ORIGINAL PAPER

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Induction of reproductive diapause via perception of photoperiod through the compound eyes in the adult blow fly, *Protophormia terraenovae*

Abstract Covering and surgical removal of the compound eyes were performed to localize photoreceptors for photoperiodic responses in the blow fly *Protophormia terraenovae* (Diptera, Calliphoridae). Adult females showed a long-day photoperiodic response to control reproductive diapause. When the compound eyes were bilaterally covered with silver paint, diapause incidence increased under diapause-preventing conditions, i.e., a long-day photoperiod and constant light, as though flies were kept under constant darkness. Neither silver painting on a medial region of the head capsule nor control painting in which both compound eyes were painted in a clear solvent caused significant effects on diapause incidence. Unilateral painting of the compound eye caused an increment of diapause incidence under constant light but no effects under a long-day photoperiod. When the compound eyes were bilaterally removed, all the flies developed their ovaries both under a long-day and a short-day photoperiod. Unilateral removal of the compound eye also caused ovarian development under a short-day photoperiod, whereas removal of one antennal lobe or all ocelli caused no effects on diapause incidence. Since *P. terraenovae* completely lost responsiveness to photoperiod after blinding of the compound eyes, it is likely that this fly perceives photoperiod through its compound eyes.

Key words Photoperiod · Diapause · Photoreceptor · Compound eye · *Protophormia terraenovae*

Introduction

Many researchers have studied the photoreceptor for the entrainment of circadian rhythms and for photoperiodic responses (Saunders 1982; Page 1985). According to the anatomical location of the photoreceptor for the entrainment of circadian rhythms, insects have been classified into two groups: in one group, including cockroaches and crickets, the compound eyes are the exclusive photoreceptor (Roberts 1965; Nishiitsutsuji-Uwo and Pittendrigh 1968; Sokolove and Loher 1975; Tomioka and Chiba 1984), and in the other group, including moths and flies, the photoreceptor is extraretinal (Engelmann and Honegger 1966; Truman 1974; Helfrich and Engelmann 1983; Helfrich et al. 1985; Helfrich 1986; Dushay et al. 1989; Cymborowski et al. 1994). In studies of photoperiodic responses, an idea that has been predominantly accepted is that insects use extraretinal pathways for the perception of photoperiod (Saunders 1982; Page 1985). Early studies involving brain transplantation showed that the photoperiodic signal was received by the brain (Williams and Adkisson 1964; Lees 1964; Claret 1966; Williams 1969; Kono 1973; Seugé and Veith 1976; Steel and Lees 1977). Furthermore, Bowen et al. (1984) and Hasegawa and Shimizu (1987) unequivocally demonstrated perception of photoperiod by the brain or brain-subesophageal ganglion complex in vitro. However, evidence has gradually accumulated that the compound eyes are also used as a photoperiodic receptor in the adult stage (Ferenz 1975; Numata and Hidaka 1983; Shiga and Numata 1996). Consequently, insects which are sensitive to photoperiod in the adult stage could also fall into two groups: *Pterostichus nigrita* (Ferenz 1975), *Riptortus clavatus* (Numata and Hidaka 1983) and *Dianemobius* (= *Pteronemobius*) *nigrofasciatus* (Shiga and Numata 1996) use the compound eyes, and *Leptinotarsa decemlineata* (de Wilde et al. 1959), *Megoura viciae* (Lees 1964), *Anacridium aegyptium* (Geldiay 1969) and *Calliphora vicina* (Saunders and Cymborowski 1996) use extraretinal receptors for the photoperiodic response.

In view of the photoperiodic perception in flies, the compound eyes seem to be dispensable both for the entrainment of circadian rhythms and for photoperiodic responses. In several eyeless mutants of *Drosophila*

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melanogaster (Engelmann and Honegger 1966; Helfrich and Engelmann 1983; Helfrich 1986; Dushay et al. 1989) and flies in which compound eyes or optic lobes were surgically removed [*Musca domestica* (Helfrich et al. 1985) and *C. vicina* (Cymborowski et al. 1994)], it has been shown that the circadian locomotor rhythms can be entrained by light-dark cycles. In addition, Saunders and Cymborowski (1996) showed that adults of *C. vicina* in which the optic lobes were removed to disrupt the nervous connections between the compound eyes and the brain respond to photoperiod in the control of larval diapause in the next generation.

The blow fly *Protophormia terraenovae* shows a photoperiodic response for the induction of reproductive diapause: adult females become reproductive under long-day conditions, whereas they enter diapause under short-day conditions (Numata and Shiga 1995). Recently, Numata and Shiga (1996) found that a white-eye mutant of this fly, which has been isolated in our laboratory, cannot discriminate photoperiod when the light intensity of the photophase is 500 lx. However, the mutant flies respond to photoperiod at a low light intensity of 0.5 lx. Deficiency of screening pigments in the compound eyes might result in the loss of the photoperiodic response at 500 lx in *P. terraenovae* (Numata and Shiga 1996). In contrast to the flies referred in the above sections, the results using white-eye mutants suggested the involvement of the compound eyes in the photoperiodic response of *P. terraenovae*. In the present study we examined the role of the compound eyes in the photoperiodic response in *P. terraenovae* by painting or removal of the compound eyes.

Materials and methods

Flies *Protophormia terraenovae* were obtained from laboratory cultures originally collected in Obihiro, Hokkaido, Japan (42°56′ N, 143°12′E). For maintenance of stock cultures we followed Numata and Shiga (1995). Female adults of *P. terraenovae* have a reproductive diapause which is controlled by a long-day photoperiodic response at moderate temperatures (Numata and Shiga 1995). All experiments were performed at 25 ± 1 °C. Larvae were raised on beef liver under a long-day photoperiod of 18 h light and 6 h darkness (LD 18:6). We used timers (Omron, Kyoto) and white

Fig. 1a–e Schematic drawings of the optic lobes and mid-brain of *Protophormia terraenovae* (a frontal section). *Closed areas* show regions which were surgically removed. For surgical operation, both compound eyes (**a**), one compound eye (**b**), three ocelli (**d**), or one antennal lobe (**e**) were removed. The operation of the compound eye (**a**, **b**) also removed the outer region of the optic lobe including the lamina (see Fig. 5). For sham operation, the frontal margin of the compound eye was incised bilaterally (broken line in **c**). *Hatched regions* indicate the mid-brain, optic lobes and retina
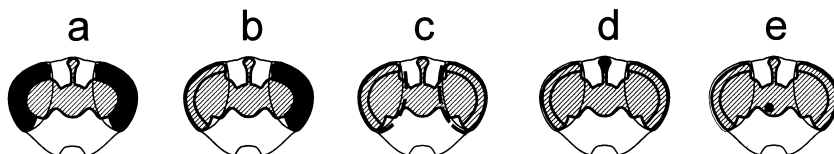
fluorescent lamps (NEC, Tokyo) to produce photoperiodic conditions. Light intensity outside of the rearing pots was about 500 lx. Adult females collected on the day of emergence (day 0) were individually placed in glass vials (3 cm diameter × 12 cm) and kept under LD 18:6 (a long-day photoperiod), LD 12:12 (a short-day photoperiod), LL (constant light) or DD (constant darkness). We used two kinds of light intensity, 500 and 0.5 lx, in the photophase. For 0.5 lx of light, the fluorescent lamp was covered with sheets of black polyethylene. The adult females were fed with granulated sucrose and water for the first 10 days, and 3% sucrose and a piece of beef liver (ca. 0.5 g) for the last 3 days. Painting or surgical operation was carried out on day 0 and the flies were dissected on day 13 to examine the ovarian development. The adults without yolk deposition in the oocytes were judged to be in diapause (Vinogradova 1987).

Before painting or operation, the flies were anesthetized with diethyl ether for a few minutes and mounted in a sealing compound (Apiezon, London) with the anterior face of the head exposed under a stereoscopic microscope. We made three painted groups, both compound eyes, one compound eye and the medial part of the head capsule, and one control group for the covering experiment. The medial part of the head included three ocelli and the dorso-posterior part of the cuticle but excluded the antennae, proboscis and compound eyes. We used a pasted silver (Nilaco Corp., Tokyo) as a paint for shielding against light. We designated flies in which both compound eyes were painted in a clear solvent of the paint as a control group in the covering experiment. We checked whether the paint remained on day 13 and flies which lost it were discarded. The adult flies in the covering experiment were reared under LD 18:6 or LL with the light intensity of 0.5 lx.

For surgical operation, we carried out five kinds of surgery, bilateral (Fig. 1a) or unilateral removal of the compound eyes (Fig. 1b), sham operation (Fig. 1c), removal of all ocelli (Fig. 1d) and unilateral removal of the antennal lobe (Fig. 1e). For removal of the compound eyes, the eyes were externally cut off with a fine scalpel and the opening was sealed with a dental paraffin wax (melting point 59.3 °C; GC Corp., Tokyo), or after cutting off the eyes the inside of the eye capsule was scraped out and only the capsule was placed back and sealed with the wax. As a sham operation, a frontal margin of the compound eye was incised bilaterally. For ocelli removal, all three ocelli were scraped off with the scalpel and the opening was sealed with the wax. For the antennal lobectomy a pool of saline (0.9% NaCl) was made to surround the whole head. Incisions were made in the anterior face of the head to expose the antennal lobes. After either antennal lobe was removed with a sharpened tungsten wire (500 μm in diameter), the cuticular opening was closed back.
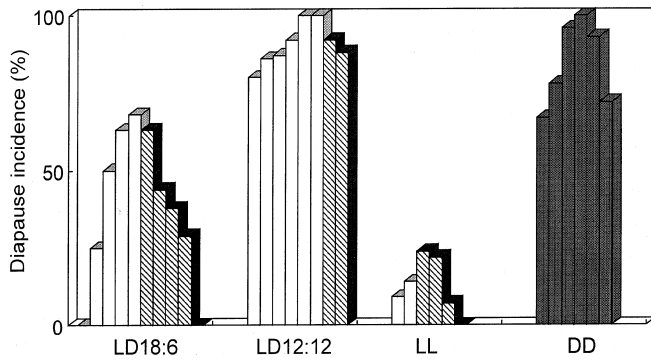
Some of the surgically operated females were sacrificed for histological examination. The head was fixed in Bodian second fixative for 2 days and embedded in Paraplast (Sherwood Med., St. Louis) to make serial sections (10 μm thickness). The sections were stained by a reduced-silver impregnation (Otsuka 1962).
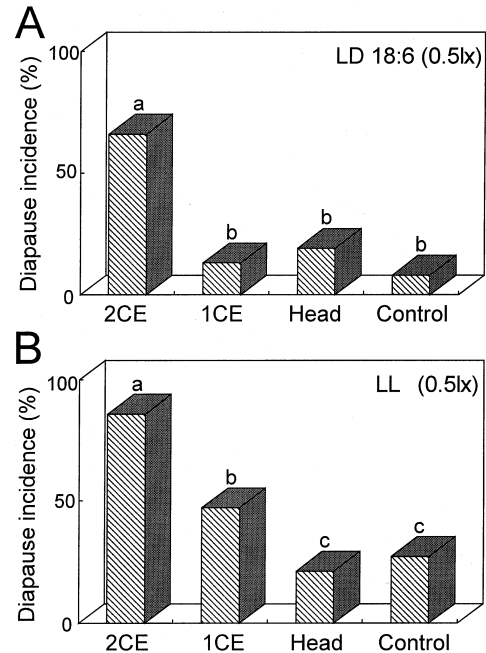
Results

Figure 2 shows diapause incidence of all the replicates in intact flies, because differences in the incidence was rather large among trials. Intact flies showed a weak photoperiodic response between LD 18:6 and 12:12. By using 500 lx of light, diapause incidence ranged from 0

a   b   c   d   e

Fig. 2 Diapause incidence in the intact flies, *Protophormia terraenovae*, reared under LD 18:6 (a long-day photoperiod), LD 12:12 (a short-day photoperiod), LL (constant light) or DD (constant darkness). Two kinds of the light intensity, 500 lx (*open column*) and 0.5 lx (*hatched column*), were used in the photophase. Two to six trials were carried out in each condition. Number of individuals in each column is 8–37

Fig. 3A, B Diapause incidence in the covering experiment of the fly, *Protophormia terraenovae*, reared under a long-day photoperiod, LD 18:6 (**A**) or constant light, LL (**B**) with light intensity of 0.5 lx. Both compound eyes (*2CE*), one compound eye (*1CE*) or a medial region of the head capsule (*Head*) were painted with a silver paste on the day of emergence. As a control, both compound eyes were painted in a clear solvent of the paint (*Control*). Number of individuals in each column is 26–64. Incidences of diapause marked by the same letter above columns are not significantly different in each rearing condition [$P > 0.05$, Tukey type multiple comparison test (Zar 1984, pp 401–402)]

to 68% under LD 18:6, and from 80 to 100% under LD 12:12. Even a typical long-day photoperiod of LD 18:6 did not always prevent diapause. Under constant conditions, however, difference in diapause incidence between LL and DD was distinctive (Fig. 2): the former prevented and the latter induced diapause. When we used the light intensity in the photophase at 0.5 lx and examined the responsiveness of the flies to photoperiod, diapause incidence ranged from 0 to 63% under LD 18:6, from 88 to 92% under LD 12:12 and from 0 to 24% under LL (Fig. 2). Thus, the flies discriminated photoperiod even when the light intensity in the photophase was lowered to 0.5 lx. Therefore, we used 0.5 lx of light in the covering experiments to prevent light from coming through the paint.

In Fig. 3, the results are given of covering experiments under diapause-preventing conditions of LD 18:6 and LL by using 0.5 lx of light. When the intact and control groups were examined simultaneously, the diapause incidences of the former and the latter were 0 and 8% under LD 18:6, and 22 and 27% under LL, respectively (Figs. 2, 3). The effect of painting in the clear solvent was not significant under both conditions ($\chi^2 = 0.654$, $df = 1$, $P > 0.05$ under LD 18:6 and $\chi^2 = 0.072$, $df = 1$, $P > 0.05$ under LL). Only when the compound eyes were bilaterally painted in silver, diapause incidence increased as though they were under DD (Fig. 2). The incidence was 66 and 86% under LD 18:6 and LL, respectively. However, covering of the medial region of the head was not effective under both conditions. When the compound eye was unilaterally painted, diapause incidence was 13 and 47% under LD 18:6 and LL, respectively. The unilateral painting of the compound eye was not effective under LD 18:6 (Fig. 3A). Under LL, however, the incidence of diapause was significantly lower than that of the flies of which both compound eyes were painted and significantly higher than that of the control group (Fig. 3B).

When the compound eyes were bilaterally removed, all the flies developed their ovaries both under LD 18:6 ($n = 10$) and under LD 12:12 ($n = 19$). Figure 4 shows the effects of different kinds of surgical operation on diapause incidence under LD 12:12 with the light intensity of 500 lx. After removal of either compound eye, only 1 out of 19 females had undeveloped ovaries. In contrast with the bilateral removal of the compound eyes, diapause incidences were high both in the sham operation (94%) and intact group (92% in Fig. 2). Removal of the ocelli or antennal lobe caused no effects: the diapause incidence was 88 and 86% in the ocelli-removal and in the antennal lobe-removal, respectively.

Figure 5 shows examples of histological examination of the operated brain. In the operation of the compound eye-removal, not only the retina but also the lamina were completely removed and the medulla and lobula complex remained. In some individuals, vacuolized areas were observed in the medulla but not in the mid-brain (Fig. 5B, C). Vacuolized areas were more often seen in the bilateral operation than in the unilateral one.

Discussion

When both compound eyes were covered or removed, adult females lost the sensitivity to photoperiod com-
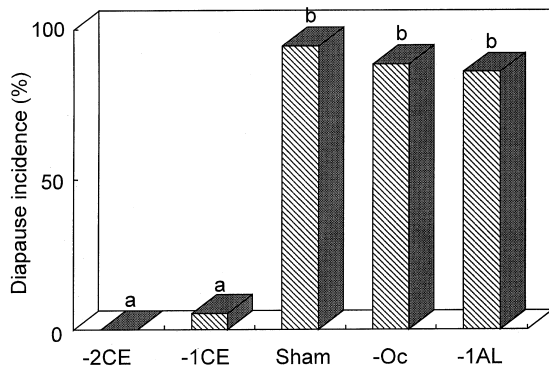
Fig. 4 Diapause incidence in the surgical operation of the fly, *Protophormia terraenovae*, reared under LD 12:12 with light intensity of 500 lx. *–2CE*: bilateral removal of the compound eyes; *–1CE*: unilateral removal of the compound eye; *Sham*: sham operation in which a frontal margin of the compound eye was incised bilaterally; *–Oc*: removal of the three ocelli; *–1AL*: unilateral removal of the antennal lobe (see Fig. 1). Number of individuals in each column is 17–21. Incidences of diapause marked by the same letter above columns are not significantly different in each rearing condition [$P > 0.05$, Tukey type multiple comparison test (Zar 1984, pp 401–402)]

pletely. These results indicate that *P. terraenovae* perceives photoperiod through the compound eyes. However, covering and surgical removal of the compound eyes gave a complete contrast in the reproductive status: the former induced diapause under diapause-preventing conditions as if flies were kept under DD, whereas the latter induced ovarian development irrespective of the photoperiod.

To interpret the results of surgical experiments, we have to take account of the effect of injury itself, because surgical injury sometimes alters endocrine activities (e.g., Maleville and de Reggi 1981; Rankin and Stay 1983). However, the following two reasons do not support the proposal that the surgical injury activated the endocrine system resulting in ovarian development apart from the reception of photoperiod:
1. There was no histological damage in the mid-brain where neurons involved in the control of ovarian development possibly reside. In *P. terraenovae*, inactivity of the corpus allatum (CA) secreting juvenile hormone (JH) causes adult diapause, and the brain inhibits the endocrine activity of the CA through the nervous pathways in diapause adults (Matsuo et al. 1997). In *Calliphora vicina* (Diptera, Calliphoridae) neurons which send the axons to the retrocerebral complex including the CA have been investigated anatomically in the brain and their cell bodies are mainly found in the pars intercerebralis and in the pars lateralis (Duve et al. 1983). In *Phormia regina* (Diptera, Calliphoridae) Hsiao and Fraenkel (1966) reported that the nucleus and perikaryon of the neurosecretory cells in the pars intercerebralis significantly increase in size after feeding with proteinaeous meal that is necessary for egg development (Orr 1964). According to these observations, cell bodies and branches of the neurons involved in the ovarian development in *P. terraenovae* must be located in the mid-brain. From our histological observation we do not

consider that surgical removal of the compound eyes directly affected neurons in the mid-brain. If surgery on the compound eye caused ovarian development due to some histological injury in the mid-brain, the removal of the antennal lobe, which locates within the mid-brain, could also cause same effects; however, this was not the case.
2. Our feeding protocol avoids the ovarian development directly caused by some temporal change in the endocrine system associated with the operation. Flies in this study were fed on only sugar for the first 10 days and liver was given on the 11th day. Because proteinaeous meal is prerequisite for the ovarian development in *P. terraenovae* (Harlow 1956) as in *P. regina* (Orr 1964), flies could not develop the ovaries for the first 10 days. Even if JH titer might increase temporally after the operation, this JH increment could not succeed in developing the ovary without proteinaeous meal.

Therefore, it is more probable that the compound eyes act as photoperiodic receptors and their removal resulted in the loss of sensitivity to photoperiod. To explain the different results between covering and surgical removal of the compound eyes, we propose that absence of the photoperiodic receptor might be physiologically different from perception of constant darkness through the photoperiodic receptor.

Involvement of the circadian system in photoperiodic responses has been demonstrated in many insect species (Beck 1980; Saunders 1982; Pittendrigh et al. 1984). In flies, abundance of evidences have suggested that the photoreceptors used in the transduction of photoperiodic information for the entrainment of the circadian pacemakers exist in the brain by using several eyeless mutants of *Drosophila melanogaster* (Engelmann and Honegger 1966; Helfrich and Engelmann 1983; Helfrich 1986; Dushay et al. 1989) and flies of which compound eyes or optic lobes were surgically removed in *Musca domestica* (Helfrich et al. 1985) and *C. vicina* (Cymborowski et al. 1994). Recently, extraretinal reception of photoperiod was also demonstrated in photoperiodic responses by Saunders and Cymborowski (1996) who showed that the adults of *C. vicina* in which optic lobes were removed retained the ability to discriminate photoperiod to control larval diapause in the progeny. These results in flies show that photoperiodic receptors exist in extraretinal regions but do not mean that the compound eyes have no relation to photoperiodic perception. We cannot exclude the possibility that the flies in the above studies use the extraretinal receptor and also the compound eyes. In *D. melanogaster* the period protein (PER) is an essential component of the circadian clock (Hall 1995). Cell bodies of PER-containing neurons lie mainly in two clusters: one is in the posterior dorsal cortex of the mid-brain and the other is in the base of the optic lobe (Siwicki et al. 1988; Zerr et al. 1990). Helfrich-Förster (1995) revealed the entire arborization pattern of the latter neurons in the optic lobe. These neurons send their fibers largely in the medulla and in the mid-brain. If they actually have a clock function, it seems appropriate for the PER-containing neurons in the optic lobe to use
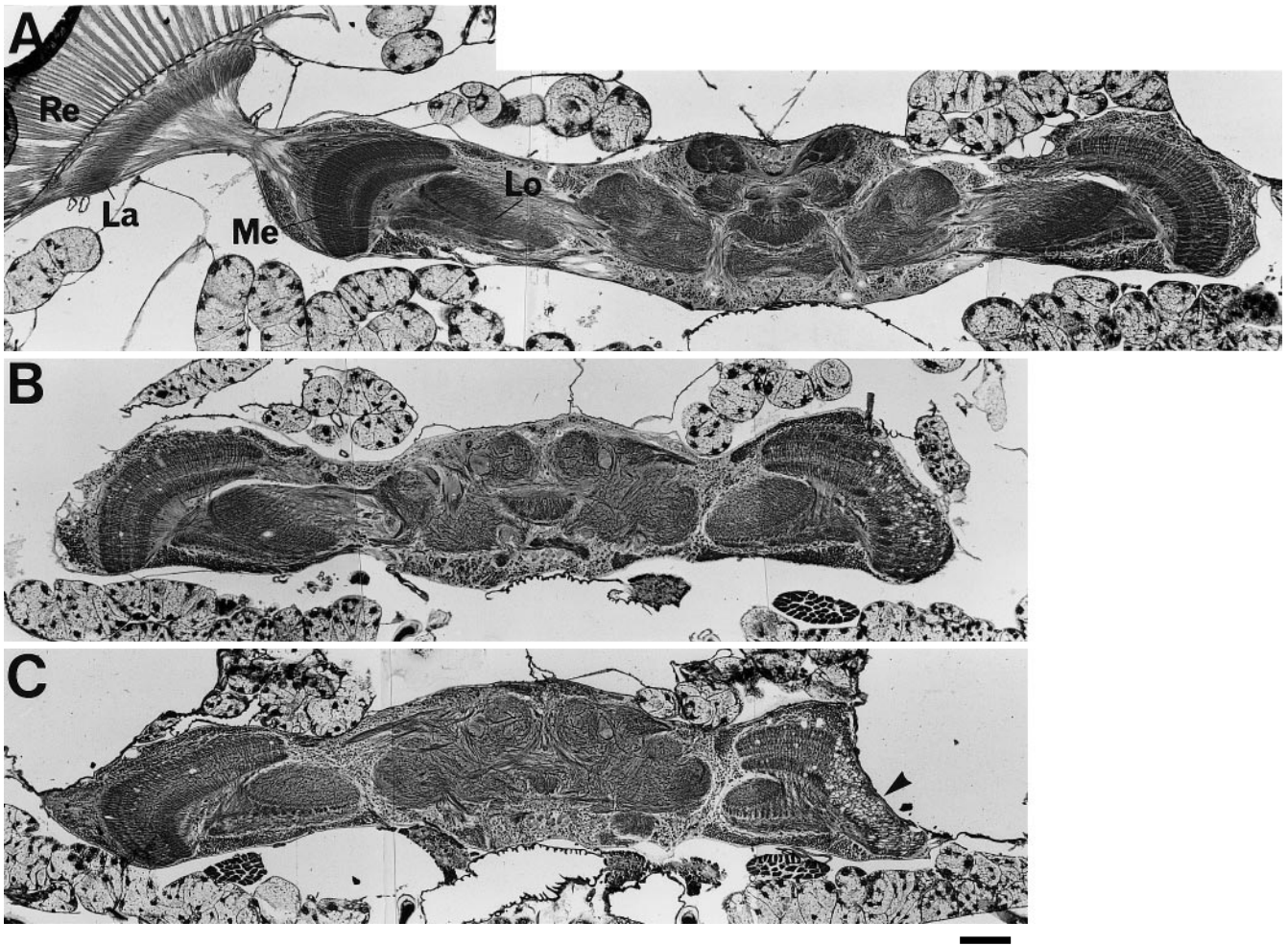
Fig. 5A–C Histological sections (10 μm) of the optic lobe and mid-brain after removal of the compound eye in *Protophormia terraenovae* (horizontal view, upper to the anterior): **A** unilateral removal of the compound eye (*right side*). The retina (*Re*) and lamina (*La*) were completely removed and the medulla (*Me*), lobula (*Lo*) and mid-brain remained intact; **B** and **C** bilateral removal of the compound eyes. **C** shows one example with the severest damage in the optic lobe. Although sometimes the medulla was partly disrupted (*arrowhead*), the lobula and mid-brain were never damaged. Scale bar 100 μm

the compound eyes in addition to the extraretinal receptor for photoperiodic perception. As *P. terraenovae* cannot perceive photoperiod when the compound eyes are covered or removed, this species uses only the compound eyes for photoperiodic perception and the extraretinal receptor in the brain might have lost sensitivity to photoperiod.

Unilateral removal of the compound eye also caused deficiency of photoperiodic perception in *P. terraenovae*. In an experiment in which the eyes are covered the same effect was observed in some individuals under constant light. After the compound eye is unilaterally covered or removed, the intact eye perceives a given photoperiod, whereas the contralateral eye perceives constant darkness because it is covered, or nothing because it is removed.

When photoperiodic information from both compound eyes is integrated, the integrated information would not necessarily reflect only the information perceived through the intact compound eye. In the band-legged ground cricket, *Dianemobius nigrofasciatus*, unilateral removal of the compound eye also caused similar effects on the photoperiodic responses (Shiga and Numata 1996).

In view of the photoreceptor in adults for photoperiodic responses, *P. terraenovae* joins an insect group including a carabid beetle, *Pterostichus nigrita* (Ferenz 1975), the bean bug, *Riptortus clavatus* (Numata and Hidaka 1983) and *D. nigrofasciatus*, (Shiga and Numata 1996), in which the compound eyes are used as a photoperiodic receptor. The present results on photoperiodic perception by the compound eyes in *P. terraenovae* clearly contrasts with previous results in flies (Engelmann and Honegger 1966; Helfrich and Engelmann 1983; Helfrich et al. 1985; Helfrich 1986; Dushay et al. 1989; Cymborowski et al. 1994; Saunders and Cymborowski 1996). The concept of a photoperiodic receptor for the entrainment of the circadian clock in *P. terraenovae* is of considerable interest and is presently under investigation.

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