

Different photoreceptor organs are used for photoperiodism in the larval and adult stages of the carabid beetle, *Leptocarabus kumagaii*

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SUMMARY

The role of the two distinct retinal photoreceptor organs in photoreception for photoperiodism was examined in the carabid beetle, *Leptocarabus kumagaii*, by surgical removal. This beetle shows long-day and short-day photoperiodic responses in the larval and adult stages, respectively. Larval diapause in the final instar is induced under short-day conditions whereas pupation occurs without diapause under long-day conditions. Adult reproductive diapause is terminated under short-day conditions but maintained under long-day conditions. The stemmata of the larvae and compound eyes of the adults were removed and the responses of the animals to photoperiod were compared to those of intact beetles. When all the stemmata were removed, larvae pupated without entering diapause under both long-day and short-day conditions, indicating that the larvae lacking stemmata were incapable of photoreception for photoperiodism. As in other holometabolous insects, the stemmata migrated into the brain during metamorphosis and remained rudimentarily in the optic lobe of the adult brain. However, these stemmata-derived organs were found to be no longer necessary for photoperiodism, because adults lacking the stemmata-derived organs responded to photoperiod normally. By contrast, removal of the compound eyes in adults resulted in the termination of reproductive diapause under both long-day and short-day conditions, indicating that photoreception for photoperiodism in the adult stage is performed by the compound eyes. Therefore, the site of photoperiodic photoreception in *L. kumagaii* appear to change from the stemmata to the compound eyes during metamorphosis.

Key words: photoperiodism, retinal photoreceptor, stemma, compound eye, *Leptocarabus kumagaii*, Holometabola.

INTRODUCTION

Like many other organisms, insects exhibit photoperiodism to synchronize their development with seasonally changing environments (Beck, 1980; Tauber et al., 1986; Danks, 1987; Saunders, 2002). To study the physiological mechanisms underlying photoperiodism, many researchers have begun with the localization of photoperiodic photoreceptors. Photoperiodic information perceived by the receptors is processed in the integrative system in the brain, involving photoperiodic clocks, and an output signal from the photoperiodic clocks probably controls a hormonal release necessary for seasonally appropriate phenotypes, such as diapause (Saunders, 2002). Identification of the photoperiodic receptor, therefore, is important in studying neural mechanisms controlling photoperiodism. Numata et al. (Numata et al., 1997) reviewed photoreceptors in insects, and suggested that compound eyes, extraretinal photoreceptors in the brain, or both types of photoreceptors are operative, depending on the species.

In insects, sensitivity to photoperiod often occurs over more than one developmental stage, suggesting that photoreceptors and neural components for photoperiodic responses can exist both in immature stages and adults. For example, the Colorado beetle, *Leptinotarsa decemlineata* (De Wilde, 1969), and the mosquito, *Culex pipiens* (Spielman and Wong, 1973), are sensitive to photoperiod for determining adult diapause in both the larval and adult stages, and in both the pupal and adult stages, respectively. Other insects, such as the bug, *Poecilocoris lewisi*, are sensitive to photoperiod in the nymph and adult stages for determining development in the respective stages (Tanaka et al., 2002). However, the ontogeny of receptors or neural components for photoperiodic response has not

been examined. In several species, including Hemiptera, it has been demonstrated that compound eyes are used as the retinal photoperiodic photoreceptor in the adult stage (Numata et al., 1997). In the nymphal stage, the importance of compound eyes for photoperiodism was first demonstrated in *P. lewisi* (Miyawaki et al., 2003). Because adult compound eyes in Hemimetabola are derived from larval compound eyes, *P. lewisi* might use compound eyes as the photoperiodic photoreceptor in both stages.

In contrast to the Hemimetabola, in holometabolous insects, the retinal photoreceptors in the larval and adult stages have different origins; through metamorphosis the stemmata, i.e. the retinal photoreceptor of the larval stage migrates into the brain and remains rudimentary whereas the compound eyes develop from the eye disc in the larva (Ichikawa, 1991; Gilbert, 1994). The role of the stemmata in photoperiodism has been examined in many lepidopterans (Tanaka, 1950; Geispits, 1957; Claret, 1966; Kono, 1970; Hayes, 1971; Seugé and Veith, 1976; Shimizu, 1982; Shimizu and Hasegawa, 1988), but a subordinate role was shown only in the large white butterfly *Pieris brassicae* (Seugé and Veith, 1976). After metamorphosis, the stemmata remain as the stemmata-derived organs that contain visual pigments and remain photosensitive during the pupal or adult stages (Ichikawa, 1991; Briscoe and White, 2005). The role of the stemmata-derived organs in photoperiodism has been examined only in pupae of the oak silkworm *Antheraea pernyi*. Hayes (Hayes, 1971) concluded that stemmata-derived organs do not play a principal role in photoperiodism because pupae of *A. pernyi* responded to photoperiod normally after surgical removal of these organs. However, the role of stemmata and stemmata-derived organs in photoperiodism has not been examined for any single

species. To examine the ontogeny of photoperiodic photoreceptors, it is important to examine insects that have sensitivity to photoperiod in both the larval and adult stages.

The carabid beetle *Leptocarabus kumagaii* Kimura et Komiya, has photoperiodism in both the larval and adult stages (Sota, 1987). In the present study, the role of the stemmata in photoperiodism in the larval stage and the role of stemmata-derived organs and compound eyes in photoperiodism in the adult stage were examined in *L. kumagaii* by surgical removal of these photoreceptors. The ontogenic change in the role of these photoreceptors in photoperiodism is discussed.

MATERIALS AND METHODS

Insects

Leptocarabus kumagaii is an autumn-breeding carabid beetle distributed in the central part of Honshu Island, Japan. Adults of this species emerge in early summer, mostly in June, and enter summer diapause. By responding to short days in early autumn, the adults terminate diapause and commence reproduction. Larvae hatch and develop in this season, and by responding to short days in autumn they enter winter diapause. Consequently, this insect has a long-day photoperiodic response in the larval stage and a short-day photoperiodic response in the adult stage (Sota, 1985; Sota, 1987).

In the present study, adults were collected with pitfall traps in June and October, 2001 at the Botanical Gardens of Osaka City University, Katano City (34°46'N, 135°41'E). They were maintained individually in transparent plastic cups (9 cm diameter and 5 cm height) containing soil (2.5 cm depth), and for food were provided with a piece of beef liver and apple every 2 days. The adults collected in June were allowed to enter summer diapause and then subjected to surgical operation (removal or incision) of the compound eyes; those collected in October were reproductive and used for the production of progeny that were subjected to surgical operation of the stemmata.

White fluorescent lamps were used for the light source and the light intensity in the photophase was kept at 200–300 lx. In the present study, long-day (16 h:8 h light:dark photoperiod) and short-day (12 h:12 h light:dark photoperiod) conditions were used. Temperature fluctuations did not exceed $\pm 1^\circ\text{C}$. To obtaining progeny, reproductive adults were reared under the short-day conditions at 15°C , and the eggs laid in the soil were collected with a medicine spoon and kept on soil in Petri dishes at 20°C . Hatching larvae were individually placed in plastic cups containing soil and fed on larvae of the cabbage armyworm, *Mamestra brassicae*, under the long-day conditions at 20°C . About 10 days after the final (second) larval ecdysis, when larvae had ceased feeding, they were transferred to larger cups (12 cm diameter and 8 cm height) containing soil (5 cm depth), so that they could make chambers for pupation. Larval photoperiodism of this species becomes clear in the final (third) instar, and if diapause is induced the duration of the third instar is markedly prolonged (Sota, 1987).

Surgical operation of the stemmata

Larvae that had been reared under long-day conditions were subjected to surgical removal or incision (sham control) of the stemmata on day 2 of the second instar. Before surgery, larvae were anesthetized with CO_2 for about 30 s and mounted sideways on a sealing compound (Apiezon, London, UK) in a plastic case (4 cm \times 6 cm \times 1.7 cm). The plastic case had a hole in which the larvae were positioned for surgery. The operation was performed under a stereo microscope, during which CO_2 anesthetization was maintained. A larva has six stemmata in a small swelling on each

side of the head. For removal of the stemmata, the swelling with stemmata was bilaterally excised with a fine scalpel. The opening was not sealed, but closed in a few hours by coagulation of hemolymph. For the sham operation, the margin of the swelling was incised bilaterally. The operated and intact larvae were placed under long-day or short-day conditions. Nineteen per cent of the stemmata-removed and 13% of the sham-operated larvae died without resuming feeding. Including these larvae, those that died without pupation during the experimental period (100 days post operation) were excluded from the results. Through ecdysis to the third instar, the stemmata did not regenerate but the opening was covered with cuticle.

Preparation of adults lacking stemmata-derived organs

The larvae that had their stemmata removed on day 2 of the second instar pupated normally under long-day conditions, and as adults lacked the stemmata-derived organs in the caudal area of the optic lobe (Fig. 1). By comparing the photoperiodic responses of these adults with intact adults, it was possible to examine the role of the stemmata-derived organs in photoperiodism. The stemmata-removed and intact larvae were reared under long-day conditions at 20°C . After adult emergence, females were continuously kept under these conditions for 30 days, and then transferred to the long-day or short-day conditions at 15°C . They were dissected for examination of their ovarian status at 60 days post transfer.

Surgical operation of the compound eyes

The adults collected in June were reared under long-day conditions at 20°C for 60 days, and then subjected to the surgical removal or incision of the compound eyes. On the day of operation, 15 females

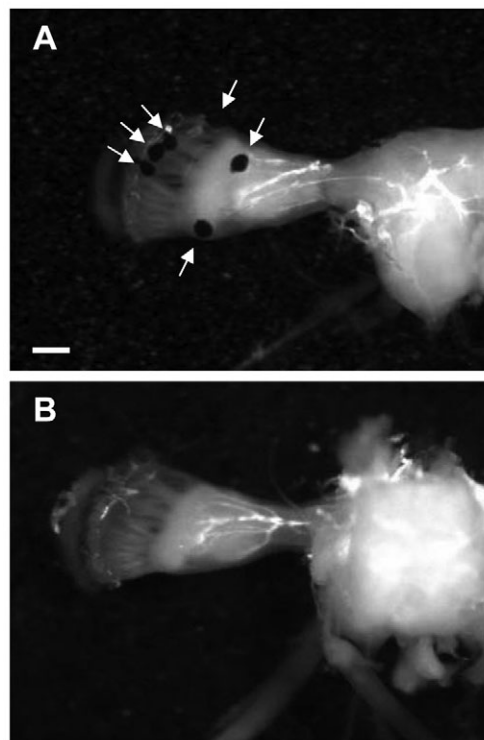


Fig. 1. The optic lobe and brain in adult *Leptocarabus kumagaii*. (A) Intact and (B) with the stemmata removed on day 2 as a second larval instar. Arrows indicate stemmata-derived organs. Scale bar, 100 μm .

were dissected for examination of ovarian status; there was no evidence of yolk deposition in the oocytes of any female, indicating that they were in diapause. Before the operation, females were fixed and mounted, following the procedure for removing the stemmata of larvae. For eye removal, the corneal lens of the compound eye was externally cut away with a scalpel, and then the retina was deliberately scraped out with sharpened forceps. For the sham operation, the marginal region of each corneal lens was incised bilaterally. The compound eye-removed, eye-incised and intact females were placed under long-day or short-day conditions at 15°C, and dissected 30 or 60 days later to examine ovarian status. Mortality in the operated insects occurred mostly within a few days of surgery and the beetles that died before dissection (5, 69 and 12% of intact, compound eye-removed and sham-operated adults, respectively) were excluded from analysis.

Histological examination of the adult brain

Some of the surgically operated females were killed and the brain examined histologically. The head was fixed with Bouin's fixative for 1 day and embedded in paraffin for the preparation of serial sections (10 µm thickness). Sections were stained with Mayer's acid-hemalaun and Eosin.

RESULTS

Effects of stemmata-removal on photoperiodism of larvae

Fig. 2 shows the development of intact and surgically operated larvae. Intact larvae showed a clear long-day photoperiodic response. Under long-day conditions, the duration of the third instar was 21.0 ± 2.0 days (mean \pm s.d.), and all of the larvae pupated. By

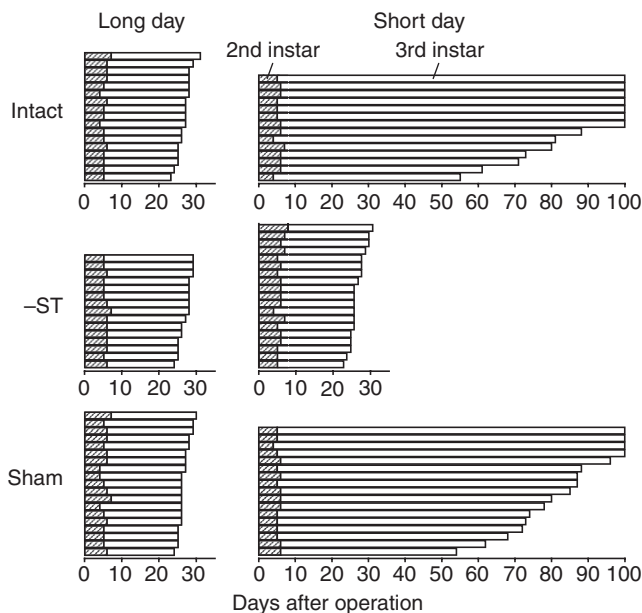


Fig. 2. Effect of stemmata removal on the photoperiodic response during larval development in *L. kumagaii*. Larvae that had been reared under long-day conditions (16h:8h L:D) were subjected to surgical removal or incision (sham operation) of stemmata on day 2 of the second instar and placed under short-day (12h:12h L:D) or long-day conditions at 20°C. Each bar indicates the development of an individual, with pupation occurring at the right end of the bars. -ST, stemmata-derived organs removed; Sham, sham operated. Seven intact and four sham-operated larvae under short-day conditions did not pupate within the experimental period. Hatched regions, second instar; white regions, third instar.

contrast, under short-day conditions the duration of the third instar was remarkably prolonged and no larvae pupated until day 51 after final larval ecdysis; even at the end of the experimental period seven of the 14 individuals were still larvae. These results indicate that diapause was induced in all larvae reared under short-day conditions. However, the stemmata-removed larvae, as for intact larvae reared under long-day conditions, pupated about 3 weeks after final larval ecdysis under both long-day conditions (21.3 ± 2.0 days) and short-day conditions (20.9 ± 1.8 days), and the difference in the third-instar duration between photoperiodic conditions was not significant (*t*-test, $P > 0.05$). As with the intact larvae, sham-operated larvae showed a clear response to photoperiod. No significant difference was observed in the duration of the second or third instar under long-day conditions among the three experimental groups (Kruskal-Wallis test, $P > 0.05$), suggesting that the injury at operation had no effect on larval development. These results indicate that *L. kumagaii* larvae become incapable of perceiving photoperiod after removal of the stemmata, and that the stemmata play an important role in photoperiodism in the larval stage.

Effects of absence of stemmata-derived organs on photoperiodism of adults

Photoperiodic response was compared between intact adults and adults in which the stemmata had been removed in the larval stage (Fig. 3). All intact female adults had mature eggs on day 60 under short-day conditions, whereas all females lacked mature ovaries under long-day conditions. Similarly, adults lacking stemmata-derived organs also showed a clear short-day photoperiodic response. These results indicate that adults of *L. kumagaii* can respond to photoperiod without stemmata-derived organs. It is thus concluded that stemmata-derived organs, which play an important role in photoperiodism as stemmata in the larval stage, were dispensable for photoperiodism in the adult stage.

Effects of compound-eye removal on photoperiodism of adults

Fig. 4 shows the ovarian development of intact and surgically operated females. The field-collected female adults show a similar short-day photoperiodic response to that of the laboratory-raised adults. Under short-day conditions most females had developed ovaries by day 30, whereas under long-day conditions all females had undeveloped ovaries after 60 days. The percentage of yolk

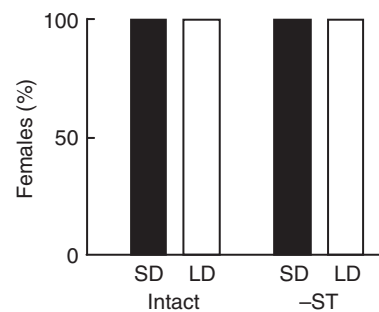


Fig. 3. Effect of the absence of stemmata-derived organs in the adult brain on the photoperiodic response of ovarian development in *L. kumagaii*. Thirty days after adult emergence, females were transferred from long-day conditions (16h:8h L:D) at 20°C to short-day (SD; 12h:12h L:D) or long-day (LD) conditions at 15°C. Insects were dissected 60 days after the transfer. -ST, stemmata-derived organs removed. White bars, no yolk deposition; black bars, mature eggs. $N=8-10$.

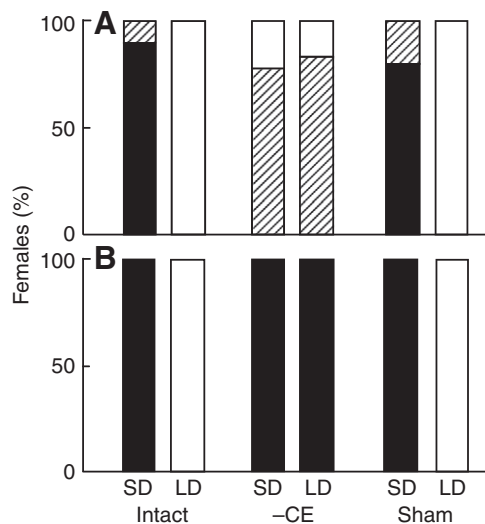


Fig. 4. Effect of compound-eye removal on the photoperiodic response of ovarian development in *L. kumagaii*. Field-collected female adults were reared for 60 days under long-day conditions (16h:8h L:D) at 20°C, then subjected to surgical operation and placed under short-day (12h:12h L:D) or long-day conditions at 15°C. Females were dissected at (A) 30 or (B) 60 days after the operation. -CE, compound eye removed; Sham, sham operated. White bars, no yolk deposition; hatched bars, yolk deposition without mature eggs; black bars, mature eggs. $N=7-11$.

deposition was significantly higher under short-day conditions than under the long-day conditions at days 30 and 60 in both intact and sham operated females (Fisher's exact probability test, $P<0.001$). In the compound-eye removed adults, however, the photoperiodic response disappeared under both short-day and long-day conditions. Although these females had developed ovaries, they were smaller than those of intact or sham-operated adults under short-day conditions. Because sham-operated adults responded to photoperiod normally, the responses of the compound eye-removed adults were not attributed to injury at surgical operation. In addition, histological observation of the brain showed that the retina is located a considerable distance from the lamina (Fig. 5A), and that surgical removal of the compound eyes was successful in removing the retina without injuring the lamina or other neuropiles (Fig. 5B). This observation ruled out the possibility that the disappearance of the photoperiodic response was due to injury of the central nervous system, in which neurons are involved in the control of photoperiodism. The surgical removal of the compound eyes neither destroyed the stemmata-derived organs nor deprived the optic lobe of the organs. Therefore, the disappearance of photoperiodic response in the compound eye-removed adults was due to the incapability of perception of photoperiod, and in *L. kumagaii* the compound eyes played an important role in photoreception for photoperiodism in the adult stage.

DISCUSSION

The role of the stemmata in photoreception for photoperiodism has been examined only in Lepidoptera (e.g. Geispits, 1957; Kono, 1970; Hayes, 1971; Seugé and Veith, 1976; Shimizu, 1982; Shimizu and Hasegawa, 1988), and demonstrated only in *Pieris brassicae*, in which the predominant role of the brain in photoperiodic photoreception is apparent and the stemmata play only a subordinate role in photoperiodism. After cauterization of the stemmata, most larvae were found to respond to a 16-h photophase of white light,

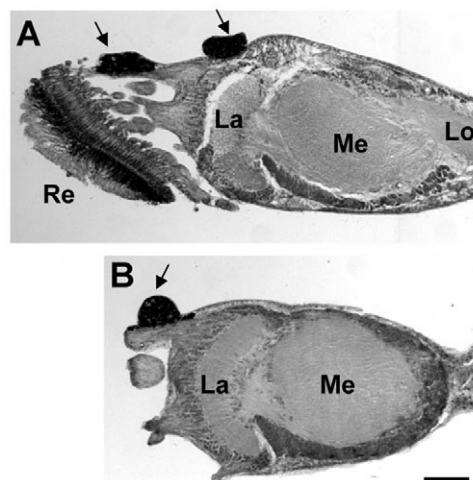


Fig. 5. Photomicrographs of the retina and optic lobe of adult *L. kumagaii* (10 μ m sections, horizontal views, frontal region to the bottom). (A) In the intact compound eye the retina (Re), lamina (La), medulla (Me), lobula (Lo) and stemmata-derived organs (arrows) can be seen. (B) After removal of the compound eye, the retina was completely ablated but the stemmata-derived organs, lamina, medulla and lobula (not in this section) remained. Scale bar, 100 μ m.

although some did not respond to a photophase of 9-h white light followed by 7h of blue, green or yellow light (Seugé and Veith, 1976). In the present study, the role of the stemmata in photoreception for photoperiodism in another holometabolous larva, *L. kumagaii*, a member of the Coleoptera, was first examined.

After adult emergence, the absence of stemmata-derived organs exerted no effect on photoperiodism, although this result does not exclude the possibility that stemmata-derived organs are involved in photoperiodism. Even if the removal of a photoreceptor had no effect on photoperiodism, there is still a possibility that two or more photoreceptors, including the removed organs, are operative and that after removal of the organs the remaining photoreceptor(s) are adequate for a photoperiodic response (Numata et al., 1997). Indeed, it has been demonstrated that multiple photoreceptors are operative in photoperiodism for two species, *P. brassicae* (Seugé and Veith, 1976) and the brown-winged green bug, *Plautia stali* (Morita and Numata, 1999). With regard to the stemmata-derived organs of *L. kumagaii*, however, this possibility can be ruled out because adults with stemmata-derived organs did not respond to photoperiod at all after removal of the compound eyes. In the carabid beetle, *Pachymorpha sexguttata*, a putative photoreceptor has been shown at the front and dorsal rim of the lamina (Fleissner et al., 1993). In the present study, histological examination clearly showed that the intact lamina remained after removal of the compound eye, indicating that the photoreceptor-like organ at the lamina is not involved in photoperiodic photoreception. It is concluded that the compound eyes are the principal photoreceptor for photoperiodism and the stemmata-derived organs are not involved in photoreception for photoperiodism in the adult stage of *L. kumagaii*. In summary, the role of the retinal photoreceptors in photoperiodism in *L. kumagaii* changes ontogenically as follows. The larval stemmata have a principle role but stemmata-derived organs in the adult do not play a role. Instead, compound eyes in the adult, which are not developed in the larval stage, have a principal role. This is the first

study that demonstrates such an ontogenic change in the role of photoreceptors in photoperiodism in insects.

The role of the compound eyes in photoperiodism in adult insects has been demonstrated in a diversity of species, including the bean bug, *Riptortus clavatus* (Numata and Hidaka, 1983), the pentatomids, *Graphosoma lineatum* (Nakamura and Hodkova, 1998) and *P. stali* (Morita and Numata, 1999), the band-legged ground cricket, *Dianemobius nigrofasciatus* (Shiga and Numata, 1996) and the blow fly, *Protophormia terraenovae* (Shiga and Numata, 1997). In the Coleoptera, the compound eyes have been shown, by surgical removal, to be involved in photoperiodism in another carabid beetle, *Pterostichus nigrata* (Ferenz, 1975). Our conclusion for *L. kumagaii*, obtained by surgical removal and histological examination, supports the results in *P. nigrata*.

Stemmata-derived organs have been electrophysiologically shown to remain photosensitive in the pupal or adult stages in some insects, such as the swallowtail butterfly, *Papilio xuthus* (Ichikawa, 1991), the mealworm beetle, *Tenebrio molitor*, the house borer beetle, *Hylotrupes bajulus* (Felisberti et al., 1997) and the Japanese fireflies, *Luciola cruciata* and *L. lateralis* (Hariyama, 2000), although in *P. xuthus* they are about 10^{-3} or 10^{-4} times less photosensitive than the stemmata in the larval stage (Ichikawa, 1991). Their role in photoperiodism has been examined by surgical removal in diapause pupae of *Antheraea pernyi*, in which spectrum sensitivity for photoperiodic termination of diapause is different from that for induction of electrophysiological responses of the stemmata-derived organs. Also, because the pupae deprived of stemmata-derived organs responded to photoperiod normally, it is considered that the stemmata-derived organs are not the principal photoreceptor for photoperiodism (Hayes, 1971). Briscoe and White (2005) demonstrated in the butterfly, *Vanessa cardui*, that two opsin mRNAs are expressed in stemmata-derived organs, and speculated that they remain functional as components of the circadian input channel. However, the role of the stemmata-derived organs in photoperiodism has not been demonstrated in any insect. As shown in the current study, this is also the case with *L. kumagaii*, in which the stemmata play an important role in photoperiodism in the larval stage.

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