec, Canada, identified the relative contributions of EIPV and SDPV to total epigenetic variation (Leung et al. 2016). EIPV was predominant in predictable environments, whereas risk-spreading SDPV prevailed

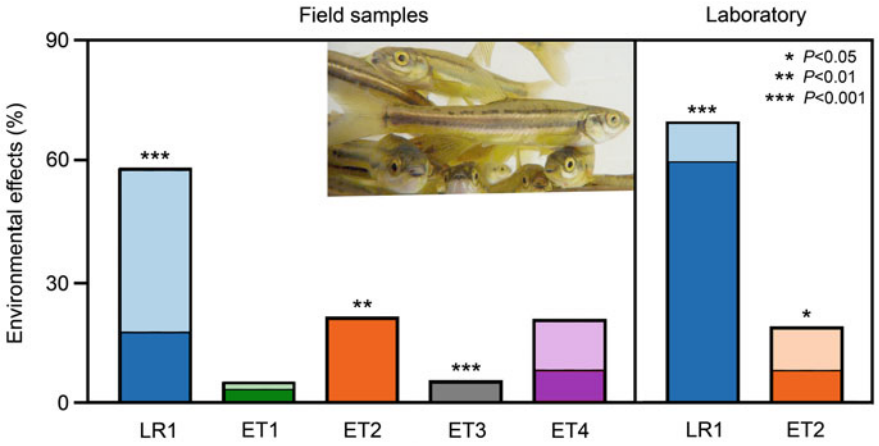

Fig. 3.16 Different roles of EIPV and SDPV in environmental adaptation of asexual fish *Chrosomus eos-neogaeus*. The pure environmental effect on epigenetic variation (darker colours in columns) is separated from the environmental plus genetic joint effect (lighter colours in columns). The remaining amount to 100% is due to developmental stochasticity. Epigenetic differences were determined by MSAP and genetic differences by microsatellites. Population LR1 is from an environmentally stable lake, and populations ET1–ET4 are from environmentally unstable streams. The animals for the laboratory experiments were sampled as larvae from the wild populations and raised for five months until adults. *P*-values refer to pure site effects. The graph shows differences of site effects on epigenetic variation between predictable (LR1) and unpredictable environments (ET1–ET4) and among lineages even if they occur in sympatry (ET3 and ET4). Site effects were highest in predictable environments, and stochastic developmental effects were highest in unpredictable environments. Comparison of LR1 and ET2 between field and laboratory suggests rapid epigenetic response to environmental change (based on Leung et al. 2016; Creative Commons Attribution License, https://creativecommons.org/licenses/by/4.0/legalcode)

in unpredictable environments (Fig. 3.16), indeed, suggesting that both strategies are differentially selected according to environmental uncertainty.

Differences in environmental effects on epigenetic variation between genetically diverse sympatric lineages (Fig. 3.16, ET3 and ET4) and genetically diverse lineages reared in similar experimental conditions (Fig. 3.16, LR1 and ET2) showed that the epigenetic response to environmental signals is strongly influenced by the genotype. Common garden experiments further revealed that the proportion of environmental effects can considerably change when clone members are transplanted into a new environment (Fig. 3.16, compare LR1 or ET2 in field and laboratory). The example of *Chrosomus eos-neogaeus* demonstrates that EIPV and SDPV always occur together but have different weighting in different environments.

3.6 Role of Epigenetically Mediated Phenotypic Variation in Environmental Adaptation

The involvement of epigenetically mediated phenotypic variation in environmental adaptation can best be studied with asexually reproducing, monoclonal populations in which confounding influences of genetic variation are minimal. Further suitable model systems are invasive groups, animals adapted to extreme environments and sessile species that cannot evade unfavourable environmental conditions. In the following, I will present examples for each of these systems.

3.6.1 Adaptation of Monoclonal Snail to Different Habitats and Conditions

The New Zealand mud snail, *Potamopyrgus antipodarum* (Fig. 3.17a), is one of the few molluscs that can reproduce asexually. Genetic and karyotypic data revealed that clonal lineages emerged repeatedly from diploid sexual ancestors by spontaneous transition from diploidy and gonochorism to triploidy and parthenogenesis (Neiman et al. 2005). Most asexual lineages in New Zealand are 20,000–70,000 years old. The mud snail has been introduced in many areas of the world including Europe (since 1859) and North America (since 1987) (Alonso and Castro-Díez 2008).

The biology and ecology of *Potamopyrgus antipodarum* is rather well investigated (Neiman et al. 2005; Alonso and Castro-Díez 2008; Wilton et al. 2013). The shell is approximately 5–12 mm long, and sexual maturity is reached after 3–6 month. There are 1–6 generations per year, and longevity is 18 months. Mud snails are ovoviviparous and produce between 20 and 120 juveniles per clutch. They live in streams, lakes and reservoirs in fresh and brackish water, feed on periphyton, macrophytes and detritus, and survive dry and cold periods buried in the mud. Population density can be extremely high amounting to many thousand individuals per m^2. *Potamopyrgus antipodarum* populations, even clonal ones, show great differences for size and fecundity that are linked to environmental parameters such as water temperature, salinity and current (Thorson et al. 2017; Verhaegen et al. 2021).

To determine the contribution of epigenetic mechanisms to phenotypic variation, Thorson et al. (2017) compared morphological traits and DNA methylation in populations from different sites in Oregon and Washington (USA). These populations originated from a single clone that was introduced in the western USA some 35 years ago, and therefore, they are genetically largely identical (Dybdahl and Drown 2011). Thorson et al. (2017) found habitat-specific differences in shell shape, which were correlated with water current speed (Fig. 3.17a). Using methylated DNA immunoprecipitation (MeDIP) and Illumina sequencing of foot pad tissue, the authors also revealed significant genome-wide DNA methylation differences between lakes and rivers (Fig. 3.17b). These data suggest that environmentally

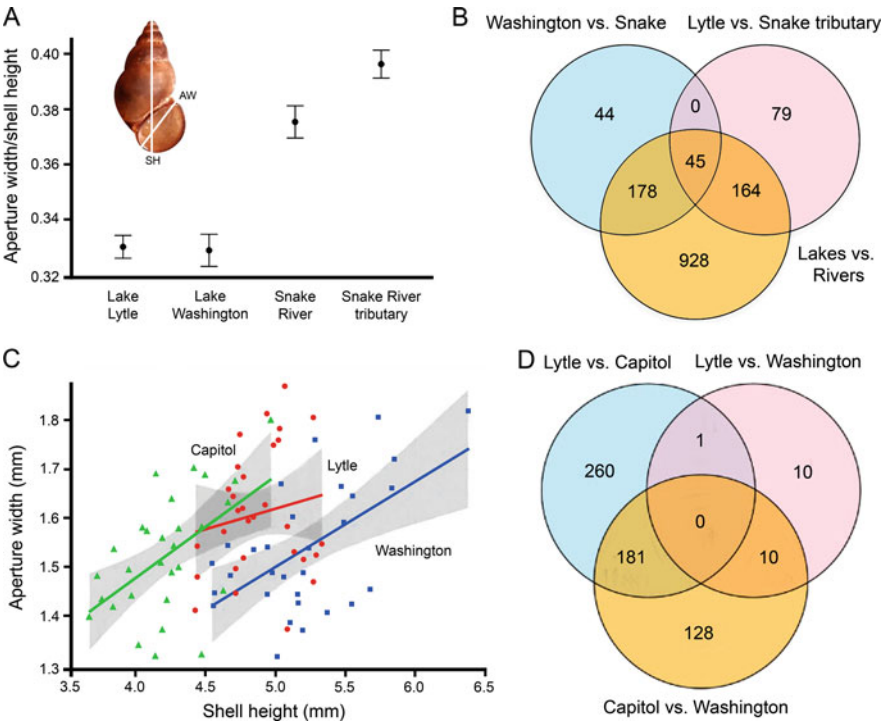

Fig. 3.17 Variation of shell shape and DNA methylation in monoclonal mud snail, *Potamopyrgus antipodarum* from different environments. (**a**) Shell shape differences between populations from distant lakes (Lake Lytle, Oregon, and Lake Washington, Washington) and rivers (Snake River, Idaho, and a tributary spring stream of Snake River, Idaho). AW, aperture width; SH, shell height. (**b**) Venn diagram showing the overlap of DMRs between lake versus river comparisons. (**c**) Differences of shell shape and relative growth rates of shell length and aperture width between pristine Lake Lytle, urban Capitol Lake and polluted Lake Washington. Shaded areas indicate 95% confidence intervals. (**d**) DMRs and their overlap between the three lakes (**a** and **b** based on Thorson et al. 2017, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/; **c** and **d** based on Thorson et al. 2019, Creative Commons Attribution NonCommercial License, http://creativecommons.org/licenses/by-nc/4.0/)

induced epigenetic diversity may underpin adaptive phenotypic diversity that has been established in less than 100 generations, despite genetic identity. The data did not support an effect of geographic distance on epigenetic signatures.

Thorson et al. (2019) then compared isogenic populations from a rural lake (Lake Lyte, Oregon) and two polluted urban lakes (Capitol Lake and Lake Washington, Washington). They measured differences in shell shape and allometric growth (Fig. 3.17c) and identified numerous differentially methylated DNA regions between the three lakes (Fig. 3.17d). A relatively high number of DMRs was shared between rural Lake Lyte and Capitol Lake characterized by high water temperature and high levels of phosphorous and faecal bacteria, and between the two urban lakes.

However, there were only a few DMRs shared between the rural lake and Lake Washington heavily polluted by heavy metals and organic xenobiotics. The presence of site-specific differences in DNA methylation between the genetically identical lake populations confirms an epigenetic response to varied environmental factors.

3.6.2 Adaptation of Sessile Corals to Adverse Conditions

In contrast to their mobile counterparts, sessile animals like Porifera, Cnidaria, Bryozoa, Bivalvia, Cirripedia, Tunicata and Pelmatozoa cannot evade unfavourable environmental conditions. Therefore, epigenetic variation could play a similarly big role in their environmental adaptation as for the sessile plants, but data are scarce.

Liew et al. (2020) analysed the association of DNA methylation patterns and phenotypic traits in adults, gametes and larval offspring of the reef-building brain coral *Platygyra daedalea* from two different environments in the Arabian Peninsula. The Abu Dhabi population lives in the Arabian-Persian Gulf under extreme temperatures (winter <19 °C and summer >35 °C) and salinities (40–46 psu) and has persisted through several major thermal stress events (coral bleaching) during the past two decades. The Fujairah population lives south of the strait of Hormuz under comparatively milder conditions (22–33 °C, 36–39 psu) and has not experienced coral bleaching in recent years. Using WGBS, the authors identified 1.42 million CpG positions (3.2% of all CpGs) that were consistently methylated in the ~800 Mb genome

Liew et al. (2020) showed that the DNA methylation patterns in the brain coral are determined by genotype, developmental stage and the parental environment (Fig. 3.18a, b). Comparison of methylation patterns in genes of adults and their sperm between the two distinct environments suggests intergenerational acclimatization to local temperature and salinity. Reproduction experiments confirmed the inheritance of genome-wide CpG methylation from adults to their sperm and larvae (Fig. 3.18a). Furthermore, genotype-independent differences of methylation levels in stress-related genes were strongly correlated with offspring survival under heat stress. These findings suggest a role of DNA methylation in environmental adaptation of corals and the transgenerational inheritance of favourable methylation marks and associated phenotypic traits.

3.6.3 Adaptation of Fish to Subterranean Habitats

The blind Mexican cave fish, *Astyanax mexicanus*, is a good example of the involvement of epigenetic mechanisms in adaptation to an extreme habitat (Fig. 3.19a). It repeatedly evolved from surface morphs with well-developed eyes starting a few million years ago. Strecker et al. (2013) analysed nuclear microsatellites and mitochondrial genes of seven cave and seven surface populations

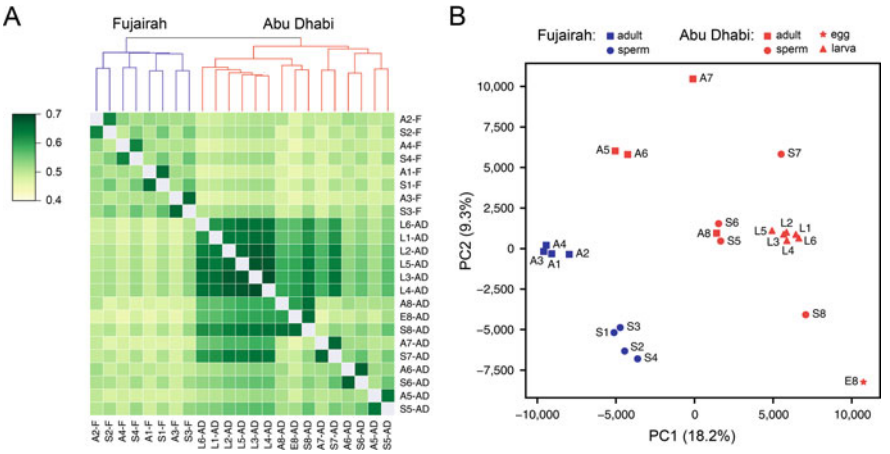

Fig. 3.18 Environmentally induced epigenetic variation and transgenerational epigenetic inheritance in brain coral *Platygyra daedalea*. (**a**) Clustering performed on pair-wise correlation of methylation data from specimens sampled at Fujairah, Gulf of Oman (F, specimens 1–4) and Abu Dhabi, Arabian-Persian Gulf (AD, specimens 5–8). Within each population, samples were collected from adults (A), their spawned sperm (S) and eggs (E), and larval offspring from reciprocal crosses between E7 and S8 (L1–L3) and S7 and E8 (L4–L6). Values on colour bar are Kendall rank correlation coefficients. The analysis demonstrates grouping of samples by environmental origin and a strong effect of inheritance on methylation patterns, because gametes cluster best with respective adults and larval samples cluster best with their parents. (**b**) Principal component analysis of the same methylated positions, showing separation of samples by environmental origin along PC1 and by developmental stage along PC2 (**a** and **b** based on Liew et al. 2020, with kind permission from Springer Nature)

and revealed that *Astyanax mexicanus* invaded northern Mexico at least three times and that populations of all three invasions adapted to subterranean habitats. There was no gene flow between surface populations and cave populations with different degrees of eye and pigment reduction, suggesting that the variability of the troglobitic phenotypes is due to repeated cave adaptations rather than to hybridization.

The evolution of eye loss in cave animals is usually explained by genetic mutations. However, in the cave morphs of *Astyanax mexicanus,* no inactivating mutations have been found in eye development genes (e.g. the crystallins *crybb1*, *crybb1c* and *cryaa*). At 36 h of development, embryos of surface and cave morphs are superficially indistinguishable with properly formed lenses and optic cups (Gore et al. 2018). After five days, degeneration of eye tissue is clearly evident, and by adulthood, eyes are completely absent in the cave morph (Fig. 3.19a). The eye development genes are significantly higher expressed in 54-h-old surface morphs than in cave morphs (Fig. 3.19b). Silencing of eye genes is apparently caused epigenetically by promoter DNA methylation (Fig. 3.19c) (Gore et al. 2018). Interestingly, the cavefish eyes could be partially rescued by injection of the DNA methyltransferase inhibitor 5-azacytidine into the embryonic eye (Fig. 3.19d). These

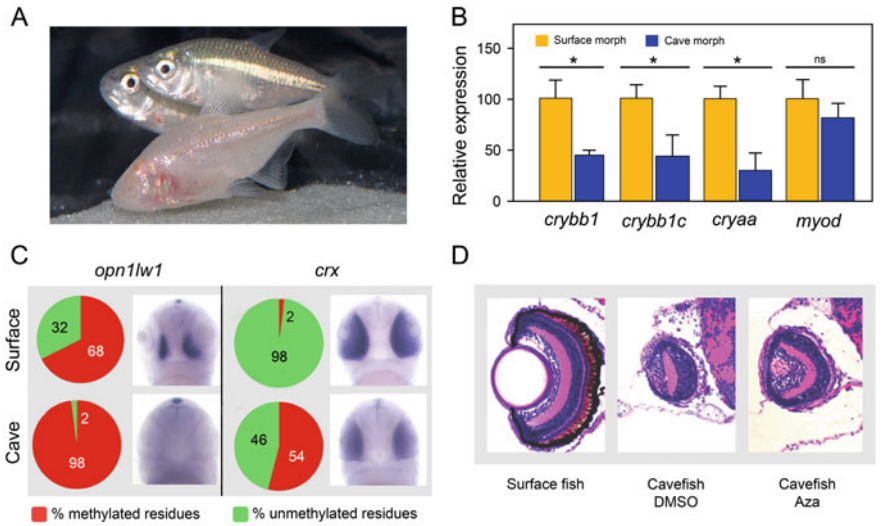

Fig. 3.19 Epigenetic and phenotypic differences between epigean and hypogean *Astyanax mexicanus*. (**a**) Two surface morphs with well-developed eyes and an eyeless cave morph. (**b**) Relative expression of eye development genes *crybb1*, *crybb1c* and *cryaa*. The gene *myod* encoding the myoblast determining protein served as control. The eye development genes show significantly higher expression in 54-h-old surface morphs than in cave morphs. Quantitative RT-PCR; * significantly different, $P < 0.05$. (**c**) Promoter CpG methylation of eye genes *opn1lw1* and *crx* in 54-h-old surface and cave morphs and whole mount in situ hybridization of corresponding larval heads, showing negative correlation between methylation level and eye expression. (**d**) Histological sections of 5-d-old surface fish eye, 5-azacytidine (Aza) injected cave morph eye and DMSO injected cave morph eye (control), showing partial eye recovery by the methyltransferase inhibitor. H&E staining (**a** photograph by Richard L. Borowsky; with kind permission; **b-d** based on Gore et al. 2018; with kind permission by Springer Nature)

results suggest that gene repression by DNA methylation can play a significant role in adaptation to an extreme environment.

3.6.4 Invasion of Diverse Biomes and Habitats by Parthenogenetic Crayfish

In the last 20 years, marbled crayfish have repeatedly been released into the wild resulting in the establishment of numerous populations in tropical to cold-temperate biomes in Europe (17 countries), Africa (Madagascar) and Asia (Israel, Japan, China and Taiwan) (references and coordinates in Vogt 2020c). These populations were shown to be genetically identical with the exception of some random mutations (Fig. 3.20a), suggesting that they all originate from a single individual (Vogt et al. 2008, 2015; Martin et al. 2015; Gutekunst et al. 2018; Maiakovska et al. 2021). In Europe, marbled crayfish was found in individual water bodies from the Netherlands

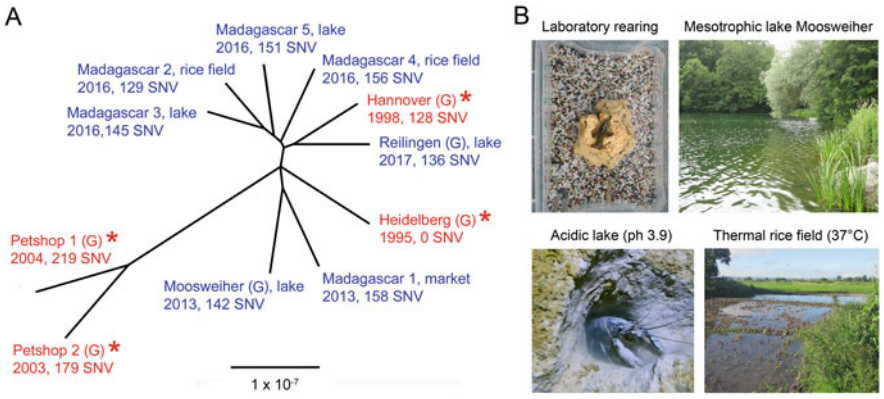

Fig. 3.20 Genetic similarity of marbled crayfish from different sources and examples of habitats. (**a**) Phylogenetic tree of 11 marbled crayfish from diverse laboratory (asterisks) and field sources in Germany (G) and Madagascar based on the comparison of ca. 20% of whole-genome sequences. The maximum genetic difference between specimens from different populations was only 219 single-nucleotide variants (SNVs). (**b**) Strikingly different habitats of marbled crayfish in different geographical regions (**a** based on Gutekunst et al. 2018, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0; **b** upper panels from Vogt et al. 2018, with kind permission from Springer Nature; lower left panel from Tönges et al. 2021a, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0; lower right panel from Andriantsoa et al. 2019, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0)

to the Ukraine and from Sweden to Malta. In Madagascar, it has spread from an initial introduction near the capital Antananarivo before 2005 over more than 100.000 km^2, mostly by human dispersal.

Marbled crayfish now occur in a broad spectrum of habitats including rivers, ponds, oligotrophic to eutrophic lakes, and acidic, thermal and polluted waters (Fig. 3.20b) (Andriantsoa et al. 2019; Vogt 2020c; Maiakovska et al. 2021). The coldest habitat in which different life stages were found was river Märstaån in Sweden. Marbled crayfish were observed crawling on the bottom in 2 °C cold water (Bohman et al. 2013). The warmest water bodies with marbled crayfish populations so far reported were a rice field at Anjingilo (Madagascar) with 37 °C (Andriantsoa et al. 2019) and thermal Lake Hévíz in Hungary with summer temperatures of 38 °C (Lőkkös et al. 2016). Marbled crayfish can build burrows as deep as 1 m as observed by Frank Lenich in Lake Murner See, Germany (Fig. 3.20b) and can survive dry periods buried deep in the mud as observed by Jones et al. (2009) in a dried-out pond in Madagascar. They can also walk overland more than 100 m (Chucholl et al. 2012).

Other extreme marbled crayfish habitats are the recultivated lignite mining sites Lake Murner See (Fig. 3.20b) and Lake Singliser See in Germany with pH 3.9–4.2 (Dümpelmann and Bonacker 2012; Tönges et al. 2021a). Lake Singliser See also has high sulphate levels (~740 mg/L) and is characterized by low biomass production (Dümpelmann and Bonacker 2012). An example of a heavily polluted marbled

crayfish habitat is Ihosy River in Madagascar that has high iron and critical aluminium levels (4792 µg/L) due to nearby mining activities (Fig. 3.22b) (Andriantsoa et al. 2019). Stable isotope analyses of field samples from three German lakes and a Hungarian stream revealed that marbled crayfish can not only inhabit highly diverse ecosystems but also is highly plastic with respect to trophic position and niche breadth, depending on habitat, the availability of food and shelter and the presence of competitors and predators (Linzmaier et al. 2020; Veselý et al. 2021).

The broad environmental adaptability is an important precondition for studying genotype–epigenotype–phenotype relationships in marbled crayfish. Other important preconditions are the availability of a fully sequenced genome (Gutekunst et al. 2018) and a genome-wide methylome (Gatzmann et al. 2018). The genome of marbled crayfish has a size of ~3.7 Gb and includes almost 22,000 predicted genes. Comparison of ca. 20% of whole-genome sequences of specimens from different laboratory lineages and wild populations in Germany and Madagascar, which are separated from each other since about 20–40 generations, revealed only small differences of 129–219 single-nucleotide variants (SNVs) (Fig. 3.20a) (Gutekunst et al. 2018). The vast majority of these SNVs were silent mutations. The maximum number of non-synonymous SNVs that change the amino acid sequence of proteins was only 4 between samples (Gutekunst et al. 2018). These data suggest that phenotypic differences between differently adapted populations must be caused by epigenetic variation rather than variation of the DNA sequence.

WGBS revealed that DNA methylation in marbled crayfish is CpG-specific and present in coding genes, intergenic regions and repeats (Gatzmann et al. 2018). Analysis of the methylome further showed that 41% of genes are heavily methylated, 33% are moderately methylated, and 26% are unmethylated (Falckenhayn 2016). Gene body methylation is highest in evolutionarily old housekeeping genes and moderately expressed genes. Repeats are mostly hypomethylated. The integrative analysis of DNA methylation, chromatin accessibility and mRNA expression patterns revealed that high gene body methylation is correlated with limited accessibility of genes in the chromatin and stable gene expression, whereas low gene body methylation is associated with higher accessibility of genes and more variable expression (Gatzmann et al. 2018).

Comparison of marbled crayfish from my laboratory and German lakes revealed considerable differences in phenotypic traits and DNA methylation. For example, specimens raised under stringent laboratory conditions for many generations reproduced well and grew old (4.5 years) but reached maximum total lengths of only 9 cm and weights of 18 g, whereas their relatives in Lake Moosweiher grew to ~12 cm and 40 g (Fig. 3.21a). Furthermore, the lake specimens had prominent sharp spines on their carapaces and chelipeds (Vogt et al. 2018), but laboratory-raised specimens of the same size lacked these spines and had only small blunt knobs instead (Fig. 3.21b). Specimens transferred from mesotrophic Lake Moosweiher to the laboratory maintained their spines through several moults until the end of life, but in the F1 progeny the spines were considerably reduced resembling members of the laboratory colony. The laboratory specimens also had significantly longer pleons and broader carapaces when compared to equal-sized specimens from Lake

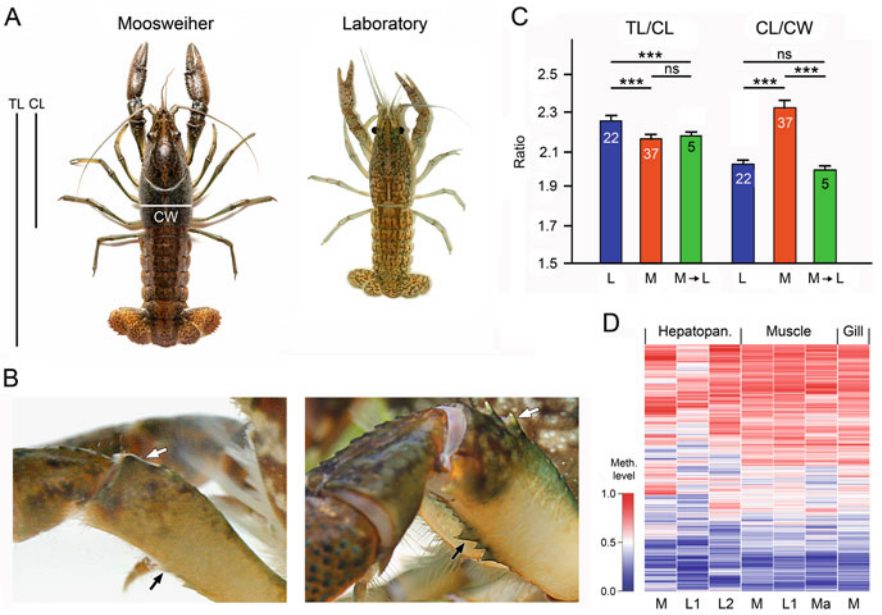

Fig. 3.21 Comparison of phenotypic traits and DNA methylation between marbled crayfish from the laboratory and Lake Moosweiher (Germany). (**a**) Comparison of maximum body size and coloration. Total length (TL) of the largest specimen captured from Lake Moosweiher exceeded TL of the largest laboratory specimen by about 30%. Coloration of the dorsal side was uniformly dark greenish-brown in lake specimens but quite variable in laboratory specimens. Total length, carapace length (CL) and carapace width (CW) were used for morphometric analysis. (**b**) Chelipeds of laboratory-raised specimen (left panel) and specimen from Lake Moosweiher (right panel), showing bigger and sharper spines (arrows) in the wild specimen. (**c**) Body proportions of marbled crayfish from the laboratory (L) and Lake Moosweiher (M), showing significant differences in TL/CL and CL/CW ratios. The laboratory-raised offspring of a female that was transferred from the lake to the laboratory (M → L) and reproduced there had a TL/CL ratio similar to the wild population but a CL/CW ratio similar to the laboratory population. Figures in columns give numbers of specimens investigated; *** significantly different ($P < 0.001$); ns, not significantly different. (**d**) Comparative analysis of 697 variably methylated genes in the hepatopancreas and abdominal musculature of two laboratory-reared specimens (L1, L2), a specimen from Lake Moosweiher (M1) and a specimen from a rice field in Moramanga, Madagascar (Ma). The heatmap shows differences in methylation patterns between individuals, particularly in the hepatopancreas, and between tissues (**a** left picture from Vogt et al. 2018, with kind permission from Magnolia Press; **b** from Vogt et al. 2018, with kind permission from Magnolia Press; **c** based on Vogt 2021, with kind permission from Springer Nature; **d** based on Tönges et al. 2021b, Creative Commons Attribution License (CC BY))

Moosweiher (Fig. 3.21c) (Vogt 2021). Interestingly, the adult offspring of a specimen that was transferred from Lake Moosweiher to the laboratory and reproduced there one year later had a total length/carapace length ratio similar to the wild population and their mother, but a carapace length/carapace width ratio more similar to the laboratory population (Fig. 3.21c). The latter feature is probably due to the

enlargement of the lateral gill chambers underneath the carapace in adaptation to the relatively low oxygen content of the water in the laboratory setting.

The analysis of global DNA methylation and methylated genes from laboratory-raised clutchmates and wild specimens revealed differences between individuals, tissues and environments (Figs. 3.12f and 3.21d) (Vogt et al. 2008; Gatzmann et al. 2018). Comparative analysis of 697 highly variably methylated genes demonstrated that inter-individual differences are highest in the hepatopancreas (Fig. 3.21d).

Andriantsoa et al. (2019) analysed five Malagasy populations from different bio-climatic regions (humid, subhumid and subarid), habitats (river, lake, pond and rice field) and altitudes (9–1491 m above sea level) and revealed marked differences in population structure despite genetic identity (Fig. 3.22a). To shed light on the association between environmental adaptation and epigenetic signatures, Tönges et al. (2021b) then compared DNA methylation of 697 variably methylated genes between two of these populations from strikingly different habitats. One population lives in a clear mountain river (Andragnaroa River) with relatively low pH and temperature and is characterized by a low population density of 20 CPUE (specimen caught by two persons per h) and a bias towards small-sized animals (Fig. 3.22a, b). The other population inhabits a turbid and polluted river (Ihosy River) with higher pH and temperature and high contents of iron and aluminium (Fig. 3.22a, b). It is characterized by high population density (152 CPUE) and comparably large animals. Principal component analysis run on the bisulphite sequencing results revealed a separation of the two populations with respect to DNA methylation (Fig. 3.22c).

Using a capture-based subgenome bisulphite sequencing approach that covered 361 variably methylated genes, Tönges et al. (2021a) then compared the Andragnaro and Ihosy river populations with two German lake populations of marbled crayfish living in acidic, oligotrophic Lake Singliser See (former lignite mining site, no fishes present) and slightly basic, eutrophic Lake Reilinger See (predatory fishes present). A total of 48 animals were analysed to achieve sufficient statistical power. The authors identified specific and highly localized DNA methylation signatures for all of these populations in both the hepatopancreas (Fig. 3.22c) and abdominal musculature that remained stable over consecutive years. Gene ontology analysis of the variably methylated genes revealed a significant enrichment of GTP-binding proteins, which transmit signals from outside into the cells, and proteins involved in regulation of transcription and translation, RNA metabolism, response to stress, and immune response to pathogens. Since no SNVs were found in the differently methylated genes of the samples, the study provides conclusive evidence for location-specific epigenetic variation that is independent from genetic variation or, with other words, the existence of epigenetic ecotypes.

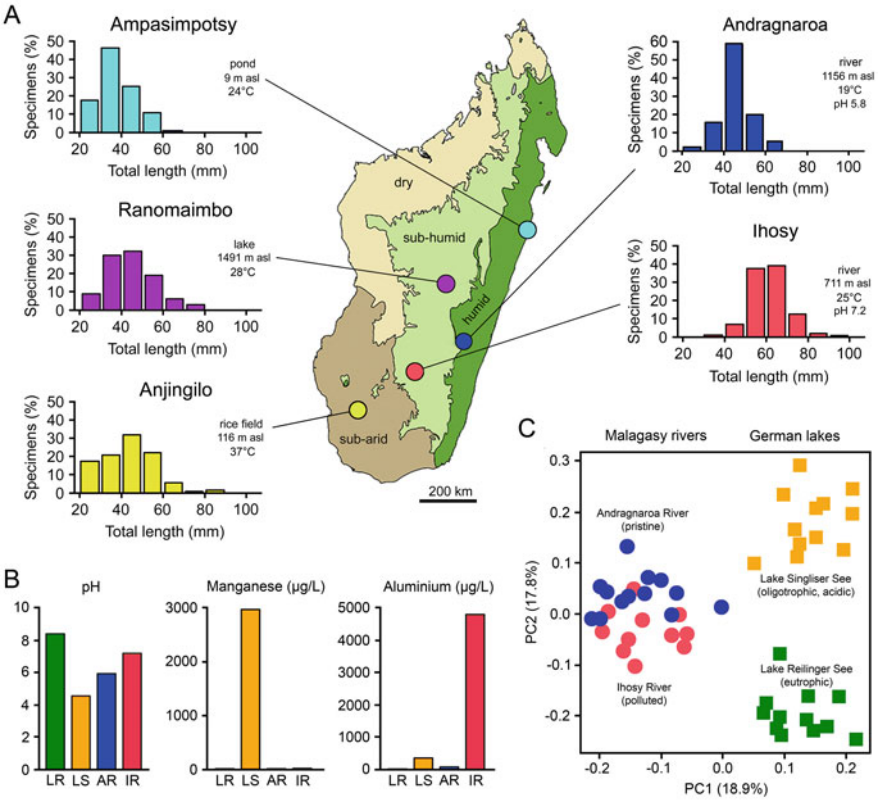

Fig. 3.22 Differences in population structure and DNA methylation patterns in marbled crayfish from Madagascar and Germany. (**a**) Size–frequency distribution of genetically identical populations from different habitats in different bio-climatic regions. Values on right side of graphs give altitude above sea level (asl) and water temperature measured at the time of sampling (8–10 a.m.) in 10 cm water depth. The particularly high water temperature in Anjingilo is caused by thermal water. Marbled crayfish were significantly larger in the Ihosy River than in the other sites ($P < 0.05$). (**b**) Comparison of physico-chemical parameters of pristine Andragnaroa River (AR) and highly polluted Ihosy River (IR) in Madagascar and oligotrophic Lake Singliser See (LS) and eutrophic Lake Reilinger See (LR) in Germany. (**c**) Principal component analysis of DNA methylation of 122 genes in the hepatopancreases of specimens from Andragnaroa River, Ihosy River, Lake Singliser See and Lake Reilinger See showing clear separation of the populations. $P < 0.05$ (**a** based on Andriantsoa et al. 2019, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/; **b** and **c** based on Tönges et al. 2021b, Creative Commons Attribution License (CC BY), http://creativecommons.org/licenses/by/4.0/)

3.7 Role of Epigenetically Mediated Phenotypes in Evolution

Domestication and polyploid speciation are particularly suitable for studying the role of epigenetics in evolution (Vogt 2017). Domestication is evolution in time laps with relatively well-known history and controlled selection of traits. Polyploid speciation is associated with intense rearrangements of the chromatin and alteration of gene expression, which requires the contribution of epigenetic mechanisms.

3.7.1 Contribution of Epigenetic Mechanisms to Domestication

Domestication of animals started ca. 30.000 years ago with dogs (Larson and Fuller 2014). A long-term, ongoing domestication experiment with silver fox started only ~65 years ago (Trut and Kharlamova 2020), and in several cultured fish species, domestication has just begun (Telechea 2018). Aside of short and rather well-known evolutionary history, domesticated species have the advantage of exceptionally broad variation in phenotypic traits like body size, coloration, physiology, behaviour and longevity. Moreover, their genetics is relatively well investigated (Wright 2015). In the early stages of domestication, selection is often targeted on tameness. Jensen (2015) emphasized that changes in this behavioural trait are to a large extent correlated to changes in gene expression, suggesting that regulatory epigenetic mechanisms might play an important role in early domestication.

In dog that descended from grey wolf, *Canis lupus*, some of the phenotypic changes related to domestication have already been linked to specific genes. For example, different alleles involved in the fight-or-flight response have been subject to strong selection and resulted in behavioural differences between dogs and wolves (Cagan and Blass 2016). Janowitz Koch et al. (2016) established that domestication of dogs has also been associated with epigenetic alterations. They analysed methylation differences in >24,000 cytosines distributed across the genomes of dog and wolves and revealed species-specific patterns of DMRs at 68 sites. The authors concluded that selection may have not only acted on genes but also on the methylation patterns.

Bélteky et al. (2018) investigated differences in hypothalamic DNA methylation between two selected lines of red jungle fowl, *Gallus gallus*, the ancestor of chicken, which were bred for either high or low fear of humans over five generations. The authors found 22 DMRs between the two lineages in genes involved in cellular metabolism and neural signalling. They concluded that selection for tameness can cause divergent epigenetic patterns within only five generations and that these changes may have had an important role in chicken domestication. Bélteky and colleagues also detected several sex-specific epigenetic changes on the autosomes

suggesting that epigenetic differences may play a role in gender-specific behavioural responses unrelated to sex chromosomes.

Anastasiadi and colleagues investigated DNA methylation and epigenetic alterations during early domestication in European seabass, *Dicentrarchus labrax*, one of the main farmed fish species in the Mediterranean (Anastasiadi et al. 2018; Anastasiadi and Piferrer 2019). A good-quality reference genome is available and selective breeding programmes are now applied to this species, which is at the beginning of domestication (Vandeputte et al. 2019). Anastasiadi and Piferrer (2019) analysed gene expression and DNA methylation changes in different tissues between wild and farmed specimens in the second generation. The number of differentially expressed genes (DEGs) ranged from 248 in the testis to 2416 in the liver, with an approximately equal number of upregulated and downregulated genes in early domesticates. Interestingly, in cultured sea bass with lower jaw malformation, a key feature of the domestication syndrome, some developmental genes were differentially expressed as well.

The number of DEGs that also contained DMRs was between 5 (testis) and 28 (muscle) as revealed by reduced representation bisulphite sequencing (RRBS). About one-fifth of the epimutations that occurred in adult domesticates were already established by the time of gastrulation and affected genes involved in developmental processes. For example, the *adamts9* gene that codes for an extracellular matrix metalloproteinase was hypomethylated in embryos of domesticated sea bass, and this pattern was maintained in the adult muscle (Fig. 3.23a), resulting in higher *adamts9* expression levels. Some of the epimutations recorded in the second generation domesticates significantly overlapped with cytosine-to-thymine mutations after 25 years of selective breeding (Fig. 3.23b), suggesting that epimutations can become integrated into the genome as genetic changes after some generations. The authors concluded from their work that epimutations in developmental genes underlie the onset of domestication in sea bass and assumed that these epimutations might be genetically fixed later on, explaining Darwin's domestication syndrome.

Konstantinidis et al. (2020) investigated the role of hydroxymethylation in domestication of Nile tilapia, *Oreochromis niloticus*, at a genome-wide level and single-nucleotide resolution and found that the muscle hydroxymethylome was changed already after a single generation of domestication (Fig. 3.23c, d). The overall decrease in hmC level in domesticated tilapia was accompanied by the downregulation of 2015 genes, mainly immune genes, whereas several myogenic and metabolic genes that affect growth were upregulated when compared to the wild specimens. There were 126 differentially hydroxymethylated cytosines between groups, which were not due to genetic variation. They were associated with genes involved in growth, immune response and neuronal pathways. The DHMCs were mostly located within gene bodies suggesting a functional role in gene expression.

Animals bred in captivity for the enhancement of sustainable fisheries or conservation often had lower fitness when compared to their wild counterparts. Artificial selection and respective genetic changes have been invoked as the most likely explanation for this reduced fitness. However, comparison of DNA sequence variation and genome-wide DNA methylation variation between hatchery-reared coho

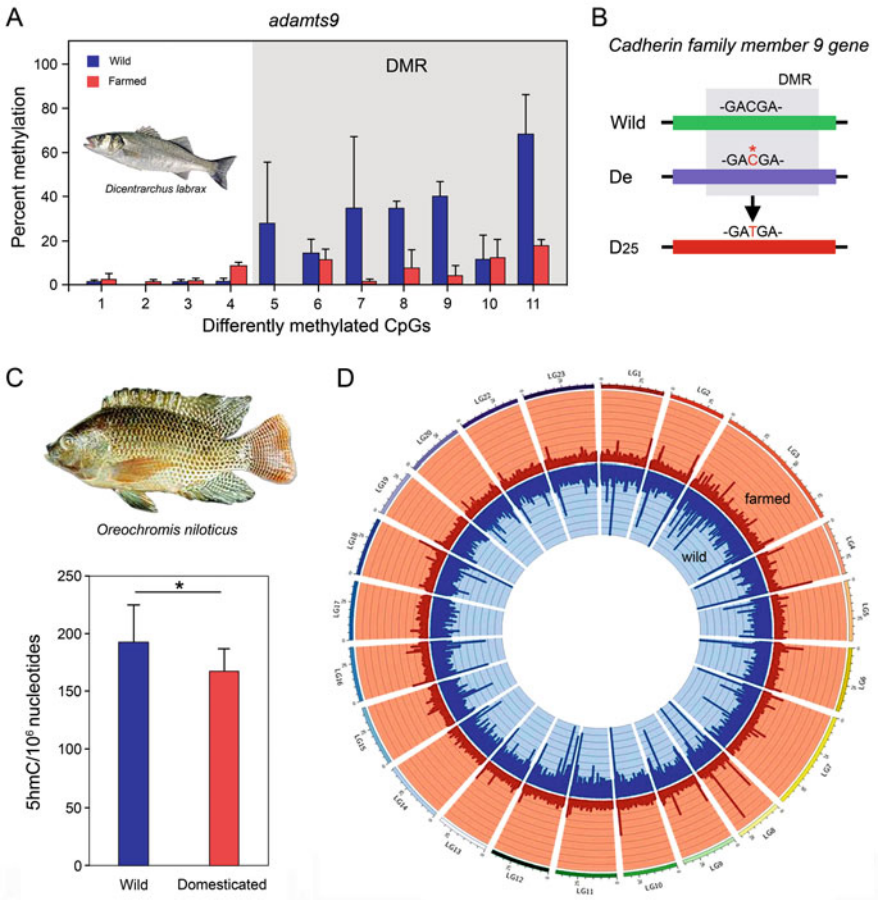

Fig. 3.23 Changes of DNA methylation and hydroxymethylation patterns in early domesticated fish. (**a**) Mean methylation of CpGs in DMR of matrix metalloproteinase gene *adamts9* in the muscle of sea bass, *Dicentrarchus labrax*, showing significantly lower methylation in farmed than wild specimens. (**b**) CpG in DMR of *cadherin family member 9* gene of sea bass that got methylated (asterisk) in early domesticates (De) and was converted into TpG after 25 years of selective breeding (D_{25}). (**c**) Difference of hydroxymethylated cytosines (5hmC) between fast muscles of wild tilapia, *Oreochromis niloticus*, and their offspring reared in captivity. Domesticates have a significantly lower 5mhC level. (**d**) Circular representation of the tilapia nuclear genome showing sites with substantial levels of 5hmC in wild specimens and their farmed offspring and differences between the two (**a** and **b** based on Anastasiadi and Piferrer 2019, Creative Commons Attribution NonCommercial License, http://creativecommons.org/licenses/by-nc/4.0/),; **c** and **d** based on Konstantinidis et al. 2020, Creative Commons Attribution-NonCommercial-NoDerivatives License, http://creativecommons.org/licenses/by-nc-nd/4.0/)

salmon, *Oncorhynchus kisutch*, and their wild parent populations revealed a highly significant proportion of epigenetic variation despite the absence of overall neutral and adaptive genetic variation (Le Luyer et al. 2017). Shared epigenetic variation

(mostly hypermethylation) between hatchery-reared salmons of different natural origin and differences to their source populations provided evidence for directional epigenetic modifications that can arise in a single generation in the hatchery environment.

3.7.2 Contribution of Epigenetic Mechanisms to Speciation

New animal species mostly arise by the divergence of allele frequencies between or within populations and the establishment of reproductive barriers. Less frequently, species originate by the duplication of entire genomes (autopolyploidy) or the fusion of two different genomes by hybridization (allopolyploidy) (Faria and Navarro 2010). All types of speciation are accompanied or followed by reproductive isolation, chromatin remodelling, alteration of gene expression and changes of life history features. These changes are particularly prominent in polyploid species and epigenetic mechanisms can apparently contribute to all of them as exemplified in the following.

The generation of new species and new higher taxa by polyploidy has played a considerable role in animal evolution (Gregory and Mable 2005; Abbott et al. 2013). A well-established higher taxon example is the vertebrates, which have experienced two rounds of polyploidy in their stem line and a further round in the stem line of the teleost fishes (Albalat et al. 2012). During these events, the copy numbers of DNMT3 and TET increased from one to three. Extant polyploid species are relatively frequent in water fleas, insects, fishes and amphibians (Gregory and Mable 2005).

Reproductive isolation is an important requirement for the separate evolution of a new species. An example for the involvement of DNA methylation in reproductive isolation is the deer mouse species complex *Peromyscus maniculatus*, in which imprinting of genes involved in placentation has led to reproductive isolation (Vrana 2007). Smith et al. (2016) found that changes in the methylome can foster the evolution of behavioural reproductive isolation between populations of tessellated darter fish, *Etheostoma olmstedi*. Laporte et al. (2019) investigated differently methylated transposable elements (TE) in the "dwarf" and "normal" whitefish, two species of the *Coregonus clupeaformis* species complex that diverged some 15,000 generations ago. They recorded an involvement of DNA methylation reprogramming and derepression of TEs in postzygotic isolation.

New polyploid genomes are usually unstable and require chromatin remodelling (Madlung and Wendel 2013). One common feature is the reduction of the DNA content. A well-investigated example is the plant *Phlox drummondii*, in which synthetic autopolyploids experienced a loss of 17% of total DNA immediately after polyploidization and a further reduction of up to 25% upon the third generation (Parisod et al. 2010). DNA loss mostly concerns redundant genes, and in extreme cases, the polyploid genome can be downsized to the diploid state. For example, after doubling of the genome in the stem line of fishes, 70–80% of duplicated genes have been lost (Inoue et al. 2015). In the hexaploid plant *Brassica rapa*, gene loss

has apparently been driven by differential DNA methylation (Chen et al. 2015). Gene copies with higher methylation levels and correspondingly lower levels of expression were more prone to loss than copies with lower methylation levels.

Alteration of gene expression is increasingly recognized as an important mechanism of speciation. According to classical concept, speciation-related gene expression changes are mainly caused by genetic mutations in promoters, enhancers and silencers. However, a substantial fraction of gene expression differences across species is apparently due to differences in DNA methylation, histone modifications and ncRNA pathways (Cain et al. 2011; Gallego Romero et al. 2012; Franchini et al. 2016).

In polyploid animals, the DNA methylation level can either be higher or lower when compared to the parent species. For example, hybrids of kangaroos *Macropus eugenii* x *Wallabia bicolor* were characterized by a genome-wide hypomethylation (O'Neill et al. 1998). Removal of DNA methylation from retrotransposons in the hybrids facilitated their amplification and caused gross changes in genome structure. An increase of DNA methylation compared to the parent species was observed in hybrids of red crucian carp *Carassius gibelio auratus* x common carp *Cyprinus carpio* (Xiao et al. 2013).

In hybrids of the frogs *Xenopus laevis* x *Xenopus muelleri*, 364 out of the 546 investigated MSAP markers exhibited differences in methylation patterns when compared to the parental species, indicating intense contribution of epigenetic mechanisms to shaping of the new genome (Koroma et al. 2011). Hybrids exhibited a significantly higher proportion of methylated fragments relative to both parental species, which may translate into changes of gene expression. Moreover, 76 methylated fragments were diagnostic of hybrids only. These new epigenetic patterns may indicate the involvement of epigenetic mechanisms in restructuring of the hybrid genome. Interestingly, female hybrids are fertile but male hybrids are sterile. Differential methylation between sexes and misexpression of genes responsible for reproduction in males may account for these differences (Malone et al. 2007; Koroma et al. 2011).

Polyploids often have life history traits that are different from those of the parent species (Xiang et al. 2006; Krois et al. 2013). Growth, number of offspring and other quantitative traits can either decrease or increase when compared to the diploid ancestors. In allopolyploids, the increase of life history traits is usually explained as the result of heterozygosity (hybrid vigour). This explanation is not applicable for autopolyploids, which have the same set of genes as their parent species. In autopolyploids, trait alteration is rather caused by changes of gene dosage, rearrangement of gene networks and modulation of gene expression. All of these changes obviously require the contribution of epigenetic mechanisms. As an example, during ancient mammalian gene duplication DNA methylation apparently played a dominant role in dosage rebalance by inhibiting transcription initiation of duplicate genes (Chang and Liao 2012).

In marbled crayfish, *Procambarus virginalis*, which originated from Floridian slough crayfish, *Procambarus fallax,* by autotriploidy and concomitant transition from gonochorism to parthenogenesis, speciation was accompanied by reproductive

isolation and marked alterations in DNA content, global DNA methylation level and life history traits (Vogt et al. 2015, 2019; Gatzmann et al. 2018). However, their morphological appearance and coloration remained very similar to the parent species. In laboratory experiments, marbled crayfish females readily copulated with males of *Procambarus fallax*, but the offspring was always pure marbled crayfish as demonstrated by microsatellite analysis (Vogt et al. 2015), indicating that reproductive isolation occurs at the cytological or genetic rather than the behavioural level.

Body size and fecundity are significantly enhanced in marbled crayfish (Fig. 3.24a) indicating superior fitness (Vogt et al. 2019). Marbled crayfish grows to a maximum total length of ~13 cm and a body weight of 52 g, whereas *Procambarus fallax* grows to a maximum of ~9 cm and 18 g. The triploid marbled crayfish has a 1.4-fold instead of a 1.5-fold increased DNA content when compared to its diploid parent species (Fig. 3.24b), suggesting loss of some DNA after polyploidization (Vogt et al. 2015). Global DNA methylation is about 20% lower in marbled crayfish (Fig. 3.24c) (Vogt et al. 2015), and there are differences in gene body methylation in hundreds of genes between both species (Fig. 3.24d) (Gatzmann et al. 2018), arguing for a considerable remodelling of the DNA methylation pattern and gene expression during speciation.

3.7.3 *Transgenerational Inheritance and Genetic Integration of Epigenetically Mediated Phenotypes*

The evolutionary role of epigenetics is very much dependent on whether epigenetically caused phenotypes and the underpinning epigenetic signatures are transgenerationally inherited or not (Burggren 2016; Jablonka 2017; Perez and Lehner 2019; Casas and Vavouri 2020; Anastasiadi et al. 2021). Since transgenerational epigenetic inheritance (TEI), the transmission of alternative phenotypic and functional states through multiple generations in the presence of the same DNA sequence, is intensely discussed in another chapter of this book, I will only shortly address this topic, mainly considering studies discussed in this chapter.

Empirical research clearly indicates that only a minor proportion of epigenetically mediated phenotypic traits is transgenerationally inherited. For example, neither the status of being a honeybee queen nor the marmoration pattern of the marbled crayfish is inherited. In these cases, it is the ability to generate these diverse phenotypes from the same genome that is inherited, and this ability enables each generation to produce them anew. In mammals, DNA methylation marks are largely erased and reprogrammed in the early developmental stages (Seisenberger et al. 2012), and this behaviour was generalized for all epigenetic mechanisms and all animal groups and raised as main argument against TEI. However, in zebrafish, *Danio rerio*, the paternal methylome is largely maintained throughout early embryogenesis, whereas the maternal methylome is maintained until the 16-cell stage and then progressively reprogrammed by parallel losses and gains of methylation marks

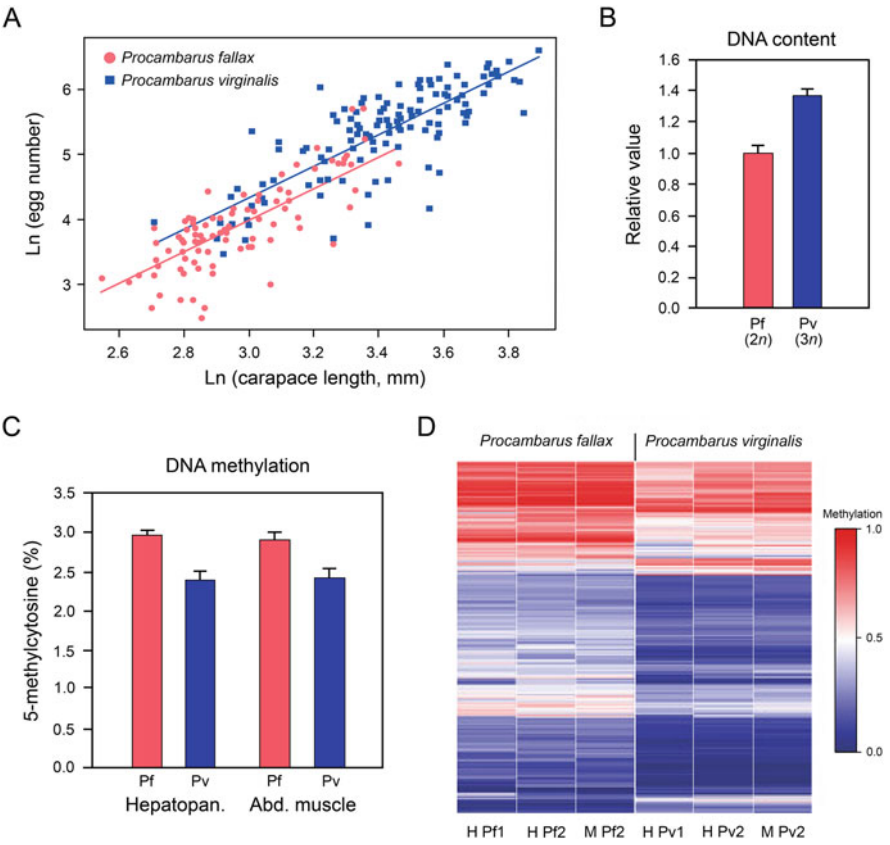

Fig. 3.24 Comparison of fecundity, DNA content and DNA methylation between triploid crayfish *Procambarus virginalis* (Pv) and its diploid parent species *Procambarus fallax* (Pf). (**a**) Pleopodal egg numbers per female and clutch. The graph shows that fecundity is on average much higher in *P. virginalis* due to bigger body size, but it is also ca. 40% higher in *P. virginalis* of equal sizes as indicated by the linear model prediction lines. (**b**) DNA content in haemocytes. Flow cytometry of two biological and three technical replicates demonstrates a ~ 1.4 fold higher DNA content in triploid *P. virginalis* (Pv) when compared to diploid *P. fallax* (Pf). $P = 1.33 \times 10^{-7}$. (**c**) Global DNA methylation levels in hepatopancreas and abdominal musculature. Mass spectrometry of the organs of three laboratory-raised females per species revealed a significant, ca. 20% lower DNA methylation level in *P. virginalis*. $P = 1.48 \times 10^{-7}$. (**d**) Heat map of gene body methylation of 2357 genes in the hepatopancreas (H) and abdominal muscle (M) of two *P. fallax* (Pf1, PF2) and two *P. virginalis* (Pv1, PV2), showing numerous differentially methylated genes. Most genes are hypomethylated in *P. virginalis* (**a** based on Vogt et al. 2019, with kind permission from Elsevier; **b** and **c** based on Vogt et al. 2015, Creative Commons Attribution License CC BY 3.0, http://creativecommons.org/licenses/by/3.0/; **d** based on Gatzmann et al. 2018, Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/)

(Jiang et al. 2013). In honeybee, there is no DNA methylation erasure in the gametes and zygote and DNA methylation marks are stably transferred from fathers to daughters (Yagound et al. 2020). The same holds for the phylogenetically basal corals (Liew et al. 2020). And even the mammalian genome can bypass epigenetic reprogramming during development and transmit information from parents to offspring via DNA methylation, histone modifications and miRNAs (Baxter and Drake 2018; Hao et al. 2021).

There is considerable evidence that epigenetic mechanisms are involved in TEI of animal phenotypes although long-term studies over dozens of generations are still missing (Casas and Vavouri 2020). Sperm seems to be particularly effective in transmitting epigenetic information to the next generation via conservation of DNA methylation and histone modification patterns and ncRNAs as exemplified in animals diverse as corals and rat (Liew et al. 2020; Beck et al. 2021). In experiments with fruit fly *Drosophila melanogaster*, Ciabrelli et al. (2017) demonstrated that TEI of phenotypes is associated with transgenerational transmission of particular chromatin states. H3K27me3 and polycomb group proteins were shown to play a crucial role in epiallele establishment, maintenance and inheritance.

Whether an epigenetic pattern is inherited or not seems to depend on trait but also on the conditions. In a well-adapted population living in a constant environment, it makes little sense to inherit epigenetic variants over many generations because it would incur costs but provide no advantage. However, if the environment changes from one stable condition to another stable condition, then new and better suited epigenetic variants may be selected and transgenerationally inherited to better cope with the new conditions. Deterministic selection models showed that newly arising epimutations are principally stable enough to respond effectively to long-term selection yielding epimutation–selection equilibria that are close to those expected for DNA sequence mutation rates (Van der Graaf et al. 2015). Kronholm and Collins (2016) showed with their adaptive walks model on asexual populations that the long-term effects of epimutations depend crucially on their stability and fitness effects relative to genetic mutations.

In sexually reproducing species, beneficial epigenetically determined phenotypes are thought to be fixed on the long term by genetic assimilation, a process by which a phenotype originally produced in response to environmental signals is later taken over by the genotype via selection on random genetic mutations with similar phenotypic effects (Waddington 1953; Pigliucci et al. 2006; Ehrenreich and Pfennig 2016). An alternative, more directional mechanism, which would also be applicable to asexually reproducing species, is the facilitated conversion of epimutations with phenotypic effects to corresponding genetic mutations. The transcription factor binding sites of genes may serve as an illustrative example. CpGs in these regions are usually unmethylated in active genes. Their methylation can block access of transcription factors to the DNA, thereby silencing the gene (Yin et al. 2017). Epigenetic silencing of a gene can change gene networks and lead to the alteration of biochemical, morphological or behavioural traits that are regulated by this network. If such a functionally important methylated CpG should mutate into TpG, which occurs with 10–50-fold higher probability than in unmethylated CpGs

(Lutsenko and Bhagwat 1999), then this site is not only temporarily and reversibly blocked but irreversibly silenced. This way, a principally reversible, epigenetically determined phenotype could become a permanent, genetically encoded phenotype, and if the epimutation-to-genetic mutation transition occurs in the germline or somatic cells that later develop into germ cells, the genetically fixed phenotype is heritable and evolutionarily established.

Evidence for this possibility comes from different sources. Firstly, the CpG-to-TpG transition is by far the most common single-nucleotide mutation in living organisms, and it is promoted by methylation of cytosines (Walser and Furano 2010). Secondly, the example of domesticated seabass described above has shown that certain methylated CpGs that were established in the second generation of domestication in response to the new environment appeared as TpGs in specimens after 25 generations of culture (Anastasiadi and Piferrer 2019). Thirdly, in bacteria, methylated cytosines were identified as hot spots for cytosine-to-thymine mutations that modulate antibiotics susceptibility (Ghosh et al. 2020), changing an important fitness trait. Last but not least, most animals display lower observed than expected densities of CpG dinucleotides in their genomes (Yi and Goodisman 2009). This feature may not just be the result of meaningless random mutations and inefficient repair mechanisms, as often believed, but may rather reflect multiple integrations of phenotypically relevant and selected epimutations into the genome, driving the evolution of species.

3.8 Discussion

The driving forces behind the production of phenotypic diversity are genetic changes of the DNA sequence, alternative splicing, developmental programmes, developmental stochasticity and environmental induction. In the latter cases, epigenetic mechanisms are among the underpinning molecular mechanisms. Of course, epigenetic processes are dependent on the information in the genome like CpG sites and genes for DNA methyltransferases, histone-modifying enzymes and ncRNAs, but they can produce significant phenotypic diversity in the absence of DNA sequence variation, contradicting the previous "one genotype maps to one phenotype" concept (discussed in Pigliucci 2010). This insight has striking consequences for biology, particularly development, ecology, evolution and applied fields like pathology and domestication.

3.8.1 Generation of Phenotypic Diversity with the Help of Epigenetic Mechanisms

Epigenetic mechanisms are crucially involved in the production of a first layer of diversity above the DNA sequence by modifying transcription and further

downstream processes, which finally results in the expression of multiple phenotypes. There is sound evidence that epigenetic mechanisms are also involved in the stabilization of phenotypes across generations, supporting the possibility of inheritance of acquired characters.

Investigations with animals ranging from Porifera to Vertebrata revealed that the phenotypic effects of DNA methylation marks depend on their location in the genome. Methylation of CpGs in promoter regions is usually associated with gene silencing, and methylation of transposons and repeats results in repression as well. In contrast, methylation of CpGs in gene bodies modulates gene expression mainly by changing accessibility of the genes in the chromatin. Gene body methylation is often negatively correlated with phenotypic variation (Schübeler 2015; Gatzmann et al. 2018; Greenberg and Bourc'his 2019). It is also thought to be involved in gene duplication, facilitating functional diversification of the genome (Branciamore et al. 2014; Asselman et al. 2016), and in removal of long-term silenced gene duplicates from the genome (Chen et al. 2015).

Histone modifications are less well investigated in animals, but they may even have greater effects on phenotypic plasticity than DNA methylation as implied from studies with honeybee queens and workers (Wojciechowski et al. 2018). Histone methylation often represses gene expression and acetylation often stimulates gene expression, depending on the site of the modification (Lennartsson and Ekwall 2009; Bannister and Kouzarides 2011; Allis and Jenuwein 2016). ncRNAs and chemical modifications of the mRNA also contribute to the generation of phenotypic variation in animals, but information is relatively scarce (Franchini et al. 2016; Gajigan and Conaco 2017; Zhao et al. 2020). Interestingly, miRNAs can transfer epigenetic information from parent to filial generation via the sperm (Chen et al. 2016b).

It is a long-running discussion whether epigenetic variation in populations is the mere consequence of DNA sequence variation or whether it can arise independently. Experiments with clonal animals clearly revealed that epigenetic variation and related phenotypic plasticity can be surprisingly broad despite the virtual absence of genetic variation, and therefore, it must be considered as a source of phenotypic variation in its own right.

Research with model and non-model animals has shown that epigenetic mechanisms are involved in quite different contexts of animal biology. They help to establish and maintain different cell types and tissues in metazoans and strikingly different life stages in holometabolous insects. These processes are deterministic (the outcome is always the same) and are not significantly influenced by other factors like the environment. Epigenetic mechanisms also mediate polyphenism, the generation of alternative phenotypes in the same life stage by environmental cues or a continuum of phenotypes in populations in response to environmental signals, which are directional processes (same cue leads to same phenotype). On the other hand, the production of different phenotypes from the same genome via stochastically established epigenetic marks is a probabilistic process. Thus, epigenetic mechanisms support quite different processes and strategies: (1) the unfolding of genetic programmes (stereotypic expression of evolutionarily shaped phenotypes), (2) the

fast phenotypic adaptation of larger proportions of a population to the prevailing conditions (directional adaptation strategy), and (3) the a priori production of a range of phenotypes around an optimized target phenotype to prepare the population for future changes of the environment (bet-hedging strategy).

3.8.2 Relevance of Epigenetically Mediated Phenotypic Variation for Development, Ecology and Evolution

Epigenetic profiles and associated phenotypes can change throughout the entire life, but their dynamics is particularly pronounced during embryonic development, when the tissues and organs are formed. First evidence suggests that global DNA methylation increases with increasing age in determinately growing animals like mammals but remains rather constant in indeterminately growing animals like crustaceans (Fraga et al. 2005; Vogt et al. 2008). The methylation level of specific sites in the genome like the binding sites of transcription factors was shown to be positively correlated with biological age in vertebrates and was therefore regarded suitable as an epigenetic clock (Bell et al. 2019; Raddatz et al. 2021).

The power and relevance of epigenetically mediated phenotypic variation in animal ecology are most convincingly shown on the example of monoclonal invaders that adapted to different environments despite genetic uniformity such as the New Zealand mud snail and the marbled crayfish (Thorson et al. 2017; Vogt 2022b). Further illustrative examples are adaptive radiations after the invasion of new geographical regions by small founder populations like the Darwin's finches on Galapagos (Skinner et al. 2014), adaptations to extreme environments (Gore et al. 2018) and the persistence of sessile species under adverse environmental conditions (Liew et al. 2020).

In asexual populations, epigenetic mechanisms are the main drivers of phenotypic variation because random genetic mutations are rare and meiotic recombination of the genome is lacking. In sexually reproducing populations, epigenetically caused phenotypic variation apparently supplements genetically caused phenotypic variation. The generation of epigenetically based phenotypic variation seems to be particularly important for species with long generation times, because evolutionary responses to environmental changes via natural selection on genetic variants may not be fast enough to mitigate such changes.

There are some striking differences in quality and function between genetically caused and epigenetically caused phenotypic variation. Epigenetically based phenotypic variants can be established in response to environmental cues in many population members within one generation, whereas a favourable genetic variant arisen by random mutation or recombination first occurs in single specimens only and requires multiplication and selection over many generations to become frequent in the population. Once established, a new genetic variant changes the population

permanently, whereas epigenetically caused phenotypic variants change the population only temporarily because they are principally reversible.

Combined with TEI, the production of epigenetically based phenotypic variation would be a perfect means to cope with transient environmental stressors and environmental changes (Burggren 2016). If the adverse conditions should disappear in the lifetime of the exposed generation or the subsequent generations, the epigenetic marks and related phenotypes could be reverted to the old state, but when the adverse conditions should become permanent the epigenetic variants could persist and get selected and genetically integrated in the long term. Thus, genetically based and epigenetically based phenotypic variations seem to have different, complementary functions in animal ecology. Interestingly, the model nematode *Caenorhabditis elegans* possesses a timing mechanism that controls the duration of transgenerational inheritance of small RNAs (Houri-Ze'evi and Rechavi 2017), which may help in the decision to propagate or reset an epigenetic phenotype.

The relevance of epigenetics for animal evolution is less well understood, but evidence from domestication and polyploid speciation suggests crucial roles in this central field of biology as well (Vogt et al. 2015; Gatzmann et al. 2018; Anastasiadi and Piferrer 2019). Examples of polyploid speciation from different animal groups demonstrated that epigenetic mechanisms help to consolidate evolutionary processes triggered by genetic change. They contribute to reproductive isolation, chromatin rearrangement and alteration of gene expression in the neospecies, finally leading to novel phenotypes (O'Neill et al. 1998; Inoue et al. 2015; Vogt et al. 2015, 2019; Smith et al. 2016). Conversely, domestication experiments demonstrated that epigenetically based phenotypic variation can already appear in the first cultured generation (Anastasiadi and Piferrer 2019; Konstantinidis et al. 2020), enabling selection of desired traits long before sufficient genetic variation is available. Selection of such epigenotypes combined with TEI could be the starting point of new evolutionary trajectories.

TEI is probably the most controversial topic of evolutionary epigenetics in animals, but evidence for its prevalence is steadily increasing (Burggren 2016; Jablonka 2017; Perez and Lehner 2019; Casas and Vavouri 2020; Anastasiadi et al. 2021). Apparently, only a minor fraction of epigenetic variants are passed on to the next generation, depending on many factors like trait, environment and selective advantage. There are new ideas aside of classical genetic assimilation (Waddington 1953) on how reversible epimutations with phenotypic effects could be integrated into the genome. The first alternative is based on the conversion of methylated CpGs into TpGs as explained above, and the second alternative is based on epigenetically activated and silenced copy number variants (CNVs) with slightly different DNA sequences (Vogt 2015a). In the latter scenario, the best suited, long-term active CNVs may duplicate over time, and long-term silenced CNVs marked by higher methylation levels may be removed from the genome, leading to genetic alteration of the genome in comparison to the initial form. In contrast to classical genetic assimilation that requires genetic variation in the population to be effective, these concepts also work in asexually reproducing populations lacking genetic variation.

In stable geological times, epigenetically triggered phenotypes may only rarely be selected, transgenerationally inherited and genetically integrated, because they provide no advantage but additional costs, and thus, their contribution to generation of phenotypic diversity and speciation may remain relatively low. However, when the conditions change between two different period of stasis, for example, via climate change and sea level rise, then epigenetically determined phenotypes may become advantageous and important. These may be selected, inherited and genetically integrated in much higher rates, speeding-up phenotypic diversification and generation of new taxa in such periods of crisis. The occurrence of EIPV-triggered and epigenetically mediated pulses of evolutionary change between periods of relative stasis may provide a mechanistic explanation of the Punctuated Equilibrium Theory of evolution proposed by Eldredge and Gould (1972).

3.8.3 Perspectives

Future research on the relationship of epigenetics and phenotypic plasticity sensu lato in animals requires suitable models for laboratory experiments and field studies, highly sensitive analytical techniques and sophisticated study designs that consider the specificities of epigenetics more carefully than before. Particularly, suitable laboratory test animals are obligatory parthenogenetic invertebrates such as *Potamopyrgus antipodarum*, *Procambarus virginalis* and *Daphnia* species and clonal vertebrates such as gynogenetic fish, parthenogenetic lizards, polyembryonic armadillos, and cloned and highly inbred mammals, in which confounding genetic variation is negligible. The genomes of most of these promising models are already sequenced and assembled, which is an important precondition for comprehensive characterization of genome–epigenome–phenotype relationships. Good field model systems are invasions with known history, adaptive radiations, animals from extreme habitats and sessile animals.

Epigenetic signatures in animals depend on many factors including genome, tissue, developmental stage, life history, environmental conditions and health state. Future studies should better consider this aspect and standardize as much influencing factors as possible to obtain meaningful results. For example, pooling of different tissues and individuals must be avoided. Moreover, sample sizes should be larger than in many earlier studies to generate statistically significant results. Previously, both requirements were difficult to implement due to limited sensitivity and high costs of the available analytical techniques. Now, this is possible by using recently developed omics techniques that enable fast analysis of small-sized tissue samples at reasonable time and costs (e.g. Simpson et al. 2017; Stuart and Satija 2019; Liu et al. 2020).

Experimentally, most challenging is the TEI issue. In order to identify the conditions under which epigenetic signatures with phenotypic effects are inherited, selected and genetically integrated, the experimental animals must be kept for many generations in both the same environment as the parents and in strikingly different

environments. Such experiments are best performed with clonal animals with known genomes and epigenomes and relatively short generation times, e.g. water fleas (*Daphnia*) or brine shrimp (*Artemia*).

Most papers on epigenetics and phenotypic plasticity in animals demonstrated correlations between epigenetic marks and phenotypes, while cause–effect relationships between the two remained largely elusive. For example, it is an open question how many single methylation polymorphisms (SMPs) are needed to change a phenotype. Single SMPs may have little phenotypic effects but many of them together constitute DMRs, which were shown to be able to cause marked phenotypic changes (Rakyan et al. 2002; Weigel and Colot 2012). The relationships between epimutations and phenotypes could be investigated in more detail by epigenome-wide association studies (Cortijo et al. 2014) and the experimental manipulation of the epigenome. The latter could be done by RNAi, pharmacological blockers of DNA methylation and histone modification, and engineering of genes and epigenetic marks of interest by CRISPR-Cas. CRISPR-Cas is suitable to modify epigenetically determined phenotypes by excising or inserting genes that encode DNA methyltransferases, histone-modifying enzymes and ncRNAs or by adding and removing individual bases and methylation marks (Xu et al. 2016; Rees and Liu 2018; Kang et al. 2019; Urbano et al. 2019).

The last perspective to be discussed is the need to integrate epigenetically based phenotypic variation into evolutionary theory. The generation of phenotypic variation, on which natural selection can act, is a central tenet of the prevailing neo-Darwinian Modern Synthesis. Traditionally, random DNA sequence mutations and other genetic alterations are considered the main sources of phenotypic variation. In this theory, the environment plays an important role, but only as a selector of the fittest phenotypes. An extended evolutionary synthesis has recently been developed that also considers non-genetic processes for the generation of phenotypic variation, which would be a paradigm shift (Laland et al. 2015). In this theory, which unifies Darwinian and Lamarckian aspects of evolution, the environment also acts as an inducer of phenotypic variation. Skinner and Nilsson (2021) have recently advocated for the integration of environmentally induced epigenetic TEI into an Extended Modern Synthesis.

I here advocate for also including SDPV into an extended evolutionary theory, which is probabilistic like genetic mutations and therefore Darwinian-like. SDPV is several orders of magnitude more frequent when compared with genetic mutations, and therefore, it is expected to induce phenotypic variation much more rapidly. On the other hand, epimutations are less stable and reversible and the average rate of their transgenerational inheritance and final genetic integration is not yet known. The integration of EIPV and SDPV into the evolutionary theory may help to explain, why animal evolution is often much faster than expected from selection on genetically caused phenotypic variation alone and why there are periods of relative stasis and fast progression in the evolution of life.

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Chapter 4
Role of Environmentally Induced Epigenetic Transgenerational Inheritance in Evolutionary Biology

Jennifer L. M. Thorson and Michael K. Skinner

Contents

Abstract The mid-twentieth century saw the incorporation of Mendelian genetics into Darwinian theories of evolution. This foundation, termed the modern evolutionary synthesis, has developed into the primary current paradigm of evolutionary biology. However, the current modern synthesis does not include a role for epigenetics in developmental modifications or any mechanisms of non-genetic inheritance. With the recent expansion of epigenetic research into non-genetic mechanisms of adaptation and inheritance, there is a need to expand the modern synthesis into a new extended evolutionary theory. The current chapter presents the role of environmentally induced epigenetic transgenerational inheritance in evolutionary biology.

Keywords Epigenetics · Epigenetic transgenerational inheritance · Phenotypic plasticity · Adaptation · Evolution

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4.1 The Modern Synthesis

The modern evolutionary synthesis is based on the theories of evolution and natural selection as described by Charles Darwin and Alfred Russel Wallace in the mid-nineteenth centuries (Jablonka 2017). In these theories, adaptive evolution occurs when four proposed postulates are met. These include: (1) variation within a population, (2) variation is heritable, (3) competition occurs between offspring for limited resources, and (4) the survival and reproduction of the offspring are not random but are associated with the heritable variation (i.e., genetic inheritance) (Darwin 1859). With these postulates of evolution by natural selection as a foundation, the discoveries of Mendelian genetics, which described how traits could be inherited as well as the discovery of the genetic materials deoxyribonucleic acid (DNA) and ribonucleic acid, provided the molecular mechanisms of inheritance of adaptive traits and the trajectory of adaptive evolution. The field of population genetics formalized the study of Mendelian genetics and the implications for inheritance and adaptation. All of these developments eventually lead to the development and formalization of the modern evolutionary synthesis in the twentieth century, with the term coined by Julian Huxley in his 1942 book (Huxley 1942).

Ideas of phenotypic plasticity and non-genetic inheritance were not incorporated into the modern synthesis. At the end of the nineteenth century, James Mark Baldwin examined the response of *daphnia* to the presence of predators in their environment. Baldwin published a paper in 1896 proposing a mechanism whereby organisms interact with a changing environment and develop adaptive traits, which were then passed on to their offspring (Baldwin 1896). This phenomenon was termed as the Baldwin effects and was most often incorporated in psychological research, though evidence has accumulated for the Baldwin effect in evolutionary biology (e.g., Crispo 2007). In the early nineteenth century, Paul Kammerer demonstrated in the midwife toad, an environmentally (i.e., arid or aquatic) induced parent-of-origin non-genetic acquired reproductive traits (Vargas et al. 2017). In the mid-nineteenth century, Conrad Waddington pioneered investigations into the phenotypic plasticity with experiments examining the effects of heat shock on *Drosophila* wing shapes in the 1940s (Waddington 1940). Waddington found that after several generations of exposure to heat shock, an adaptive wing shape became "canalized" in the population, by which he meant the trait was retained in a population regardless of the genotype or environment. These results lead Waddington to coin the term "developmental epigenetics" to describe the phenotypic response to the environment (Waddington 1940). The initial genetic terminology used to describe effects such as those observed by Baldwin, Kammerer, and Waddington was genetic assimilation, where heritable changes occur in response to a novel environmental pressure (Crispo 2007). Despite early evidence for these phenomena, interest soon waned in favor of strictly genetic inheritance of traits in the absence of any non-genetic mechanisms. When the modern synthesis was formalized, ideas of soft inheritance, described by Ernst Mayr as "gradual change of the genetic [hereditary] material itself, either by use or disuse, or by some internal progressive tendencies, or through

the direct effect of the environment" (Mayr 1980) were strictly left out of the modern synthesis without a specific molecular mechanism to be considered (Jablonka 2017).

Aside from the evidence supporting the Baldwin effect and genetic assimilation, and epigenetic phenomenon proposed by Waddington, there are other phenomena long accepted by the evolutionary community to serve as mechanisms of inheritance. The first being maternal effects, which have long been documented in both plant and animal breeding and quantitative genetics (Falconer 1996). The maternal environment can affect offspring development and fitness, which can influence adaptation across generations (Mousseau and Fox 1998). Maternal effects on offspring fitness are both non-genetic and heritable, so are a form of adaptive non-genetic (i.e., intergenerational) inheritance. Moreover, epigenetic inheritance is implicated as a part of the parental effects inherited by offspring (Danchin et al. 2019; Skinner 2015). There has been recent interest in two additional non-genetic forms of inheritance. Prions are proteins which have the capacity to incorporate changes that last over many cycles of mitosis and meiosis and thus serve as a non-genetic mechanism of inheritance (i.e., intergenerational) (Harvey et al. 2018). Prions may even serve as facilitators of other forms of epigenetic inheritance, for example, altered chromatin states (Harvey et al. 2020). If prion-mediated alterations lead to adaptive phenotypic change, this is an alternative route to non-genetic inheritance (i.e., intergenerational) of adaptive traits. Finally, horizontal gene transfer is a common phenomenon in bacteria and may even influence eukaryotic organism's nutrition, protection, and adaptation to extreme environments (Husnik and McCutcheon 2018). While horizontal gene transfer does involve alterations and inheritance of genetic material, it is outside the typical vertical inheritance described in the modern synthesis and is therefore a candidate to be incorporated as a novel mechanism of inheritance (i.e., intergenerational).

The recent research and evidence for the phenomena described above has led to the proposition of an extended evolutionary synthesis (EES) (Pigliucci 2007; Pigliucci and Muller 2010). The EES would take the tenets of the modern synthesis and build upon them, adding what has been demonstrated in evolvability, phenotypic plasticity, epigenetics and epigenetic inheritance, and evolution on adaptive landscapes (Pigliucci 2007). The authors who originally proposed these ideas were careful to argue that this EES would not be a "paradigm shift" as none of the new evidence directly opposes the original modern synthesis, but instead propose a shift from the population genetic-centered view that originally characterized the modern synthesis (Pigliucci and Muller 2010). While this debate continues in the evolutionary biology community (Baedke et al. 2020; Futuyma 2017; Muller 2017), there is sufficient evidence to suggest that non-genetic forms of inheritance are implicated in all aspects of evolution (Adrian-Kalchhauser et al. 2020; Bonduriansky et al. 2012; Richards 2006; Stajic and Jansen 2021; Sultan 2017). In particular, epigenetic inheritance of environmentally influenced alterations is implicated in adaptive evolutionary change (Nicoglou and Merlin 2017; Nilsson et al. 2020; Norouzitallab et al. 2019; Skinner 2015).

4.2 Molecular Epigenetic Mechanisms

The regulation of gene expression and genome activity requires a variety of molecular epigenetic mechanisms. The most extensively studied epigenetic mechanism is DNA methylation. DNA methylation involves the attachment of a small methyl group to DNA which produces 5-methylcytosine (5mC). This attachment occurs primarily at the cytosine base when it is adjacent to a guanine residue (Singer et al. 1979). Other chemical modifications of cytosine and adenine bases in DNA can occur and are far less frequent potential mechanisms of non-genetic adaptation.

DNA is wrapped around histone proteins to form the nucleosome, and these histone proteins can be chemically modified to alter gene expression. These histone post-translational modifications act to facilitate downstream functions in chromatin (Rothbart and Strahl 2014). The downstream effects of histone modifications include changing chromatin structure, recruiting transcriptional cofactors to regulate gene expression, and even repressing gene expression in heterochromatin regions of the genome. The variety of forms and effects of histone modifications is extensive and complex (Bartova et al. 2008; Taylor and Young 2021). Additional possible sources of epigenetic variation can be found in the presence of histone variants, in the spacing between nucleosomes and the position of chromatin in the nucleus (Margueron and Reinberg 2010). The modulation of these components is critical for the regulation of gene expression through determination of accessibility and sequential recruitment of regulatory factors to the DNA sequence (Quina et al. 2006). In the male germline, the sperm histone retention is also critical for the early embryo and involved in epigenetic inheritance (Ben Maamar et al. 2021).

The action of non-coding RNA molecules as epigenetic factors has been explored extensively (Huang et al. 2014; Wei et al. 2017). Non-coding RNAs are small and long, and do not code for any protein. They instead function as regulatory toward gene expression (Kornfeld and Bruning 2014). These RNA molecules are considered epigenetic factors as they are not dependent on DNA sequence and do not rely on a complimentary nucleotide sequence to function. Epigenetic modifications can occur on RNA molecules, which then affect translation and gene expression (Sibbritt et al. 2013). Methylation of adenosine to form N6-mA is the most common modification to the internal sequence of mRNA, and this reversible modification is associated with post-transcriptional gene expression regulation (Fu et al. 2014; Yue et al. 2015). Sperm ncRNAs are postulated as important molecular mechanisms that can transmit gene regulatory information across generations and in response to environmental pressures (Sharma 2017).

Since all these epigenetic processes can be altered in the germline (i.e., sperm and egg), following fertilization they can impact the early embryo epigenetics and transcriptomes to influence the offspring and subsequent generations. The repeated demonstration of epigenetically facilitated transgenerational inheritance of altered phenotypes suggests that this molecular mechanism plays a significant role in ecology and evolution, and should be included in evolutionary processes and theory (Angers et al. 2020; Herman et al. 2014; Sarkies 2020; Skinner 2015).

Transgenerational inheritance has been repeatedly demonstrated in model organisms in a laboratory setting. Further research is needed among field populations of non-model organisms responding to natural selection pressures (Hu and Barrett 2017; Sarkies 2020; Vogt 2015). For example, observations have been provided in Darwin finches for a role for epigenetic transgenerational inheritance and evolution (McNew et al. 2017; Skinner et al. 2014).

West-Eberhard proposed a process by which environmental pressures result in the selection of novel phenotypic traits which then result in genetic alterations and ultimately speciation (West-Eberhard 2003). This theory has been coined "genes as follower," and epigenetic variation is a strong candidate to explain the molecular mechanisms at play (Banta and Richards 2018; Jablonka 2006, 2017; Vogt 2021). Interestingly, environmentally induced epigenetic transgenerational inheritance has been shown to increase genetic mutations in the transgenerational generations (Skinner et al. 2015). Therefore, epigenetic inheritance promotes not only adaptive phenotypic variations, but also genetic variation on which the modern synthesis is based (McCarrey et al. 2016).

4.3 Epigenetic Transgenerational Inheritance

There are several different types of exposure to selection pressures, an organism can experience that could lead to altered epigenetics and a resultant altered phenotype. Direct exposure to any selection pressure involves the specific organism directly experiencing the exposure (Maynard 2000). An example of direct exposure would include a significant alteration in the seasonal temperature regime, such as that resulting from human-mediated climate change. Multigenerational exposure involves the organism experiencing the exposure and the germ cells that organism carries inside them (Skinner 2008). For example, when an organism is exposed to altered nutrition or a significant increase in temperature outside the normal seasonal regime, their sperm or egg cells are also exposed to that shift (Nilsson et al. 2018). These environmental pressures and exposures can alter the epigenetics to impact the developmental trajectory of the organism and subsequent offspring development due to the exposed germ cells, termed as intergenerational epigenetic inheritance (Skinner 2015). Finally, transgenerational phenomena are those in which an organism does not have continued direct exposure to the environmental stressor, but there is a permanent reprogrammed germ cell epigenetic inheritance of the epigenetic-induced phenotypic alterations resulting from the direct exposure of their ancestors, Fig. 4.1 (Anway et al. 2005; Nilsson et al. 2018; Skinner 2008). An example of environmentally induced epigenetic transgenerational inheritance could involve a single intense episode of heat shock that is experienced by an F0 generation, the F1 germ cells and the F2 germline within the F1 generation fetus. If a phenotypic shift is observed among the F3 generation, a generation that did not directly experience the heat shock, there is an epigenetic transgenerational inheritance phenomenon, Fig. 4.1 (Nilsson et al. 2018; Skinner 2008). Examples of transgenerational

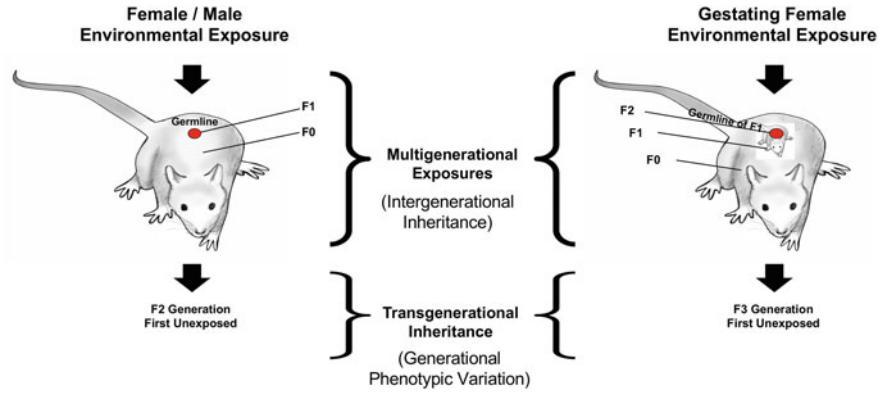

Fig. 4.1 Environmentally induced transgenerational epigenetic inheritance: schematic of environmental exposure and affected generations for both gestating female and adult male or female. The multigenerational direct exposures are indicated in contrast to the transgenerational generation without direct exposure. Modified from (Nilsson et al. 2018)

inheritance in human and animal models have been reviewed (Aiken and Ozanne 2014; Jirtle and Skinner 2007; Nilsson et al. 2018).

The epigenetically mediated inheritance of an environmental shock or alteration in selection pressures fits well with the original postulates of natural selection. The alteration in selection regime may yield novel variation in the population (postulate 1) (as described by West Eberhard 2003 (West-Eberhard 2003)). The novel phenotypes are heritable (postulate 2) (Anway et al. 2005; Bohacek and Mansuy 2015; Holland and Rakyan 2013; Legoff et al. 2019). Competition between offspring results in differential survival based on the phenotype of individuals (postulate 3), and the differential fitness of phenotypes is not random, but is explained by inheritance of the adaptive phenotype (postulate 4) (Sarkies 2020; Skinner 2015; Sudan et al. 2018; Weyrich et al. 2018). The alternative route to adaptation mediated by epigenetic alterations leading to inherited phenotypes is supported as an important avenue of evolutionary change.

It should be noted that, as a "rapid path" to adaptive change, epigenetic transgenerational inheritance of epigenetically mediated phenotypes may not always be adaptive (Skinner 2015). When the environment is shifting rapidly, an adaptive response may involve phenotypic switching by epigenetic inheritance rather than by genetic mutation (Burggren 2016; Skinner 2015). The capacity for epigenetic changes and resulting phenotypic changes to occur rapidly and even transiently may be the most adaptive path in some circumstances. Whether by transient phenotypic switching in changing environments or long-term alterations in response to phenomena such as climate change, epigenetic transgenerational inheritance provides a pathway toward adaption.

4.4 Examples of Epigenetic Transgenerational Inheritance Impacts on Evolution

The role of heritable epigenetic variation induced by environmental changes has been demonstrated in plant systems (Becker and Weigel 2012; Bossdorf et al. 2008; Cubas et al. 1999; Hirsch et al. 2012; Richards et al. 2010). While plant species are known to exhibit a high level of developmental plasticity in changing environments, heritable epigenetic variation is proposed as a major mechanism influencing this developmental plasticity and ultimately the adaptation and evolution of plant species (Miryeganeh and Saze 2019; Sudan et al. 2018). Plant species may be more prone to epigenetic inheritance through environmentally altered epigenetic states. This may be a result of their modes of reproduction and the lack of a sequestered germ line (Quadrana and Colot 2016). The plant group has served well for initial observations of adaptive epigenetic variation and evolutionary change. Notable examples of environmental-induced adaptive phenotypic change were documented in *Taraxacum officinale* (Ferreira de Carvalho et al. 2016; Wilschut et al. 2016) and *Arabidopsis* (Luo et al. 2020; Schmid et al. 2018).

Heritable epigenetic variation has been demonstrated in many animal species as well (E. Nilsson et al. 2018). *Caenorhabditis elegans* is one of the most studied animal species in the investigation of mechanisms of epigenetic inheritance (Fabrizio et al. 2019; Greer et al. 2011; Rechavi et al. 2011). The inheritance of epigenetic mechanisms, such as histone modifications or heritable small RNAs, can alter adaptive ancestral response among *C. elegans* (Rechavi and Lev 2017).

Empirical tests of the proposed idea that epigenetic mechanisms can contribute to environmental adaptation and evolution have been found in clonal laboratory lineages, monoclonal invasive animal species, and adaptive radiations (Vogt 2017). Natural animal populations have been found in general to contain higher epigenetic variation than genetic variation. The invasive house sparrow (*Passer domesticus*) exemplifies this pattern (Liebl et al. 2013). This example also demonstrates a pattern among invasive animal species whereby the higher amount of epigenetic variation is proposed as a mechanism by which rapid phenotypic change and adaptive evolution are facilitated by the enhanced epigenetic variation (Carneiro and Lyko 2020; Vogt 2017). Animal lineages that are not reliant on genetic variation, such as clonal lineages, are also prime candidates for the investigation of adaptation through environmentally induced epigenetic variation. The asexual clonal snail *Potamopyrgus antipodarum* is a widespread invasive species in the North America. Adaptive phenotypic variation in these invasive populations was found to be associated with epigenetic variation, providing support for the proposed mechanism of adaptation through environmentally induced epigenetic variation (Thorson et al. 2017, 2019). *Chrosomus eos-neogaeus* is a hybrid clonal fish, which inhabits both the predictable (lakes) and unpredictable (intermittent streams) environments. Significant differentiation in epigenetic phenotype has been documented in this hybrid (Massicotte and Angers 2012), and this variation is associated with the divergent environments (Leung et al. 2016). The invasive house sparrow populations exhibit

morphological variation which is associated with epigenetic variation between subpopulations in the Middle East (Riyahi et al. 2017) and among distinct introductions in Australia (Sheldon et al. 2018). These successful invasive species, which exhibit significant epigenetic variation, provide natural empirical investigations into the potential for environmentally induced epigenetic variation and inheritance to act as a source of adaptive phenotypic variation.

Adaptive radiations provide additional empirical examples of epigenetically mediated evolutionary change. Epigenetic changes were found to be more common than genetic changes among five closely related species of Darwin's finches (Skinner et al. 2014). Moreover, epigenetic variation was correlated with urban and rural populations of two of the Darwin finch species, suggesting environmentally induced epigenetic inheritance in this adaptive radiation (McNew et al. 2017). The examples of *Chrosomus eos-neogaeus*, *Passer domesticus*, and Darwin's finches support the role of epigenetic variation particularly among population with depleted genetic variation which can include invaders, founding populations, clonal lineages, and adaptive radiations (Vogt 2017). From these natural empirical examples, strong support for the proposed "soft inheritance" hypotheses (i.e., epigenetic inheritance) has been developed.

Laboratory populations have also shown significant evidence of induced epigenetic change and transgenerational inheritance of altered phenotypes. The evidence for epigenetic transgenerational inheritance of environmentally induced epigenetic changes in mammalian species has been reviewed (Legoff et al. 2019). Laboratory lineages of *Rattus norvegicus* have demonstrated numerous cases of epigenetic transgenerational inheritance of altered phenotype induced by an environmental perturbation and accompanied by epigenetic alterations and epigenetic transgenerational inheritance (Anway et al. 2005; Nilsson et al. 2018; Nilsson and Skinner 2015). Laboratory manipulations and environmental exposure experiments provide important support for the proposed mechanism of epigenetic inheritance and phenotypic change. Other human-mediated alterations to selection regimes include captive breeding programs and hatcheries. Hatchery and wild populations of Steelhead trout (*Oncorhynchus mykiss*) exhibit extensive phenotypic differences in growth and maturation rates. When examined for epigenetic differences, significant differential methylation was found in somatic and germ cells of these hatchery and wild populations (Nilsson et al. 2021).

4.5 Conclusion: Integration of Epigenetic Transgenerational Inheritance and Evolutionary Biology

Overall, the evidence for a functional role of epigenetic variation and the various mechanisms of epigenetic variation in all organisms investigated, such as plants (Chang et al. 2020; Hauser et al. 2011; Lamke and Baurle 2017) and animals is

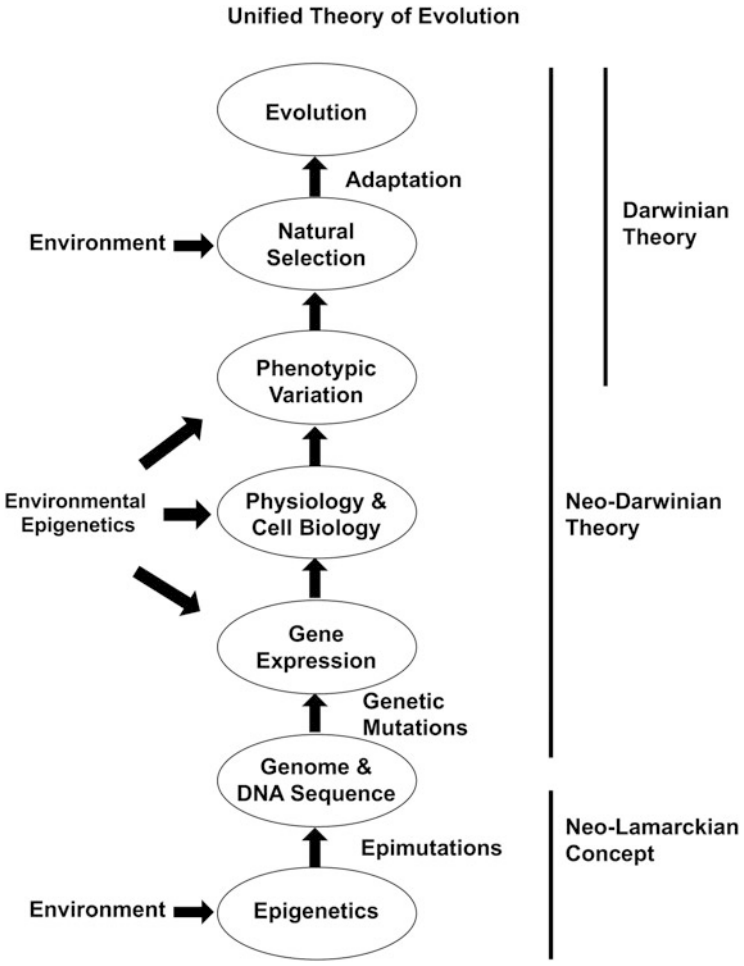

Fig. 4.2 Schematic of the unified theory of evolution. No dominance is suggested by the appearance of specific circles (e.g., epimutations versus genetics) such that all are equally important components. Modified from (Skinner 2015)

compelling (Nilsson et al. 2018; Skvortsova et al. 2018; van Otterdijk and Michels 2016; Xu and Xie 2018). With a proposed epigenetic mechanism for non-genetic inheritance, there is significant support for the previously discarded ideas of "soft inheritance" (i.e., epigenetic inheritance) from the late nineteenth and early twentieth centuries (Skinner 2015). Epigenetic inheritance has been described as a redemption of the ideas of Jean Baptiste Lamarck, who was the first to suggest the inheritance of acquired characteristics (Nilsson et al. 2020; Skinner 2015; Wang et al. 2017), Fig. 4.2. This new evidence suggests that a revision of the ideas set forth during the establishment of the modern synthesis is required. The impacts of epigenetic transgenerational inheritance and epigenetic variation on the evolutionary and

adaptive trajectory of species are supported as relevant and crucial (Jablonka 2017; Skinner 2015). The four postulates of natural selection are supported by the evidence of epigenetic inheritance and phenotypic change, such that alteration of the modern synthesis need to focus on the integration of the non-genetic and genetic forms of inheritance involved in phenotypic variation, adaptative, and evolution.

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Chapter 5
Transgenerational Epigenetic Programming

Naim M. Bautista

Contents

Abstract Ever since the theory of natural selection was proposed, the study of how characters are inherited across generations has become a principal paramount in biology. These studies have focused on deciphering how phenotypic variation and plasticity across generations contribute to population maintenance and evolution. In this regard, studying how the experience of environmental conditions of a parental population influences offspring phenotypic characteristics through epigenetic processes has gained substantial attention in the past decades. In particular, the mechanisms underpinning this type of transgenerational acclimation include maternal provisioning, microbiome transfer, inheritance of epigenetic markers (e.g., DNA methylation, small RNAs, and histone modifications), and behavioral and cultural processes. These phenomena can result in the programming of the next generation and influence their survival and adaptability to changing environmental conditions. To better understand this topic, in the first part of this chapter I will introduce the reader to the scientific framework on which transgenerational epigenetic

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programming, and non-genetic inheritance in general, finds its roots. In the second part, I revised the concepts of 'epigenetics', 'transgenerational inheritance', and, 'programming', with the purpose of building a solid ground on which we can base an integrated and deeper discussion in the subsequent sections. The third part of this chapter is focused on discussing the connection between these three concepts, as well as to delve into the tight, but complex, link between 'transgenerational epigenetic programming' and developmental biology. After revieing the concept and providing examples of its complexity, I discuss the potential evolutionary implications of transgenerational epigenetic programming in the fourth part of the chapter. Finally, I posit a list of topics and approaches that warrant further research in this scientific field and provide future directions that will help to elucidate knowledge gaps.

Keywords Non-genetic inheritance · Transgenerational acclimation · Parental effects · Developmental programming · Evolution · Epigenetics

5.1 Introduction

Ever since the theory of natural selection was proposed by Darwin and Wallace (Beddall 1968), the study of how traits are inherited has become a principal paramount in evolutionary biology. This field of study has been dominated by the approaches embraced within a conceptual framework known as the 'Modern Evolutionary Synthesis'. The Modern Synthesis emerged early in the twentieth century and the term was coined by Julian Huxley—the grandson of Thomas Henry Huxley, 'Darwin's bulldog'—in his book 'Evolution: The Modern Synthesis' (Huxley 1942). This framework arose from the fusion between Darwinian-Wallace evolution by natural selection, a population-level approach, with Mendelian genetic inheritance, a mechanistic-molecular approach, which resulted in the development of population genetics as a field (Dickins and Dickins 2018; Mayr 1993; Provine 2020). However, it was further constructed, developed, and popularized with the work of recognized scientists such as Theodosius Dobzhansky, Ernst Mayr, and Douglas Futuyma, among others (Dobzhansky 1982; Futuyma 2015; Mayr 1991).

The modern synthesis posited a well-funded scientific conceptual framework for evolutionary biology. However, as it is true for any other scientific framework, the pillars that sustain the concept were, and continue to be, challenged by new discoveries, and the modern synthesis has been continuously improved. For an in-depth review of this topic, the reader is referred to references (Dickins and Dickins 2018; Dobzhansky 1982; Jablonka and Lamb 2020; Mayr 1982, 1991, 1993; Provine 2020). Of particular importance for this chapter is that when the modern synthesis was first proposed it failed in acknowledging the existence of two phenomena that can influence adaptation and evolution. The first is that environmental experiences can lead to the inheritance of characters (soft inheritance)

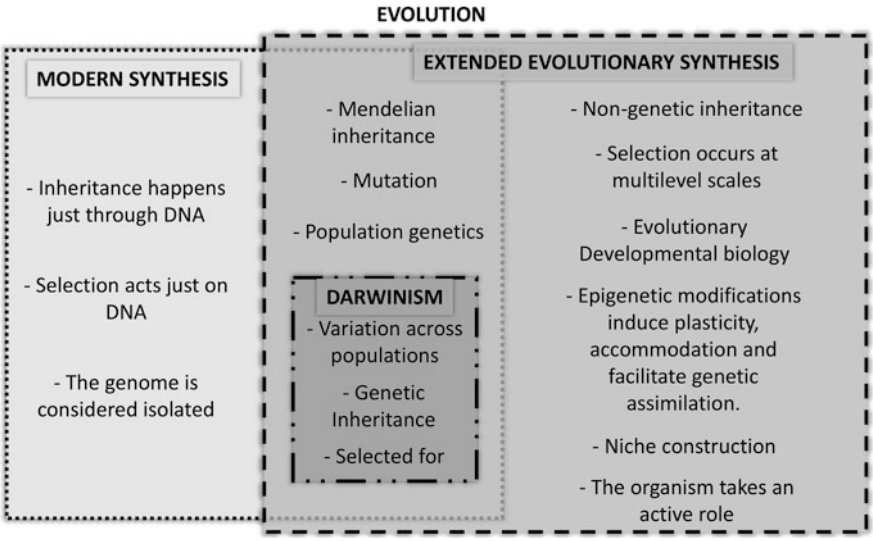

Fig. 5.1 Relationship between the Modern Evolutionary Synthesis (dotter square) and the Extended Evolutionary Synthesis (dashed square). The Extended Synthesis does not contradict nor deny the Modern Synthesis, in fact both frameworks share common core assumptions (overlapping area) such as classical Darwinism (dashed double dotted square), Mendelian inheritance, mutation, and population genetics. In comparison with the Modern Synthesis, the Extended framework considers that inheritance can happen through non-genetic processes, that selection occurs at multilevel scales and not just on DNA and that the genome is not isolated from the processes happening at the physiological, organ, system, and whole-individual level. Furthermore, The Extended Synthesis considers that the organism plays an active role on selection and that it is not only a product of it

(Jablonka and Lamb 2015, 2020; Jablonka and Raz 2009; Lacal and Ventura 2018; Moore 2015; Noble 2015; Richards 2006). The second is that changes acquired at developmental stages, without altering DNA sequences can affect later life stages, and can also be passed on to next generations (Jablonka and Lamb 2014, 2020).

With the advent of technology and new laboratory techniques, the unraveling of molecular mechanisms led to a revision and expansion of the scientific framework of the modern synthesis and led to the proposal of a newer, expanded approach known as the 'The Extended Evolutionary Synthesis'. Principal proponents of this new framework have put together a comprehensive manuscript on its core principles (Laland et al. 2015)—for a deeper review, please refer to (Gilbert et al. 1996; Gould 2002; Jablonka and Lamb 2014, 2015, 2020; Jablonka and Raz 2009; Noble et al. 2014; Pigliucci and Müller 2010). Noteworthy is the fact that the Extended Evolutionary Synthesis does not contradict or deny the Modern Synthesis, nor does it invalidate the work that has been developed under its framework. Instead, and as is implicit in its name, it *extends* the approach by considering and integrating biological processes that are subject to selection and that can have evolutionary implications (Fig. 5.1). For instance, the Extended Synthesis found particular scientific support

from the fields of evolutionary biology, developmental plasticity, inclusive inheritance, and niche construction theory—for extensive literature on this topic refer to (Bonduriansky and Day 2018; Laland et al. 2016; Moczek et al. 2011; Odling-Smee et al. 1996; Sultan 2015; Uller and Laland 2019; West-Eberhard 2003). Overall, this 'extended conceptual framework' has gained scientific approval and increased consideration by scientists around the world. Figure 5.1 depicts the relation between the Modern and the Extended Evolutionary Synthesis (Fig. 5.1).

Particularly important for this Chapter on Transgenerational Epigenetic Programming are two observations brought by the framework of the extended synthesis. The first is to study how phenotypic variation can be influenced by processes occurring during development and the second focuses on how phenotypic plasticity elicited by those processes contributes to evolution by means of phenotypic accommodation and potential genetic assimilation. In this regard, the study of how the experience of environmental challenging conditions—biotic, abiotic, or anthropogenic—during early developmental life stages can influence juvenile and adult phenotypes and its evolutionary consequences, has gained considerable attention.

To better understand this topic, in the first part of this chapter I will first revisit the concepts of 'epigenetics', 'transgenerational inheritance', and 'programming', with the purpose of building a solid ground on which we can base a more integrated and deeper discussion in the subsequent sections. Then, I will focus on discussing the connection between these three concepts, as well as to delve into the tight, but complex, link between 'transgenerational epigenetic programming' and developmental biology. After reviewing the concept and provide examples of its complexity, I discuss the potential evolutionary implications of transgenerational epigenetic programming. Finally, I finish this chapter by providing a list of topics and approaches that warrant further research in this scientific field and provide future directions that will help to elucidate knowledge gaps.

5.2 'Epigenetics', 'Transgenerational Inheritance', and 'Programming': What Do we Mean by Them?

'Transgenerational epigenetic programming', the title of this chapter, is built from three words whose individual definitions have been under debate during the past decades. Throughout the scientific literature, these words have been used by researchers from different areas of expertise and fields of work. Not surprisingly, the use of these words is varied and inconsistent, resulting in scientific debates that have been accentuated and expanded as a result of the later discovery of biological mechanisms that occur within-generationally (molecular to organismal level responses along a life span) and/or transgenerationally (e.g., inheritance). However—at least from this author's perspective—most of the opposing views and misunderstandings have mainly arisen from the existing mismatch between the semantics of the words and the actual in vivo biological processes. Although this

is not a review on the semantics of the topic, it is worth giving a short look at terminology to have a common ground of understanding from which we can stand and follow a more in-depth, integrated discussion of transgenerational epigenetic programming. Overall, transgenerational effects can arise from phenomena happening at multiple levels of organization; thus, it is imperative to emphasize that all these processes play a crucial role in transgenerational epigenetic inheritance and can have evolutionary consequences as I shall discuss below.

5.2.1 Epigenetics

The term 'epigenetics', as first proposed by Waddington in *The epigenotype* (Waddington 1942), referred to the discipline dedicated to the study of the causal mechanisms by which the genes influence phenotypes. Since Waddington coined the term, its definition has varied broadly (Burggren 2016; Tollefsbol 2017b). However, a common theme across those definitions is the acknowledgment of the role of the environment as selective pressure that can induce changes on gene expression and thus result in changes of phenotypic traits. This possibility of modifying gene expression is, not surprisingly, of main interest in the field of medical research where it has been successfully used in therapeutic treatments, for example against cancer (Ghasemi 2020; Søreide 2017). Although the medical/health focus has overshadowed the study of the role of epigenetic phenomena as a factor for inducing phenotypic variability and in its potential role for evolution, during the past decade this interest has re-emerged—since Lamarck's days—and several research areas from theoretical to experimental biology are now factoring epigenetic phenomena in their designs.

Epigenetic mechanisms are variable and diverse across species and across levels of organization. Among the most studied processes, at the molecular level, are DNA methylation and hydroxy-methylation, posttranslational histone modifications, microRNAs, non-coding RNAs, nucleosome remodeling, parental imprinting, paramutations, parental care, maternal provisioning, social learning, etc. (Qureshi and Mehler 2018; Richards 2006; Tollefsbol 2017a). Furthermore, epigenetic phenomena at the molecular and the whole-individual levels can be studied within- and transgenerationally, with neither dimension being mutually exclusive. Noteworthy is the fact that both within- and transgenerational epigenetics refer to phenotypic modifications that result from changes in gene expression derived from the action of one or more of the molecular epigenetic mechanisms mentioned above. In this chapter, I will use within-generational effects to refer, in particular, to the changes occurring along the lifespan of an organism, while transgenerational effects will describe on the action of epigenetic mechanisms occurring across generations under the scope of *transgenerational inheritance*, a topic I shall now discuss.

5.2.2 *Transgenerational Inheritance*

In biology, the concept of 'inheritance' is used to refer to the transmission of information from parents to their offspring—or beyond—through genetic material. This use of the word is clearly under the umbrella of Mendelian genetics, the classic framework of scientific research for decades. However, this concept has been challenged as non-genetic mechanisms of inheritance have been (and still are) uncovered. Not surprisingly, the concept of 'transgenerational inheritance' has also been under debate with the core of the discussion rooted in the mismatch between semantics and actual in vivo biological process. One side focuses on the argument that for the process of transgenerational inheritance to be properly called 'transgenerational', the transmission of information from parents to offspring has to occur, undoubtedly, through the germline—an approach clearly influenced by Weismann's germ plasm theory—(Nilsson et al. 2020; Weismann 1893), commonly referred to as 'meiotic epigenetic inheritance' (Skvortsova et al. 2018). This approach argues that for transgenerational inheritance to be true, a trait elicited by a particular environment experienced only by the parental generation (P_0) must be also seen in the F_3 generation and beyond (Fig. 5.2). This scenario is commonly exemplified with a gestating female who encounters a particular environment or stressor (Fig. 5.2a).

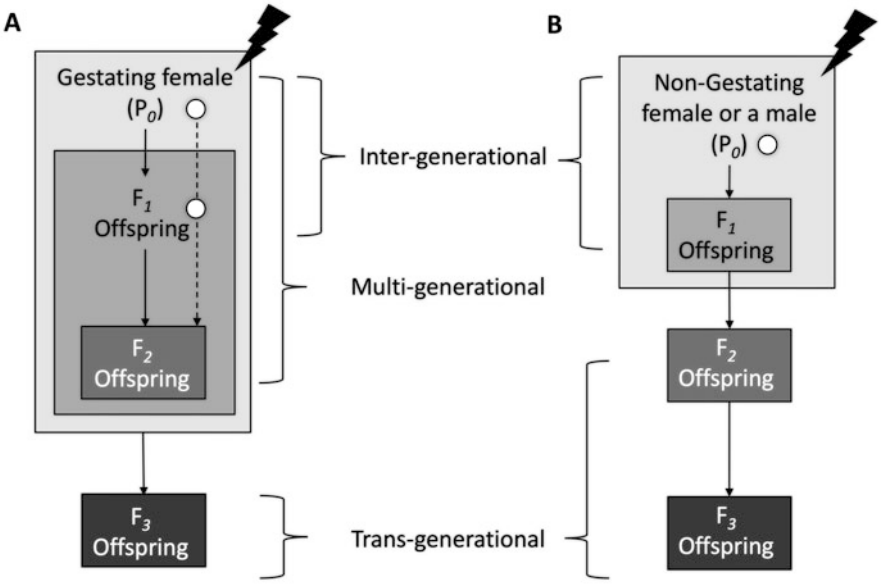

Fig. 5.2 Different scenarios of effects involving more than one generation. (**a**) Effects considering that the parental generation is represented by a pregnant female. (**b**) Effects considering that the parental generation is represented by a unpregnant female or a male. The black shinning represents exposure to a stressor. White circles represent direct exposure of germline cells in both scenarios

In other words, the P_0 female exposure will have an effect on its germline, and during reproduction, the epigenetic mark will be transmitted to the F_1 offspring epigenome and lead to phenotypic modifications (Szyf 2015; Tollefsbol 2014). Therefore, if the epigenetic-gamete modification is not removed during the processes of germ cell differentiation and reprogramming in the F_1, then the phenotypic change could be evident also on the F_2 phenotype. However, it is possible that after reproduction of the F_2 generation takes place, the epigenetic marks can be erased during differentiation and reprogramming of the germ cell in the F_3, then, the epigenetic mark cannot lead to phenotypic effects on the F_3 offspring or beyond (Fig. 5.2a) (Szyf 2015; Tollefsbol 2017a). On the other hand, however, this strict definition of transgenerational phenomena leaves out important mechanisms through which the phenotype of more than one generation can be modified. For example, a *multigenerational* effect can occur when the exposure event on a gestating female will affect somatic and germline cells of the F_1 generation, leading to phenotypic effects on the F_2 (Nilsson and Skinner 2014; Szyf 2015). However, in this scenario, the effect on the F_1 germline was also the result of the direct exposure that occurred simultaneously when the P_0 female was exposed and was not transmitted from the P_0 to the F_1 offspring through the germline. These effects can potentially be erased from the F_2 germline during cell differentiation reprogramming and thus do not induce any phenotypic change in the F_3 (Fig. 5.2a) (Lacal and Ventura 2018; Szyf 2015).

Noteworthy is that the strict definition provided above is not only excluding multigenerational effects. For example, it is possible that when the gestating female experiences a particular environment, the effect will lead to changes only on the F_1 somatic cells, inducing a change on its phenotype while not affecting the fetus' germline (Fig. 5.2b). In this scenario, as the change is not inherited through the germline, the effect is referred as '*prenatal exposure*', '*transplacental epigenetic effect'*, '*cross-generational inheritance*', or '*inter-generational inheritance*', (Aiken and Ozanne 2014; Burggren 2016; Lacal and Ventura 2018; Szyf 2015; Tollefsbol 2014). Furthermore, these modes of inheritance are excluded from the 'transgenerational inheritance' dimension because the phenotypic effect induced by the acquired epigenetic mark is not transmitted to a second F_2 or third F_3 generation through the germline. Worth mentioning is that, if instead of considering a path where the exposure to the particular environment affects and starts with a gestating female, we focus on a path starting with an adult unpregnant female or a male; then, the exposure will affect the somatic and germ cells of the P_0. Furthermore, if the acquired epigenetic marks are not erased during differentiation and reprogramming of the F_1 germline, it is possible that the F_1 will exhibit phenotypic modifications (Fig. 5.2b) (Lacal and Ventura 2018). Under this scenario then, if the exposure leads to a phenotypic change in the F_1, arguably, it be also seen as the effect of direct exposure. However, in this case, if the effect is also seen in the F_2 generation, the effect can be called 'pure' epigenetic inheritance (Fig. 5.2b) (Lacal and Ventura 2018).

Strict definitions in biology have to be taken with a grain of salt, as described above, that definition of transgenerational inheritance is restrictive and should not be generalized or taken as the norm, because the effects are context dependent and may

apply differently to species based on variation of particular life traits such as the species' reproductive and fertilization mode (Bautista and Crespel 2021). For instance, in species with external fertilization in which both parental gametes are deposited into the environment the pure epigenetic effect may be seen as soon as the F_2 generation exhibits the phenotypic modification. In addition, these scenarios can be somewhat confusing because the presence of the phenotypic change in the F_3 generation implies that the inheritance of the epigenetic trait occurred though the germline; hence, the change was also present on the F_1 germline and transmitted to the F_2 and F_3 germline. Therefore, it becomes clear that the best way to be confident of the presence of pure transgenerational effects is by implementing molecular techniques aimed at determining the presence of the epigenetic modifications in the germline of each generation, though this may not be feasible because of the high costs of the techniques.

On a more liberal side, the broadest definition of 'transgenerational inheritance' refers simply to 'the transmission of information from parental generation to its immediately following F_1 offspring generation and/or beyond (F_n), without alterations in DNA sequence' (Burggren 2016; Tollefsbol 2017a). This general definition fails to provide directionality regarding the role of the germline during the transmission of information, but it is open to include other processes, such as the social and cultural aspects of species, that can result in the modification of epigenetic marks. Those epigenetic changes can be transmitted from one generation to the next and are of particular interest for discussing transgenerational epigenetic programming as we shall now see.

5.2.3 Programming

The term *programming* has the connotation of an unchangeable, pre-meditated, and scheduled set of instructions that will result in actions or a series of events. However, under the umbrella of the field of epigenetics, the term 'epigenetic programming' refers to the influence that stable epigenetic alterations resulting from the exposure or experience of a particular environment during early developmental stages will have on modifying organismal phenotypes at later developmental stages (Fig. 5.3a) (Cantone and Fisher 2013; Zapata-Martín Del Campo et al. 2018). There are two points of particular interest in this concept. The first is that the term epigenetic programming focuses on the exposures during early development, specifically on sensitive periods of development (Zapata-Martín Del Campo et al. 2018). The second is that the definition applies only to phenomena happening at the frontier between within-generational effects and the broadest definition of transgenerational effects (Fig. 5.3a). Not surprisingly, this area of research has been of main focus within the human medical/health field of research because of the opportunities and potential therapeutic applications (Hanif and Shah 2018; Lewis et al. 2015). Nonetheless, the definition is subject to debate because it assumes that after 'programming' has occurred, the environment will remain constant and thus neglects the

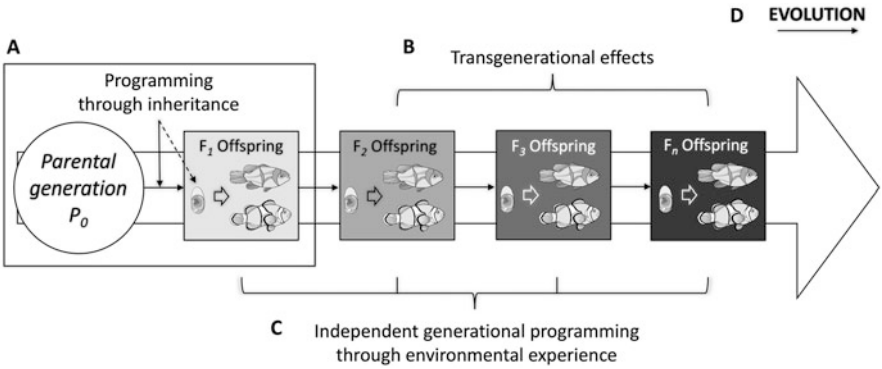

Fig. 5.3 Epigenetic programming. (**a**) Epigenetic programming through the germ line. The dashed arrow represents the common understanding that programming occurs at the very early stages of development and the effects will be seen at later developmental stages. The solid line between generations represents the change inherited through the germline. (**b**) Programming can induce transgenerational effects in populations. (**c**) Each generation can be programmed independently of inheritance if the environmental conditions elicit an epigenetic change. (**d**) Evolutionary change can occur if programming leads to a change in the allelic frequencies of a species with time. This genetic component is represented by the large white background arrow

possibility that inheritance-independent environmentally induced changes in the epigenome occurring at later developmental stages can also result in changes of phenotypic traits (Fig. 5.3b-c). As mentioned above, with the advent of new technology and the continuous uncovering of molecular mechanisms, scientific dogmas and terms are in need of revision, as is the case of 'programming'. In fact, some researchers have expressed their disagreement with the use of the term. For example, in his book *The developing genome,* David S. Moore stated 'Personally, I don't like the use of the word "programming" here, because it implies a sort of automation that unfolds in a context-independent and inevitable way' (Moore 2015), in a passage referring to one of the most known studies of epigenetic programming by maternal behavior. In addition, scientific findings also highlight the need for reviewing this term and its implied meaning of being a pre-meditated and scheduled list of instructions. Researchers around the world have recorded evidence proving that changes in the epigenome resulting from the experience of environmental conditions during early developmental stages can be reversed at later stages (Dolinoy et al. 2007; Feil and Fraga 2012; Vickers and Sloboda 2012; Vickers et al. 2005; Weaver 2005; Weaver et al. 2004b).

5.2.4 Epigenetic Programming Is Dynamic

In a broad sense, epigenetic programming refers to the study of how the molecular epigenetic mechanisms, acquired during sensitive periods of early development,

influence phenotypic traits in later developmental stages (Zapata-Martín Del Campo et al. 2018). These effects represent the environment–genome interactions and occur through the modification of transcriptomic profiles (gene expression) due to the activation or deactivation of epigenetic mechanisms (e.g., DNA methylation, histone methylation and acetylation, structural modifications, microRNA, small RNAs (Nicholson et al. 2015; Tollefsbol 2017a). These phenomena have been reported across distinct animal taxa including mammals (Li and Zhang 2014; Migicovsky and Kovalchuk 2011), birds (Bautista et al. 2021; Frésard et al. 2013; Guerrero-Bosagna et al. 2018), reptiles (Hammond et al. 2016; Ruhr et al. 2021), amphibians (Bian et al. 2009; Sarma et al. 2020), fish (Bautista et al. 2020; Cavalieri and Spinelli 2017; Jiang et al. 2013), and invertebrates (Ardura et al. 2017; Díaz-Freije et al. 2014; Vaiserman 2014) and have been shown to induce changes at different organismal levels such as behavior, whole-individual physiology, organ, tissue and cells physiology and metabolism (Bautista et al. 2021; Skinner et al. 2008; Smith et al. 2012; Tollefsbol 2017a; Van Cauwenbergh et al. 2020; Vinci et al. 2013; Weaver et al. 2004a). In addition, these processes have been identified as responsible mechanisms of caste differentiation in social insects and found associated with alternative splicing (i.e., mature mRNA is formed by the junction of different combinations of exons from the same gene); for an introduction to this topic, please refer to (Chittka et al. 2012; Elango et al. 2009; Vaiserman 2014; Weiner and Toth 2012). However, most of the studies on epigenetic programming focus on human health areas (Egger et al. 2004; Langley-Evans 2006; Van Cauwenbergh et al. 2020; Zhu et al. 2019), or fall into within-generational effects. Consequently, relatively little attention has been given to understanding epigenetic programming phenomena at the transgenerational scale (Fig. 5.3d).

Arguably, the most well-known example of epigenetic programming in vertebrates is that of the stress response in rats induced by lack of maternal care. Briefly, in a series of studies, Michael J. Meaney, and Ian Weaver, and their teams characterized and described that the maternal behavior experienced by rat pups can alter their hypothalamic–pituitary–adrenal response to stress when they reach maturity (Meaney 2001; Meaney and Szyf 2005; Weaver et al. 2004a). Specifically, in comparison with adult offspring rats that experienced low licking, grooming, and arched back maternal behavior during the first week of life, offspring that experienced high levels of these behaviors from their mothers exhibited increased expression of hippocampal glucocorticoid receptors (*Nr3c1* gene), as well as enhanced glucocorticoid sensitivity (Francis et al. 1999; Liu et al. 1997; Weaver et al. 2004b). These effects are reflected as a decrease in the expression and synthesis of hypothalamic–corticotropin-releasing factor, resulting in a smaller hypothalamic–pituitary–adrenal response to stress (De Kloet et al. 1998; Meaney and Szyf 2005). These results were later supported by cross-fostering experiments where offspring from high licking and grooming mothers raised by low licking and grooming mothers resembled the responses of biological offspring (Caldji et al. 1998; Francis et al. 1999). These experiments suggested that the environment in which the rats were raised dictated their stress response in later developmental stages; hence, maternal behavior 'programs', by means of non-genetic transmission, the reactivity

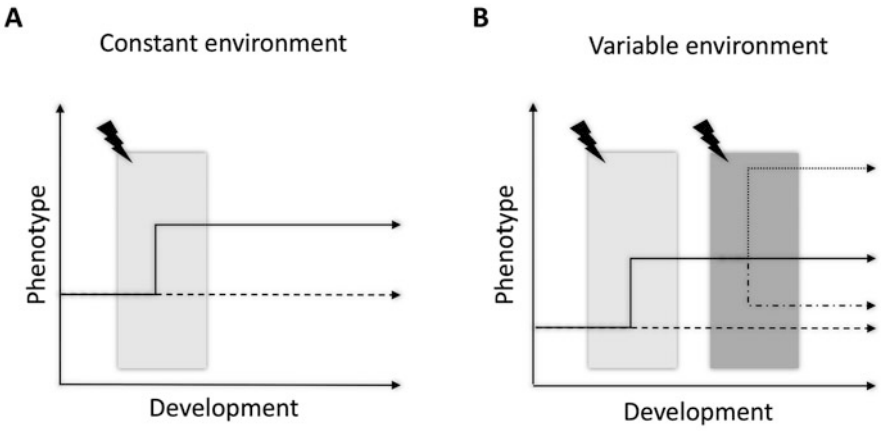

Fig. 5.4 Epigenetic programming under two distinct scenarios. (**a**) Constant environment. Epigenetic programming will occur when a particular environment induces a change in a phenotypic trait early in development. The change can be seen or not during early development, in either case, the change is present at later developmental stages. (**b**) Variable environment. After epigenetic programming by environment has occurred, the experience of a different environment at later developmental stages can modify the already programmed phenotype (dashed/dotted line and dotted line)

to stress across generations (Fleming et al. 1999; Meaney 2001; Meaney and Szyf 2005). Besides studying the phenotypic effects, Meaney and colleagues were able to connect those results with their underpinning molecular mechanisms. The team reported that in comparison with the adult rat offspring from high licking and grooming mothers, the adult offspring from low licking and grooming females expressed lower levels of hippocampal exon 1_7 mRNA transcripts (Weaver 2005; Weaver et al. 2004b). These same rats also exhibited hypermethylated 1_7 GR promoter, hypoacetylation of histone H3-lysine K-9, and reduced binding to the transcription factor egr-1 (Weaver 2005). Not surprisingly, adult offspring from high licking and grooming mothers exhibited opposite results. However, of main importance for this chapter is the fact that Meaney and colleagues also reported that the methylation and acetylation states—which were elicited by the experience (or lack) of maternal licking and grooming during early life stages—can be reversed in adult rats (Weaver 2005). Worth mentioning is that this reversal of epigenetic states was induced pharmacologically, by infusion of methionine and the histone deacetylase inhibitor (HDAC) trichostatin A (TSA), for details refer to (Weaver 2005; Weaver et al. 2004b).

These experiments clearly illustrate that epigenetically induced traits are dynamic, and thus, adult phenotypic traits elicited by epigenetic marks—acquired at early developmental stages as a response of environmental experiences—can be environmentally modified, reversed, or induced at later developmental stages (Fig. 5.4a,b). Noteworthy, those experiments are a clear example of epigenetic programming occurring at the within-generational scale. More importantly is that

the observed effects were elicited by epigenetic markers acquired in born rat pups, a long time after fertilization, and not while the fetuses were in the womb when early developmental processes (e.g., epigenetic reprogramming) have already occurred. Particularly important to remember here is the fact that epigenetic programming at the transgenerational dimension requires that the parent-to-offspring transmission of the acquired epigenetic marks occur through the germline. Moreover, these epigenetic marks must be stable, and stay present even after germline reprogramming has occurred, during the very first stages of early development.

5.3 Transgenerational Epigenetic Programming

Beyond understanding the within-generational implications of epigenetic programming, its ability for modifying transcriptomic profiles and its tight relationship with individual responses to environmental stimuli highlight the need for studying its long-term effects on species resilience and population maintenance. The molecular mechanisms underpinning epigenetic programming, elicited by the interaction between genome and environment, are involved in the differentiation and development of cells and tissues by regulating gene expression in almost all organs and cell types (Anway et al. 2008; Jiang et al. 2004; Zhu et al. 2019) At the cellular level, the primordial germ cells—the precursors of sperms and eggs, and the only cell type than can pass information into the next generation—are of particular interest for understanding transgenerational epigenetic programming because it is during their development (sensitive periods) that environmental conditions can induce epigenetic modifications with the potential of inducing long-lasting and transgenerational effects.

As the number of existing epigenetic mechanisms is large and varies phylogenetically and functionally, and because of the existence of a considerable amount of information and material to develop on this topic, in this section, I have placed the artificial limit of focusing on DNA methylation which is the most studied and understood epigenetic mechanism.

5.3.1 Epigenetic Programming and Reprogramming in the Next Generation

After encountering a particular environment, epigenetic programming through DNA methylation in animals occurs commonly when a methyl group ($-CH_3$) from S-adenosylmethionine is transferred to the carbon-5 position of the cytosine ring in a cytosine-guanine dinucleotide (i.e., CpG) (Chavatte-Palmer et al. 2018; Moghadam et al. 2015; Veland and Chen 2017). Noteworthy is that the effects that DNA methylation will induce in the organism differ depending on the different

regions where the methyl group gets attached (e.g., promoters, genes, enhancers, introns, exons), as well as the cell type where they occur (Sarda et al. 2012; Suzuki and Bird 2008). Noteworthy is the fact that some epigenetic modifications, such as DNA methylation, can influence the processes of transcription (increasing exon inclusion), and splicing of RNA (Burggren 2017; Flores et al. 2012; Vaiserman 2014).

Those effects, which will ultimately lead to the enhancement or silencing of gene expression (Tate and Bird 1993), can have consequences for the survival of the organism. However, as mentioned above, to induce a transgenerational effect the epigenetic mark must be stable in germ cells. In other words, DNA methylation must be maintained, established (de novo), or transferred, during germ cell differentiation and development. Nevertheless, DNA methylation can also be removed (Wu and Zhang 2017). DNA methylation processes are performed by a family of enzymes called DNA methyltransferases (DNMTs, writers, readers, and erasers); however, other epigenetic tools may be also involved (e.g., 5-methylcytosine binding domain or 'MBD' protein family, and ten-eleven translocation protein family TETs, erasers) (Bergman and Cedar 2013; Ginder and Williams 2018; Hu et al. 2015; Jurkowska and Jeltsch 2016; Veland and Chen 2017; Wu and Zhang 2017). In fact, the wide pool of enzymes involved in all epigenetic processes, and currently widely known as 'writers, readers, and erasers', are directly and indirectly related on a functional level (Biswas and Rao 2018; Nicholson et al. 2015; Torres and Fujimori 2015). Therefore, it is clear that DNA methylation patterns can be read, written, or erased during the organism's lifetime.

In animals, the stability of DNA methylation marks and their further transgenerational effects are modulated by at least one event of genome-wide DNA demethylation and reprogramming during early development. In mammals and marsupials, these events can happen pre- or postnatally (Ishihara et al. 2019). Additionally, in some species, these reprogramming events appear to vary between sperms and oocytes (Jiang et al. 2013; Ortega-Recalde et al. 2019). In fact, the epigenomic states of sperm and oocytes in mammals are incredibly different; consequently, the embryonic epigenome from the maternal or the paternal side might behave differently during development (Cantone and Fisher 2013; Wang et al. 2014). However, this variation in reprogramming events is not limited to mammals and has been also reported in fish. In the zebrafish (*Danio rerio*); for example, the epigenome of the germline does not undergo the process of genome-wide demethylation reprogramming, and the DNA methylation states of sperm and oocytes differ (Jiang et al. 2013; Ortega-Recalde et al. 2019). Briefly, while the paternal epigenome of primordial germ cells is maintained throughout embryonic development, the maternal side undergoes a demethylation step (Jiang et al. 2013). This demethylated state of the maternal epigenome is maintained up to the 32-cell stage (about 1.8 h post-fertilization). After this stage (from the morula stage onward), the oocyte DNA methylation level resets and then is followed by gradual increase to match a similar methylation level as in the sperm (Ortega-Recalde et al. 2019; Potok et al. 2013; Wang and Bhandari 2019; Wang and Bhandari 2020). This level is then maintained throughout the rest of development.

The variation in reprogramming processes is not limited to sex differences of germ cells; it can also occur across species of the same animal group. This can be illustrated with a different experimental animal model, the medaka fish (*Oryzias latipes*), which expresses a different DNA methylation reprogramming compared to the zebrafish. In medaka, prior fertilization, the sperm genome is in a hypermethylated state while the oocyte genome is hypomethylated (Wang and Bhandari 2019). Soon after fertilization during the first cell cycle, the sperm genome undergoes erasure of the epigenetic methylation marks. This hypomethylated state is maintained up to the 16-cell stage; beyond this stage and up to the early gastrulation, the methylation level increases gradually until reaching hypermethylation (Wang and Bhandari 2019). The hypermethylated level is maintained throughout gastrulation, but it decreases during the gastrula-to-neurula transition. Noteworthy is the fact that the DNA methylation dynamics of medaka resemble the mammalian DNA methylation reprogramming processes, making this fish a suitable model for comparison (Guo et al. 2014; Wang et al. 2014; Wang and Bhandari 2019, 2020).

In mice (*Mus musculus*), two events of genome-wide demethylation occur post-fertilization in both, the maternal and paternal genomes. The first takes place during differentiation and development of the primordial germ cells; the second occurs during early embryogenesis around E.5 to E13.5 developmental stage (Dean et al. 2003; Seisenberger et al. 2013; Wang et al. 2014). However, soon after fertilization, at the zygote stage, the global genomic DNA methylation level of both, the paternal and maternal genomes, decreases and reaches its lowest level by the blastula stage (Smith et al. 2012, 2014). Nonetheless, while the maternal methylation level decreases gradually, the paternal methylation level undergoes a rapid decrease during cleavage (Peat et al. 2014; Smith et al. 2012; Wang et al. 2014). After reaching the lowest methylation level, the mammalian genome experience re-establishment of methylation, and around the E9.5 stage, the first demethylation phase occurs, followed by a short plateau period before the second demethylation phase takes place by the E10.5 stage and up to the E13.5 stage (Wang et al. 2014; Wang and Bhandari 2020). The low levels of methylation in oocytes are maintained even after birth, but not in sperm (Lee et al. 2014; Ortega-Recalde et al. 2019).

5.3.2 Within- and Transgenerational Epigenetic Programming beyond Nuclear DNA Inheritance

Perhaps, the first mechanism that cross our mind when thinking about inheritance of traits is the transfer of nuclear DNA from the oocyte and the sperm to the offspring, which is subject of Mendelian laws of inheritance. However, nuclear genomic transfer is not the only mechanism through which the phenotype of subsequent generations can be altered. For instance, extranuclear/non-nuclear inheritance of mitochondrial DNA (mtDNA) can occur through cytoplasmic inheritance (Cummins 1998; Seidel 2002; Wallace 2016). Mitochondrial DNA encodes for

22 transfer-RNAs, 13 different protein subunits that are essential in the electron transport chain, endogenous peptides, and for at least two ribosomal RNA molecules. In addition, both mitochondrial and nuclear cell integrity are co-dependent and maintained through the constant crosstalk between the expression of nuclear DNA and mtDNA (Gyllenhammer et al. 2020; Wallace 2016). Therefore, it has been proposed that mtDNA inherited through the cytoplasm can have important implications for offspring fitness and offer an additional pathway underlying developmental programming (Gyllenhammer et al. 2020). In fact, studies in animal models and humans have demonstrated that gestational and pre-conceptional exposures to pollutants, oxidative stress, dietary quality and quantity, maternal obesity, and even psychosocial stress (Alfaradhi et al. 2014; Andreas et al. 2019; Fetterman et al. 2013; Minocherhomji et al. 2012; Peterside et al. 2003; Zander-Fox et al. 2015) can lead to developmental programming of mitochondrial function that reduces the capacity to meet cellular bioenergetic demands. These effects include reduced mitochondrial content, impaired oxidative phosphorylation and REDOX balance, and increased production of reactive oxygen species (Vriens et al. 2017). Furthermore, it has been suggested that both reactive oxygen species and antioxidant balance play a role in controlling gene expression and can act as signaling molecules for epigenetic control (Alfaradhi et al. 2014; Hitchler and Domann 2007; Vriens et al. 2017; Zander-Fox et al. 2015). For an introductory review on developmental programming through the mitochondria, please refer to (Darr 2020; Gyllenhammer et al. 2020; Wallace 2016).

Beyond the within-generational implications of mitochondrial developmental programming, the effects mentioned above can also be persistent transgenerationally (Aiken et al. 2015; Hanafi et al. 2016; Saben et al. 2016). In addition, and particularly important for understanding transgenerational epigenetic programming, is the fact that mitochondria are maternally inherited—although cytoplasmic inheritance of paternal mitochondrial DNA can also occur (Sharma et al. 2016; Stearns 2001)—offering a venue for better understanding the extent to which maternal inheritance can influence their offspring, as well as for understanding the limits—if any—of paternal inheritance.

In addition to mtDNA, a relatively new line of research has been focused on determining the role of other epigenetic modifications, such as histone modifications, in developmental programming and their transgenerational implications. For example, it was believed that histone modifications (e.g., acetylation, methylation, phosphorylation, etc.) were completely erased during gametogenesis and thus had no role in transgenerational inheritance (Chen and Zhang 2011). However, recent studies in mice (Inoue et al. 2017) have demonstrated imprinting of gene expression through histone-mediated mechanisms, and fish, birds, and other mammals have demonstrated that early embryos can retain somatic histones from oocytes and from sperm (Ausió et al. 2014; Duffié and Bourc'his 2013); therefore, it is possible that these epigenetics modifications have a role in transgenerational programming of gene function in the offspring. Similar to histone modifications, small RNAs can play a role in transgenerational inheritance. For example, it has been demonstrated that inheritance of *miR*-34c can affect gene expression and further physiology in *Caenorhabditis elegans* (Liu et al. 2012); however, controversy exists regarding its

effects at the transgenerational scale (Chen and Zhang 2011). For an introductory read on this extensive topic, the reader is referred to (Tollefsbol 2019).

Although not all the specific mechanisms that work as a template for epigenetic modifications are fully understood, these mechanisms interact with each another and influence the heredity of traits that will ultimately affect individual survival and fitness. Overall, epigenetic modifications are involved in mechanisms such as genomic imprinting, transcriptional regulation, gene expression, development, and cell differentiation (Aiken and Ozanne 2014; Jiang et al. 2013; Kass et al. 1997; Li et al. 1993). Additionally, these modifications are transmissible from parents to their offspring, and their effects will influence how the subsequent generations respond to environmental stimuli. Therefore, there is opportunity for transgenerational acclimation, and even, evolutionary adaptation to occur.

5.4 Evolutionary Implications of Transgenerational Epigenetic Programming

Epigenetic programming at the within-generational scale can induce phenotypic plasticity that equips organisms with the tools to cope with environmental challenges. However, population and species maintenance and resilience depend upon acclimation and further adaptation across generations that will allow them to overcome environmental challenges in the long-term (Bautista and Crespel 2021). Evolution (genetic adaptation) happens when the allelic frequencies of a population shift across generations as a response to environmental pressures (Bernatchez 2016; Manhard et al. 2017). Noteworthy is the fact that micro-evolutionary changes can happen fast, across a small number of generations (Bell and Aguirre 2013; Carroll et al. 2007; Hairston et al. 2005; Reznick et al. 2019). However, theory predicts, and empirical results support, that the evolutionary potential of a particular species will be low if the species' adaptive rate is outpaced by that of the change in environmental conditions (Morgan et al. 2020). Nevertheless, the adaptability to new environments can also happen from one generation to the next by means of non-genetic inheritance (Cavieres et al. 2020; Ryu et al. 2018).

Understanding the implications of non-genetic inheritance for adaptation and evolution is a daunting challenge because of the large diversity of mechanisms with different timescales (Klosin and Lehner 2016). Although the role of non-genetic inheritance, including transgenerational epigenetic programming, for evolution is still under debate, (Bautista and Crespel 2021; Charlesworth et al. 2017; Day and Bonduriansky 2011; Laland et al. 2014), transgenerational epigenetic effects can be stable and influence organism's responses to environmental stimuli (Jablonka and Lamb 2020; Klironomos et al. 2013; Ryu et al. 2018; Yin et al. 2019). Importantly, the effects of epigenetic programming can lead to positive and negative effects on physiological function (Langley-Evans 2006; Ruhr et al. 2021). Consequently, its advantages and disadvantages for species resilience are yet to be

determined and more research is warranted. Notwithstanding, theory predicts that transgenerational acclimation will be particularly advantageous when two conditions are met. First, that the rate of change of environmental conditions is slow. Second, that the correlation between environmental conditions experienced by the parental and the offspring population is high (Bautista and Crespel 2021; Klironomos et al. 2013; Klosin and Lehner 2016; Uller et al. 2015).

In addition to conveying advantageous responses to environmental change, it is possible that a high proportion of a population will experience epigenetic modifications that can help them to bridge the environmental disturbance (Burggren 2016). Therefore, transgenerational acclimation by means of non-genetic inheritance can weaken the strength of selective pressures and can delay the rate of genetic adaptation (Donelson et al. 2019; Huey et al. 2009). For instance, organisms with diverse genotypes can exhibit similar fitness due to the phenotypic plasticity of traits orchestrated by the epigenetic modifications. Furthermore, if the environment is constant across generations, it is possible that the mean of the phenotype of interest will shift closer to the new fitness optima imposed by the conditions (Bautista and Crespel 2021; Falconer and Mackay 1981; Forsman 2015; Ghalambor et al. 2007; Wild and Traulsen 2007). However, more research is still needed to better understand this process and to determine if transgenerational acclimation and epigenetic programming can lead to genomic assimilation.

Overall, both mechanisms of inheritance can significantly impact population maintenance and species resilience; nonetheless, modeling suggests that if selection imposed by environmental conditions acts simultaneously on both genetic and non-genetic mechanisms, the rate of adaptation is significantly faster in comparison with the rate attained when just one mechanism is affected (Klironomos et al. 2013).

5.5 Conclusion: Research Challenges and Future Directions

The inherent complexity of understanding the role of within- and transgenerational epigenetic programming for phenotypic evolution under ecologically relevant scenarios highlights the need for interdisciplinary efforts. These efforts will face a large number of problems because in reality, the feasibility of empirical evolutionary approaches in the wild may be limited by abiotic (e.g., funding, resources, technology, time, and space) and biotic factors (e.g., organismal life span, time to reach sexual maturity, low number of offspring per clutch). For those problems, following August Krogh's principle—'For a large number of problems there will be some animal of choice, or a few of such animals, on which it can be most conveniently suited' (Krogh's principle for a new era 2003; Krebs 1975)—as well as delving into mathematical modeling and statistical approaches for analysis, might prove to be the most effective strategy. In addition, although the role of scientists is not to discuss the semantics of specific words, because the existing terminology and semantics vary widely across disciplines, it is essential to offer a clarification of the one being used.

Furthermore, in this chapter I advocate for studying the effects of transgenerational epigenetic programming beyond the human health field. Of course I am not trying to diminish nor question the value of all the research that has been performed in this field, but simply following the bias of my scientific training and planting the seed of the idea that studying epigenetic programming, as a part of non-genetic inheritance, under an evolutionary umbrella may hold discoveries for better understanding evolution in general terms.

Finally, below I have provided a list of topics that warrant further investigation in this field, these directions are followed by a recommendation of how they can be addressed in future experimental designs.

- The most obvious field of experimentation is the uncovering of the molecular mechanisms that underlie transgenerational epigenetic phenomena. Although the advent of new technology has provided tools for discovering specific mechanisms, our understanding of the exact steps of how epigenetic inheritance occurs is still limited. This field can find strong support from approaches in biochemistry and structural biology aimed to uncover the specific binding properties of amino acids and proteins, and the constrains for their inheritance.
- The capacity of transgenerational epigenetic phenomena to promote genomic fixation and induce evolutionary change has yet to be revealed. In spite of the fact that experimental approaches have provided some insights on the role of transgenerational epigenetic inheritance across a few generations, the current understanding of its evolutionary capacity is limited. This results from the fact that it is not yet completely understood how some epigenetic marks, acquired during programming, remain stable while others do not last after the reprogramming events. In other words, the specific mechanisms of inheritance are not fully understood. However, although comprehension of the mechanistic basis is needed, interpretations of its role at the evolutionary scale require examination at the population and species level. Therefore, this topic could be approached by performing experimental designs that consider and can control for artificial selection events. Additionally, those experiments should focus on demonstrating that transgenerational acclimation as a product of epigenetic marks can induce a shift in the mean of a phenotype toward the phenotypic optimum imposed by the new environmental condition. Nonetheless, as mentioned above, this type of experiments may not be always feasible, and thus, the use of animal models, as well as mathematical and statistical modeling, can provide more accurate predictions.
- How development affects the outcomes of transgenerational epigenetic programming is a major tenet in the field that deserves more attention. Transgenerational epigenetic programming is closely related to development; therefore, it is almost always assumed that this phenomenon happens during the very early stages. However, epigenetic states can be acquired and modified also during later life stages. If the acquired modifications by the parental population are stable in their offspring even after the reprogramming events, then it is essential to also consider how epigenetic marks acquired at each of the different developmental stages of a

parental population can affect each specific developmental stage of the offspring. Experiments inquiring about these topics may find a guide and more accurate predictions by prioritizing focus on 'developmental rate' rather than 'chronological time' for analyzing specific traits. Furthermore, the use of genetic tools, such as knock-out organisms, can help to examine the effects of specific mechanisms at the tissue, organ, and system levels.

- How life-history traits affect the outcome of transgenerational epigenetic programming is a topic commonly neglected in empirical approaches. Consideration of the basic biology of the organisms has been relatively put aside because the main focus or modern research has been that of unrevealing the underlying mechanistic processes of transgenerational epigenetic programming, and inheritance in general. However, to better understand their evolutionary implications the phenology of life-history traits must be taken into account. In particular, those studies could consider related species representing opposite extremes of any life-history trait. This approach can provide a framework for comparison. For example, considering species with short generational times may prove more suitable for experiments aimed at understanding evolutionary processes. In contrast, long-lived species may be more useful when addressing within-generational plastic responses. These studies can also render more precise information for determining the interplay between within- and transgenerational epigenetic phenomena.
- Natural conditions are the product of interacting environmental factors such as temperature, oxygen concentration, UV radiation, and pollutants. These conditions are constantly fluctuating and vary in magnitude, duration, and periodicity. Furthermore, these interactions between factors can induce additive, synergistic, or antagonistic effects on the individuals within populations. However, these interactions are rarely considered in experimental designs. Individuals can cope with some of those effects within-generationally through plastic responses; however, for population maintenance and species survival, transgenerational acclimatory and—if possible—adaptive responses are needed. Consequently, studies aimed to understand the effects of environmental stressors on individuals and populations should consider multi-stressor interactions as well as different time scales and lengths of exposure to the conditions. These designs should be followed by determination and comparison of the epigenetic marks induced by the exposure regimes.

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Chapter 6
Epigenetics, Evolution and Development of Birds

Carlos Guerrero-Bosagna, John Lees, Daniel Núñez-León, and João F. Botelho

Contents

Abstract In this chapter, we summarise the contribution of research in birds to the field of epigenetics and development, both from the Waddingtonian and molecular perspectives. We compiled the most relevant bird research in the field, starting with the Aristotelian concept of epigenesis, describing the preformation versus epigenesis dichotomy of the renaissance and finally presenting state-of-the art developmental and molecular research. We also summarise the main environmental influences known to affect bird's development, including hypoxia, temperature and toxicants as well as their phenotypic effects. We present current research in birds describing molecular epigenetic changes in response to common environmental exposures, such as to stressors. In parallel, we also explore the relevance of epigenetics to understand evolutionary process, describing both relevant classical publications and current research in birds. We also present cases of transgenerational epigenetic

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inheritance and explore the contribution of birds to understand genomic dynamics in evolution.

Keywords Birds · Epigenetics · Evolution · Development · Egg · Waddington · Genomic Variation · Environmental Exposures

6.1 Introduction: Birds and the Conceptual Development of Epigenetics

Embryonic research in birds has been fundamental for the conceptual development of the term epigenetics. In the mid-twentieth century, Conrad H. Waddington named epigenetics as a new discipline defining it as 'the branch of biology that studies the causal interactions between genes and their products that produce the phenotype' (Waddington 1952). In this chapter, we will call this definition as '*Waddingtonian epigenetics',* in order to distinguish it from the most modern usage of the term, which we will refer to as *'Molecular epigenetics'.* This distinction between these two definitions of epigenetics has previously been employed by Jablonka and Lamb (2002).

Waddingtonian epigenetics is linked to the Aristotelian idea of epigenesis, namely the generation of animals from homogeneous matter through a gradual succession of forms (Van Speybroeck 2002). Aristotle's views of embryonic development were shaped by the systematic examination of chicken embryos, and occasional observation of embryos from other bird species such as doves, partridges and ostriches, in which he observed that '[the] simultaneous formation of the parts . . . does not happen: some of the parts are clearly to be seen present in the embryo while others are not.' (Aristotle. 1943). This epigenesis Aristotelian view prevailed during the Middle Ages and influenced scientists during the renaissance. In 1651, William Harvey, best known for describing the role of the heart in the circulation, published an embryological treatise comparing his predecessors' ideas to his own conclusions obtained from observations of chicken development. He used the term epigenesis in a similar fashion as proposed by Aristotle, i.e. describing the 'gradual, part by part' development observed in higher animals: 'The generation of the chicken from the egg is the result of epigenesis [. . .] and all its parts are not created simultaneously, but emerge in due sequence and order; . . . some parts supervene on other, from which they become distinct' (Harvey 1952).

In the following century, Marcelo Malpighi offered the first detailed illustrations of chick development. Interestingly, his work was used by some scientists as evidence of preformation, an idea opposing epigenesis, which proposed instead that unfertilised eggs contained already preformed chicks (Correia 1997). Albrecht von Haller, for instance, believed that tiny, preformed chickens grew, inside eggs, from the flow of fluids pumped through the heart. He pointed to the continuity between the membranes involving yolk and gut as evidence of preformation (Roe

1981; Roger 1971). Caspar Friedrich Wolff disagreed with Haller's interpretation of the continuity between the membrane surrounding the hen's egg yolk and the future digestive tube (Roe 1979). Similar interpretations deriving from the analyses of chick embryos were made in the first half of the nineteenth century by Von Baer and Pander (Churchill 1994). They described the formation of germ layers and morphogenetic movements such as gastrulation and neurulation, laying the foundations of modern embryology as an epigenetic process.

In the early nineteenth century, according to Waddington himself, the concept of epigenesis '*had more or less passed into disuse*' (Waddington 1968) after the reporting of Mendelian inheritance, the discovery of DNA structure and the publication of influential works on population genetics. In this context, Waddington joined together the concepts of epigenesis and genetics for an integrative understanding of early developmental processes, in which heredity would also play a role (Van Speybroeck 2002). Even before molecular biology offered light on how gene regulation works, *Waddingtonian epigenetics* depicted genes interacting during developmental processes, proposing genetic redundancy, macro-mutational effects and environmental influences acting together towards the formation of the phenotype.

Experiments in birds were of particular interest and influence to Waddington's ideas, as birds were a very important model for embryology since Aristotle, and for medical research on viral infections in the early twentieth century (Kain et al. 2014). Proof of that influence is his book '*The Epigenetics of Birds*', published in 1952, in which Waddington summarised the research performed during the late nineteenth and early twentieth centuries on morphogenetic processes during early bird development (Waddington 1952).

Inspired by Waddington's ideas, concepts such as '*epigenetic mechanisms*' and '*epigenetic effects*' started to be used by evolutionary developmental biologists to refer to the interactions such as between cells, tissues, organs, organisms and the environment, or even behavioural (Alberch 1980, 1982; Hall 1983; Ho and Saunders 1979). An updated definition of Epigenetics that is in accordance with Waddington's legacy is that of Hall (1992) who has defined epigenetics as '*the sum of the genetic and non-genetic factors acting upon cells to selectively control the gene expression that produces increasing phenotypic complexity during development.*'

The second meaning of epigenetics, which we call *Molecular epigenetics*, emerged in the 1990s to initially focus on '*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*' (Riggs et al. 1996) and more recently on chromatin chemical modifications that regulate gene expression and are maintained after cell divisions (Skinner et al. 2010). *Molecular epigenetics* relates to concepts such as 'epigenetic control regions', 'epigenetic inheritance', 'DNA methylation' and 'histone modifications'. Additionally, epigenetic modifications started to be studied in response to environmental factors, giving rise to the field of 'environmental epigenetics' (Jirtle and Skinner 2007).

Birds have also been fundamental to nurture knowledge in relation to *Molecular epigenetics*. Key work in chickens during the 1980s revealed the effects of estrogen

on promoter DNA methylation and expression of the vitellogenin gene in laying hens (Wilks et al. 1984). This work led Australian geneticist Donald MacPhee to hypothesise that endocrine-disrupting chemicals could interfere with such an estrogen-dependent mechanism and act like epimutagens (MacPhee 1998). A few years later, American pharmacologist John McLachlan also hypothesised that estrogens or endocrine-disrupting chemicals could affect gene programming or imprinting through persistent changes in DNA methylation (McLachlan 2001). Since then, endocrine effects on epigenetic mechanisms, such as DNA methylation, have been reported numerous times in different experimental models, making the study of epigenetic effects of endocrine-disrupting chemicals one of the main drivers of the field of environmental epigenetics (Nalvarte et al. 2019). In addition, as we will see later in this chapter, research in birds is currently contributing to the understanding of genomic and epigenomic dynamics in evolution, and the molecular basis of behavioural variability.

6.2 Waddingtonian Epigenetic Research in Relation to Bird Development and the Environment

The development of the avian within the egg has lured the attention of scientists for centuries. Oviparity, being the ancestral mode of reproduction for vertebrates is by no means rare; however, birds are relatively unique in their ability to expedite development through incubation. Although this behaviour is present occasionally in some other species of reptile and amphibian, relative to other amniotes, birds demonstrate rapid embryological development, facilitated in part by the stable elevated temperatures resulting from parental care. A comparison of avian incubation periods (11–85 days) to those estimated in non-avian dinosaurs (2.8–5.8 months) illustrates the stark influence of incubation upon development (Erickson et al. 2017). For non-avian sauropsids, the impact of the external environment upon eggs is critical, for example with temperature determining sex ratio in numerous species of turtles and crocodilians, as well as cognition, morphology, physiology and hatching success (Siviter et al. 2017; Siviter et al. 2019). As a result, many species have evolved strategies that maximise the stability of the egg's environment in the absence of parental care (Siviter et al. 2017, 2019). Temperature is, of course, also critical for avian development. However, birds have evolved physiological and behavioural mechanisms that buffer the impacts of external environment on embryos developing within eggs. This characteristic of birds (which is also present in some reptile and amphibian species) arose early in their evolution, with fossil evidence indicating some level of parental care of eggs in ancestral non-avian theropods (Bi et al. 2021), although Mesozoic birds were likely too heavy to incubate their eggs by brooding (Deeming and Mayr 2018). The parallel evolution of larger, stronger eggs in addition to the traits necessary for flight (feathers, reduced body weight, increased metabolic capacity) then made true brooding possible. Indeed the

evolution of incubation behaviours is also intricately linked to the evolution of endothermy and, to some extent, a requirement if egg temperature is to be consistently maintained above ambient levels (Farmer 2000). The beneficial consequences of incubation for offspring development are clear, with reduced developmental times in birds compared to many other extant amniotes, creating greater plasticity to environmental change (Erickson et al. 2017). In addition, predation is likely reduced through expedited development and the fact that parents are able to defend their offspring from predation.

Eggs offer unique insights into the epigenetic mechanisms underlying development environmental manipulations can be directly executed and its effects observed, unlike in mammals where embryos develop inside the womb, which complicates the assessment of the effects of environmental exposures in vivo (Edwards et al. 2021). Understanding how the avian embryo responds to the external environment is important not only to developmental biology but also to numerous connected fields within physiology, molecular and evolutionary biology (Bednarczyk et al. 2021; Burggren et al. 2016; Durant et al. 2013). Outside of the laboratory, such work is also critical in assessing the impact of human-induced environmental changes (both chemical and climatic) on birds (Sauve et al. 2021). In addition, the economic value of poultry as protein source has increased the attention towards understanding the influence of incubation conditions upon post-hatch development and growth. As a result, avian literature is plentiful but somewhat biased towards galliform species. At the time of this publication, a PubMed search of 'avian development' yields 63,000 results, while only 'chicken development' generates 42,000 results.

The avian egg offers an excellent model to investigate the epigenetic influence of the environment upon embryonic development, as maternal effects can be restricted to egg composition. Eggs can vary in factors such as yolk, hormone, RNA and mitochondria composition as a result of variation in the maternal environment (Johnson 2015). Differences in egg composition (yolk environment) and size can be dramatic, and for example, underpin the large phenotypic differences observed between chicken breeds (Ho 2014). One notorious example relates to maternal effects altering offspring's sex ratio, which may result from differential segregation of sex chromosomes or alteration of maternal sex steroids (Johnson 2015). The role of steroids (mainly androgens) has received much attention, with numerous investigations showing that manipulation of egg hormone levels produces marked effects upon brain development behaviour (Groothuis and Schwabl 2008). Prenatal testosterone is shown to affect behavioural traits such as social rank (Schwabl 1993), hatch time and begging behaviour (Groothuis and Schwabl 2008), in addition to post-hatch growth, immune development and survival (Groothuis and Schwabl 2008). Mechanistically, hormones deposited in the embryo may act early in development via androgen and estrogen receptors in the extraembryonic membranes (Kumar et al. 2019). In addition to the strong maternal effects on embryonic development, paternally derived factors in ejaculates may also play an important role. Paternal age is known to impact offspring fitness and ageing, for example influencing telomere length (Bauch et al. 2019).

By manipulating the *in ovo* environment and quantifying the embryonic response, we have learnt much of how regulatory systems are shaped during development in a truly Waddingtonian sense of the word epigenetics. This is, however, a complex field as there are numerous external (climatic, parental incubation investment) and internal (water, nutrients, hormones, carotenoids, vitamins, nucleotides) Waddingtonian epigenetic factors which can influence development differently dependent upon embryonic age, breed, genetic and molecular epigenetic background as well as the length, magnitude and interaction of exposures (Boleli et al. 2016; Reed and Clark 2011). As a result, the literature concerning this topic is vast and often conflicting due to difficulties in standardisation of the many confounding factors. Much of the data have been obtained from various breeds of domestic chickens, for both commercial and practical reasons. However, investigations are increasingly aimed at determining how these factors play out in the wild under the influence of unpredictable climatic conditions and variable parental investment. Given the burgeoning interest in the influence of abiotic factors upon avian development, here we will focus attention upon the impacts of the most potent environmental forces to which eggs may be exposed, namely oxygen, temperature and environmental toxicants.

6.2.1 Hypoxia

Oxygen is, unsurprisingly, central to embryonic development and oxygen availability has profound influences upon the phenotype. Atmospheric oxygen enters the egg through diffusion across the egg-shell and depending on the age of the embryo is transported either via the yolk sac, the chorioallantoic membrane (CAM) or the lungs (Mueller et al. 2015). Tissue concentrations of oxygen vary in normal embryonic development and tissue hypoxia is a fundamental part of avian embryonic development (Carroll et al. 2021). A combination of increased oxygen consumption, a small gas cell and low membrane permeability means that the embryo experiences less than 5% oxygen in the first few days of development. This hypoxia is critical in driving vascular development, haematopoiesis and chondrogenesis (Carroll et al. 2021; Haron et al. 2021).

The influence of developmental hypoxia is broad and varies greatly depending on its timing and magnitude. In birds, this can be investigated by manipulating oxygen levels during incubation. Chronic hypoxia (often around 15–17% environmental oxygen) largely results in increased lethality and surviving chicks often display late hatching with malformations, cardiovascular pathologies and growth retardation (Grabowski 1964; Metcalfe et al. 1984). Although there is a remarkable amount of plasticity in the system (Amit Haron et al. 2021), hypoxia has a profound influence on cardiovascular development and fluid balance in the developing embryo. At the organ level, hypoxia influences respiratory and cardiovascular development, likely in-turn impacting the organ systems they supply. For example, chronic hypoxia causes enlargement of the heart and beta-adrenergic desensitisation in hatchlings

(Lindgren and Altimiras 2011) and increases the extent of the CAM (Burggren et al. 2016; Druyan and Levi 2012). Interestingly, hypoxia decreases the metabolic rate of developing embryos in an apparently regulated manner, involving downregulation as opposed to the passive result of lower oxygen availability (Haron et al. 2021; Rohlicek et al. 1998).

Although acute periods of hypoxia may be tolerated by the embryo, prolonged but sub-chronic hypoxia exerts developmental effects and is dependent upon species and the timing of exposure. For example, quail embryos incubated under 16% oxygen die at ED 9 as a result of cardiovascular malformations and also show ventricular hypertrophy in response to hypoxic incubation (Nanka et al. 2008). In chickens, however, the survival is 64% when embryos are incubated at 15% oxygen up to ED3.5 (Sharma et al. 2006), while chronic exposure at 17% oxygen during E16–18 has no effects on survival, although resulting in morphological and physiological effects at hatch (Haron et al. 2017). As one might expect, there are certain developmental 'critical windows' in which sensitivity to hypoxia differs. By exposing chicken embryos to 15% oxygen during early (ED1–6), middle (ED6–12) or late (ED12–18) development, Chan and Burggren (2005) demonstrated that these critical windows vary dependent on the organ systems. Beak and eyes growth were most strongly impacted early in development whereas the opposite was true for the CAM, which increased in mass during ED12–18 hypoxia. Interestingly, in a normoxic ED12–18, a number of tissues investigated were not affected by any preceding hypoxia, suggestive of the notion that compensatory mechanisms allow for the 'recovery' from any abnormal development. This plasticity has also been seen in quail (Burggren and Elmonoufy 2017) and is very much in line with Waddington's ideas of canalisation in which 'normal' development is resilient as systems are buffered from perturbations, perhaps in this case by compensatory (homeorhetic in a Waddingtonian sense) mechanisms in cardiovascular development that maintain optimal tissue partial pressures of oxygen. Although epigenetic mechanisms may facilitate plasticity in the developmental apparatus, which compensates for reduced oxygen during development, the resultant physiological adaptations may have consequences for adult performance, particularly when their physiological systems are put under stress. For example, chickens incubated under hypoxic conditions show blunted thermogenesis (Azzam et al. 2007) and altered chemosensitivity (Ferner and Mortola 2009).

6.2.2 Temperature

From an epigenetic perspective, temperature is perhaps one of the most interesting environmental factors which can influence development. Avian embryos are poikilothermic, requiring an external heat source (usually in the form of a parent) for development and correct metabolic function (Mueller et al. 2015). Because temperature during incubation will strongly influence avian embryonic development (Decuypere and Michels 1992), there is an important role for ambient temperature

to which eggs are exposed, which will vary over various time courses (daily, annually, millennia), with different geographical locations, and in response to variations in parental care of eggs. Investigating the developmental responses of embryos to altered temperatures and their subsequent post-hatch development is therefore of huge evolutionary relevance as it offers a very tangible example of how epigenetic changes may impact future generations, either through parental effects or through genetic assimilation (Sauve et al. 2021). The results of temperature studies also provide a valuable insight into the plasticity that these epigenetic influences impart on species, thus buffering them against the effects of recent accelerated climate change.

Chronic hypothermia and hyperthermia exert a strong influence over development. Besides the effects of temperature on reaction rates, temperature also influences the solubility of oxygen in embryonic blood (decreasing oxygen solubility as temperature increases). In addition, carbon dioxide solubility and the resultant effect on pH will also be altered, in turn changing erythrocyte oxygen affinity. Chronic hypothermia results in impaired growth, delayed hatching and disturbance of embryonic development, particularly of the heart, which becomes hypertrophic with altered pacemaker activity (Vostarek et al. 2016; Warbanow 1970). Wild birds may experience more intermittent periods of hypothermia, and appear to have evolved a remarkable tolerance to intermittent, acute hypothermia. For example, a study exposing eggs from five bird species to 10 °C for 6 hours per day revealed no effect on hatching success in four of the five species (Zhao et al. 2017). Detailed physiological indices were, however not measured. Hyperthermia also exerts pathological effects on the development of the cardiovascular system. Just 2 days of 3–4 °C temperature elevation in chicken embryos results in vascular abnormalities such as abnormal branching, pathological leakage and perivascular oedema (Nilsen 1984). Clearly, the impacts of these vascular abnormalities are likely to be global. van den Brand et al. (2021) exposed ED8 chicken eggs to hyperthermia as well as varying carbon dioxide concentration. CO_2 (between 0.1 and 0.8%) did not affect embryonic development; however, a 1 °C elevation in temperature decreased egg weight, embryo size and heart weight of the embryo, thus affecting hatchability of chicks.

Changes in incubation temperature at specific developmental windows can elicit different epigenetic effects later in life. Ducks but not turkeys show altered thermoregulatory phenotypes in response to altered temperatures late in incubation (Nichelmann 2004). Ducks reared in cold late conditions preferred lower ambient temperatures post-hatch and maintained lower core body temperatures. This 'thermal conditioning' in which thermoregulatory responses may be epigenetically set during development is also seen in broiler chickens, which after just 24 hours of elevated temperature (38 °C) at ED5 have increased food intake and growth as well as improved heat tolerance post-hatch (Yahav and Plavnik 1999). However, broilers incubated at 39.5 °C from E7-E16 showed reduced hatchability and body weight despite having improved thermotolerance, illustrating that timing is key and that physiological changes emerging from environmental challenges influence the biological system as a whole (Piestun et al. 2008a). Similarly, inter-specific differences

in responses to acute or sub-chronic temperature variation may lie in the timings of development of important thermoregulatory centres such as the thyroid and adrenal axes (Piestun et al. 2008b). Thermal-induced alterations in physiological and neuroendocrine traits during development may appear to be resolved upon hatching but the extent to which other important traits, such as behaviour, are affected is becoming increasingly known (Bertin et al. 2018).

The consequences of short-term effects of altered temperature upon development may be stark on a species level, illustrating the possibility for short-term developmental plasticity (i.e. Waddingtonian epigenetic influences) to become fixed over time. A good example of this comes from North American migratory birds. A study involving over 70,000 individuals from 52 species revealed a significant decline in body size over the past 40 years, coincident with increasing temperatures (Weeks et al. 2020). In parallel, wing lengths were seen to increase, likely as a response to the increasing energetic demands of migratory flight with decreased size. Although small changes in size between generations may appear insignificant, over longer time periods, important behavioural (e.g. migration), physiological (e.g. metabolism and food requirements) and reproductive (e.g. egg size and number) changes may occur.

The mechanistic underpinnings of developmental responses to changes in temperature are varied but heat shock proteins are one significant contributor. Toth and collaborators (Toth et al. 2021) assessed the effect of heat stress in 1-day-old Transylvanian naked neck chicks by the expression of heat-shock proteins in the later mature chickens. Heat-shock proteins HSP90 and HSF4 increased significantly in heat-treated female gonads, but HSF2 and HSF3 showed substantially lower expression. HSP70, HSF1 and HSF3 expression levels increased in male gonads (Toth et al. 2021). These consequences in the gonads suggest that heat stress could be a potential factor for inter- or transgenerational effects in birds. The effects of environmental perturbations are also intimately connected and often centre on the interaction between oxygen, temperature and cellular metabolism. For example, Vimmerstedt et al. (2019) showed that oxygen limitation severely impacts embryonic heat tolerance, likely as oxidative phosphorylation cannot meet the increased ATP needs of enzymes at higher reaction rates.

Given the temperature sensitivity of embryonic development, it is likely that the accelerated rises in global air and water temperatures will impact development in oviparous species. Animals that do not brood their eggs may be able to mitigate such impacts through altered timing and location of egg laying (Telemeco et al. 2009). However, the long-term effectiveness of these strategies in light of forecasted raises in temperatures is unclear. For terrestrial oviparous ectotherms without temperature-dependent sex determination, it may seem intuitive that warming temperatures will speed embryonic development, because chronic elevated temperatures increase developmental rate in ectotherms (Burraco et al. 2020). However, more realistic recreations of temperatures incorporating stochastic thermal fluctuations, such as unpredictable spikes in temperature, reveal a negative impact upon embryonic survival (Hall and Warner 2018).

Compared to non-brooding oviparous species, the incubation behaviours and parental care seen in birds may put the avian embryo at a diminished risk from rising fluctuating global temperatures. Indeed, evidence shows that parental birds are able to compensate for temporal, geographical and human-induced fluctuations in temperature through behavioural alterations that influence embryonic temperature, such as egg rotation patterns, incubation frequency, nest insulation and timing of lay (Du et al. 2019). However, many species of precocial ground-dwelling bird do not brood during early development, making them vulnerable to the effects of environmental temperature fluctuations (Reyna and Burggren 2017).

In any case, in the event the effects of developmental temperature variation cannot be compensated by parental behaviour, plastic responses will then play an important role. Although phenotypic plasticity in response to temperature has been documented extensively, such tolerance is irrelevant to adaptation if not heritable and influential upon offspring fitness (Burggren 2018). In this sense, future research should investigate the ability of this plasticity to be inherited, particularly concerning the current context of global warming.

6.2.3 Toxicants

Another well-studied factor that affects egg development is exposure to environmental toxicants. Pesticides, for example, seem to alter egg-lying and developmental traits. Maybe the most notorious example is the poisoning case of bald eagles (*Haliaeetus leucocephalus*) by DDT environmental contamination that occurred in the early twentieth century and the further accumulation of DDE, the resulting degradation product. These insecticides slowly degrade in the environment and interfere with the calcium uptakes in birds (Lundholm 1997; Peakall 1969). The high concentration of these pesticides in the water during the mid-twentieth century poisoned fish-eating birds such as Osprey, pelicans, herons, ibises and cormorants (King et al. 1978). Both DDT and DDE were reported as the cause of the thinning of bald eagle egg shells, nearly causing their extinction (Stokstad 2007). This case, portraited in the 1962 book *Silent Spring* by Rachel Carson (1962), helped to change legislation in the USA in relation to the use of pesticides in the environment.

Another pesticide, dieldrin, has shown dissimilar results in agriculture areas and experiments in birds. Although exposure of gallinule eggs to this pesticide is not shown to alter hatchability (Fowler et al. 1971), this exposure causes reproductive problems in golden eagles and is lethal to brown pelicans (Blus et al. 1974; Stickel 1973). In domesticated birds, the accumulation of dieldrin in eggs (of pheasants and chickens) from hens consuming this compound in their diets was initially reported already in the 1960 (Atkins and Linder 1967; Graves et al. 1969). Additionally, reproductive effects were observed in eggs and chicks from hens consuming dieldrin. While consumption of 6 mg/week of dieldrin by hens' pheasants led to reduced egg production and egg weight, no effects were observed in egg survival or hatchability (Atkins and Linder 1967). In chicken hens, no effects in egg production or

hatch were observed with diets containing up to 5 ppm dieldrin. The effects of dieldrin consumption in hen pheasants were also investigated in for two generations, in which some offspring were exposed only in the first generation and others in both generations. Although mortality was unchanged in hens exposed in the first generation, it was increased in all the hens exposed again in the second generation, which also exhibited reduced feed consumption (Baxter et al. 1969). Additionally, second generation hens exposed via the egg to dieldrin residues laid eggs with decreased fertility and hatchability (Baxter et al. 1969).

Pesticides can also alter behavioural aspects in birds. In bobwhite quails (*Colinus virginianus*), control animals made fewer errors in discriminatory behaviour than those treated with as little as 20 mg/kg of DDT in their diets (James and Davis 1965). Also, aberrant territorial breeding behaviour has been reported among sharp-tailed grouse (*Pedioecetes phasianellus*) in a field study designed to determine response of group to single oral doses of Dieldrin and Malathion (McEwen and Brown 1966).

The broad, indirect and often interconnected actions of diverse environmental perturbations upon development, as well as inter-specific and inter-individual differences in response, elicit widespread developmental and phenotypic variation. Considering the Waddingtonian concept of *Epigenetic landscape* (Waddington 1957), early environmental exposure can bias the development towards specific outcomes that become hard wired with time. In the molecular sense, a fruitful area for future research lies in understanding the tissue-specific molecular modifications resulting from environmental conditions experienced at a given developmental timepoint.

6.3 Molecular Epigenetic Research in Relation to Bird Development and the Environment

Birds have been used as important models to investigate the consequences of environmental exposures during embryonic or post-hatching stages on molecular epigenetic mechanisms. Birds are particularly good models for this for many reasons: some bird species have well annotated genomes, such as great tits, Darwin finches and chickens; birds possess nucleated red blood cells, which allow for longitudinal experiments aiming to investigate environmental exposures and lifelong epigenomic effects in these cells; in birds the egg environment can be directly and precisely manipulated to investigate post-natal molecular epigenetic consequences; birds are susceptible to climate variations; chickens, in particular, are a species of high economic importance as it represents the largest consumed meet source worldwide (OCDE-FAO 2021).

Although many epigenetic mechanisms have been described to date, the most studied of them in birds is DNA methylation, in relation to life long, inter- and transgenerational consequences of early exposures. The epigenetic mechanism called DNA methylation involves the enzymatic addition of methyl groups to

nucleotides in the DNA, mainly cytosines neighbouring a guanine; these dinucleotides are known as CpG sites (Bestor 2000; Singal and Ginder 1999). When methyl groups attach to cytosines, they can regulate the activation of a gene. DNA methylation is also related to genomic imprinting. Although in most cases, methylation patterns are equivalent between the maternal and paternal alleles in any given cell, a few genomic regions exhibit differential methylation among these, leading to also differential regulation of gene expression in each of these alleles; when these allelic methylations, and consequently gene expression differences, are related to parent-of-origin, this is known as genomic imprinting (Plasschaert and Bartolomei 2014).

Although well-described in mammals, in birds the occurrence of genomic imprinting is still unconfirmed (Fresard et al. 2013). One study performed with sufficient number of individuals and using appropriate corrections showed parent-of-origin QTLs in alleles of chromosome 1 of chickens (Rowe et al. 2009). Interestingly, these regions are orthologous to imprinted genomic regions in human and mouse. However, investigation of other genomic regions in birds that are orthologous to well-known imprinted genes in mammals has mostly shown the absence of imprinting (Colosi et al. 2006; Shin et al. 2010; Yokomine et al. 2005). More recent studies employing RNA Next Generation Sequencing have also shown the absence of imprinting. When considering both transcripts and non-coding RNAs, Wang et al. (2015) did not observe imprinting in the brain of 1-day-old chicks. Zhuo et al. (2019) in turn, investigated the liver and brain of 12-day-old chicken embryos to find that although allelic-specific expression was common, it was not related to parent-of-origin effects. The use of modern genomic techniques to identify imprinting in mammals will help to elucidate whether this phenomenon occurs also in birds (Fresard et al. 2013).

Recent research in birds has been of high relevance to elucidate the influence of early environment and the role of the epigenome in behaviour and neurodevelopment. In chickens, it has been demonstrated that the methylome of red blood cells (nucleated in birds) reflects previous rearing condition, i.e. cages or open aviaries (Pertille et al. 2017). These conditions associate with differential fearfulness and cognitive abilities (Brantsaeter et al. 2016; Tahamtani et al. 2015). Also in chickens, exposure of 4-day-old male chickens to social isolation stress, incrementally for three weeks, is shown to produce DNA methylation changes in red blood cells (obtained at the end of the treatment) compared to a barren, control condition (Pertille et al. 2020). In great tits, early life stress caused by experimental manipulation of brood size altered DNA methylation in red blood cells 14 days after hatching, with larger effects between siblings of enlarged or reduced broods compared to controls (Sepers et al. 2021).

Studies in birds have also provided clues to understand the epigenetic basis of neurobehavioural variability. In a very homogenous laying chicken population, it was found that DNA methylation variation in the nidopallium (brain region relevant for decision-making tasks) correlates with naturally emerging variable behavioural patterns and also different cellular functions (Guerrero-Bosagna et al. 2020). In zebra finches, exposure of eggs to songs from conspecifics, hetero-specific closely related birds (Bengalese finch) or hetero-specific farther related birds (pin-tailed whydah)

altered genome-wide methylation in the auditory forebrain of embryos, with higher methylation levels incrementally observed with songs from more phylogenetic distant birds (Antonson et al. 2021).

In birds, the effects of parental exposure on their offspring are reported to some extent. In chickens, early stress (social isolation) has been shown to affect gene expression in the thalamus/hypothalamus of their offspring (Goerlich et al. 2012). Interestingly, transcriptomic changes in the hypothalamus of chickens raised with unpredictable light exposure are also observed in their offspring (Natt et al. 2009). These effects observed in the offspring of exposed parental birds suggest transmission to future generations of germline epigenetic alterations induced postnatally. The mechanisms through which parental exposures would affect epigenetic marks in the gametes are not known. However, it is known that important changes in DNA methylation, histones and chromatin structure take place during adult spermatogenesis (Rajender et al. 2011; Vlachogiannis et al. 2015). On other hand, the inheritance of mitochondrial alterations after developmental or adult exposures is an exciting field in relation to epigenetic inheritance. Mitochondria act as environmental sensors, integrating the complex internal cellular milieu into metabolic response, which can influence nuclear gene expression, both directly and indirectly, through molecular epigenetic mechanisms (Whelan and Zuckerbraun 2013). Although mitochondrial transgenerational transmission has been widely assumed to take place exclusively via maternal inheritance, in chickens evidence exists for the inheritance of paternal mitochondrial DNA (Alexander et al. 2015).

Some of these effects are even shown to be perpetuated across multiple generations besides the immediate offspring. A study in ducks showed that maternal methionine deficiency affected body weight and lipid metabolism in their grand-offspring (Brun et al. 2015). In chickens, exposure of hens to either viral or bacterial infections altered the body weight at 1 day of age in their offspring and grand-offspring (Liu et al. 2018). In quails, injection of eggs with genistein, an endocrine disruptor naturally available in soy, produced effects that were observed after three generations of breeding without further injection. Several traits were transgenerationally affected, such as body weight (reduced at 3 weeks of age with the ancestral exposure), abdominal fat weight (increased with the ancestral exposure), age of the first egg (delayed by 8 days with the ancestral exposure), egg number (reduced with the ancestral exposure) and birds' reaction to social isolation (reduced with the ancestral exposure) (Leroux et al. 2017).

The phenomenon of transgenerational epigenetic inheritance has been described in many organisms to date (Jacobs et al. 2017). However, in rodents, an important mechanistic aspect known is the interference of sensitive periods of major epigenetic resetting in the migration of primordial germ cells (PGCs) towards the gonads during the development (Hackett and Surani 2013; Lees-Murdock and Walsh 2008; Reik et al. 2001). It is expected that a similar event of epigenetic reprogramming in the migration of PGCs occurs in chickens. At least the migration of PGCs in chickens is well described. After laying, chicken PGCs migrate outwards from the anterior part of the embryo (germinal crescent) towards the extraembryonic tissue, while blood vessels are being formed (Nakamura et al. 2007). Once the circulatory system starts

to be active, PGCs migrate inside the embryo through the newly formed blood vessels, finally reaching the genital ridges at around 60 hours after egg laying (De Melo Bernardo et al. 2012; Nakamura et al. 2007). Changes in the gene expression of DNA methyltransferases suggest the occurrence of a major epigenetic reprogramming of PGCs during their migration in chickens (Rengaraj et al. 2011). In birds, however, transgenerational experiments involving controlled exposure of eggs during the migration of PGCs are lacking.

6.4 Waddingtonian Epigenetic Research in Relation to Bird's Evolution

Despite astounding diversity in morphology, physiology and behaviour amongst modern day birds, all members share clear defining characteristics. Waddingtonian epigenetics, as the causal study of development, clarifies the evolution of some avian characteristics that depend on the interaction of cells, tissues and behaviour. Feathers are one of the defining features of birds and display huge functional diversity, providing insulation, waterproofing, camouflage and lifting surfaces for flight (Gill 2007). Additionally, feathers have sensory and sexual roles (Prum 2017). Modified keratinous integumentary structures are likely to have evolved from scales, which are still present in many body locations in modern birds (Di-Poi and Milinkovitch 2016). Early feathers were first present in non-avian theropods and evolved into the specialised structures we see today. The modern flight feather is highly specialised, composed of a stiff central rachis flanked by interlocking vanes, whereas down feathers have open vanes and no rachis (Stettenheim 2015). The evolution of feathers followed a hierarchical sequence of transformations causally constrained on how feathers develop. Filamentous and branched feathers like those found in non-avian theropods are epigenetic prerequisites for the development of closed pennaceous vanes (Prum 1999; Sawyer and Knapp 2003). Interestingly, these structures allowed the correct prediction of intermediate morphologies even before they were actually discovered in the fossil record (Xing et al. 2016).

Flight is a challenging biological process in many respects, with adaptations towards flight permeating the avian form. From a biomechanical perspective, forward flight requires the generation of lift and thrust forces in excess of those of drag and weight. Birds, therefore, display adaptations that maximise the former (lift and thrust) and minimise the latter (drag and weight). Weight adaptations are seen in the form of hollow, often pneumatised bones, the replacement of a heavy jaw and teeth with a keratinised rhamphotheca and in the general reduction in size of birds compared to other groups. Feather morphology and its influence over wing shape and stiffness are the dominant influence over lift, varying according to species-specific requirements in flight style and speed (Lees et al. 2017). Whereas the power requirements of gliding are relatively low, the flapping flight mode of most modern birds requires high power. Much of the power is provided by the extensive pectoralis

muscles which attach to a keeled sternum, which is characteristic of flying birds. The metabolic requirements of these muscles are met by a four-chambered heart, and an efficient respiratory system consisting of uni-directionally ventilated lungs connected to a network of air sacs of variable number.

The ability to generate endogenous heat in birds (endothermy) is at the same time dependent on the increase of musculature size, and necessary for the metabolic demands of sustained flight. Although a mechanistic link between endothermy and increased metabolic capacity is far from clear, when comparing animals of similar body mass, aerobic capacity is orders of magnitude higher in endotherms, facilitating metabolically demanding activities regardless of external temperature (Bennett and Ruben 1979). Flight, body size and endothermy are interlinked by reciprocal effects on the physiology and morphology of birds. During embryonic development, the interaction of the immense appendicular musculature shapes the skeleton in which it is inserted, just as the reduction of axial musculature is associated with vertebral fusions. Physiological requirements of highly aerobic activities such as increased mitochondrial density, higher ventilation and oxygen extraction rates at the lungs, improved cardiovascular performance and blood oxygen carrying capacity, facilitated the ability to thermoregulate (Bennett and Ruben 1979). Endothermy might have evolved in parallel with the miniaturisation of theropod dinosaurs through avian evolution, scaling several other biological processes (Grady et al. 2014; Lee et al. 2014; Rezende et al. 2020). By providing their young with warmth, endotherms can speed juvenile development, and might have had a direct impact upon the development of homeostatic structures in the offspring, such as in the hypothalamic-hypophyseal axis, reinforcing the metabolic effects across generations. Faster developmental rates influenced the diversification of birds in the precocial to altricial spectrum (Botelho and Faunes 2015; Starck and Ricklefs 1998). Together with size reduction, faster developmental rates might be associated with the evolution of the paedomorphic morphology of modern avian skull (Bhullar et al. 2012). Even though it might be impossible to disentangle the evolutionary sequence of all these transformations, they have mutually influenced each other through epigenetic processes, facilitating and constraining the evolution of birds.

The evolution of the skeleton of birds is characterised by the reduction and fusion of elements. Most bones of the skull roof are completely fused during development (Smith-Paredes et al. 2018) and the shape of the skull roof itself is strongly influenced by the larger brain and eye of modern birds compared to their ancestors (Fabbri et al. 2017), which is possibly due to inductive or topological influences of brain vesicles during development. All modern birds, fossil or living, are toothless (Louchart and Viriot 2011). Classic tissue recombination experiments have shown that the ectoderm of birds' mouth is competent to develop teeth when induced by the oral mesoderm of mammals or lizards (Kollar and Fisher 1980; Mitsiadis et al. 2003). In many species, two to six thoracic vertebrae are fused forming a rigid structure called notarium (Newton and Gadow 1896). The fusion of the last thoracic, lumbar and sacral structures forms the synsacrum at late embryonic development, with the last caudal vertebrae fusing to each other and forming the pygostyle much earlier. Avian wings have fused digits (Vargas and Fallon 2005), and fused carpal

bones. In addition, birds possess a keeled sternum formed by mechanical stress during development occurring in the sites of attachment of the large pectoralis and supracoracoideus muscles (Bellairs et al. 1960; Raikow 1985). The avian leg evolution is strongly influenced by epigenetic factors (Botelho et al. 2016; Hampe 1958; Hampé 1957; Müller and Streicher 1989; Wolff and Hampe 1954). The fibula is distally reduced (Botelho et al. 2016), metatarsal bones fuse to each other and to distal tarsals forming a single bone called tarsometatarsus (Namba et al. 2010), and the development of an opposable hallux in perching birds is caused by the twisting of the metatarsal cartilage due to embryonic muscular activity (Botelho et al. 2015).

6.5 Molecular Epigenetic Research in Relation to Bird Evolution

Modern day birds demonstrate incredible phenotypic diversity. The early adaptive radiation of birds coincided with a complex array of phenotypic traits both unique (e.g. feather development) and convergent (endothermy, flight) amongst extant animals. Although the precise origins of many of these traits remain contentious, they appeared over a period of over 100-million years during the transition from theropods through to the Neornithes which represent modern bird species (Brusatte et al. 2015; Prum et al. 2015). Particularly in relation to DNA methylation, higher levels of DNA methylation and CpG content exist in anamniote (fish and amphibians) compared to amniote (reptiles, birds and mammals) vertebrates, suggesting a 'methylation transition' appearing with the latter (Jabbari et al. 1997).

Reconstructing the molecular bases of the evolutionary transition to birds from the fossil record alone is daunting. However, a combination of both reductionist and systemic approaches in extant birds continues to provide valuable mechanistic insights into the origins of this diverse vertebrate group. Furthermore, with the advent of rapid, affordable and precision sequencing technologies, we are now beginning to uncover the role of the epigenome in selective processes essential for the evolution and development of complex avian traits. Important genomes from model bird organisms have been sequenced and updated in recent years, notably the chicken (Rubin et al. 2010), Darwin finches (Lamichhaney et al. 2015) and great tits (Laine et al. 2016).

Genome-scale analysis of modern birds shows a rapid radiation of 36 lineages occurring within 10–15 million years, which is suggested to have been caused by the opening of new niches following the environmental destruction caused by an asteroid impact (Jarvis et al. 2014). The question that emerges then is what role new niches played in this rapid genomic and phenotypic diversification. One of the options is that new environments might have triggered genomic variability mediated by epigenetic changes differentially induced by specific new niches. Interestingly, some evolutionary genomic studies point to the importance of the epigenome in the genomic diversification of birds. Some of these studies have shown correlations

between methylomic changes and phylogeny or life history. For example, in the diversification Darwin finches, it has been reported that epigenetic changes in red blood cells match more closely the phylogeny of selected Darwin finches than genetic changes (copy number variations) (Skinner et al. 2014). In red jungle fowl chickens, gene expression and methylomic changes emerge in the hypothalamus after only five generations of divergent selection for high or low fear of humans (Belteky et al. 2018). In great tits, CpG methylation is higher and non-CpG methylation is lower in the brain in selective sweep regions (Laine et al. 2016). Additionally, genes with low levels of methylation in their TSS and gene bodies evolve slower than genes with higher levels of methylation (Laine et al. 2016).

These studies show important correlations between somatic epigenetic marks and genomic variability during evolutionary processes. However, causation between epigenetic changes and genomic evolutionary novelties can only be established when the germline methylome is investigated (Guerrero-Bosagna 2020). One important reason for which the epigenome could influence genomic variability is the approximately 12-fold increased mutability of cytosines to thymines when a cytosine is methylated (Huttley 2004; Tomatsu et al. 2002; Tsunoyama et al. 2001; Ying and Huttley 2011). Importantly, this increased mutability is even higher in the germline (Kong et al. 2012). Because of this, CpG to TpG mutations are more frequent than other point mutations, leading with time to CpG deficiency in vertebrate genomes (Simmen 2008; Sved and Bird 1990). Such CpG deficiency has led to functional consequences in the genome, being important, for example, for the evolution of transcription factor binding sites in tetrapods (He et al. 2015).

Besides affecting point mutations, CpG methylation can also influence the appearance of large genomic rearrangements known as Copy Number Variations (CNVs). The way this happens is through the repressive role that CpG methylation plays on transposable elements (TE) (Adelson et al. 2015; Luo et al. 2014). When de-repressed, TE can translocate or replicate in a genome, affecting the copy number of a specific region, either by excising (deletion) or producing multiple copies (e.g. duplications) of that region. CNVs can have important consequences for genomic evolution. In great tits, for example, a low frequency but large inversion has been found encompassing most of Chr1A (approximately 1000 genes). Interestingly, this inversion harbours a CNV of approximately 2.8 Mb in its downstream breakpoint (da Silva et al. 2019). Indeed, CNV breakpoints exist in nearly half of the great tits' genes, are CpG-rich, locate prominently at repetitive (segmental duplications) and regulatory regions and overlap with transcription start sites (da Silva et al. 2018).

In birds, a possible causation role for epigenetic changes in relation to genotypic variability has been recently investigated in the male germline. A study that tracked mutation dynamics in domesticated chickens in relation to the sperm methylome of their closest ancestral relative, red jungle fowl revealed that the methylomic levels in the sperm of the ancestor, and its inter-individual variability, correlated with different types of mutations such as Single Nucleotide Polymorphisms (SNPs) or Copy Number Variations (CNVs) in the domesticated breeds. Moreover, the further away a breed was from the ancestor, the more the variation accumulated in CpG sites

(Pertille et al. 2019). These results in chickens and the above results in great tits show the importance of addressing the specific mechanisms in which CpG methylation regulate retro-transposition to promote genomic variability in evolution.

6.6 Conclusion

Research in chickens has enormously contributed to the generation of epigenetic knowledge both from the Waddingtonian and the molecular perspective. These two approaches, however, should not be seen as competing, rather complementary. In the Waddingtonian sense, it is important to understand how systemic changes at various stages of embryonic development drive subsequent development. If the concept of *Epigenetic landscape* is invoked (Waddington 1957), it could be said that pre-molecular epigenetic researchers have marvelously contributed with the theory that describes the dynamics of such epigenetic landscape. Recent molecular epigenetic research, in turn, has given empirical support to the concept of epigenetic landscape, specially to describe how the genome (not only genes) expresses to enable a realm of developmental possibilities in such a landscape. The empirical support of the concept of epigenetic landscape certainly has consequences for evolutionary theory, since it helps to explain the origin of the range of developmental and genomic possibilities that are evolutionarily maintained in organisms. Waddington himself changed the Darwinian concept of the 'Survival of the fittest' for the 'Survival of the adaptable' (Waddington 1957), and recently, one of us (CG-B) has proposed the 'Survival of the non-unfit' (Guerrero-Bosagna 2017a, b) to highlight that we should not only focus of the fittest forms as those perpetuating in evolution. Understanding that cross-talk between the Waddingtonian and molecular epigenetic approaches will be key to determine how the environment shapes the wide variety of forms and species observed throughout evolutionary history. Additionally, we will be able to understand how the *epigenetic landscape* not only is influenced by genes but also affects the genome.

Since the modern synthesis, scientists have relied on a gene-centric view to understand evolutionary processes. Historically, the divergence of genomes in relation to speciation has been studied in association with changes in allele frequencies resulting from natural selection and adaptation (Schluter and Conte 2009; Wolf et al. 2010). Recent molecular evidence, however, shows that the genome is much more malleable and complex than previously thought, with many other factors besides changes in allele frequencies having an important role in shaping genetic diversity (Wolf and Ellegren 2017). Additionally, the molecular mechanisms underlying the formation of phenotypes depend on a plethora of genomic processes in addition to the canonical view of gene expression regulation; these include long distance regulation of genes by elements such as enhancers (Ong and Corces 2011), the action of repetitive elements (Rodic and Burns 2013), alternative splicing (Ben-Dov et al. 2008) or post-transcriptional actions of small RNAs (Ambros and Chen 2007). Since molecular epigenetic mechanisms are currently known to play a

fundamental role in regulating genome stability, conformation and expression (Alabert and Groth 2012; Pal and Tyler 2016), evolutionary processes cannot be completely understood without the epigenetic component. Following its epigenetic legacy, future research in birds will certainly contribute enormously to this new understanding of evolution, in which epigenetics will play a major role.

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Chapter 7
Epigenetics and the Extreme Stress Response

W. Aline Ingelson-Filpula, Tighe Bloskie, and Kenneth B. Storey

Contents

Abstract Environmental conditions can be highly unfavorable for many organisms, imposing a variety of extreme stresses onto animal inhabitants. Winters are typically synonymous with shorter photoperiods, lack of food resources, and subzero temperatures. While some species migrate to avoid these conditions, many others have evolved defensive responses to combat these otherwise lethal situations. Such strategies can be classified into major categories including: freeze tolerance, freeze avoidance, anoxia tolerance, diapause, and hibernation. These types of strategies have been documented in a range of organisms including soil microfauna, intertidal

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marine invertebrates, insects, mammals, and various ectothermic vertebrates including some turtles, snakes, salamanders, and frogs. Extreme survival responses are possible thanks in part to metabolic rate depression (MRD), in which animals dramatically suppress energy expenditure and production to varying degrees. MRD necessitates holistic changes to the transcriptome of these specialized species. Unsurprisingly, recent research is showing that epigenetic mechanisms are invaluable contributors to stress adaptation, as is also true of gene silencing by noncoding RNAs. Epigenetic controls are a collection of regulatory mechanisms that alter gene expression without changing the DNA sequence itself, thereby making them ideal for implementing rapid, transient changes in phenotype as is characteristic of seasonal MRD. The current review will summarize the recent literature regarding epigenetic regulatory mechanisms, MRD, and adaptation to extreme environmental conditions. We also document where current research is directed, and what the most consequential and pressing inquiries are in the field.

Keywords Epigenetics · Freeze tolerance · Torpor · Hibernation · Hypoxia · Anoxia · Estivation · Dehydration

Abbreviations

5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
bp	Base pair
ChIP-seq	Chromatin immunoprecipitation sequencing
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EA	Early arousal
EN	Entrance (into torpor)
ET	Early torpor
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1 alpha
IA	Interbout arousal
KAT	Lysine acetyltransferase
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KMT	Lysine methyltransferase
LT	Late torpor
miRNA	MicroRNA
MRD	Metabolic rate depression

mRNA	Messenger RNA
nt	Nucleotide
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-PCR	Real-time PCR
SAM	*S*-adenosyl methionine
snoRNA	Small nucleolar RNA
T_b	Body temperature
TET	Ten-eleven translocation
tRNA	Transfer RNA
WAT	White adipose tissue

7.1 Introduction

Many organisms live in environments where seasonal conditions can vary widely and can impose extreme stresses onto animal inhabitants, i.e., extreme cold or heat, oxygen limitation, and dehydration, among others. For example, winters are typically synonymous with shorter photoperiods, lack of food resources, and subzero temperatures. While some species can migrate to avoid seasonal extreme conditions, many others have evolved defensive responses to elude lethal situations, using behavioral, physiological or biochemical strategies including freeze tolerance, hibernation, estivation, and anaerobiosis (Storey and Storey 2010a, 2012a, 2017; Krivoruchko and Storey 2015). Such strategies have been documented in a range of organisms including soil microfauna, insects, intertidal marine invertebrates, mammals, and various ectothermic vertebrates including turtles and frogs (Ring 1982; Murphy 1983; Thomashow 1999; Costanzo et al. 2008; Holmstrup 2014; Storey and Storey 2017). Extreme survival responses are possible thanks in part to the phenomenon of metabolic rate depression (MRD), by which animals dramatically suppress energy expenditure and descend into a hypometabolic (torpid) state. MRD necessitates holistic changes to the transcriptome of these specialized species. Not surprisingly, recent research is showing that epigenetic mechanisms are valuable contributors to stress adaptation; these mechanisms include epigenetic transcriptional controls on DNA and the histone proteins that guard DNA, as well as translational silencing by noncoding microRNA. Epigenetic controls are a collection of regulatory mechanisms that alter gene expression without changing the DNA sequence itself, making them ideal for implementing rapid, transient changes in phenotype as are needed to achieve both global control of MRD and short-term adaptive adjustments to changing environmental conditions. The current review

summarizes recent literature regarding epigenetic regulatory mechanisms, MRD, and adaptation to extreme environmental conditions.

7.1.1 MicroRNA

While there is some contention about whether microRNA should be classified as a form of epigenetic regulation, these small molecules nonetheless constitute a highly conserved, vital method of post-transcriptional control of gene expression that has been documented across eukaryotic organisms and plays major roles in regulating the translation of gene transcripts. MicroRNAs (miRNA) are short, single-stranded, noncoding RNA of 21–24 nt in length that bind to mature mRNA transcripts to suppress their translation by mediating either the degradation or sequestration of mRNA transcripts (Bartel 2004). Since the discovery of the first miRNAs *let-7* and *lin-4* in 1993, hundreds of miRNAs have been identified across animal and plant species, all of which exhibit very high conservation between species. Several important features of miRNA:mRNA binding have been established: (a) miRNAs bind to gene transcripts through complementarity with the seed sequence, a stretch of 8 nt at the 5′ end of the miRNA sequence that corresponds with the 3' UTR of the mRNA sequence; and (b) perfect complementarity leads to cleavage and degradation of the mRNA transcript, whereas imperfect binding leads to translational suppression via isolation of the mRNA transcript into p-bodies or stress granules (Bartel 2004). MiRNAs are synthesized via the miRNA biogenesis pathway (Fig. 7.1). Given the high conservation of miRNAs observed among vertebrates, and the possession of unique features that make miRNA ideal for implementing reversible, transient phenotypes, miRNAs are a robust mode of post-transcriptional regulation that play a critical and dynamic role in allowing organisms to endure extreme environmental stresses. The miRNA studies which will be discussed in this chapter are laid out in Table 7.1.

7.1.2 DNA Methylation

DNA methylation is one of the three main mechanisms of conventional epigenetic control of DNA expression, the other two being histone acetylation and deacetylation, and histone methylation and demethylation. Table 7.2 displays the available DNA methylation studies on extreme environmental stress responses, while Table 7.3 highlights those on histone modifications. DNA methylation is the transfer of a methyl ($-CH_3$) group from *S*-adenosylmethionine, catalyzed by the DNA methyltransferase (DNMT) family of enzymes, to the 5′ carbon of a cytosine base to form 5-methylcytosine (5mC). Such 5mC methylation patterns often occur

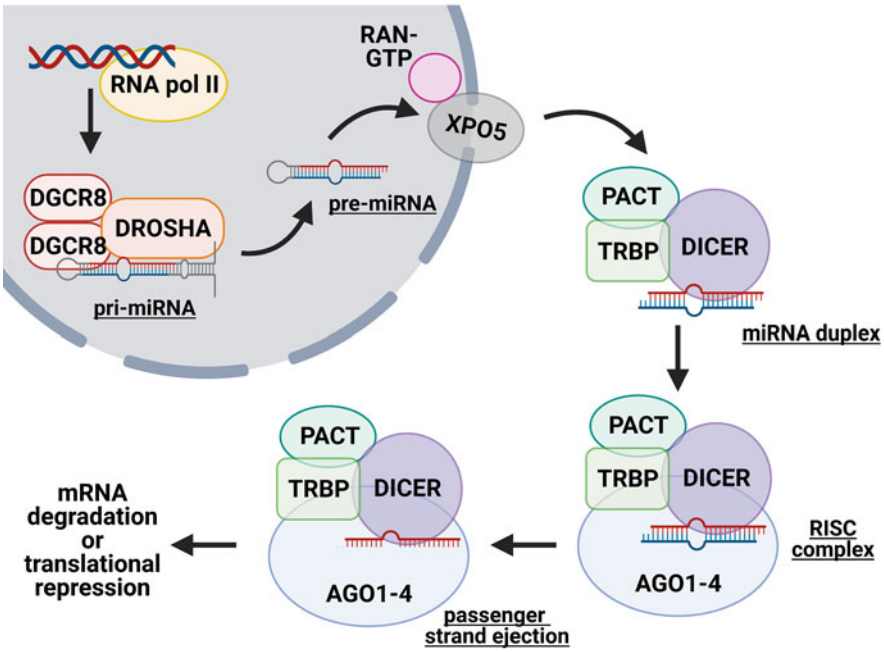

Fig. 7.1 The canonical miRNA biogenesis pathway. miRNAs are transcribed in the nucleus by RNA polymerase II to form the ~70 nt double-stranded primary miRNA (pri-miRNA), where the mature sequence is enclosed within a hairpin turn. Hairpin cleavage by the microprocessor complex (DROSHA/DGRC8) forms the double-stranded precursor-miRNA (pre-miRNA). With the help of RAN-GTP, the pre-miRNA is exported out of the nucleus via Exportin 5 (XPO5) and in the cytoplasm the RNase III enzyme DICER along with cofactors TAR RNA-binding protein (TRBP) and protein activator of PKR (PACT) process the pre-miRNA into the 21–24 nt long duplex miRNA. The 3′ end of the duplex miRNA has a two-nucleotide overhang which is used to load the duplex miRNA onto the Argonaute (AGO) proteins. The strand holding the mature miRNA sequence is kept whereas the other strand is discarded (named passenger strand ejection), and this structure constitutes the miRNA-induced silencing complex (RISC) which is fully prepared to target mRNAs. Figure created with BioRender.com and adapted from Ingelson-Filpula and Storey 2022

on CpG residues: a cytosine and a guanine nucleotide separated by a single phosphate group (Bird 1986). Regions of high CpG density are called CpG islands and these are commonly associated with the promoter region of genes. Hypermethylation of CpG islands correlates with transcriptional silencing of the downstream gene by (1) direct blockage of transcription factor binding, and/or (2) recruitment of repressive methyl-CpG-binding proteins which include MBD1, MBD2, and $MeCP_2$ (Bogdanović and Veenstra 2009; Moore et al. 2013). These three "reader" proteins bind methylated CpG regions, and then recruit chromatin remodeling complexes (like histone deacetylases) to block access of the transcriptional machinery to promoter elements (Nan et al. 1998).

Table 7.1 The collection of miRNA studies as discussed in this chapter

Stress	Animal	Tissue	Method	References
Freeze tolerance	*Rana sylvatica*	Brain	Western blot, RT-PCR, bioinformatics	Hadj-Moussa and Storey (2018)
		Heart	qPCR	Bansal et al. (2016)
		Skeletal muscle	qPCR	
	Dryophytes versicolor	Liver	Western blot, bioinformatics	Ingelson-Filpula and Storey (2022)
		Skeletal muscle	Western blot	
		Kidney	Western blot	
	Eurosta solidaginis	Whole larvae	RT-PCR	Lyons et al. (2016) Lyons et al. (2015)
Hibernation/ torpor	*Ictidomys tridecemlineatus*	Liver	RT-qPCR	Lang-Ouellette and Morin (2014)
		Skeletal muscle	RT-qPCR	
		Liver	RT-PCR	Wu et al. (2016)
		Heart	RT-PCR	
		Skeletal muscle	RT-PCR	
	Microcebus murinus	Liver	RT-PCR	Biggar et al. (2018)
		Skeletal muscle	RT-PCR	Hadj-Moussa et al. (2020)
	Ursus arctos	Skeletal muscle	RT-qPCR	Luu et al. (2020)
Hypoxia and anoxia	*Orconectes virilis*	Hepatopancreas	RT-qPCR, bioinformatics	English et al. (2018)
		Tail muscle	RT-qPCR, bioinformatics	
	Trachemys scripta elegans	Liver	RT-PCR	Biggar and Storey (2017)
		White muscle	RT-PCR	
		Spleen	RT-PCR	
		Kidney	RT-PCR	
Dehydration/ estivation	*Xenopus laevis*	Liver	RT-PCR	Wu et al. (2013)
		Skin	RT-PCR	
		Kidney	RT-PCR	
		Brain	RT-qPCR	Luu and Storey (2015)
		Heart	Bioinformatics, RT-qPCR	Hawkins and Storey (2020)
	Otala lactea	Foot muscle	qPCR	Hoyeck et al. (2019)

DNMT functions fall into two major categories: (1) maintenance methyltransferases, like DNMT1, that bind hemi-methylated DNA to copy methylation patterns onto a newly replicated DNA strand and (2) de novo

Table 7.2 The collection of DNA methylation studies discussed in this chapter

Stress	Animal	Tissue	Method	References
Freeze tolerance	*Rana sylvatica*	Liver	Western blot, activity assay, methylation kit	Zhang et al. (2019)
		Skeletal muscle	Western blot, activity assay, methylation kit	
		Brain	Western blot, activity assay	Bloskie (2021)
Hibernation/ torpor	*Ictidomys tridecemlineatus*	Liver	PCR, methylation kit	Alvarado et al. (2015)
		Skeletal muscle	PCR, methylation kit	
Hypoxia and anoxia	*Trachemys scripta elegans*	Liver	Western blots, activity assay, methylation kit	Wijenayake and Storey (2016)
		White muscle	Western blots, activity assay, methylation kit	
		Heart	Western blots, activity assay, methylation kit	

methyltransferases like DNMT3A and DNMT3B that place new methyl marks onto DNA (Lyko 2018). DNMT3L is a noncanonical DNMT given that it possesses no catalytic activity, but instead forms complexes with DNMT3A and DNMT3B, as well as other epigenetic enzymes, to regulate their activity (Chédin et al. 2002; Suetake et al. 2004).

Methyl marks are in turn removed through a two-step process via Ten-Eleven Translocation (TET) enzymes, which oxidize the 5mC to form 5-hydroxymethylcytosine (5hmC) before removing the methyl group altogether (Shi et al. 2017). Alternate pathways involve oxidation to 5-carboxycytosine (5caC) or 5-formylcytosine (5fC) before removal of the methyl group.

7.1.3 Histone Modification

Histones are small positively charged proteins that make up the functional unit of chromatin, the nucleosome. The nucleosome consists of ~200 bp of DNA wrapped around a histone octamer, made of pairs of histones H2A, H2B, H3, and H4 that enable intense condensation of genetic material within cell nuclei. Like other proteins, histones are subject to post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, citrullination, and serotonylation. Among these, the acetylation/methylation of lysine residues on N-terminal tails are the best studied, particularly due to their functional consequences on nearby gene transcription. Chromatin remodeling is a characteristic effect of histone modification, involving the dynamic interconversion between transcriptionally permissive euchromatin and repressive heterochromatin (Kouzarides 2007).

Table 7.3 The collection of histone modification studies as discussed in this chapter

Stress	Animal	Tissue	Method	References
Freeze tolerance	*Rana sylvatica*	Liver	Western blot, activity assay	Hawkins and Storey (2018)
		Skeletal muscle	Western blot, activity assay	
		Brain	Western blot, activity assay	Bloskie (2021)
Hibernation/ torpor	*Tamias asiaticus*	Liver	ChIP-seq	Tsukamoto et al. (2017; Tsukamoto et al. (2018)
	Ictidomys tridecemlineatus	Skeletal muscle	Western blot, activity assay	Morin and Storey (2006; Hawkins and Storey (2017; Rouble et al. (2018)
		White adipose tissue	Western blot, activity assay, acetylation kit	Rouble and Storey (2015)
		Brown adipose tissue	Western blot, activity assay, acetylation kit	Rouble et al. (2018)
		Liver	Western blot, activity assay, methylation kit	Watts and Storey (2019)
		Skeletal muscle	Western blot, activity assay, methylation kit	
Hypoxia and anoxia	*Trachemys scripta elegans*	Skeletal muscle	Western blot, activity assay, PCR	Krivoruchko and Storey (2010)
		Liver	Western blot, activity assay, PCR	
		Heart	Western blot, activity assay, PCR	
		Liver	Western blot, activity assay	Wijenayake et al. (2018 Wijenayake and Storey (2020)

The acetylation of lysine residues on histone tails is facilitated by lysine acetyltransferases (KATs), a name that reflects their ability to also act on a variety of nonhistone protein targets, whereas histones are deacetylated by histone lysine deacetylases (HDACs). KATs transfer acetyl groups from acetyl-CoA donors onto side chain ε-amino residues, and HDACs generate acetate as a by-product. KATs are subdivided into GNAT, MYST, and p300/CBP families (Berndsen and Denu 2008) whereas HDACs exist in four major classes: zinc-dependent class I, II, and IV, and NAD-dependent class III, which are also called sirtuins (SIRTs) (Haberland et al. 2009). Acetylation is tightly linked to gene activation, sometimes referred to as "permissive" to transcription, through (1) locally relaxing the tight electrostatic

interactions of positively charged histone proteins with negatively charged DNA, (2) providing binding sites for bromodomain "reader" proteins to recruit transcriptional machinery, and (3) preventing positions from being occupied by silencing modifications, since multiple modifications cannot co-exist at the same lysine residue.

Conversely, histone lysine methylation has much more variable outcomes depending on the particular methyl-lysine binding effector proteins that are recruited. Histone lysine methylation involves the addition of one, two or three methyl groups onto side chains of lysine residues. This epigenetic mechanism is reversible; marks are added by "writer" lysine methyltransferases (KMTs), removed by "eraser" lysine demethylases (KDMs), and interpreted by "reader" Chromo, Tudor, PWWP, PHD, WD or MBT domain proteins (Hyun et al. 2017). Through chromatin immunoprecipitation sequencing (ChIP-seq) analysis, most methyl-lysine marks have been shown to be strongly associated with actively or lowly transcribed genes (Barski et al. 2007; Mikkelsen et al. 2007). Methylation of H3K4 is linked to gene activation (H3K4me1 to primed enhancers, H3K4me3 to active promoters), whereas methylation of H3K9 and H3K27 is associated with repression that provides recruitment sites for heterochromatin protein 1 (HP1) (Bannister et al. 2001) and polycomb repressive complexes (PRCs), respectively. Mono-methylation of H4K20 is also generally deposited near start sites of actively transcribed genes (Evertts et al. 2013). Most KMTs contain the consensus Su(var) 3–9, Enhancer of zeste, and Trithorax (SET) domain as their key catalytic site and use methyl groups from the donor substrate, S-adenosyl methionine (SAM), to methylate the side chain amino group of lysine residues.

Epigenetic regulation including DNA methylation, histone lysine acetylation, and histone methylation coupled with post-transcriptional control via miRNAs are all potent tools for widespread, reversible methods of phenotypic variation. We have posited that animals, which transition into hypometabolic states as part of their survival strategy for enduring harsh environmental conditions, may use epigenetic modifications as an ideal mode of regulation to rework their metabolic needs without committing to lifelong changes in the genome. This introduction, as well as other chapters in this book, lay the groundwork for the multiple examples of environmental stress-mediated MRD that contributes to natural adaptive strategies including: freeze tolerance, freeze avoidance, hibernation/torpor, hypoxia/anoxia, and dehydration endurance.

7.2 Freeze Tolerance

Freeze tolerance is a survival strategy that has been documented for many species living in seasonally cold environments, including several species of vertebrates (frogs, salamanders, hatchling turtles) and many invertebrates (insects, molluscs) (Murphy 1983; Storey and Storey 1988; Storey and Storey 2017). Freeze tolerance is typified by the formation of ice in extracellular and extra-organ spaces and the

complete cessation of heartbeat, breathing, and movement. While this allows animals to mitigate harsh winter conditions including subzero temperatures, scarcity of food, and short photoperiods, freeze tolerance brings with it a collection of physiological dangers that require attention. Of primary importance is the prevention of intracellular ice crystal formation that poses mechanical threats of rupturing cell membranes and destroying subcellular architecture. Therefore, strategies are employed that prevent intracellular ice formation and restrict freezing to extracellular compartments only. Extracellular ice formation leads to exclusion of solutes from the growing ice lattice such that remaining extracellular fluid becomes hyperosmotic and draws water out of cells. Hence, cells risk both dehydration and extreme shrinkage. To counteract this, freeze-tolerant animals synthesize high levels of low molecular weight cryoprotectants that are packed into cells (Ring 1982; Storey and Storey 2017). The cryoprotectant used varies depending on the species and includes a variety of small molecules: polyhydric alcohols (e.g., glycerol, sorbitol), sugars (e.g., glucose, trehalose), and small nitrogenous compounds (e.g., urea).

Another consequence of ice formation in extracellular and extra-organ spaces (e.g., abdominal cavity, between skin and muscle) is ischemia caused by the interruption of blood flow with the consequence of hypoxia/anoxia as oxygen is depleted (Storey and Storey 2017). Gas exchange via the lungs is halted, kidneys do not remove waste, and skeletal muscle may atrophy from lack of use. To survive using only their own endogenous fuel reserves, cells/organs switch their metabolism and ATP generation from aerobic respiration to anaerobic fermentation, with the accumulation of end-products such as lactate and alanine (Storey and Storey 2017). On a holistic level, prevention of widespread damage severe enough to cause cell/organ death must be successfully managed by changes in antioxidant defenses, chaperone proteins, and antiapoptotic measures (Storey and Storey 2017). Recent studies of epigenetic regulatory mechanisms have illuminated the role of these controls in both downregulation of nonessential genes and processes as a part of MRD, and in facilitating pro-survival mechanisms in response to the threats posed by whole body freezing. The following section highlights studies of this nature, and a brief graphical overview of epigenetic influence during freeze tolerance is given in Fig. 7.2.

7.2.1 MiRNAs in Freeze Tolerance

Freeze tolerance among vertebrate species has been extensively studied in the main model for this process: the wood frog *Rana sylvatica*. During the winter months, wood frogs can endure the freezing of ~65% of total body water as extracellular ice when temperatures drop below about −2 °C (Costanzo and Lee 2013). MiRNA regulation of gene expression has been identified as a significant regulator of both entrance into and maintenance of freeze tolerance. In wood frog brain, miRNAs may serve a protective role by stabilizing existing, crucial neural networks, thereby acting as neuroprotectants (Hadj-Moussa and Storey 2018). They are synthesized via the

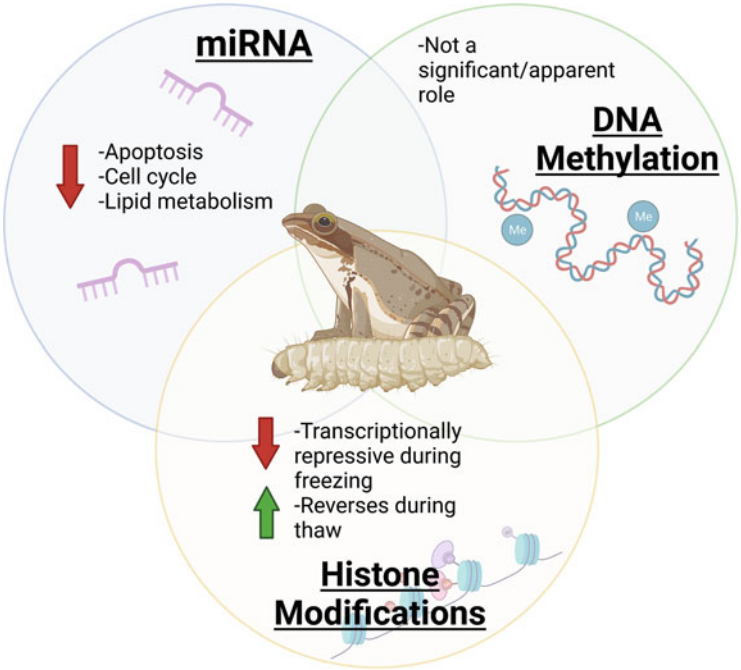

Fig. 7.2 An overview of epigenetic influences during freeze tolerance. Figure created using BioRender.com

miRNA biogenesis pathway (Fig. 7.1) and, in brain, protein levels of four members of the pathway decreased significantly during freezing. This indicated reduced synthesis of miRNAs in brain during freezing and may infer that widespread translational repression by miRNAs is not occurring (Hadj-Moussa and Storey 2018). However, it is possible miRNAs are acting in a cryoprotective manner by stabilizing existing, crucial neural networks. Furthermore, 113 miRNAs were quantified in wood frog brain via RT-PCR, with 24 of these exhibiting differential expression during freezing (Hadj-Moussa and Storey 2018). Nearly all of these miRNAs were downregulated save for one, miR-451-5p. Significantly, miR-451-5p has been previously characterized as a glucose-sensing switch which leads to downstream suppression of the PI3K/AKT pathway and activation of mTOR (Godlewski et al. 2010). The PI3K/AKT/mTOR network is a wide-ranging pathway with regulatory effects in actin cytoskeleton, apoptosis, autophagy, cell cycle progression, cell survival, DNA repair, epigenetic regulation, genetic stability, ion transport, metabolism, protein synthesis, regulation of gene expression, and ribosomal RNA synthesis (Ersahin et al. 2015). As mentioned, this pathway has many processes that we observe being differentially regulated during freeze tolerance, including cell cycle progression (an energy-expensive process that may be downregulated), cell survival processes including apoptosis and DNA repair, and epigenetic components. A study by Zhang and Storey hypothesized that AKT may

be playing an antiapoptotic role, demonstrating that AKT was inhibited in skeletal muscle, kidney, and heart after 24 h freezing exposure with a reversal after thawing (Zhang and Storey 2013). It is possible that miR-451-5p upregulation is downregulating energy- and metabolism-related processes of the AKT pathway, instead focusing on AMPK. AMPK is colloquially known as the energy sensor of the cell, and is responsible for fuel use switches by increasing glucose uptake and promotes fatty acid oxidation by phosphorylating ACC and decreasing malonyl-CoA production (Ke et al. 2018). Under conditions of glucose withdrawal, miR-451 downregulation is necessary for AMPK pathway activation, leading to suppressed proliferation rates and increased cell survival. Glucose is the cryoprotectant used by wood frogs with levels rising from ~5 μmol/g wet weight (gww) in unfrozen frogs to over 200 μmol/gww in frozen animals. It is possible that strong upregulation of miR-451-5p effectively targets the suppression of genes that would otherwise be upregulated by high glucose levels, including those that would funnel glucose into glycogen storage or use glucose as a metabolic fuel to support anabolic biosynthesis (Rider et al. 2006). Hence, the unexpected novel response to freezing by miR-451 may be a crucial factor in the ability of wood frogs to inhibit metabolizing glucose so it can be used as cryoprotectant. By contrast, many of the downregulated miRNAs affected genes/proteins involved in signal transduction and RNA processing and were linked with regulating intracellular signaling pathways, coupled with decreased miRNA biogenesis. This may infer that continued function of these signaling pathways is critical to survival during freeze tolerance, thereby highlighting a potential role of miRNAs in brain tissue to support freezing survival.

Studies on miRNA regulation over the freeze/thaw cycle in *R. sylvatica* have also been undertaken for heart and skeletal muscle, in which qPCR was used to quantify levels of 53 miRNAs in these tissues (Bansal et al. 2016). In heart, only one miRNA was upregulated whereas four were downregulated during freezing, although larger subsets of twenty were downregulated after 8 h thawing (Bansal et al. 2016). The widespread downregulation of miRNAs during thawing may signify that many cellular processes need to be reactivated after thawing to cope with any accumulated damage from freezing. Indeed, selected miRNAs from the group that was analyzed are known to play roles in heart function, such as miR-145 and miR-208 that are overexpressed in various heart diseases (Cooley et al. 2012). Skeletal muscle showed an alternate trend, with 16 miRNAs upregulated and one downregulated during freezing, as well as six remaining upregulated after thawing (Bansal et al. 2016). The miRNAs affected in skeletal muscle targeted genes in the cell cycle and apoptosis, thereby suggesting that these processes are suppressed during freezing and remain this way throughout the thaw. Bioinformatic prediction of pathways affected by these miRNAs yielded targets including actin cytoskeleton, PI3K-Akt, and MAPK signaling as being disproportionately affected by miRNAs (Bansal et al. 2016). The focus on intracellular signal transduction has been observed during freeze tolerance in wood frogs previously, suggesting a more global theme for the functions of miRNA in regulating signaling pathways during freezing.

Emerging data from our lab also show the miRNA responses by another freeze tolerant amphibian, the gray tree frog, *Dryophytes versicolor* (Ingelson-Filpula

2021). Members of the miRNA biogenesis pathway (Fig. 7.1) were differentially regulated across three tissues between control and frozen states, with liver showing upregulation of miRNA synthesis proteins and skeletal muscle/kidney exhibiting downregulation of some biogenesis proteins, thereby indicating suppression of miRNA synthesis. Noteworthy was the increased expression of ribonucleases DICER and DROSHA in hepatic tissues, and the strong reduction of RNA-binding argonaute proteins in frozen *Dryophytes* kidneys and muscle. Like wood frogs, *D. versicolor* produced copious amounts of cryoprotectant in liver, this organ being the most metabolically active tissue during freezing and the last organ to be affected by freezing. Skeletal muscle and kidney are less important with respect to freeze tolerance, and downregulation of miRNA biogenesis may be contributing to global MRD in these two tissues in order to conserve cellular energy. To further elucidate the functions of miRNA in liver tissue, unpublished data involved bioinformatic analysis of a small RNA dataset to filter out all non-miRNA reads (e.g., rRNA, tRNA, snoRNA, etc.), comparing control versus frozen states Ingelson-Filpula (2021). A subset of miRNAs were differentially regulated, both up and down, in response to freezing. Targets for these miRNAs appeared to center around downregulating intracellular signal transduction, apoptosis, and nuclear processes.

Many insects are also freeze-tolerant and, indeed, most tolerate temperatures far lower than frog species can, often to −40 °C or even lower (Denlinger and Lee 2010; Storey and Storey 2012b). Studies into insect freeze tolerance are currently limited to miRNAs, and these will be introduced herein. A major model for studies of the metabolic adaptations used for insect freeze tolerance is the goldenrod gall fly, *Eurosta solidaginis*, whose larvae overwinter inside galls on the stems of goldenrod. To survive, the larvae accumulate high levels of glycerol and sorbitol for cryoprotection, as much as 400 mM glycerol and 150 mM sorbitol in their tissues. MicroRNA also contributes to regulating freeze survival in this species. A study of freezing-associated miRNAs monitored responses over a time course of 3 weeks at 5 °C followed by 3 weeks at −5 °C, and finally by 3 weeks at −15 °C. A group of 24 miRNAs were differentially regulated at −15 °C, with four downregulated and 20 upregulated (Lyons et al. 2016). Lipid metabolism seems to be a focus of regulation by miRNAs in these insects, given miR-1-3p can regulate the expression of Liver X receptor alpha and modulate levels of lipogenic enzymes in humans (including fatty acid synthase and acetyl-CoA 1 carboxylase) (Zhong et al. 2013). In cold-hardy insects, selected lipid metabolism-related enzymes are known to be downregulated in *E. solidaginis* (Lyons et al. 2016). Although the larvae enter the winter season with huge lipid reserves, they are a poor fuel for winter metabolism given the need for oxygen to produce ATP from fatty acid catabolism (not an option when larvae are frozen). Instead, lipid catabolism is suppressed in winter and lipids are largely reserved for the spring pupation, emergence, mating, and egg laying by nonfeeding adults. MiR-14-3p was also bioinformatically predicted to be upregulated in this study and has been associated with stress responses and fat metabolism in *Drosophila melanogaster*. Other miRNA functions may include activation of the hypoxia-inducible transcription factor 1 alpha (HIF-1α), as evidenced by upregulation of miR-31a-3p under −15 °C conditions (where the

larvae are solidly frozen) and other work supporting HIF-1α activation during freezing in *E. solidaginis* (Morin and Storey 2005; Lyons et al. 2016). Further study by Lyons et al. revealed quantification of miR-8 and its relevance to freeze tolerance in *E. solidaginis*, given the miR-8/miR-200 family have been found to influence other models of hypometabolism (discussed elsewhere) by modulating expression of signaling pathways including Wnt, Toll, and PI3K (Lyons et al. 2015). An upregulation of miR-92b was also reported and suspected to play a role in regulating PTEN which is a regulator of cell growth (Lyons et al. 2015).

7.2.2 DNMT Enzymes in Freeze Tolerance

DNA methylation has been assessed in freeze-tolerant wood frogs to investigate whether hypermethylation of the genome is a potential contributor to inducing and maintaining a hypometabolic state during the winter. Because wood frogs utilize glucose as their primary cryoprotectant during freezing, this has the side effect of creating extreme hyperglycemia. Therefore, a study by Zhang et al. used glucose-loading as an experimental condition to investigate whether glucose cryoprotectant itself caused measurable changes in the expression/activity of the DNA methylation machinery and compared this with the effects of freezing alone (Zhang et al. 2019). A marked difference in the responses of two tissues, liver and skeletal muscle, to both freezing and glucose-loading was seen. In liver, DNA methylation appeared to be less important during freezing given that freezing did not affect DNMT protein levels, whereas total DNMT activity fell to ~25% of the control (saline) value after 24 h freezing and 5mC genome methylation decreased by about 25% (Zhang et al. 2019). The lone exception was upregulation of DNMT3L during thawing. DNMT3L possesses no catalytic activity and has various roles as a cofactor, both with DNMT enzymes as well as other epigenetic enzymes including HDAC1 (Deplus 2002). It is possible that DNMT3L was associating with other proteins that are not directly causal to DNA methylation, thus leading to the overall minimum trend in DNA methylation observed during freeze/thaw.

During glucose-loading of wood frogs (mimicking the hyperglycemia of the frozen state), the situation was somewhat different. In liver, DNMT1 and 3A were downregulated but DNMT3L once again was upregulated. Global 5mC and 5hmc levels were unchanged as was total DNMT activity. This fell in line with the trends observed during freezing: DNA methylation appeared to be downregulated in liver during glucose-loading with the lone exception of DNMT3L, which may be serving other regulatory purposes as a cofactor. It can be postulated that liver, being the most metabolically active organ and the last to freeze, needs to have its DNA accessible to transcribe genes that are crucial to survival and, hence, hypermethylation of the genome may disrupt pro-survival mechanisms.

Wood frog skeletal muscle showed somewhat different responses; both DNMT1 and DNMT3L were upregulated in response to 24 h freezing, whereas DNMT3A/3B remained unchanged along with DNMT activity (Zhang et al. 2019). Global 5mC

levels increased, perhaps reflecting DNMT1 upregulation, in contrast to liver (Zhang et al. 2019). After an 8 h thaw, DNMT1 and 5mC had returned to control levels whereas DNMT3L increased even further. Interestingly, total DNMT activity was strongly suppressed in muscle after 8 h thawed. With regard to the glucose-loading condition, DNMT1 was downregulated whereas both DNMT3A and 3B were upregulated in skeletal muscle. There were no changes in genomic 5mC levels but total DNMT activity decreased to less than 40% of the control (saline) value. DNMT1 is a maintenance methyltransferase, responsible for methylating hemi-methylated DNA following a round of DNA replication (Lyko 2018). As mentioned earlier, DNMT3L is not a canonical DNMT and does not possess any methylation capabilities, but its action as a cofactor allows greater affinity for DNA and therefore more efficient function. However, glucose-loaded frogs showed upregulation of the *de novo* methyltransferases DNMT3A or 3B suggesting that the global increase in 5mC may be due to increased DNA replication in muscle, necessitating DNMT1 function. This may be counterintuitive given that skeletal muscle is one of the first tissues to freeze and has a low metabolic activity during freezing, thus bringing the necessity of DNA replication and cell division into question. Further study will be needed to elucidate the complete function of DNA methylation in this tissue.

Investigation of DNA methylation during freeze tolerance in *R. sylvatica* has been extended to brain tissue (Bloskie 2021). Preliminary results suggest that during freezing, only levels of DNMT3B increase, while the other DNMTs remain unchanged. Thawing resulted in decreased expression levels of DNMT3A and DNMT3L. Total DNMT activity decreased during freezing and decreased further during thaw.

7.2.3 Histone Modifications Accompany Freeze Tolerance

Despite its energetically costly mechanism, transcriptional suppression is an important characteristic of hypometabolic states (Bocharova et al. 1992; Van Breukelen and Martin 2002). Like DNA methylation, a recent study has highlighted histone methyl-lysine patterns indicative of a repressed chromatin state in two tissues of wood frogs in response to freezing (Hawkins and Storey 2018). In both liver and skeletal muscle, hypomethylation of the H3K4 residue was identified, along with reduced levels of transcriptionally permissive H3K4me1. Analysis of the relevant KMT enzymes indicated that reduced expressions of SMYD2 and ASH2L were the contributing activities (Hawkins and Storey 2018). In liver, repressive H3K36me2 also appeared to be involved, but underlying mechanisms are currently unknown. H3K27me1, an intragenic-deposited permissive mark, was reduced in skeletal muscle during freezing but enriched during liver freeze-recovery. Similar to arousal from hibernation (as described in the next section), thawed recovery after freezing appears to facilitate transcriptional activation. This is evidenced in wood frog brains, where hypomethylation of H3K9 is observed during freeze-thawing (Bloskie 2021). Additionally H3K9me3, a chromatin mark highly associated to suppression of

nearby gene transcription, was significantly reduced in thaw recovery, which may be attributed to decreased expression of catalyzing enzymes SUV39H1 and ESET.

Unfortunately to date, the specific transcriptomic implications of these histone modifications have not yet been elucidated. However, several studies have highlighted the freeze-induced transcription of a number of genes (*li16, fr10,* α/γ *fibrinogen, glut2, ADP/ATP translocase, pyruvate kinase, ribosomal phosphoprotein* P_0) (Cai and Storey 1997a; Cai et al. 1997; Cai and Storey 1997b; Wu and Storey 2005; Sullivan and Storey 2012; Rosendale et al. 2014; Al-attar et al. 2020), which are expected to be, at least partly, due to epigenetic controls. Hepatic transcriptomic analyses in the related freeze-tolerant Cope's gray treefrog, *Dryophytes chrysoscelis*, show several DNA damage repair and heat shock response genes to be activated in cold-acclimated and frozen frogs, whereas those involved in cellular responses to oxidative stress and oxygen limitation were either downregulated or unchanged (Do Amaral et al. 2020).

Overall, freeze tolerance is a highly complex phenomenon, involving the implementation of multiple adaptations that address a variety of factors: (a) metabolic rate depression to halt or minimize metabolic processes that are not needed in a regulated manner, (b) tolerance of anoxia/ischemia to deal with lack of breathing and blood circulation while frozen, (c) a tolerance of cell and tissue dehydration due to water loss into extracellular and extra-organ ice formation, and (d) accumulation and tolerance of extreme concentrations of low molecular mass metabolites that provide colligative protection of cell volume and of macromolecular structures (e.g., glucose in wood frogs). All of the aforementioned phenomena are alien to the human condition but occur as one or more survival strategies in diverse organisms. For example, metabolic rate depression underlies hibernation, estivation, and anaerobiosis. Estivation also requires mechanisms to minimize cell and tissue dehydration (particularly in animals with highly permeable skins such as amphibians) by elevating the levels of compatible solutes like urea. Anoxia/hypoxia tolerance requires not just MRD but also pathways of ATP generation that are not oxygen-dependent. All of these survival strategies come together in freeze tolerance but they are also utilized by many other species and are regulated, at least in part, by conserved epigenetic controls on gene expression. The following sections analyze the roles of epigenetic mechanisms in some of these strategies.

7.3 Torpor/Hibernation

For mammals, homeothermy is a "double-edged sword" providing key advantages (e.g., regulated warm body temperature, ability to remain active in cold environments, fast locomotion, etc.) and disadvantages (a need for high food intake to fuel a high metabolic rate). The latter is a particular problem for small mammals, where a consistently high metabolic rate demands a huge daily food intake. As a result, many species implement energy-conserving strategies such as: (a) daily torpor—a reduction in metabolic rate during the inactive nonforaging hours, or (b) hibernation—

seasonal entry into prolonged multiday torpor to survive the winter (Jansky et al. 1986; Körtner and Geiser 2000). In both strategies, body temperature can fall to near ambient, although regulation is re-initiated if the body cools to near 0 °C (Ruf and Geiser 2015). Metabolic energy expenditure during hibernation can be reduced to as low as 1–5% of euthermic rates (Carey 2003). Prolonged torpor bouts in hibernating species are interspersed with short periods of arousal where T_b rises back to euthermic levels (near 37 °C) for several hours during which restorative actions occur before animals sink into another bout of torpor (Carey 2003). Regulation of torpor/arousal involves global controls that are used to reorganize an animal's metabolic needs including actions at physiological, biochemical, and molecular levels that downregulate nonessential processes during torpor. Such regulation, and its reversal during rewarming, includes controls at transcriptional (Srere et al. 1992; Morin and Storey 2006), translational (Wu and Storey 2012), and post-translational levels (Morin and Storey 2006; Abnous et al. 2012) including activation of selected transcription factors that have pro-survival roles (Tessier and Storey 2010; Tessier and Storey 2012). Given that all these molecular and physiological changes are transient in nature and need to be reversed during arousal back to euthermia, it is reasonable to assume that epigenetic regulation of gene expression plays a key role in facilitating cellular adaptations for daily torpor and hibernation.

Both daily torpor and seasonal hibernation have physiologically discrete phases that require distinct metabolic actions (Carey 2003). Additionally, the physiological characteristics of torpor/arousal can vary greatly from one species to the next. Henceforth, our discussion of 13-lined ground squirrel hibernation will involve these phases: EC designates euthermic control animals in the 5 °C cold room that have a stable body temperature (T_b, ~37 °C) and could enter torpor, but had not done so for at least three days. When triggered to enter a torpor bout, body temperature falls over time during the entrance (EN) phase (T_b = 18–31 °C) before T_b stabilizing at 5–8 °C; animals sampled after 1 day at this T_b are termed early torpor (ET). Late torpor (LT) is defined as T_b = 5–8 °C for >5 days into the torpor bout. Squirrels can remain in torpor for many days but, ultimately, arouse back to euthermia for short periods of time. The early arousal (EA) period is characterized by a rising T_b with an increase to 9–12 °C being indicative of a full arousal to come. Interbout arousal (IA) typically lasts ~18–24 h during which T_b stabilizes at euthermic values. Since EA and IA periods are characterized by high metabolic rates, re-establishing euthermic values during IA before decreasing again into another torpor bout, this presents the unique challenge for the animals that need to implement and reverse MRD multiple times over the hibernation season, adding another level of intricacy onto metabolic reorganization and regulatory control. Figure 7.3 highlights the major ways that epigenetics underlies torpor and hibernation.

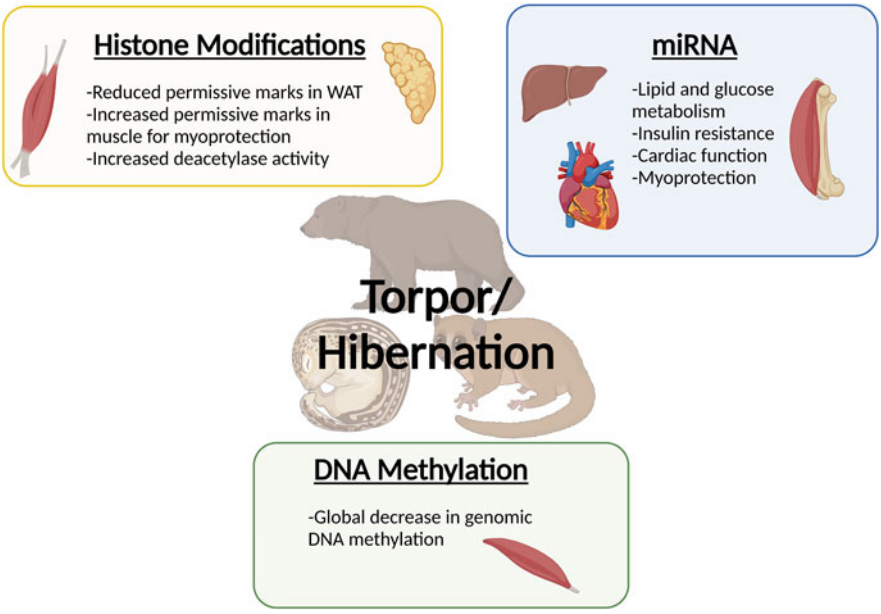

Fig. 7.3 A graphical depiction of the main biological processes affected by epigenetics during torpor and hibernation. Figure created using BioRender.com

7.3.1 MiRNA Involvement in Torpor and Hibernation

A first foray into analyzing the role of microRNAs in mammalian hibernation used RT-qPCR to evaluate the responses by microRNAs in liver and skeletal muscle of 13-lined ground squirrels, *Ictidomys tridecemlineatus* (Lang-Ouellette and Morin 2014). Increased levels of miR-29a were observed in liver of hibernating animals and were linked to functions including reduced glucose production via G6Pase and PGC-1α. Additionally, the fatty acid synthesis pathway appeared to be highly regulated given the reduced expression of fatty acid synthase in liver coupled with the overexpression of miR-195, a regulator of the fatty acid synthesis pathway. Other miRNAs have been reported to target the fatty acid synthesis pathway, confirming that lipid metabolism plays a key role during torpor (Lang-Ouellette and Morin 2014). Tangentially related were FOXO1 and SR-BI, targets of miR-223, that were also elevated in liver; these have ties to oxidative stress and glucose metabolism and high-density lipoprotein cholesterol (Greer and Brunet 2005; Wang et al. 2013).

The study of miRNA involvement during hibernation of *I. tridecemlineatus* was greatly expanded by Wu et al. with an analysis of 117 miRNAs assessed in liver, heart, and skeletal muscle across four stages of torpor/arousal in this squirrel species (Wu et al. 2016). In heart and skeletal muscle, enriched miRNAs targeted pathways related to cell growth, microtubule cytoskeleton organization, and active transport. Liver showed a similar trend, with miRNAs linked to downregulation of

energy-intensive processes including endosome transport, growth factor receptor signaling, mitosis and nuclear division, and glycolysis regulation. With regard to specific miRNAs, miR-208b was strongly upregulated in heart during LT and IA, and its action is known to be directly linked to regulating cardiac arrhythmias. Hence, miR-208b was hypothesized to play a role in facilitating the major decrease in heart rate from ~300 bpm in euthermia to ~10 bpm in torpor. In skeletal muscle, however, miR-208b was downregulated. Other known functions of this miRNA involve muscle remodeling, leading to the proposal that suppression of miR-208b, as a negative regulator of gene expression, may facilitate some needed changes in muscle contractile proteins at cold temperatures such as may also contribute to the shivering thermogenesis that aids rewarming of the squirrel body during arousal from torpor. Finally, insulin resistance appeared to be regulated via miR-181a overexpression specifically in liver during ET.

MiRNAs involved in insulin sensitivity were also overexpressed in brown adipose tissue in hibernating *I. tridecemlineatus* (Logan and Storey 2021). These miRNAs targeted nearly all major genes in the glycolysis pathway, thus downregulating them, while KEGG pathway analysis predicted enrichment of gluconeogenesis (Logan and Storey 2021). This inhibition was continued for major enzymes in the electron transport chain and possible anaerobic metabolism via L-lactate dehydrogenase.

Several novel miRNAs were predicted from small RNA-sequencing data, screened against database miRDeep, and experimentally validated to be significantly altered during hibernation in liver, skeletal muscle, and heart of *I. tridecemlineatus* and revealed roles for miRNAs in metabolism and signal transduction cascades (Luu et al. 2016). The metabolism-focused miRNAs in liver reinforced the switch to lipid oxidation from glucose consumption, which strengthens the results of Lang-Ouellette and Morin (Lang-Ouellette and Morin 2014). Downregulation of miRNAs in skeletal muscle and heart also corroborated the findings of Wu et al. (Wu et al. 2016) since the observed downregulation of miRNAs may facilitate myoprotective roles and skeletal muscle remodeling in response to decreased mobility (Luu et al. 2016).

Studies of the gray mouse lemur, *Microcebus murinus*, native to Madagascar, provide further insights into the roles of microRNA in hypometabolism using an animal that commonly undergoes daily torpor with a relatively small decrease in T_b but can also exhibit multiday hibernation in the cool, dry winter season (Schmid and Kappeler 1998). As a primate, this species has the closest evolutionary link to humans of any hibernator and this makes studies of its torpor capacity more relevant for potential discovery of metabolic mechanisms that can be employed to induce metabolic rate depression in humans. A study by Biggar et al. (Biggar et al. 2018) measured novel and conserved miRNA in *M. murinus*, and found 122 conserved miRNAs along with 44 novel miRNAs in liver. Of these, 16 conserved miRNAs were upregulated in liver during torpor, whereas 30 were downregulated. Similarly ten novel miRNAs were upregulated during torpor while only one displayed significant downregulation (Biggar et al. 2018). Interestingly, miR-222 (downregulated in *M. murinus*) has been found in white adipose tissue of hibernating ground squirrels

and may allow for metabolic adaptation in insulin-sensitive tissues, such as liver and adipose (Wu et al. 2014). Pathways under increased translational repression via miRNAs involve cell differentiation and growth, whereas pathways "enhanced" by reduced levels of miRNA during torpor include immune processes and G-protein-coupled signaling.

In skeletal muscle of *M. murinus*, 20 miRNAs amid a group of 234 conserved miRNAs were significantly altered during torpor (Hadj-Moussa et al. 2020). Eleven were significantly upregulated, and nine were significantly downregulated. Key members of the myo-miR family were among those downregulated; suppression of this group may act to limit muscle growth and differentiation which are very metabolically expensive and not congruent with MRD during torpor (McCarthy 2011). Moreover, two myomiRs (miR-1 and miR-133) directly target other potentially crucial processes including apoptosis, where reduced levels of miR-1 and/or miR-133 have been shown to favor survival (Xu et al. 2007). Of the upregulated miRNAs, many were related to cell growth including miR-2478 and miR-889 that target TGFβ1, a receptor primarily linked to cell proliferation and differentiation; these, energy-expensive processes are typically suppressed during torpor/hibernation (Li et al. 2017).

The small marsupial, *Dromiciops gliroides*, is unique in that is the only hibernating marsupial in South America and the last living relative of the Order Microbiotheria (Bozinovic et al. 2004). *D. gliroides* undergoes daily torpor in response to environmental stress and is also capable of prolonged hibernation in the winter. Hibernation-responsive miRNAs have been studied in liver and skeletal muscle of this animal, which continued to draw parallels to torpor-sensitive processes with a heavy emphasis on signaling-related pathways (Hadj-Moussa et al. 2016). In liver, signaling including MAPK, mTOR, and PI3K/Akt protein kinases was enriched due to downregulation of relevant miRNAs, whereas skeletal muscle appeared to overexpress miRNAs that regulate the ErbB and mTOR signaling pathways. The tissue-differentiated response of miRNAs between liver and skeletal muscle has been robustly demonstrated in primates by Biggar et al. (Biggar et al. 2018) and Hadj-Moussa et al. (Hadj-Moussa et al. 2020). Recall that miRNAs are generally downregulated in skeletal muscle of *I. tridecemlineatus* to contribute to myoprotective roles, so the upregulation observed in skeletal muscle of *M. murinus* and *D. gliroides* may signal a unique, species-specific role for miRNAs in primates and marsupials as compared with rodents, or a potential molecular difference between low-T_b versus high-T_b hibernation.

Another "warm hibernator", that is, a hibernator that maintains T_b at or near euthermic levels during hibernation, is the brown bear *Ursus arctos*. Muscle atrophy, or the lack thereof during hibernation, is a primary area of study and may be due in part to the MEF2A (myocyte enhancer factor 2A) signaling pathway, that is responsible for skeletal muscle development, maintenance, and regulation (Taylor and Hughes 2017). An investigation by Luu et al. used RT-qPCR to analyze 36 miRNAs linked to MEF2A in muscle samples from hibernating versus summer-active bears (Luu et al. 2020). Three miRNAs under MEF2A regulation were increased during hibernation and their corresponding mRNA target transcript levels decreased

in turn (Luu et al. 2020). Another 18 miRNAs involved in skeletal muscle regulation were also quantified, six of which were upregulated in hibernating bears. Finally, 11 members of the myomiR family which play roles in skeletal muscle atrophy and regeneration were studied, and three were upregulated (miR-23a-5p, miR-221–3p, and miR-31-5p) whereas two were downregulated (miR-199a-5p and miR-223-5p) (Luu et al. 2020). Taken together, these results implicate miRNAs in facilitating MRD and skeletal muscle maintenance during hibernation, at least partially through upregulation of MEF2A. Other functions of miRNAs involved decreased glucose utilization and uptake along with decreased fatty acid oxidation/lipid metabolism (Luu et al. 2020).

7.3.2 DNMT Enzymes in Torpor

The role of DNA methylation was assessed in the model hibernator, the 13-lined ground squirrel, *I. tridecemlineatus*. Global methylation levels, mRNA transcript levels of DNMT1 and DNMT3B enzymes, and mRNA transcript levels of "reader" proteins MBD1–3 and $MeCP_2$ were measured in liver and skeletal muscle (Alvarado et al. 2015). Significant changes in DNA methylation patterns in the liver were seen only during the IA phase of torpor, and while there was altered expression of *dnmt* transcript levels during various stages of hibernation, they were not correlated with changes in genomic DNA methylation at the corresponding timepoints. In muscle, genomic DNA methylation decreased strongly during LT, EA, and IA stages of hibernation (Alvarado et al. 2015). This decrease in genomic methylation may represent implementation of global MRD in muscle, supported by the overall transcriptional activity observed in the skeletal muscle of hibernating mammals (Bocharova et al. 1992; Storey and Storey 2004; Morin and Storey 2006). Furthermore, no significant changes in *dnmt* expression were observed across the torpor-arousal cycle of hibernation in skeletal muscle despite decreases in global DNA methylation. As mentioned above, other trans-acting mechanisms known to regulate methylation include post-translational (Kang et al. 2001) and post-transcriptional events that may affect DNMT enzymes to alter the final methylation state of the genome; this may explain the contradictory results observed in skeletal muscle.

7.3.3 Histone Modifications during Torpor

Research on the role of epigenetic mechanisms in mammalian hibernation has largely focused on two models, the Siberian chipmunk (*Tamias asiaticus*) and 13-lined ground squirrel *(I. tridecemlineatus*). Histone modifications have been shown to be integral to the torpor-mediated knockdown of hibernating proteins HP20, HP25, and HP27 in the liver of Siberian chipmunks (Tsukamoto et al. 2017; Tsukamoto et al. 2018). HP20/25/27 are highly homologous, belonging to

the C1q and tumor necrosis factor (C1q/TNF) superfamily (Kondo and Kondo 1992; Kishore et al. 2004). They form a 140 kDa complex in circulating blood. Using chromatin immunoprecipitation analysis, researchers demonstrated that permissive histone modifications, H3K9ac, H3K14ac, and H3K4me3, were all reduced in the HP25 promoter during torpor. The data suggested that this is due, in part, to decreased DNA binding capacity from putative "writer" enzymes KAT3A, NCoA-1, KAT2B, and SETD1A that are triggered by disabled binding of the coactivator, hepatocyte necrosis factor 4 (HNF4), to the HP25 promoter by the small heterodimer partner (SHP) (Tsukamoto et al. 2017). Subsequent work showed similar repression at HP27 and HP20 promoters via decreased binding of the coactivators, USF2 and/or HNF1 (Tsukamoto et al. 2018).

Histone modifications are also implicated on a global scale during the torpor-arousal cycle of ground squirrels. Deacetylation was initially suggested as a contributor to transcriptional suppression in torpor where a reduction of transcriptionally permissive H3K23ac, along with increased activity and expression involving HDAC1 and HDAC3 were noted in the skeletal muscle of hibernating animals (Morin and Storey 2006). These results were later supported by data showing increased total class I/II HDAC activity in the skeletal muscle of torpid ground squirrels (Hawkins and Storey 2017). Torpor-mediated suppression of skeletal muscle KAT3A and hepatic KAT2A also occurred (Rouble et al. 2018). The complexity of acetyl-histone mechanisms in mammalian hibernation has become increasingly evident with continuing research, often with tissue-specific but sometimes unclear results. Tissue differences were particularly apparent when acetyl-histone profiles of brown (BAT) and white adipose tissue (WAT) were compared. In BAT, increased levels of H3K9ac, likely a result of increased KAT2A expression and total histone acetyltransferase activity, provided evidence of a more transcriptionally permissive state during ET and LT (Rouble et al. 2018). Analysis of WAT suggested a different pattern, since H3K9ac was reduced during ET, along with decreased global HAT activity and KAT1 expression in late torpor, suggesting the reverse. Another study showed increased SIRT2 levels during LT in WAT (Rouble and Storey 2015). The contrast between these results is likely explained, in part, by differences in function required of BAT and WAT during hibernation. Metabolically active BAT must oxidize lipid stores to support nonshivering thermogenesis to prevent body temperature from falling below 0 °C and to reheat the body during arousal, whereas a less active WAT provides fatty acids' fuels to other tissues during the winter. Hence, the markedly different functions of the two adipose tissues undoubtedly lead to differing requirements for gene transcription. Acetylated histone residues, that support active transcriptional states, were also linked to early arousal states (Tessier et al. 2017; Rouble et al. 2018). In skeletal muscle during EA, global H3K14ac and H3K18ac were elevated (Tessier et al. 2017). KAT2A and KAT2B protein levels were also upregulated in the liver during EA (Rouble et al. 2018), although KAT2A expression remained high across all torpor stages. This preliminary evidence suggests that induced transcription during early arousal might facilitate essential pro-survival mechanisms across the torpor-arousal transition.

Analysis of histone lysine methylation also showed marked trends during ground squirrel hibernation (Watts and Storey 2019). In both liver and muscle, permissive H3K4me1 marks peaked during entrance into and arousal from torpor and this was suggested to result from increased KMT2 complex enzyme (ASH2L, RBBP5) expression leading to myoprotective roles. G9a methyltransferase, a mediator of repressive H3K9me2/3, was also induced during these transitions and SMYD2 targeted H3K4 and H3K36 to allow transcriptional activation during these states in both tissues. A heightened need for myoprotective factors at times of higher metabolic activity (i.e., EA and IA) can be postulated to explain this, although gene-specific methyl-lysine dynamics are still being investigated.

7.4 Hypoxia and Anoxia

Hypoxia is defined as low (suboptimal) availability of oxygen, whereas anoxia is a complete lack of oxygen. Both of these conditions arise when the cellular need for oxygen is greater than the accessible supply. Many animals experience hypoxia/anoxia as a result of their environmental conditions, particularly among various aquatic species such as those that undergo breath-hold diving (e.g., turtles such as the red-eared slider turtle *Trachemys scripta elegans*) or gill-breathing species that experience seasonal depletion of oxygen in water (e.g., crayfish *Orconectes virilis*, or fish such as Crucian carp and goldfish) or are deprived of oxygen during each low tide (Nilsson and Renshaw 2004; Storey 2007). Hypoxia/anoxia stress is also one component of freeze tolerance due to the lack of blood flow and gas exchange in frozen animals. Most species that experience routine hypoxia/anoxia show regulated metabolic rate depression to lower their energy needs when oxygen is depleted and, coupled with cold water during the winter season, most can survive for many weeks using anaerobic pathways of metabolism alone. For example, red-eared sliders can survive for 12–18 weeks in cold water without breathing oxygen (Jackson 2002). Metabolism switches from aerobic to anaerobic, high glycogen stores are slowly consumed by tissues, antioxidant defenses are upregulated to combat reactive oxygen species (ROS) formation and damage, and end products of anaerobic glycolysis are excreted or buffered (Storey 2007). For example, turtles store lactate in their shells using Ca^{2+} and Mg^{2+} bicarbonate ions released from the shell to buffer acidosis.

All the hallmarks of MRD and hypometabolism hold true for animals that survive hypoxia/anoxia. Metabolic rate is often lowered to ~10% or less of the aerobic rate, and nonessential/energy-expensive processes are downregulated. Energy usage is reprioritized to survival mechanisms including antioxidant defenses, antiapoptotic mechanisms, and anaerobic glycolysis. Epigenetic and post-transcriptional controls on gene expression can assist with the implementation of these survival strategies, and this section highlights some relevant studies performed on anoxia-tolerant animals. For a visual depiction of the primary hypoxia- and anoxia-responsive epigenetic effects, see Fig. 7.4.

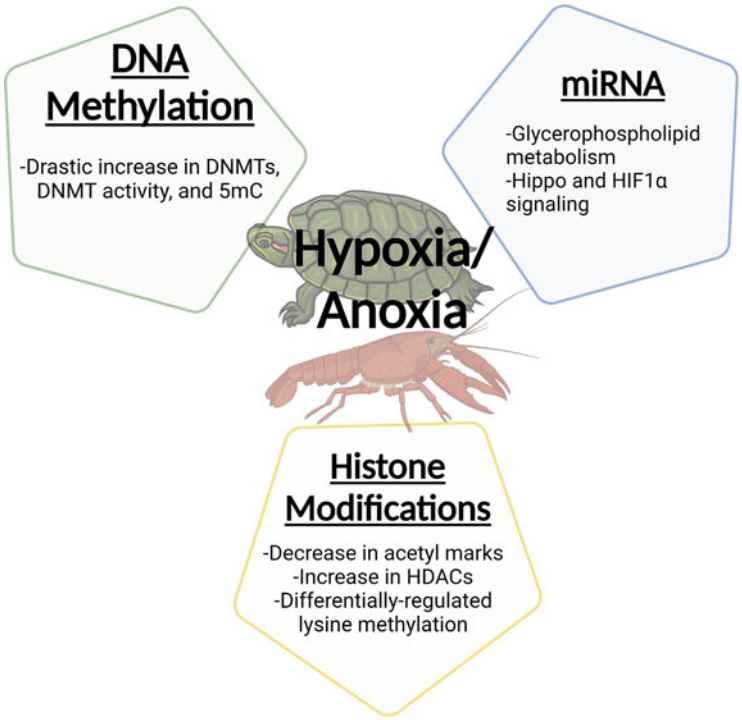

Fig. 7.4 A layout of the primary epigenetic roles during hypoxia and anoxia. Figure created using BioRender.com

7.4.1 MiRNAs in Anoxia

The northern crayfish *O. virilis* must contend frequently with hypoxic and anoxic water arising from high heat in shallow water in summer or ice-locked waters in winter. Whereas the molecular mechanisms by which *O. virilis* endures these conditions have not been well-studied, it is hypothesized that crayfish can enter a hypometabolic state similar to other anoxia-tolerant animals. Differential microRNA expression appears to contribute to their survival. An analysis of 76 miRNAs using RT-qPCR compared crayfish responses under acute (2 h) or chronic (20 h) anoxia exposures in two tissues, hepatopancreas and tail muscle (English et al. 2018). Interestingly, hepatopancreas metabolism appeared to be strongly regulated by miRNA action with 21 mRNA species downregulated under acute anoxia, whereas tail muscle showed significantly altered levels of only two miRNAs (one up- and one down-regulated) as well as two significantly upregulated in chronic anoxia (English et al. 2018). Bioinformatic analysis of the miRNAs altered in hepatopancreas suggested that the Hippo, JAK-STAT, and MAPK signaling pathways were particular targets under anoxia along with glycerophospholipid metabolism and mucin type O-glycan biosynthesis. The Hippo pathway has strong links to the hypoxia

stress response given that decreased signaling by this pathway promotes hypoxia-responsive genes indirectly via HIF-1α (Morin et al. 2005). Moreover, cell growth and proliferation are suppressed by Hippo under hypoxia stress, strengthening the importance of miRNA regulation of this pathway during anoxia. Many of the specific miRNAs predicted through bioinformatic analysis proved to be either direct or indirect regulators of HIF-1 itself, shedding more light on the importance of this transcription factor during anoxia in crayfish.

The importance of miRNAs in anoxia tolerance of turtles (*T.s. elegans*) began with RT-PCR quantification of a select group of miRNAs in liver, white muscle, spleen, and kidney in response to both acute (5 h) and chronic (20 h) anoxia exposure (Biggar and Storey 2017). Tissues showed variable expression of the miRNAs chosen for assessment. In liver, five miRNAs were upregulated under anoxic conditions, whereas three were downregulated. White muscle showed six upregulated and only one downregulated, kidney had six upregulated and three downregulated, and spleen showed five upregulated and one downregulated (Biggar and Storey 2017). Only one miRNA, miR-20a, showed similar anoxia-responsive upregulation across all four tissues and its gene targets center around cell division and proliferation. This signifies that increased expression of miR-20a helps to suppress the energy-expensive cell cycle during periods of oxygen deprivation (Biggar and Storey 2017). Another miRNA that was similarly expressed in muscle, kidney, and spleen was miR-21, which may mediate an antiapoptotic role in these tissues.

7.4.2 DNMT Enzymes under Anoxia

Altered DNA methylation also contributes to anoxia tolerance in *T. s. elegans*, as reported by Wijenayake and Storey (Wijenayake and Storey 2016). Enzymes responsible for reading, writing, and erasing DNA methyl marks were differentially regulated in both a tissue-specific manner and over time under anoxia stress. For example, in liver, DNMT1 and DNMT2 protein levels were strongly upregulated by 4- and 2- fold, respectively, in response to 5 h submergence in nitrogen-gassed water, before declining again to near control levels after 20 h anoxia. Liver MBD1 and MBD2 proteins also increased by ~three-fold after 5 h anoxic submergence but were partially reduced again after 20 h. Total DNMT activity and global 5mC levels also increased significantly in liver after both 5 h and 20 h of anoxia exposure. In white muscle, the primary responses were by DNMT3a and 3b whose protein levels increased by ~three-fold after 5 h anoxic submergence and DNMT3b remained high after 20 h anoxia whereas DNMT3a fell to below control levels. MBD1 protein showed no change over both anoxia conditions and DNMT3B levels also rose during 5 h anoxia, and increased even further after 20 h anoxia. Total DNMT activity was increased in both 5 h and 20 h anoxia, and global 5mC methylation rose in the 5 h anoxia condition but declined somewhat after 20 h anoxia but remained higher than control values.

In heart, DNMT1 protein levels did not change across anoxia but DNMT3A rose after 5 h anoxia and DNMT3B increased strongly after 20 h anoxia. Total DNMT activity in heart was essentially unchanged as were global 5mC levels.

It is noteworthy that DNMT activity was upregulated across all three tissues in response to 20 h anoxia exposure, with two tissues (liver and white muscle) also showing this elevated activity after 5 h anoxia. The protein expression levels of various DNMT enzymes were more varied: whereas some were upregulated after 5 h anoxia, others remained unchanged or were even downregulated after 20 h anoxia exposure. This would suggest that the cell's primary method of regulating DNMTs is not strictly from increased or decreased protein synthesis, which would strain available cellular resources, but another mechanism which affects DNMT activity and supports more efficient modulation of methyltransferase activity. Global 5mC levels reflected this increase in activity in both liver and white muscle, whereas 5mC levels in heart remained unchanged after 5 h anoxia (corresponding to the unchanged DNMT activity in this condition) and remained consistent during 20 h anoxia even though DNMT activity was increased at this timepoint.

7.4.3 Histone Modifications during Anoxia

Both histone lysine methylation and acetylation have been examined as parts of the anoxia tolerance response of red-eared sliders (*T. s. elegans*) (Krivoruchko and Storey 2010; Wijenayake et al. 2018; Wijenayake and Storey 2020). In terms of methylation, permissive H3K4me1 and repressive H3K9me3 levels were both elevated during prolonged (20 h) anoxia. The expression of corresponding methyltransferases ASH2L and G9a changed in agreement with their respective histone target residues, implying contributing roles. Global KMT activities at H3K4 and H3K9 were also increased under anoxia exposure (Wijenayake et al. 2018). Overall, this study suggested that lysine methylation plays a complex role in the gene regulation of anoxia survival, likely promoting transcription of anoxia-responsive genes, while actively suppressing nonessential pathways. Liver transcriptomic studies found mRNA significantly increased in pathways related to DNA damage repair and metabolic reprogramming (Biggar et al. 2019). Their results suggest that heightened succinate metabolism may be utilized during turtle anoxia to combat lactate accumulation, which is characteristic of other established models.

Equally intricate mechanisms were found upon investigation into histone acetylation. In a related study, H3K14ac, a hallmark of active promoters, was found to be consistently reduced across both short- and long-term anoxia stress conditions in turtle liver (Wijenayake and Storey 2020). KAT3A protein levels, an enzyme involved in H3K14ac catalysis, as well as global nuclear lysine acetyltransferase activity were similarly depressed, suggesting their involvement. KAT1 expression was also reduced during prolonged anoxia. This study built on previous work on deacetylases, which highlighted attenuated H3K9ac and H3K23ac levels in both liver and muscle tissues of turtles (Krivoruchko and Storey 2010). That study also

showed that a variety of HDACs were upregulated at both transcript and protein levels in response to anoxia. These results implied that transcriptional suppression may be mediated by deacetylation of key histone lysine residues, made possible by hypoactive acetyltransferase and hyperactive deacetylase activities that contribute to metabolic depression under anoxic conditions.

7.5 Estivation/Dehydration

Dehydration stress has been discussed above as a subcomponent in freeze tolerance, where cells lose liquid water that exits to join extracellular and extra-organ ice masses. However, more commonly, dehydration is a response to extreme heat and/or drying of the environment. Species with a poor capacity to resist water loss across their body surface are particularly vulnerable and often seek shelter underground including various species of frogs, toads, and lungfish. Some can minimize dehydration stress (at least initially) by constructing mucus or shed skin cocoons around their bodies or, for many amphibians, by slowly resorbing water from a very full bladder (a skill not found in other vertebrates) as well as retaining nitrogenous waste (urea) to elevate the osmolality of body fluids (Storey and Storey 2010b). Also key to survival in hot dry environments is metabolic rate depression, termed estivation that occurs widely among both vertebrates and invertebrates; for example, the milk snail, *Otala lactea*, is a well-studied model. These snails also limit water loss by constructing a mucus membrane across the aperture of the shell to minimize water evaporation, accumulating high concentrations of urea to provide colligative resistance against tissue water loss, and suppressing metabolic rate to only about 30% of their nonestivating rate (Bell et al. 2012). Estivating species can typically maintain aerobic metabolism for a long time but ultimately, as water loss progresses, blood plasma volume decreases (concentrating blood cells) and the workload on the heart increases to maintain circulation. Decreasing oxygen transport triggers an increased dependence on glycolysis for ATP generation resulting in lactate accumulation. To cope with this hypoxic and water-restricted state over a long period of time with no nutrient consumption, dehydration-tolerant animals then resort to metabolic rate depression to minimize substrate and ATP consumption. Figure 7.5 highlights the main functions of miRNA control during estivation and dehydration.

7.5.1 MiRNAs in Dehydration

As for other systems of MRD described above, a suppression of nonessential processes during estivation is key to survival and microRNA can play a significant role in this. Using the African clawed frog, *X. laevis*, as the model organism, a study of dehydration-induced changes in miRNA patterns in liver, skin, and kidney showed significant changes consistent with hypometabolism (Wu et al. 2013). An

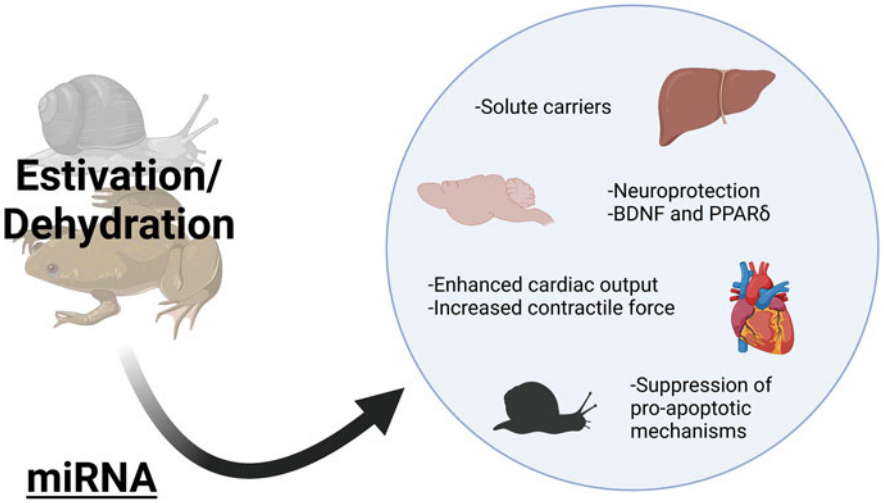

Fig. 7.5 The critical miRNA influences during estivation and dehydration between *X. laevis* and *O. virilis*. Figure created using BioRender.com

analysis of ten miRNAs revealed that three were downregulated and three upregulated in liver in response to the loss of >30% of total body water. Kidney showed three different miRNAs upregulated and skin showed only two upregulated whereas other miRNAs analyzed remained unchanged (Wu et al. 2013). MiR-203 was upregulated in both kidney and skin but unchanged in liver, whereas miR-34a was upregulated in skin and liver but not kidney. The downregulated miRNAs in liver were those that target genes for solute carriers. Various solute carriers have been observed to be upregulated during anoxia and hibernation, suggesting their general importance for MRD (Wu et al. 2013). There were other links to hibernation, e.g., upregulation of miR-29b which targets p85α, the catalytic subunit of the Akt pathway, which is reduced during hibernation (Abnous et al. 2008).

A second study that evaluated the responses of 43 miRNAs from brain of *X. laevis* via RT-qPCR revealed 12 that were downregulated during dehydration and none that showed upregulation (Luu and Storey 2015). Predicted functions for the downregulated miRNAs involved genes associated with axon guidance and long-term potentiation, which could be enhanced as a result and therefore worth investigating as coping mechanisms for dehydration tolerance. Other neuroprotective pathways may be activated given that downregulated miRNAs of interest in this study have also been shown to suppress neuroprotective factors such as BDNF and PPARδ in other species (Yin et al. 2010; Gao et al. 2015).

A further study of miRNA regulation during dehydration in *X. laevis* evaluated heart with a bioinformatics-centered study that identified 24 miRNAs that were differentially regulated in response to dehydration stress (Hawkins and Storey 2020). Of these, 21 were significantly downregulated whereas the remaining three were upregulated. The large number of downregulated miRNAs suggested a

facilitated upregulation of genes and processes with pro-survival actions. These could include actions that increase the contractile force of the heart to sustain circulation in the face of an increased thickening of blood due to water loss from the plasma as evaporative dehydration progresses (Hillman 1978). MiR-99b-5p showed the greatest change during dehydration with reduction to 15% of control values, and it was hypothesized that downregulation of this miRNA is linked to enhanced cardiac output. Functions of the collective group of differentially expressed miRNAs also related to RNA/DNA/transcription factor binding, with particular emphasis on proteins involved in other facets of post-transcriptional regulation (Hawkins and Storey 2020). The KEGG pathway Cardiac Muscle Contraction was the most significantly enriched, reflecting knowledge that heart function must increase to cope with the reduction in blood volume and increase in viscosity caused by water loss during dehydration, as mentioned earlier. Ion transporters were also targeted by specific miRNAs predicted to be downregulated in the study, and the Glycolysis/Gluconeogenesis pathway was also enriched. This is in line with known features of hypometabolic states, including increased reliance on anaerobic metabolism for ATP production.

The estivating snail *O. lactea* also showed differential miRNA expression as a facet of its stress tolerance (Hoyeck et al. 2019). A selection of 75 miRNAs were detected in foot muscle, of which 26 were upregulated during estivation and none were downregulated. The significantly upregulated miRNAs were implicated in regulating cell survival mechanisms revolving around antiapoptosis, tumor suppression, and muscle maintenance responses. The miR-2 family were among those upregulated and these suppress pro-apoptotic mechanisms, which would be crucial during estivation (Gennarino et al. 2012). Other antiapoptotic miRNAs upregulated in foot muscle included miR-153 and miR-124, further highlighting the importance of antiapoptotic measures in this animal (Hoyeck et al. 2019).

7.6 Conclusions

In this chapter, we provided a comprehensive look into modes of epigenetic regulation and how they interplay with extreme environmental stress conditions including freeze tolerance, torpor, hypoxia/anoxia, and estivation/dehydration. The main themes are outlined as follows:

During freeze tolerance, miRNA regulation seems to be greater in liver which is highly metabolically active, and therefore requires stricter control over essential processes. However, miRNA biogenesis seems to be downregulated in less critical tissues to aid in the theme of global MRD and hypometabolism and save on energy resources. The emphasis on signaling pathways (namely PI3K) in both invertebrate and vertebrate models of freeze tolerance may insinuate that miRNA is especially vital in maintaining intracellular transduction during this stress. Other functions may vary according to tissue-specific need, given the observed variation in expression in liver versus skeletal muscle and kidney, whereas closer analysis of miRNAs in brain

revealed neuroprotective roles, and heart showed cardioprotective roles. The rise of bioinformatic prediction tools and large-scale sequencing efforts may help shed more light on the functions of miRNAs across species in response to freezing stress and help elucidate their exact roles in enabling survival of this extreme environmental stress.

DNA methylation also appears to be less important in liver during freeze tolerance, so it is possible that miRNA is predominant in regulating cellular processes in this tissue in response to freezing stress. DNA methylation exhibited tissue-specific variations during freeze tolerance. Strong upregulation of DNMT3L was observed for three freeze-tolerant species, potentially signifying its importance as a cofactor to interact with many different forms of epigenetic control. The upregulation of DNMT1 also merits note, although downregulation in liver and upregulation in muscle (*R. sylvatica*) were opposite to the upregulation in liver and downregulation in muscle (*D. versicolor*). DNMT enzymes are also subject to a variety of post-transcriptional and post-translational modifications, all of which affect downstream activity and final methylation patterns of the genome. More research will uncover specific mechanisms and functions by which DNA methylation interplays with MRD and hypometabolism as a whole.

In torpor, miRNAs appear to play cross-species roles in muscle maintenance. Insulin resistance and lipid metabolism are also themes, with miRNAs targeting glycolysis and insulin-related pathways appearing in both *I. tridecemlineatus* and *M. murinus*, species with diverse profiles of torpor/hibernation use. DNA methylation during hibernation is understudied and with current preliminary data, it is hard to draw hypotheses regarding its function in any species. However, future research may elucidate a specific role, if any, for DNA methylation during hibernation.

Studies in hypoxia/anoxia strongly suggest a role for miRNAs and DNA methylation to suppress the cell cycle, as shown in *T.s. elegans*. Northern crayfish *O. virilis* appeared to utilize miRNAs in antioxidant defense, as evidenced by interactions with HIF1, which was echoed by antioxidant defense and protection against ROS observed in *T.s. elegans.* Overall, signaling pathways and antiapoptotic mechanisms have important roles across both anoxia-tolerant species, highlighting an area for future research in all aspects of epigenetic control.

Of all environmental stresses discussed, estivation/dehydration has received the least research. The model organisms explored so far have been *X. laevis* and *O. lactea,* each living in very different habitats that limits our ability for pattern-establishment between these models. The importance of miRNAs in regulating the expression of solute carriers appeared in *X. laevis,* as well as the need for increased cardiac contractility to cope with reduced blood volume. Analysis of *O. lactea* highlighted the importance of miRNA action in regulating antiapoptotic mechanisms.

The metabolic reorganization needed to both facilitate and maintain MRD is extensive and requires tight regulatory oversight through many factors, including by epigenetic and other modes of post-transcriptional control. The diversity of animals which use hypometabolism as a survival strategy for dealing with severe environmental conditions ranging from extreme cold to extreme heat is expansive, making it

all the more impressive that epigenetic and post-transcriptional mechanisms are crucial players in all the studies discussed in this chapter. The following years will see an increase in understanding of all these molecular mechanisms, and bring us closer to learning how animals ranging from molluscs to primates have evolved to survive and thrive in environmental conditions humans have yet to tolerate.

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Chapter 8
Epigenetic Adaptation to Local Ecologies as a First Step toward Gene: Culture Co-evolution

Gillian Ragsdale and Robert A. Foley

Contents

Abstract The sum of individual biases in cognition and behaviour can influence the development of culture within a population. One of the biological influences contributing to such bias is gene–culture coevolution. Adaptation of culture to local ecologies could also be influenced relatively rapidly by epigenetic–cultural coadaptation and coevolution. This process could provide the very earliest biases in behaviours that go on to become cultural traits; that is, it can explain how cultural norms and differences first arose in prehistoric human populations (and by extension, continues to influence culture today). It could explain how these processes can come about rapidly—more rapidly than could be accounted for by gene–culture coevolution alone—even in the face of entirely novel triggers. Epigenetics can also explain one of the central challenges to the feasibility of this process: how traits such as behaviour and personality can be both adaptive and heritable. There are several possible routes by which epigenetic regulation of human genes might influence behaviour and consequently culture. This chapter discusses the potential role of an example of an epigenetic influence on the brain and behaviour, changes in diet. The specific example of 'social trust' as an epigenetically regulated cultural trait is then discussed as well as options for future research.

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8.1 Introduction

The tension between heritability and variation is a broad problem in evolution. Maintaining the fidelity of genetic transmission across generations while permitting enough variation to allow evolution to occur must be a balancing act for different processes. This is true for both the genetic and cultural evolution. Just as genetic variation must exist in order for selection to bring about genetic evolution, so must cultural diversity exist in order for culture to evolve. Similarly, there is also a tension between the benefits of cohesive cultural practices, necessary for group co-ordination, and the need to adapt over time as conditions change.

Gene–culture coevolution is a process by which local ecologies can select for specialisations that then have cultural consequences, and the cultural practices that arise can then bias natural selection (Kolodny et al. 2018; Laland and Brown 2002). However, the differing time scales for genetic vs. cultural variations are a problem for models of gene–culture coevolution. How can variants that have no genetic basis lead to changes in genes, given the relatively smaller time scale of the former and the longer time scale of the latter. Epigenetics, we argue, can bridge these time scales, bringing them into closer alignment. Cultural adaptation to environmental changes can then interact with epigenetic influences in a feedback loop.

The triggers for epigenetic changes typically include different kinds of stress, for example: ecological stress such as climate change, changes in quality and/or quantity of food, and changes in social stress such as competition for resources. Similarly, the nature of the response can also manifest at different levels, as outlined below, from biological adaptations to diet to behavioural adaptations to social stress.

This chapter firstly reviews epigenetics and the role it can play in evolutionary processes more broadly. Evidence is then presented for how one common environmental influence, changes in diet, can influence the brain and behaviour via epigenetic processes. Social trust is then discussed as an example of a specific cultural trait that could be influenced by epigenetically regulated expression of genes in the brain. Finally, some recommendations for future research are suggested.

8.2 Epigenetics as an Accelerator and Multiplier of Possible Adaptations

Changes in the gene coding are a possible source of genetic variation, but they are permanent and therefore, do not accommodate response to rapid or reversible changes. Epigenetic mechanisms mediate the regulation of gene expression by

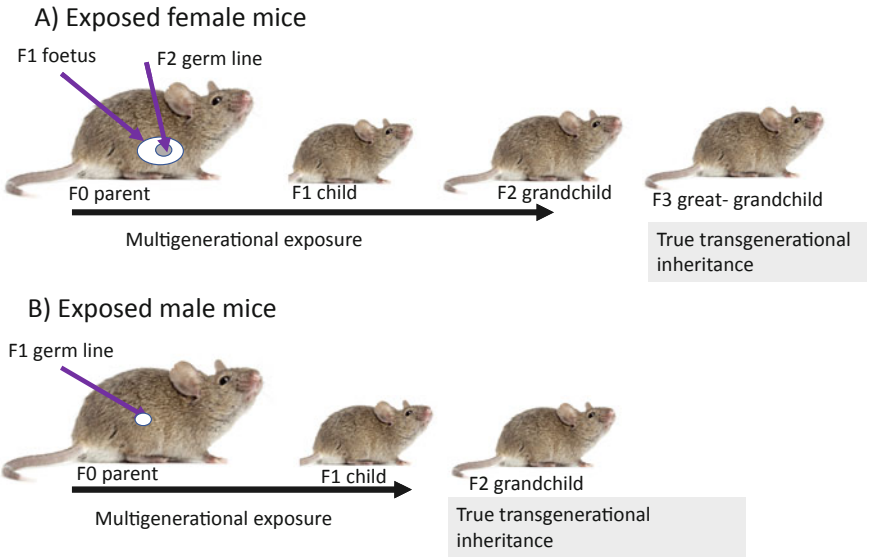

Fig. 8.1 Epigenetic processes can begin at conception and potentially influence the entire life of the organism. (**a**) In a pregnant female mammal exposed to an epigenetic trigger such as stress, a female foetus born with a complete set of eggs may be similarly influenced, so that two generations may be epigenetically programmed simultaneously. (**b**) In males, current triggers epigenetically modify offspring via the sperm. In both the cases, the possibility of true transgenerational inheritance is still under debate (Lacal and Ventura 2018)

processes such as DNA methylation, histone acetylation and regulation by non-coding RNAs, without the need for changes in DNA coding. These chemical modifications and regulatory elements can up- or down-regulate the expression of genes, turning them completely on or off, or something in between. Crucially, these processes are rapid and reversible. Figure 8.1 shows the maximum time range for an epigenetic trigger occur during foetal development. Epigenetic processes peak during a developmental window which is, as yet, not well defined but tends to decline into adulthood (Boyce et al. 2020).

Epigenetic modifications are themselves facilitated by specific gene products and DNA elements which may be under selection, and epigenetic processes are triggered by both the internal and external environmental triggers. For example, our immune response, as mediated by the highly polymorphic major histocompatibility complex (MHC), is further amplified by epigenetic processes (Suarez-Alvarez et al. 2010).

Additional layers of possibility and complexity can occur where epigenetics is combined with allelic variation, such that the expression of one allele in response to an environmental trigger is different to another. Epigenetic differences may also be sex specific. For example, sex differences in epigenetic regulation partially account for different responses to stress (Brivio et al. 2020). Epigenetic gene regulation thus drives developmental plasticity which can extend evolutionary explanations beyond the role of genes and natural selection (Uller et al. 2019).

Selection for changes in epigenetic regulation can itself be relatively rapid. In that most rapid of all evolutionary arms races that between disease pathogens and immune response, epigenetics is an efficient multiplier of possible responses. Antibiotic resistance in bacteria, for example, has been found to be faster than can be accounted for by spontaneous DNA mutation, and is mediated by stochastic changes in epigenetic regulation of the expression of existing genes (Adam et al. 2008). It has been proposed that such stochastic epigenetic variation could enable selection for phenotype variance without necessarily changing the mean phenotype (Feinberg and Irizarry 2009). Applying this model to epigenetic regulation of mental processes would enable human cognition and behaviour to adapt to changes in the environment as a kind of mental immune response. In other words, by regulating mental processes, and hence their output (thoughts and actions), new mental variants can appear or be exposed, and thus change as conditions change.

Following the examples from disease and immunity, the cognitive (and thus cultural and behavioural) response to entirely new environmental triggers is of particular interest, that have not previously existed.

There is a great deal of available bandwidth when it comes to epigenetic regulation. A major difference between adaptation via epigenetics and adaptation via allelic variation is that the former does not necessarily require natural selection. In the previous example of evolving bacterial antibiotic resistance, natural selection was involved because the epigenetic response did not already exist—it was selected from stochastic changes altering existing epigenetic regulation. However, in many cases, adaptation occurs via triggers acting on existing epigenetic processes.

It is also possible that existing epigenetic responses may be triggered by novel environmental agents—and this could be either advantageous or not. Taking an example of a trigger from modern human culture, Vassoler et al. (2013) found that paternal cocaine used in rats resulted in cocaine-resistant male (but not female) offspring. The resistance to cocaine use was mediated epigenetically by histone acetylation of the brain-derived neurotrophic (*Bndf*) factor gene promotor. It seems reasonable to assume that the murine environment of evolutionary adaptiveness did not include a period of heavy cocaine use—so this response is being co-opted from its original (as yet unknown) target—although in this case it is still highly adaptive. The authors speculate that the response may be acting to minimise the increased brain plasticity brought about by cocaine-induced elevations in *Bndf*. This raises some important questions for future research on the pivotal role of *Bndf* in brain development and life-long health, and how this responds to environmental triggers—some of which may be relatively novel and/or culturally specific.

The possibilities with regard to changing the phenotype can range from the very subtle to those mimicking speciation level changes: an example of the latter occurs in locusts (Boerjan et al. 2011; Burrows et al. 2011). The two naturally occurring phases possible in a locust's life are the solitary and gregarious forms. These two forms differ dramatically not only in their social behaviour, but also in their morphology, including their brain structure—to the extent that you would not normally expect such different forms to belong to the same species, let alone the same individual insect. These changes are brought about by epigenetic regulation

triggered principally by changes in serotonin levels—which, in turn, is a response to population density. Clearly, epigenetics has the potential to bring about radical changes in the phenotype of an organism even within its own lifetime.

Epigenetic regulation of gene expression also allows variation in the influence of genes that are, by necessity, too highly conserved to evolve allelic variation. Epigenetics still comes at a cost to the organism, however. A common epigenetic mechanism involves the down regulation of one of a pair of alleles, effectively eliminating the genetic influence of one parent and making the individual vulnerable to errors in the expressed allele that cannot be compensated for by the silenced allele (genomic imprinting: Bartolomei et al. 2020, Tucci et al. 2019). This ability to compensate for one faulty allele in a pair is one of the great advantages of sexual reproduction. For selection to favour a mechanism such as epigenetics that decreases or eliminates this advantage, the alternative fitness benefits must be greater. In fact, the phenotypic plasticity and increased variation can facilitate natural selection of changes in the genetic code, both reducing and increasing variation (for a comprehensive account of how epigenetics influences evolution see the special issue as introduced by Ashe et al. 2021). This linkage between epigenetics and natural selection is critical for maintaining the integrated homogeneity of the evolutionary process as a whole.

A particularly striking and relevant observation is that epigenetically regulated genes are over-represented in the brain, and there is clear evidence for epigenetic regulation affecting the brain and behaviour (Grayson 2017). Svrakic et al. (2009) have proposed that personality disorders are better described as 'adaptation disorders' arising through person–environment interaction via epigenetic processes. Epigenetic differences between identical twins have implicated differential gene methylation in risk-taking behaviour (Kaminsky et al. 2008). Histone methylation and acetylation are implicated in major depression (Sun et al. 2013). Taking a focus on social behaviour, epigenetic regulation of the serotonin and oxytocin pathways is of particular interest since they have been shown to have major influence on sociability, empathy, theory of mind and antisocial behaviour (Aghajani et al. 2018; Craig et al. 2021; Hiroaka et al. 2021; Kumsta et al. 2013; Krol et al. 2019).

The internal and external environmental triggers for epigenetic regulation include diet, hormones, sex of the cell and various forms of stress providing several possible routes by which epigenetic regulation of human genes might influence behaviour and consequently culture.

8.3 Changes in Diet as Epigenetic Triggers

A natural human experiment at the end of World War 2 provided the first evidence that dietary changes could drive changes in epigenetic regulation. In the autumn of 1944, the Allied forces were preparing a final push across the Rhine to end the war. To prepare for this, the exiled Dutch government called for a rail strike to block German army supplies and in retaliation the Germans stopped food reaching western

Holland. However, the Allies were not able to cross the Rhine, and effectively starved by both sides, the resulting famine became known as The Dutch Hunger Winter. Food intake dropped to 4–500 calories per day—half the amount recommended for a one-year-old child.

Even in the darkest times, women go on conceiving and bearing children, and despite poor health, many children were born during and after the famine. This was a very rare opportunity to study the effect of poor nutrition during pregnancy in a population that was otherwise reasonably well fed. These children have been monitored regularly throughout their lives and clearly something happened in the womb that has had life-long consequences: there has been foetal programming. A foetus experiencing famine via its mother went on to regulate calorie intake as though the famine continued, even if it had stopped at birth. As adults, they also suffered from a range of health issues including increased susceptibility to some mental health conditions. Eventually, it was determined that the long-term effects on calorie regulation were brought about epigenetic changes to the genes regulating the insulin pathway (Conradt et al. 2018).

The field of foetal programming has established the link between dietary intake and epigenetics, but the focus of this chapter is on cultural changes associated with qualitative rather than quantitative changes in diet. For example, the proportions of basic food categories in the pre-agricultural human diet vary dramatically by general climate and season. The pre-modern Australian Aboriginal diet, for example, varied from 25 to 80% protein, depending on climate (arid, semi-arid and tropical), season (wet and dry) and proximity to the coast (White 2001).

There is increasing evidence of epigenetic influences on the brain and behaviour triggered specifically by diet (Dauncey 2013; Leroy et al. 2020; Pizziorusso and Tognini 2020). For example, with regard to language skills, epigenetics is implicated in the models of specific language impairment, and prenatal folic acid supplementation has been associated with reduced risk of severe language delay at three (Rice 2012). For the purposes of this discussion, however, it is important to tease out epigenetic influences on cognition and behaviour in the *normal* range.

As an example of an essential dietary component with epigenetic influence, protein in the diet supplies methyl groups for gene methylation. Protein deficiency leads to Kwashiorkor which is associated with changes in the 'Big Five' personality traits: increased neuroticism and decreased extraversion, conscientiousness, openness and agreeableness (Galler et al. 2013). Galler et al. describe the brains of children with Kwashiorkor as being adapted to a 'world of scarcity'. The implication is that these changes are not simply the result of an insult to brain metabolism—they constitute an adaptation to that insult. If this is the case, epigenetic regulation triggered by protein deficiency could be a means of bringing about specific changes. For example, the global demethylation that would result from lack of protein mimics the response to stress generally, heightening stress sensitivity and vigilance as adaptations to a relatively stressful environment (Hing et al. 2014). This example illustrates a link between the direct influence of diet; here a scarcity of protein and hence methyl donors, and an adaptive response to the general environmental stress that poor nutrition is usually associated with. In mice, protein deficiency has been

associated with over expression of genes regulating the dopaminergic pathway resulting in altered reward processing and hyperactivity suggesting a similar profile to that of ADHD in humans (Vucetic et al. 2012).

When assessing the influence of nutritional items, there very often appears to be a U-shaped goldilocks effect—both too much and too little have negative consequences. You can have too much methylation; for example, *increased* methylation of the promoter region of the serotonin type 1A gene is associated with both the schizophrenia and bipolar disorders (Carrard et al. 2011). Moreover, there is evidence of *decreased* methylation of genes in the human brain, compared to other primates, associated with a risk for schizophrenia (Hyeonsoo et al. 2021).

8.4 Epigenetics, Diet and Cultural Psychology

The 'Big Five': extroversion, openness, agreeableness, neuroticism and conscientiousness have correlations with other psychological traits, such as the association between high extroversion and high sociability, between high openness and low conservatism, and between high neuroticism and low emotional stability. Cross cultural psychology uses combinations of similar factors to describe and compare different cultures, such as complexity, tightness, collectivity and individualism (Triandis and Suh 2002).

Discussion of personality traits differing within and between cultures is controversial, largely because it could be taken as evidence that human nature is less universal than many psychological or anthropological models presuppose. In Montiglio et al.'s (2013) paper on social niche specialization, the authors make the case for research 'on the coevolution of personality and niche specialization and its consequences on the social structure of mammal populations'. While humans as a species have a broad niche, each population is capable of high levels of niche specialisation (Odling-Smee et al. 2003; Laland et al. 2016). As such, humans are the ultimate niche specialists: humans not only respond to their local ecology, but they also partially create their own niches, resulting in escalating specialisation. Large-scale differences in ecology will bias the niche profiles of human populations living in those conditions: i.e., differences in culture.

Given evidence for epigenetic regulation of both the physiological and behavioural traits, the possibility exists that local ecological conditions may trigger epigenetic changes in personality and behaviour that are not just the side effects of relevant physiological changes, but are actually adaptive to those conditions. In humans, this further raises the possibility that the characteristics of cultures may be biased to some degree by the accumulated influences of individuals whose behaviour is influenced by epigenetic regulation of the brain. The availability of food resources, together with foraging and/or hunting strategies, is a major determinant of social structure and groups comprising mixed, rather than uniform, behavioural phenotypes may be advantageous. This kind of adaptive, heritable phenotypic heterogeneity could be well maintained by a combination of low-level allelic variation combined

with epigenetic regulation as exemplified by the regulation of the serotonin transporter gene, discussed below.

That such epigenetic regulatory processes exist is not the issue—the question is whether they are systematic in influencing the cultural practices and modes of human populations and whether such biases are adaptive, either historically or currently. There has been much speculation on the maladaptation of the human Stone Age mind to modern life (Baron-Cohen 1997). Major transitions, for example, to hunting, cooking and farming are likely candidates as drivers of epigenetic changes that might impact culture. The complexity of modern cultures, for example, with regard to diet and migration between cultures, creates a whole new library of possible adaptation and maladaptation via epigenetic processes.

Epigenetics as an evolutionary mechanism has itself been under selection to enable relatively rapid adaptation to local triggers—but the range and rapidity of change experienced increasingly by post-agricultural humans outstrip even the relatively rapid response of epigenetics. This is clearly illustrated by the association of obesity and diabetes, with foetal programming. As mentioned above, poor maternal nutrition triggers epigenetic changes in expression of foetal genes associated with insulin and glucose metabolism that persist across several generations. The adaptation of one generation to conditions of relative famine is a susceptibility to diseases of affluence when food is abundant (Stevenson et al. 2020).

The range of epigenetic responses to diet illustrate the potential for ecological triggers to influence the brain and behaviour, enabling individuals to adapt, for example, from times of feast to those of famine, and vice versa. Some of the behavioural consequences, such as hyper-vigilance, distrust, anxiety and aggression, may not appear adaptive to modern eyes, since the criterion for evolutionary adaptiveness is reproductive fitness rather than happiness, but in less modern contexts they are likely to have been. If competition for food is fierce, an individual who lived alone in an isolated food patch might live a relatively long and stress-free life, but would also form an evolutionary cul-de-sac compared to one who lived fast, died young but left offspring.

8.5 Epigenetic Influences on Social Trust

Just as individuals differ in their tendency to trust versus mistrust, whole cultures can differ on measures of social trust (Rothstein and Uslaner 2005). In a regular World Survey of social trust, countries have been found to differ consistently over the time period of the survey (since 1991), for example, China and Sweden are generally among the highest scoring nations while Brazil and Columbia are among the lowest. While measures are generally stable, changes are evident; for example, US citizens report steadily declining trust over the last 40 years. Higher social trust scores correlate with several national measures such as higher GDP, lower income inequality, higher levels of education, less violence and greater political stability (Ortiz-Ospina and Roser 2016).

For individual mammals generally, trust can be influenced by developmental epigenetic responses to stress such that prosocial, more trusting behaviours are favoured under conditions of low stress where competition, and hence the threat of aggression between conspecifics is lower (Cunliffe 2016).

Levels of oxytocin and serotonin strongly influence trusting behaviours with higher levels promoting trusting behaviours. Oxytocin is the central regulator of a hub of neurotransmitters (including serotonin) and hormones influencing trust (Riedl and Javor 2011). In turn, levels of these chemicals are influenced by many kinds of stress, social support and genetics. Stress reduces trust and social support increases the level of trust, as well as buffering the effect of stress (McQaid et al. 2016).

There are several genes that also influence the levels of these chemicals such as the oxytocin receptor gene (OXTR) and the serotonin transporter gene (SERT). Both the genes are polymorphic, that is, they have more than one common allele—and these different alleles are associated with different levels of influence on trusting behaviour (Feldman et al. 2016; Iurescia et al. 2016).

Having, for example, two possible alleles in a population means there are two possible kinds of influence on the phenotype. If conditions change favouring one allele over the other (in terms of reproductive fitness), the frequency of that allele will increase, enabling the population to adapt to the new conditions. This process of natural selection is not quick, however, requiring many generations, depending on the strength of the advantage. As discussed, an allele that is also epigenetically regulated can influence phenotypic change more quickly—potentially bringing about changes in one generation that would otherwise take many more. If one allele is epigenetically regulated while the other is not, then the ability to adapt is itself a population variable that can be selected for: some conditions may favour phenotypic stability over plasticity and vice versa. With regard to the OXTR and SERT genes, both processes occur for both genes, i.e., there are common alleles of both genes and at least some of these alleles are also epigenetically regulated (Iurescia et al. 2016; Kumsta et al. 2013).

The OXTR and SERT genes expressed in the brain are regulated in response to stress and are 'socially sensitive', i.e., their expression is altered in response to social stress and this, in turn, influences social cognition and behaviour. These processes of responding to social stress have a long evolutionary history with added complexity in highly social species such as humans (Feldman et al. 2016). Social stress, in turn, is influenced by environmental pressures on resource availability and competition, mortality and population density thereby linking triggers such as climate change and diet to changes in social behaviour sufficient to lead to biases in individual behaviour, such as, levels of trust and openness, hence generating different patterns of cultural norms.

The SERT gene illustrates just how much variation in adaptation is possible given a gene that is both polymorphic and epigenetically regulated. There are two common alleles: one shorter (S) and one longer (L). The S carriers are more vulnerable to depression because the S allele is epigenetically regulated—it is methylated in response to stress in early life (Caspi et al. 2003). Furthermore, the degree of risk was found to be sex-specific: there is an almost eight-fold increase in risk for females

carrying two short versions of the SERT gene. Although some studies have not supported the epigenetic regulation of SERT in this way (Risch et al. 2009), further evidence in support continues to accumulate (Ryan and Ancelin 2019; Soga et al. 2021).

When the L carriers grow up with, for example, physical abuse, harsh maternal treatment and/or sexual abuse—they are more resilient. By comparison, when the S carriers experience severe childhood maltreatment, *two thirds of them* are likely to have an episode of major depression as adults. This reflects a substantial effect size for this epigenetically mediated gene–environment interaction (Uher et al. 2011). The S carriers are also less trusting and more discriminatory to outgroups—especially under stress.

Rather than viewing the S allele as a 'risk' gene, however, it is better described as a 'plasticity' gene that facilitates sensitivity, that also has a number of positive consequences including some improved cognitive abilities such as better decision making. The S carriers are more sensitive to social signalling generally—and derive more benefit from social support (Way and Lieberman 2010). Social support is an effective buffer against the increased sensitivity to stress experienced by the S carriers. Without differing levels of stress in their lives, the different effects of the S and L alleles in the carriers would be undetectable. Cultures where individuals with the S allele are predominant or dominant may be expected to be more biased towards collective social support systems, capitalising on social trust and prosocial behaviours, than cultures where the L carriers are more common leaning towards notions of individual self-reliance. As the variation is likely to be as much within as between populations, these cultural traits may fluctuate over time in response to changing conditions.

The S allele is by no means rare, and polymorphism at this site appears to have been selected for several times in primates. Taking a comparative approach, Dobson and Brent (2013) propose that S and L type alleles have been under balancing selection and that S type alleles are favoured when conditions, in particular social competition, fluctuate in short term. In this case, hypervigilance during periods of intense social competition may have survival benefits—as long as it does not persist when competition is relaxed. Being more sensitive to positive changes in social signals means that they are able to switch more flexibly from hypervigilance to more trusting, prosocial behaviour. The L type alleles are favoured when social competition tends not to vary so much. Some support for this comes from primate studies such as those on macaques, who have populations with differing allele frequencies and different profiles of competition and aggression. In macaques, the S allele frequencies are higher in groups with greater variance in intra-group competition (Dobson and Brent 2013). This implies that there can be population differences in the S and L alleles associated with differences in affiliative group behaviour.

How might this operate in human groups—and how does trust manifest itself at the group level? 'Social trust' is one measure used to describe different cultures as more or less trusting. There is a lot of research interest in social trust, largely because of its relationship to economic development and wealth. Making a transaction or doing work and expecting payment is only sustainable with supporting levels of

social trust. The world historian Yuval Noah Harari (2016, p. 203) writes that: 'Credit is the economic manifestation of trust'.

In cross-cultural psychology and economics, there is a phenomenon called extreme response style as a cultural response to harsh, demanding climates and economies—that response is characterised by low-social trust, risk avoidance and intolerance of uncertainty or ambiguity. However, this depends also on the context in terms of resources, i.e., wealth. It seems that the ability to make good on the social capital of trust depends on the availability of resources, via wealth, to meet ecological demands such as climate stress (He et al. 2017). Cognitively, this requires a reappraisal of the risks as challenges depending on the context and a move away from intolerance of uncertainty and ambiguity (Braunstein et al. 2013; Troy et al. 2010).

Adding SERT allele frequencies to this analysis suggests that this relationship actually only applies to populations who are low S carriers, that is, carry less of the socially sensitive allele (Kong 2015, 2016). It is in these populations that trust is higher in challenging habitats—and this is mediated by increased tolerance for uncertainty. A possible explanation for this extrapolates tendencies seen in individuals to the group level so that under stress, the S carriers are more likely to view the situation as negative and threatening, and tend towards risk minimising, avoidance behaviours. The L carriers are more likely to view demands more flexibly, depending on other available resources and so in the 'high wealth' conditions, risks are reappraised as positive challenges, facilitating trust and co-operation.

Recall that the S carriers had some superior cognitive skills such as better decision making—but although they are better at financial decisions in economic games in general, they are more risk averse, and it may be that the S carriers are less likely to reappraise risks as challenges or what some life coaches like to rephrase as 'opportunities'.

Global population differences in the S and L allele frequencies are well documented (Minkov et al. 2015). The S frequencies are consistently higher in the East Asian populations than in the N European (70–80% S carriers vs. 40–45%). This raises a number of questions: have population differences in the S and L allele frequencies come about by neutral processes or selection? Is this an example of different solutions to the same problem (stress)—or different solutions to different problems (different kinds of stress, e.g., ecological vs. social)? Collectivism in Asia is also associated with the high S-allele frequency and there is some evidence that the increased social support in collectivist cultures might buffer against the increased risk of stress vulnerability and depression (Way and Lieberman 2010). The Asian collectivist cultures appear to have lower prevalence of mental health issues, especially depression despite having higher frequencies of an allele known to be a risk factor for depression (for more on this controversial topic, see Chiao and Blinsinksy 2010; Hofmann et al. 2010; Hofmann and Hinton 2014; Juhasz et al. 2012). It may be that the social support aspect of collectivist cultures buffers against this risk—and has other cultural consequences such as influencing the interplay between social trust, ecological stress and economics. For the S carriers, it may be less about trust in general than trust radius—*who* do you trust?

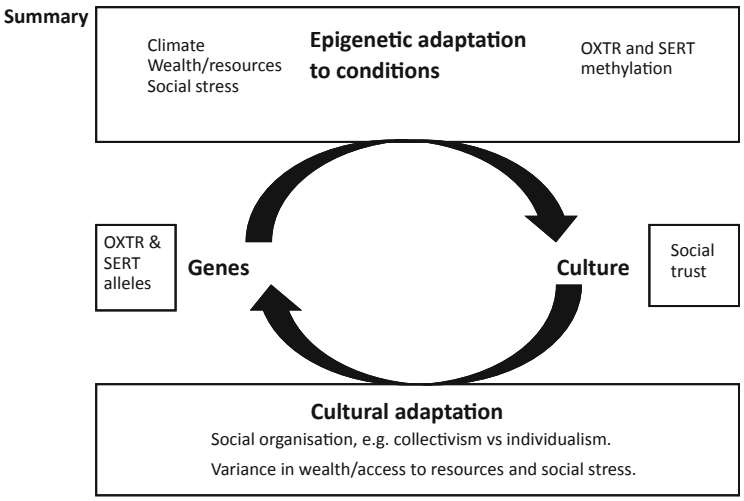

Fig. 8.2 Epigenetic regulation of the SERT and OXTR genes as an example of epigenetics facilitating gene–culture coevolution. Differential regulation of gene alleles, such as the S and L SERT alleles, enables selection to favour one allele relative to the other, resulting in changing population frequencies of the alleles

OXTR allele frequencies also vary East to West in a similar way, i.e., with socially sensitive alleles becoming more common from West to East (Kim et al. 2010; Luo and Han 2014), further supporting a gene–culture coevolution model involving social trust (Fig. 8.2). An intervention in the US providing social support to at risk families has been found to be more effective with the S carriers who derive more benefit from social support—and in this case there is also less OXTR methylation (Beach et al. 2018). So there appears to be a network of genes that are epigenetically sensitive to stress and possibly social stress in particular; as many cultural systems are predicated on ways of managing risk and trust under different social and environmental conditions, it follows that these epigenetically regulated systems may provide the link between broad human gene–culture coevolution and the fine tuning necessary for groups to track local circumstances and their changes.

8.6 Directions for Future Research

The potential influence of gene–culture coevolution on mental processes is well recognised (Lumsden et al. 1981). With the discovery of epigenetic processes, researchers in behavioural genetics have advocated incorporating epigenetics into models of, for example, the evolution of complex human social behaviours such as altruism and mate choice (Rushton et al. 1986). In their discussion, Rushton et al. proposed that 'epigenetic rules bias individuals to preferentially use culture traits in accord with their particular genotype to shape their social development'. Since these

processes were first proposed, research establishing the epigenetic regulation of behaviour in response to social triggers has been well documented (Rozanov 2012).

A next step might be research into the missing link that first predisposes a population to be biased toward one type of behaviour over another—the very first steps toward one culture differentiating itself from another. Can local ecological conditions influence population shifts in behaviour, rapidly and directly via triggers such as food resources and climate? In time, ecological conditions are bound to influence behaviour—but is it possible some adaptive behaviours can be favoured, if not within the lifetime of an individual, then certainly within one generation, without necessarily depending on the gradual transmission of cultural trends over many generations?

One approach to answering this question is a test of support for the first principle, i.e., that changes in ecological stress, could *in principle* select for gene–culture coevolution of, for example, social trust, i.e., is there any support for this process in analysis of the relevant data. An appropriate anthropological and ecological dataset could be assembled from existing sources. Choice of locations and time periods might be driven by the quality of the least available but most informative data. For example, data are relatively abundant for the period associated with the Neolithic shift to agriculture (as opposed to earlier in prehistory), and this period includes a time of relatively intense, fluctuating ecological stress. Ancient DNA studies are expanding to include ancient epigenomics (Llamas et al. 2012; Pedersen et al. 2014; Zhenilo et al. 2016). There are also several complete human genome studies from this era that could provide data on 'socially sensitive' genes in these populations (Der Sarkissian et al. 2015; Skoglund and Mathieson 2018).

A second approach might focus on whether epigenetically mediated gene–culture coevolution is currently an influence in shaping modern human culture, for example, as reflected in levels of social trust. A targeted study with specified and stratified population samples could investigate the relationship between social traits, such as trust, and the epigenetic status of, for example, 'socially sensitive' alleles. The feasibility of such a study is demonstrated by Beach et al. (2018), where a psychosocial intervention was associated with altered epigenetic status of two such genes: SERT and OXTR. There is already informative relevant research in this area such as evidence for differential gene–environment interactions associated with the same allele in different populations (Comings and MacMurray 2014; Kitayama et al. 2015). An understanding of these processes can inform social policy and interventions for vulnerable individuals and groups.

In the modern context especially, there are a number of limitations and constraints to consider. Compared to their earlier evolutionary environment modern humans now live in a much more complicated environment with regard to environmental triggers generally, including nutrition. Although whole cultures can be differentiated by diet, within-culture differences are very large. In modern cultures, there are two processes to consider. One is the use of existing epigenetic processes which are being triggered by dietary items, some of which may be novel to the human system. The current adaptiveness of any consequent behavioural changes is likely to be questionable. In order for the processes themselves to adapt, there must be changes

in the regulatory sequences governing the epigenetic response—which depends on the same selective pressure of reproductive fitness that drives evolution generally. It is unclear, at present, how the rate of evolution of epigenetic processes themselves compares to the evolution of DNA sequences associated with genes: in the example of epigenetic evolution in bacteria, above, the rate appeared to be faster. Furthermore, the ability to respond in this way differs dramatically across modern human populations depending on whether and how reproductive fitness is (or is not) controlled within cultures. Epigenetics is also likely to play a role in adaptation to future climate change (McGuigan et al. 2021).

8.7 Summary

Applying evolutionary principles to the study of psychology has highlighted the universal nature of human cognition and behaviour. Gene–culture coevolution then opened a dialogue between social and biological anthropologists in explaining persistent differences in culture without detracting from that universal ideal. Epigenetic–cultural coevolution can go further and provide the very earliest biases in behaviours that go on to become cultural traits. It explains how these processes could come about rapidly and reversibly even in the face of entirely novel triggers. It should be possible to find the signature traces of these processes by combining cross-cultural studies with behavioural ecology and the epigenetic response to triggers such as stress and changes in diet.

The suggestion that epigenetic regulation of socially sensitive genes such as SERT and OXTR can influence group level behaviour raises the possibility of epigenetically mediated gene–culture coevolution whereby ecological stress and/or social stress changes the trust radius of the group depending on allele frequencies, which then impacts cultural traits such as social trust. However, context, culture and gender are all influencing factors, and the simple categories of ‘collectivist’ and ‘individualist’ are unlikely to be sufficient to account for the global population frequencies of socially sensitive alleles. The initial association thus serves as a ‘smoking gun’ for further research, alongside related findings. Indeed, it is likely that the detection of the influence of single alleles is made possible because the phenotypic measure actually reflects selection at multiple related loci, and identifying this epigenetically regulated network will be the ultimate goal of research in this area.

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