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Hatching controlled by the circatidal clock, and the role of the medulla terminalis in the optic peduncle of the eyestalk, in an estuarine crab *Sesarma haematocheir*

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Summary

Embryos attached to the female crab *Sesarma haematocheir* hatch synchronously within 1 h. Hatching is also synchronized near the time of the expected nocturnal high tide. These events are governed by a single circatidal clock (or pacemaker) in the female crab. The present study examined the role of the optic peduncle of the eyestalk on hatching and hatching synchrony. Surgery was performed either from the tip of the eyestalk [to remove the region of the optic peduncle from the compound eye–retina complex to the medulla interna (MI)] or from a small triangle 'window' opened on the eyestalk exoskeleton [to create lesions on the medulla terminalis (MT) of the optic peduncle]. Neither hatching nor hatching synchrony was affected by removal of the region of the optic peduncle from the compound eye–retina complex to the MI: the circatidal rhythm also remained. Removal of the MI probably caused damage to the sinus gland and the bundle of axons running from the sinus gland to the X organ. Nevertheless, maintenance of highly synchronized hatching indicates that the X organ–sinus gland system is not related to hatching. Hatching and hatching synchrony were not affected by dorsal-half cuts of the MT: the timing of hatching was not affected either. By contrast, transverse and ventral-half cuts of the MT caused severe damage to most females; hatching of many females was suppressed, while hatching of some females was either periodic, at intervals of approximately 24 h, or arrhythmic for a few days. The bundle of neuronal axons is tangled in the MT, and the axons inducing hatching pass through the ventral half of the MT. Complete incision of these axon bundles may have suppressed hatching. Incomplete incision of the axon bundle or partial damage to the neurons may have caused periodic or arrhythmic patterns of hatching. There are two possible roles for MT in hatching. One possibility is that neurons in the MT only induce hatching under the control of the circatidal pacemaker located in a site somewhere other than the optic peduncle. Another possibility is that the circatidal pacemaker is actually present in the MT. The second possibility seems more plausible. Each embryo has a special 48–49.5 h developmental program for hatching. This program could be initiated by the circatidal pacemaker in the female, and hatching synchrony may also be enhanced by the same pacemaker.

Key words: circatidal pacemaker, estuarine crab, gentle-release behavior, hatching synchrony, medulla terminalis, optic peduncle, eyestalk, neuronal pathway, vigorous-release behavior, *Sesarma haematocheir*.

Introduction

Activities of a number of terrestrial organisms are synchronized with day/night and seasonal cycles and show rhythmic patterns. Some of these rhythmic patterns persist for many days in constant conditions in the laboratory, indicating that they are controlled endogenously. Endogenous rhythms are governed by a circadian clock or pacemaker (Pittendrigh, 1960, 1981; Saunders, 1976). The circadian pacemakers of a variety of animals have been traced to the nervous system; e.g. the optic lobe or the brain in several insect species (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Loher, 1974; Roberts, 1974; Truman, 1974; Page, 1981), the eyes in a mollusc (Block and McMahon, 1984), the pineal in reptiles and birds (McMillan, 1972; Underwood, 1981) and the suprachiasmic nuclei in mammals (Rusak, 1977; Aguilar-Roblero et al., 1997).

Aquatic crustaceans also exhibit circadian rhythms in a variety of behavioral and physiological events; e.g. hormone level (Keller, 1981), serotonin content (Castañón-Cervantes et al., 1999), retinal structure (Arikawa et al., 1987), electroretinogram (ERG) amplitude (Aréchiga and Wiersma, 1969; Larimer and Smith, 1980; Barlow, 1983; Aréchiga et al., 1993) and locomotor activity (Fanjul-Moles et al., 1996). In

the horseshoe crab *Limulus polyphemus*, many physiological events are modulated by retinal function. These events in the retina are generated by the efferent input from a circadian pacemaker located in the brain (Barlow et al., 1977, 1987). In the locomotor activity of crayfish *Procambarus clarkii*, the circadian pacemaker may be located in the supraesophageal ganglion (Page and Larimer, 1975). However, in the ERG circadian rhythm, the pacemaker is suggested to be present in the eyestalk–protocerebrum complex (Barrera-Mera and Block, 1990).

Marine crustaceans also show rhythmic activity patterns in locomotion, swimming and reproduction. However, the tidal cycle, as well as the day/night cycle, affects the timing of the activity in the marine environment. Accordingly, the activity of marine crustaceans is often synchronized not only with the day/night cycle but also, more or less, with the tidal cycle and demonstrates complex patterns (e.g. Saigusa, 1981, 2001; Saigusa et al., 2002). Much work on the timing systems has been conducted on the locomotor activity of intertidal and estuarine crabs (see Palmer, 1995). However, very few specimens show well-demarcated rhythmic patterns in constant conditions in the laboratory (e.g. Honegger, 1976). The lack of clear endogenous rhythmicity in these crabs has interrupted elaborate experimental studies in the laboratory. Most experimental results obtained from a number of crab locomotor activities were not reproducible. Therefore, hypotheses proposed on the timing systems of the circatidal rhythms are not acceptable. For example, the circatidal rhythms have been explained in terms of an interaction between circadian and circatidal clocks (see Palmer, 1995). However, no clear evidence supports this hypothesis (see Saigusa, 1986, 1988). Information on the anatomical location of the pacemaker controlling circatidal rhythms is also conspicuously lacking.

Well-demarcated synchrony with the tidal cycle has been observed in the larval-release activity of a number of intertidal and estuarine crabs (Saigusa, 1981, 1982; Christy, 1986; Paula, 1989; Queiroga et al., 1994). In contrast to the locomotor activity, a free-running tidal rhythm of the larval-release activity is very clear in constant darkness or in 24 h light:dark (L:D) cycles in the laboratory (Saigusa, 1986, 1992a; Saigusa and Kawagoye, 1997). This suggests that the larval-release activity is clearly controlled by an internal clock (circatidal clock) or a pacemaker (circatidal pacemaker). The larval-release rhythm is well suited for experimental analysis of the timing system in marine organisms.

In an estuarine terrestrial crab *Sesarma haematocheir*, each female incubates 20 000–50 000 embryos in her incubation chamber. Hatching occurs prior to the larval release; all of the embryos hatch within 1 h in the female's incubation chamber. Hatched zoea larvae are immediately liberated into the water by the vigorous fanning behavior of the abdomen, which lasts for only 4–5 s (Saigusa, 1982). The circatidal system of hatching in *S. haematocheir* is characterized by (1) highly synchronous hatching of embryos attached to the female and (2) synchrony of hatching with nocturnal high tide. These events are controlled by the circatidal clock of the female (Saigusa, 1992b, 1993).

The present study investigated whether the circatidal pacemaker controlling hatching and hatching synchrony is present in the optic peduncle of the female eyestalk and, if so, where it is located within the optic peduncle. Ablation of both eyestalks can easily answer this question (e.g. Page and Larimer, 1975). However, ablation results in serious damage to the animals, especially in terrestrial crabs, which may affect the interpretation of the results. Furthermore, the optic peduncle of the eyestalk contains the X organ and the sinus gland, i.e. the major neuroendocrine system in crustaceans, and the expectation is that the tidal rhythm is generated through the neuroendocrine system. In view of this possibility, it is important to maintain at least blood circulation after surgery. In the present study, surgery on the optic peduncle was performed either from an incision at the tip of the eyestalk, i.e. to remove the region of the optic peduncle from the compound eye–retina complex to the medulla interna (MI), or from a triangle 'window' opened on the eyestalk exoskeleton, i.e. to create lesions on the medulla terminalis (MT). This study indicates that synchronous hatching among embryos and synchrony with the tide are lost when the ventral half of the MT is damaged.

Materials and methods

Collection of animals and maintenance of crabs

The red-handed crab *Sesarma haematocheir* de Haan inhabits thickets along a small estuary. Ovigerous females were collected at two habitats (Kasaoka and Ushimado) in Okayama Prefecture, Japan in the summers (July–September) of 1987–1989 and 2000. Kasaoka and Ushimado are 70 km away from each other, and the phase of the tidal cycle is shifted, at most, 20 min between these two locations. After collection, crabs were immediately transferred to the laboratory and were kept in several plastic containers (70 cm×50 cm×40 cm, length × width × depth) containing a small quantity of diluted sea water (1–5‰). The plastic containers were placed in the experimental room in which light and temperature were controlled.

The 24 h L:D cycle is critical for maintaining the phase of the circatidal rhythm of *S. haematocheir* (see Saigusa, 1986, 1992a). In the field, the time of sunset shifts from 19:20 h to 18:15 h, and that of sunrise shifts from 05:00 h to 05:45 h, from early July to mid-September, respectively. So, the photoperiod in the field is 14.3 h:9.7 h L:D in early July and 12.5 h:11.5 h L:D in mid-September. In the laboratory, we employed similar photoperiods and phases to those observed in the field, i.e. a 15 h:9 h L:D cycle (light off at 20:00 h and on at 05:00 h) or a 14 h:10 h L:D cycle (light off at 19:00 h and on at 05:00 h). The intensity of illumination on the floor was 700–1200 lux in the light phase and <0.05 lux in the dark phase. Temperature was constant at 24±1°C. In these conditions, the larval-release activity of the population clearly shows the free-running tidal rhythm, the phase of which roughly coincides with the time of

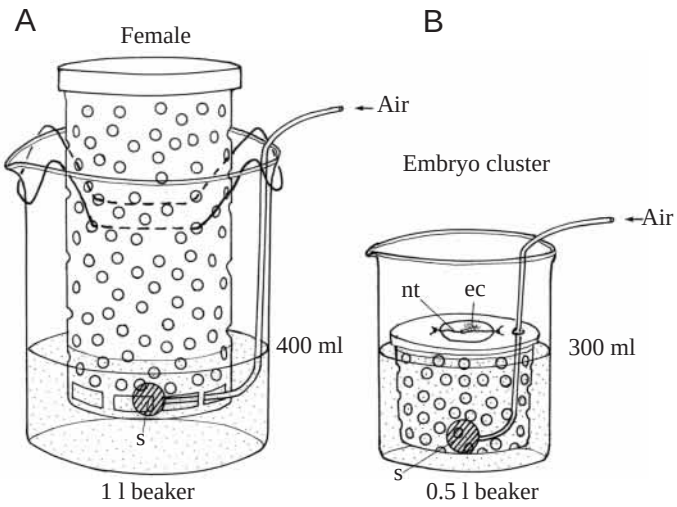


Fig. 1. Water-exchange method used to monitor (A) hatching of embryos attached to the female and (B) hatching of embryos detached from the female. Abbreviations: s, air stone; nt, nylon thread; ec, embryo cluster.

nocturnal high tide in the field for at least one month after collection.

Monitoring hatching and assessment of hatching synchrony

Hatching of the embryos (embryos attached to the female and detached embryos) was monitored by either of the following two methods.

Water-exchange method

Ovigerous females were individually placed in perforated plastic cages (7 cm×14 cm, width × height), with small holes in the sides. Each cage was then suspended by a fine wire from the rim of a 1 l glass beaker containing 400 ml of diluted, clean seawater (salinity 10‰) (Fig. 1A). Only the bottom of the plastic cage was immersed in the water. The water was strongly aerated with an air stone. At intervals of 30 min, the plastic cage was transferred to another beaker containing the same quantity of diluted (10‰) seawater. Exchange of the beaker was carried out under a red light in the dark phase of the L:D cycle (Saigusa, 1993). The original beaker was removed from the experimental room, and the number of hatched zoeas was counted with the aid of a pipette. This method detected the temporal distribution of the number of zoeas hatched. Moreover, if the embryos attached to the female hatch synchronously, zoeas are released by the vigorous fanning behavior of the abdomen, which lasts for 4–5 s ('vigorous-release behavior'). As the water was exchanged every 30 min, it was easy to judge when this behavior occurred.

Photoelectric-switch method

Ovigerous females were individually placed in a perforated plastic cage (the same cage as shown in Fig. 1A). The cage was suspended from the rim of a glass beaker containing 400~ml of diluted (10‰) seawater, and the bottom of the cage was immersed in the water (to approximately 1~cm depth). The beaker was set in the recording apparatus used to detect larval release, which consisted of a sensor unit (infrared source and receiver; E3S-2E4, Omron Co. Ltd, Japan) placed on both sides of the beaker and a controlling unit (S3S-A-10, Omron Co. Ltd, Japan). The infrared beam passed through under the plastic cage. When the zoeas were released by the female, the beam was partially interrupted by swimming zoeas, and this interruption was detected by the controlling unit placed outside the experimental room. Furthermore, the output of the controlling unit was monitored by an event recorder (R17-H12T, Fuji Electric Co. Ltd., Japan) (for a figure of this apparatus, see Saigusa, 1992a). This method was effective when hatching occurred synchronously and zoeas were liberated by vigorous-release behavior. It was also less labor-intensive than the water-exchange method. When hatching synchrony deteriorated and hatch time was prolonged, zoeas were liberated by a gentle pumping movement of the abdomen ('gentle-release behavior). Time of hatching associated with gentle-release behavior was not accurately detected by the photoelectric-switch method, because larval release often continued for hours. Thus, hatching associated with gentle-release behavior was monitored by the water-exchange method.

Hatching of detached embryos

A female *S. haematocheir* has four pairs of abdominal appendages, each of which bears one plumose seta and one ovigerous seta. Embryos are attached to many ovigerous hairs arranged along the ovigerous seta (Saigusa, 1994). One ovigerous seta with its attached embryos was cut at its base, and bleeding was stopped using a soldering iron. The embryo cluster was then tied by a cotton thread to the nylon thread in the center of a small plastic, perforated container (8 cm×6 cm diameter × depth) with small holes in the sides. This container was placed in a 500 ml glass beaker containing 200 ml of diluted sea water (salinity 10‰), and the water was strongly aerated with an air stone (Fig. 1B). At intervals of 30 min (or 1 h), the plastic cage was transferred to another beaker with the same quantity of diluted sea water, and the number of hatched zoeas was counted with the aid of a pipette. Exchange of the beaker was carried out under a red light in the dark phase of the L:D cycle.

Discrimination of mature zoeas from immature zoeas

Hatching (breakage of the egg envelope) occurs just before releasing zoeas. The egg case of *S. haematocheir* abruptly cracks, and the dorsal thorax of the zoea appears (Saigusa and Terajima, 2000). The zoea vigorously bends and stretches its body, with strong vibration of the appendages. Via this process, the larva can shed the sticky embryonic exuvia and is transformed to a mature zoea. In our experiments, some of the mature zoeas swam, but others did not. These zoeas were submerged at the bottom of the beaker. Furthermore, immature zoeas (the larvae that were still folded by the embryonic exuviae) were sometimes released. They could easily be discriminated from mature zoeas under a stereomicroscope. In

this study, zoeas were divided into three types: ‘immature zoea’, ‘mature zoea that cannot swim’ and ‘swimming mature zoea’.

Assessment of the degree of hatching synchrony

In the water-exchange method, the degree of hatching synchrony was assessed using a ‘synchrony index’ (SI). This index was defined as the maximum value (%) of hatching divided by the ‘number’ of bars arranged at intervals of 30 min that contain 47.5% above and below the median value, respectively. For example, in Fig. 4A (top panel), the maximum value is 87.7%, and the number of divisions containing 95% hatching (enclosed by the broken line) is 2. So, SI is 43.9 (87.7 divided by 2).

Surgery on the optic peduncle of both eyestalks

Eyestalk ablation

One of the eyestalks or both eyestalks were removed with a knife, and bleeding was stopped with a small soldering iron. Hatching was monitored either by the water-exchange method or the photoelectric-switch method.

Removal of the compound eye–retina complex, the medulla externa (ME) and the medulla interna (MI)

Ovigerous females were individually buried in an appropriately sized crushed-ice bed, with the head facing up so as to expose both eyestalks. A knife blade (100 μm thick) was made by splitting the edge of a razor blade and was attached to the knife holder (Handaya Co., Tokyo, Japan). A new blade was used for each incision.

Fig. 2A shows a schematic representation of the optic peduncle of *S. haematocheir*. First, the cornea (Fig. 2B) was cut along the edge of the exoskeleton of the eyestalk and removed. Next, the compound eye–retina complex was carefully removed using a paper towel. The lamina ganglionalis–ME complex was visible after the removal of the compound eye. These ganglia were cut and removed under the stereomicroscope. Removal up to the MI was also carried out in the same way (see Table 3). After surgery, bleeding was stopped using a small soldering iron (Fig. 2C).

Lesions of the medulla terminalis (MT)

Surgery towards the MT was difficult to perform from the tip of the eyestalk, so a right-angled triangle was cut into the exoskeleton of both eyestalks under the stereomicroscope (Fig. 2D). A cluster of neurosecretory cells (Fig. 2F) was readily visible as a white spot from the triangle ‘window’ (Fig. 2E). After determining the position of this cluster of neurosecretory cells (i.e. white spot), it was possible to locate the correct position to make an incision. The MT was cut three different ways (Fig. 3): cutting transversely from the dorsal part to the ventral part along the hypotenuse (transverse cut), cutting the upper half of the MT perpendicular to the optic peduncle (dorsal-half cut) and cutting the ventral half of the MT perpendicular to the optic peduncle (ventral-half cut; see Table 5). After each operation, the triangular exoskeleton was returned to the original site, and the wound was sealed using the soldering iron. Under these conditions, the optic peduncle remained alive throughout the experiment (compare Fig. 2G with Fig. 2B).

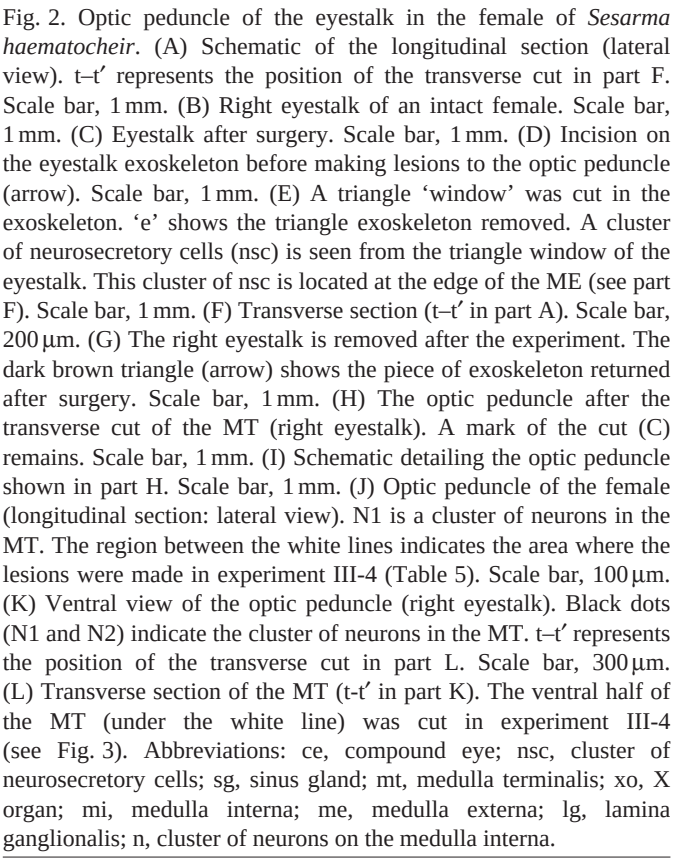


Fig. 2. Optic peduncle of the eyestalk in the female of *Sesarma haematocheir*. (A) Schematic of the longitudinal section (lateral view). t–t′ represents the position of the transverse cut in part F. Scale bar, 1 mm. (B) Right eyestalk of an intact female. Scale bar, 1 mm. (C) Eyestalk after surgery. Scale bar, 1 mm. (D) Incision on the eyestalk exoskeleton before making lesions to the optic peduncle (arrow). Scale bar, 1 mm. (E) A triangle ‘window’ was cut in the exoskeleton. ‘e’ shows the triangle exoskeleton removed. A cluster of neurosecretory cells (nsc) is seen from the triangle window of the eyestalk. This cluster of nsc is located at the edge of the ME (see part F). Scale bar, 1 mm. (F) Transverse section (t–t′ in part A). Scale bar, 200 μm. (G) The right eyestalk is removed after the experiment. The dark brown triangle (arrow) shows the piece of exoskeleton returned after surgery. Scale bar, 1 mm. (H) The optic peduncle after the transverse cut of the MT (right eyestalk). A mark of the cut (C) remains. Scale bar, 1 mm. (I) Schematic detailing the optic peduncle shown in part H. Scale bar, 1 mm. (J) Optic peduncle of the female (longitudinal section: lateral view). N1 is a cluster of neurons in the MT. The region between the white lines indicates the area where the lesions were made in experiment III-4 (Table 5). Scale bar, 100 μm. (K) Ventral view of the optic peduncle (right eyestalk). Black dots (N1 and N2) indicate the cluster of neurons in the MT. t–t′ represents the position of the transverse cut in part L. Scale bar, 300 μm. (L) Transverse section of the MT (t-t′ in part K). The ventral half of the MT (under the white line) was cut in experiment III-4 (see Fig. 3). Abbreviations: ce, compound eye; nsc, cluster of neurosecretory cells; sg, sinus gland; mt, medulla terminalis; xo, X organ; mi, medulla interna; me, medulla externa; lg, lamina ganglionalis; n, cluster of neurons on the medulla interna.

In this study, surgery on the optic peduncle was performed while ovigerous females were individually buried in a crushed-ice bed. However, it is possible that contact with ice might have an effect on the phase of the circatidal rhythm. For the control experiment, 26 females were immersed in ice water for 10 min at various times on a given day, and the timing of hatching was compared with that of the control females that had not been immersed in ice water.

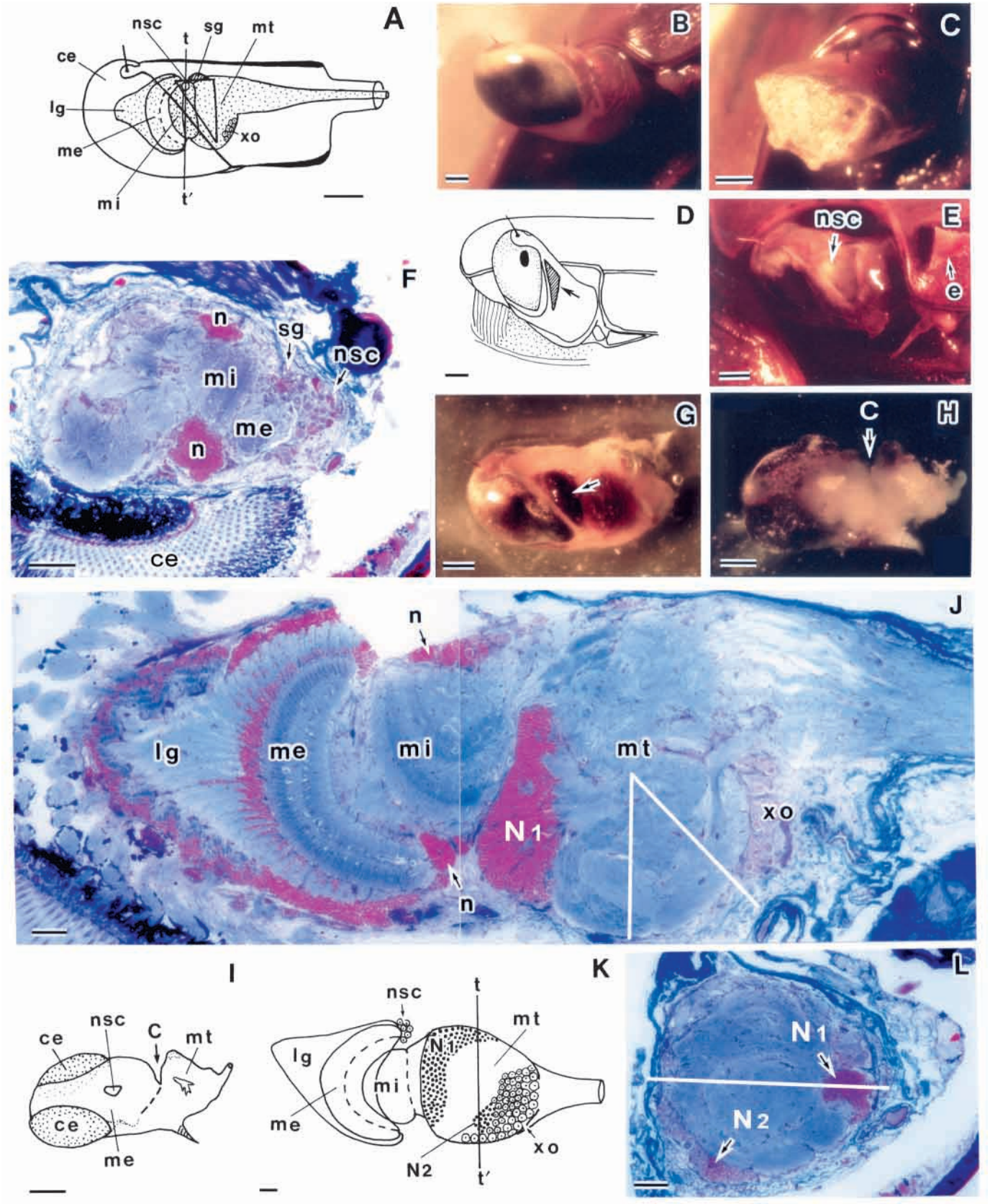
Assessment of the success of surgery on the MT

After the conclusion of each experiment, both eyestalks were separated (Fig. 2G), the optic peduncle was excised, with care being taken not to damage it, and the success of the operation was assessed under the stereomicroscope (Fig. 2H). Although detailed histological studies may be required to specify the region of tissues of the MT that was damaged by the surgery, inspection of the optic peduncle under the stereomicroscope could roughly determine what region of the peduncle was cut by the surgery.

Results

Synchrony of hatching in attached and detached embryos

Fig. 4 shows the hatching profiles of two females. All hatching was monitored by the water-exchange method.



Female 1 (Fig. 4A) frequently moved around in the plastic cage and immersed her body into the water from 01:30 h to 02:00 h on 3 September 2000, when 11.5% of zoeas appeared in the water (top panel). This female liberated most zoeas (87.7%) by vigorous-release behavior between 02:00 h and 02:30 h. Zoeas were all mature and swam. The

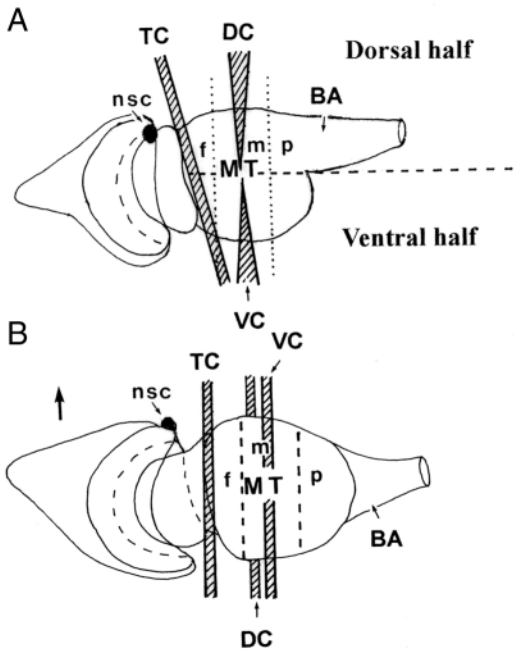


Fig. 3. The optic peduncle of the right eyestalk. (A) Lateral view. This side shows the front of the crab. (B) Ventral view. The arrowhead shows the front of the crab. The dotted lines indicate the boundaries between the frontal, middle and posterior regions of the MT. Abbreviations: MT, medulla terminalis; nsc, cluster of neurosecretory cells; f, frontal region of the MT; m, middle region of the MT; p, posterior region of the MT; BA, base of the optic peduncle; TC, transverse cut of the MT; DC, dorsal-half cut of the MT; VC, ventral-half cut of the MT.

median time of hatching distribution was 02:15 h on 3 September. The synchrony index (SI) was estimated as 43.9 in this female. A cluster of embryos that had detached from this female (detachment at 12:10 h on 2 September; middle panel) also hatched. Hatching peaked between 02:30 h and 03:00 h (median 02:15 h) on 3 September. Hatching synchrony deteriorated to an SI of 6.7. A second embryo cluster that had detached from the same female (detachment at 19:00 h on 1 September; bottom panel) also hatched and all zoeas swam. Hatching peaked at 02:30–0300 h (median 02:45 h) on 3 September. The SI further deteriorated to 3.7.

The hatching of embryos attached to Female 2 (Fig. 4B, upper panel) peaked at 02:00–02:30 h (median 02:15 h) on 1 September (87.5%). Larvae were liberated by vigorous-release behavior. The remaining 12.5% appeared in the water at 02:30-03:00 h on 1 September. (A small quantity of zoeas often remains after vigorous-release behavior. Such zoeas are often liberated during a second episode of vigorous-release behavior.) An embryo cluster that had detached at 13:35 h on 31 August all hatched (middle panel), with hatching peaking at 03:45 h on 1 September (SI=7.0). An embryo cluster that had detached at 17:35 h on 30 August all hatched on 1 September, with all zoeas swimming (bottom panel). However, their hatching was delayed and peaked at 05:45 h on 1 September (SI=3.6). As shown in these two females, the embryos that detached at least 1 day before larval release all hatched and swam. In contrast, no embryo cluster that had detached more than 2 days before larval release hatched in aerated water (not shown).

Table 1 summarizes hatching synchrony of the embryos attached to the female and that of detached embryos. In attached embryos, the SI of all females was >25. Hatching was always associated with vigorous-release behavior. In contrast, hatching synchrony of detached embryos deteriorated. Deterioration increased for the embryos detached one day before larval release. The difference in SI between the embryos detached on the day of larval release and those detached one day before larval release was significant at the 1% level (*t*-test; P=0.0017, N=23).

Synchrony of hatching with nocturnal high tides

Ovigerous females (73 specimens) were collected on 16 August 1988 and were randomly separated into two groups. In one group (47 females), hatching of the embryos attached to the female was monitored by the photoelectric-switch method (Fig. 5A, open circles). Hatching of all embryos occurred around the time of nocturnal high tide. The mean shift (± S.D.) of hatching from nocturnal high tide was 0.91±0.61 h. Clear correlation of hatching with the nocturnal high tide lasted for at least 3 weeks in non-tidal laboratory conditions.

A second group (26 females) was immersed in ice water for 10 min at various times on 20 August. Zoeas were always released by vigorous-release behavior, so their hatching was monitored by the photoelectric-switch method (Fig. 5A, filled triangles). Hatching of these females also coincided with the nocturnal high tide. The mean shift (± S.D.) of the time of release from the nocturnal high tide was 1.05±0.83 h, indicating that the timing of hatching was not disturbed by ice water.

Table 1. *Comparison of hatching synchrony in female-attached embryos and detached embryos*

Experimental conditions	Number of females or embryo clusters detached	Degree of hatching synchrony
Embryos attached to the female	14	28.9≦SI≦99.7 (mean 77.3) (0.5 h≦*t*≦1.0 h)
Embryo cluster detached on the day of larval release	10	1.5≦SI≦11.6 (mean 6.7) (2.5 h≦*t*≦8.0 h)
Embryo cluster detached 1 day before larval release	13	1.2≦SI≦10.7 (mean 3.7) (3.0 h≦*t*≦8.5 h)
Embryo cluster detached ≥2 days before larval release	No hatching	–

SI, synchrony index; *t*, period of hatching (h).

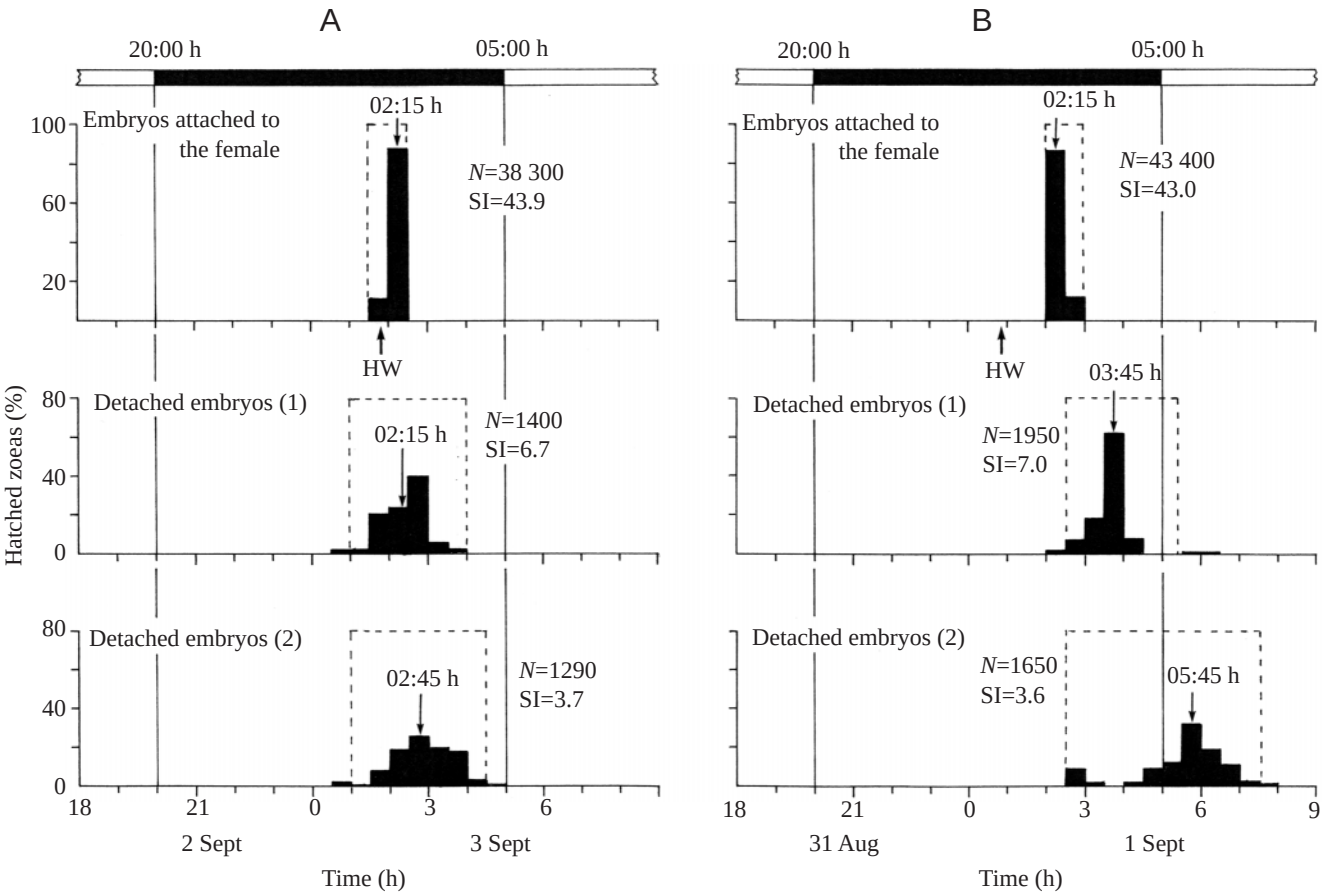


Fig. 4. Hatching profiles of two intact females (A and B) and the embryo cluster detached from each female. Top panel: hatching of the embryos attached to the female. Middle panel: hatching of the embryos detached on the day of larval release from the female. Bottom panel: hatching of the embryos detached one day before larval release. The area depicted by broken lines shows 95% hatching, while the downward arrow shows the median time in hatching distribution. The 24 h L:D cycle is shown by the horizontal bar at the top of the figure (the filled section represents the dark phase). Vertical solid lines indicate the times of light on and light off in the experimental room. *N* is the total number of zoeas hatched. SI, synchrony index. HW, predicted time of high tide.

Ablation of the eyestalk

Experiment I-1: ablation of one eyestalk

Fifty ovigerous females were collected in July–August 2000. One of the eyestalks was cut, and hatching of most females was monitored by the photoelectric-switch method, while that of others was monitored by the water-exchange method. Hatching of all females in which the eyestalk had been cut did not differ from that of intact females. Zoeas were liberated by vigorous-release behavior and swam (Table 2).

Table 2. *Effect of ablation of the eyestalk on hatching*

Results	Control (*N*=50 females)	Ablation of one eyestalk (*N*=50 females)	Ablation of both eyestalks (*N*=60 females)
Hatching occurred			
≤2 days	12	6	14
≥3 days	38	44	2*
Hatching did not occur			
Death	0	0	24
Ecdysis	0	0	5
Embryo cluster dropped	0	0	6
Incubation maintained	0	0	9

*A small number of zoeas hatched and swam.

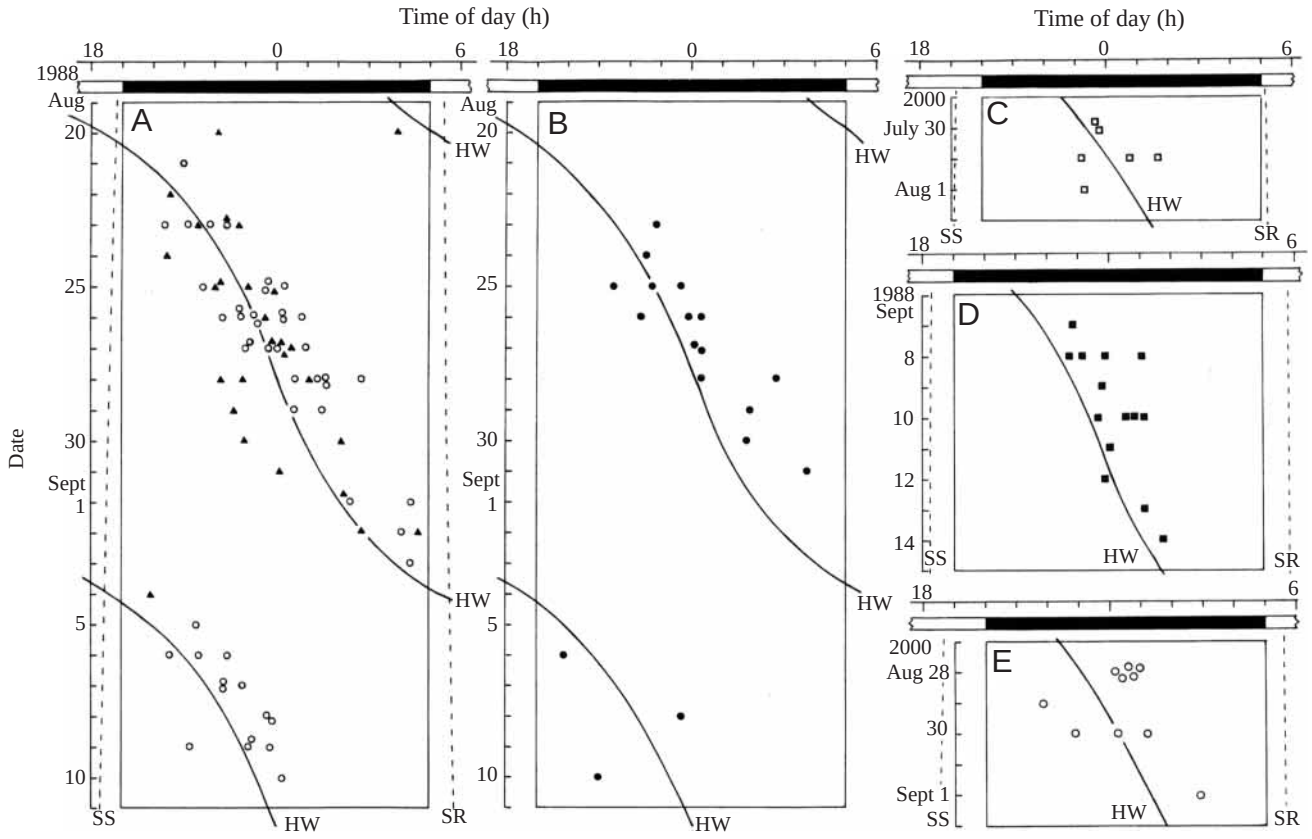


Fig. 5. Timing of hatching monitored in the 24 h L:D cycles. (A) Control groups. Open circles indicate the time of hatching in intact females, and solid triangles indicate the time of hatching in the females immersed in ice water for 10 min. (B) Hatching and larval release of females from which the compound eye–retina complex was removed (experiment II-1; Fig. 2C). (C) Hatching and larval release of females from which the optic peduncles had been removed up to the medulla externa (ME) (experiment II-2; Fig. 2C). (D) Hatching and larval release of females from which the optic peduncles had been removed up to the medulla interna (MI) (experiment II-3; Fig. 2C). (E) Hatching and larval release of females from which a triangle window on the eyestalk exoskeleton had been removed and replaced (experiment III-1; Fig. 2E). The 24 h L:D cycle is shown by the horizontal bar at the top of the figure (the filled section represents the dark phase). HW connects the times of predicted high tide in the field. SS and SR indicate the times of sunset and sunrise in the field, respectively.

Experiment I-2: ablation of both eyestalks

Ovigerous females (110 specimens) were collected on 23 August 1987 and were randomly separated into two groups. The eyestalks of one group (50 females) were left intact (control experiment) while those of the second group (60 females) were ablated on 25 August. Hatching was monitored by the photoelectric-switch method.

In the control group, all females released larvae within 2 weeks of collection (Table 2). For the group with ablated eyestalks, locomotor activity decreased remarkably and their appetites became voracious. Embryos of 14 females hatched within 2 days of ablation. Embryos of these females had already started the hatching program before eyestalk ablation, so their embryos all hatched despite ablation of both eyestalks. Hatched zoeas were liberated by the gentle-release behavior. The results of 46 other females are shown in Table 2. More than half of the females died in the course of the experiments. Five females molted with living embryos, and six females discarded embryos without hatching. A small number of zoeas hatched from only two females until 25 September (marked with an asterisk in Table 2).

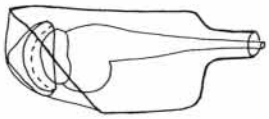
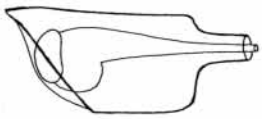
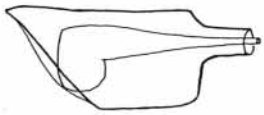
Surgery from the tip of the eyestalk

Experiment II-1: Removal of the compound eye–retina complex

Seventeen ovigerous females were collected on 25 June 2000 and the compound eye and retina were removed. Hatching was monitored by the water-exchange method (Table 3). No females died after surgery. Five females had already started the hatching program before surgery (see Table 6). Hatching of 12 other females occurred within 1 h, and zoeas were liberated into the water by vigorous-release behavior. The mean shift (± S.D.) of hatching from the nocturnal high tide was 1.1±0.7 h, indicating that the timing of hatching is not affected by the surgery.

Eighteen ovigerous females were collected on 16 August 1988, and the same surgery was performed on 20 August (data not shown). Hatching was monitored by the photoelectric-switch method (Fig. 5B). No females died after surgery. Hatching of the females coincided with nocturnal high tides.

Table 3. *Effects of removal of the optic peduncle from the compound eye–retina complex to the medulla interna*

Experimental group	Operation procedure	Number of animals[a]	Post operative observations: Days of observation	Shadow reflex	Synchrony index	Mean deviation from the nocturnal high tide
II-1		12 (5)	14 days	No	25<SI (mean 74.7) (0.5 h≦*t*≦1.0 h)	1.1±0.7 h
II-2		6 (0)	5 days	No	25<SI (mean 90.7) (0.5 h≦*t*≦1.0 h)	0.84±0.4 h
II-3		6 (4)	5 days	No	25<SI (mean 69.9) (0.5 h≦*t*≦1.5 h)	1.1±1.2 h

Data are taken from experiments carried out in 2000.
[a]The number of females that had started the hatching program before surgery is shown in parentheses (see Table 6).
SI, synchrony index; *t*, period of hatching (h).

Thus, the pattern of the larval release activity was the same as that of control groups (Fig. 5A), indicating that the circatidal rhythm of these females is maintained.

Experiment II-2: Removal up to the medulla externa

Six ovigerous females were collected on 25 July 2000, and both optic peduncles were removed up to the ME on 28 July. No females died after surgery. Hatching was monitored by the water-exchange method. No females had started the hatching program when the operation was made. Hatching of each female occurred within 1 h, associated with vigorous-release behavior (Fig. 5C). The mean deviation (± S.D.) of hatching from nocturnal high tide was 0.8±0.4 h (Table 3), indicating that the timing of hatching is not affected by the operation.

Experiment II-3: Removal up to the medulla interna

Ten ovigerous females were collected on 25 July 2000, and both optic peduncles were removed up to the MI on 28 July (five females) and on 9 August (five females), respectively. Hatching was monitored by the water-exchange method. No females died after surgery. Four females had already started the hatching program before surgery (Table 6). Hatching of six other females occurred within 5 days, and the larvae were liberated by vigorous-release behavior (data not shown). The mean deviation (± S.D.) of hatching from nocturnal high tide was 1.1±1.2 h (Table 3), indicating that the circatidal rhythm of these females is still maintained.

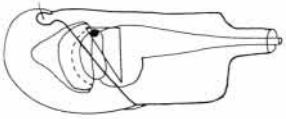
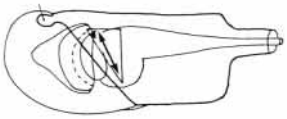
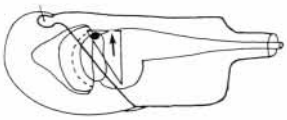
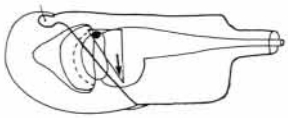
18 ovigerous females were collected on 2 September 1988, and both optic peduncles were removed up to the MI. No females died after surgery. The hatching of 14 females was normal, although the hatching of two other females was extremely delayed (release at 20:40 h on 22 September and at 23:50 h on 24 September). The time of hatching of these 14 females could be monitored by the photoelectric-switch method (Fig. 5D). The mean deviation (± S.D.) of hatching from nocturnal high tide was 0.9±0.6 h, indicating that neither hatching nor hatching synchrony of these females are affected by the removal of the optic peduncle up to the MI and that the circatidal rhythm of these females is still maintained. However, for two other females, mature zoeas hatched every night from 20 September to 27 September (data not shown). Very few

Table 4. *Four types of hatching patterns observed after lesions of the medulla terminalis*

Type 1	Synchrony index (SI)>25 in all females. Zoeas were liberated by vigorous-release behavior. Pattern of hatching was the same as that of intact females (Fig. 3A,B, top panels).
Type 2	Hatching synchrony deteriorated. Hatching was periodical at intervals of approximately 24 h. Zoeas were mature, but most did not swim. Zoeas were liberated by gentle-release behavior.
Type 3	Hatching synchrony deteriorated. Periodicity blurred. Zoeas were mature, but most did not swim. Immature zoeas were often liberated. Zoeas were liberated by gentle-release behavior.
Type 4	In many females, very few zoeas hatched. Hatched zoeas were generally mature. Females dropped embryos without hatching.

For each pattern, see Figs 6, 7.

Table 5. *Effects of incision on the medulla terminalis in the optic peduncle*

Experimental group	Operation procedure	No. of animals[a]	Post-operative observations: Days of observation (max)	Shadow reflex	Hatching profile: Type 1[b]	Type 2	Type 3	Type 4
III-1		10 (0)	15 days	Yes	10 (1.3±0.5 h)	0	0	0
III-2		11 (3)	15 days	No	3 (0.8±0.2 h)	1	2	5
III-3		5 (7)	10 days	Yes	5 (1.7±0.9 h)	0	0	0
III-4		11 (3)	22 days	No	2 (2.2 h)	1	0	8

[a]The number of females that had started the hatching program before surgery is shown in parentheses (see Table 6).
[b]Mean deviation (h) from nocturnal high tide is shown in parentheses.

zoeas could swim. The hatching synchrony of these two females was obviously affected by the surgery.

Lesions of the medulla terminalis (MT)

The surgery on the MT through the triangle window of the eyestalk exoskeleton (Fig. 2E) was performed on 50 females. Hatching was monitored by the water-exchange method. As shown in Table 4, the effects of this operation could be classified into four types (types 1–4).

Experiment III-1: control group

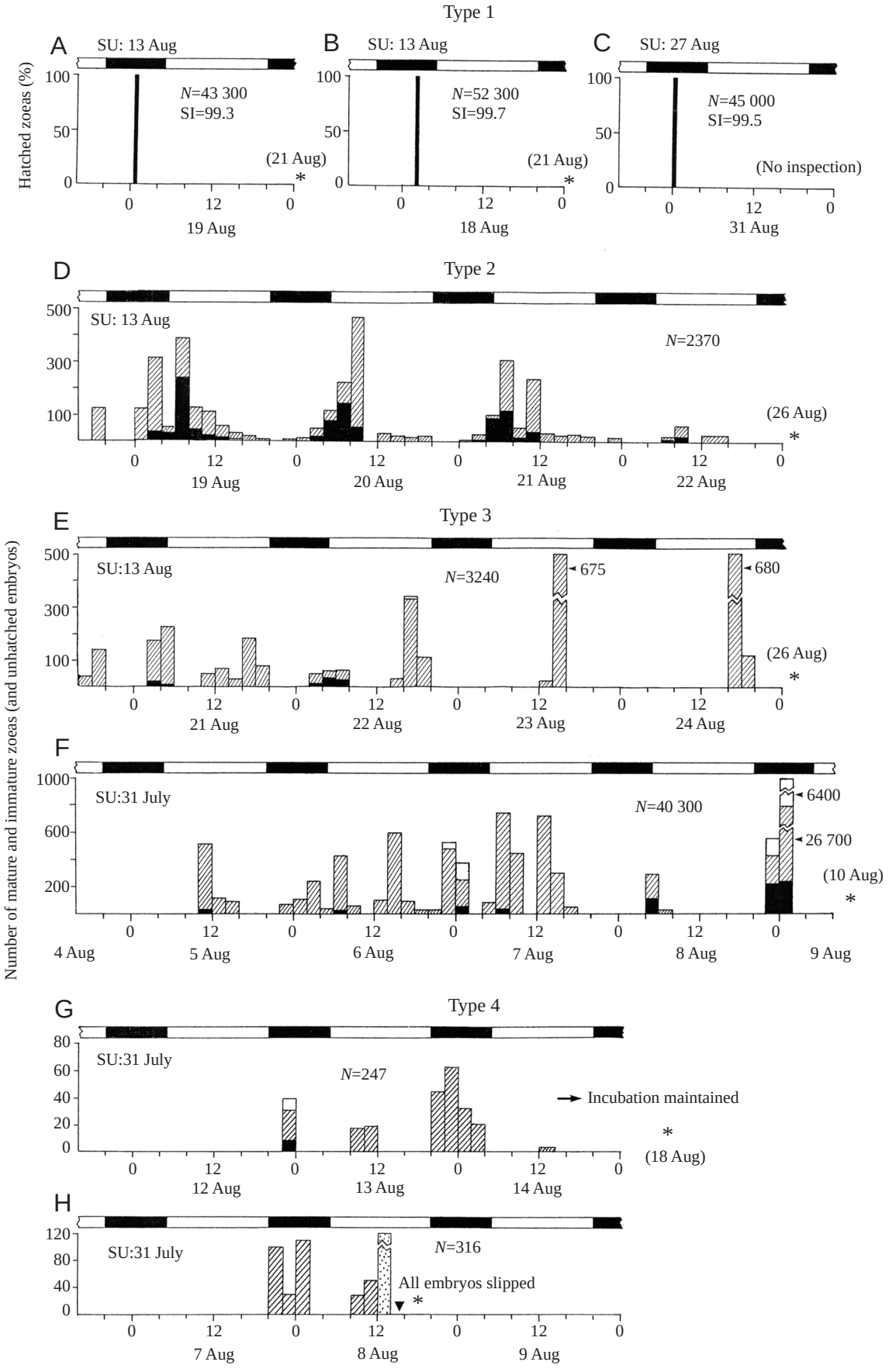
Ten females were collected on 10–12 August 2000. On 18 August, the triangle window was cut in the eyestalk exoskeleton (Fig. 2E), and the piece of the exoskeleton was returned to the original site without incision of the optic peduncle (Fig. 2G). No females died. All females released mature zoeas until 2 September. Hatching of all females was classified as type 1 (Table 5). Hatching occurred around nocturnal high tide (Fig. 5E). Zoeas were liberated by vigorous-release behavior. The mean deviation (± S.D.) of hatching from nocturnal high tide was 1.3±0.5 h (Table 5), indicating that the circatidal rhythm of these females is maintained.

Experiment III-2: transverse cuts of the MT

The surgical transverse cuts performed through the triangle window (Fig. 2E) were made in 14 females. In 5 females (collected on 25 July 2000) surgery was performed on 31 July, while in the remaining nine females (collected on 10–12 August) surgery was performed on 13 August and 27 August. No females died after surgery. Three females had already started the hatching program when the cuts were made (see Table 6).

Hatching of three out of the remaining 11 females (Fig. 6A–C) was not different from that of the control group (type 1). The SI was >99.0, suggesting that neither hatching nor hatching synchrony in these females is affected by the surgery. Mean deviation (± S.D.) from the nocturnal high tide was 0.8±0.2 h, indicating that the circatidal rhythm of these females is maintained. However, hatching synchrony in the other eight females was affected to varying degrees (Table 5; types 2, 3 and 4). Zoeas hatched from these females were liberated by gentle-release behavior.

Fig. 6. Pattern of hatching after transverse cuts were made on the medulla terminalis (MT; experiment III-2). The effects of the lesions were classified into four types (see Table 4 for details of each type). Parts (A)–(H) represent the pattern of hatching in individual females; data represent either the percentage of hatched zoeas (A–C) or the number of hatched zoeas (and that of embryos dropped without hatching) (D–H). Filled bars represent the number of mature, swimming zoeas; hatched bars represent the number of mature, but non-swimming, zoeas; stippled bars represent the number of embryos dropped from the female without hatching. The 24 h L:D cycle is shown by the horizontal bar at the top of each graph (the filled section represents the dark phase). SU indicates the date when the surgery was performed. *N* is the total number of zoeas hatched. The asterisks indicate the date when the optic peduncles were inspected under the stereomicroscope. In (H), the arrowhead indicates the time when the incubation was stopped.


Type 1
A
SU: 13 Aug
N=43 300
SI=99.3
(21 Aug)
19 Aug
B
SU: 13 Aug
N=52 300
SI=99.7
(21 Aug)
18 Aug
C
SU: 27 Aug
N=45 000
SI=99.5
(No inspection)
31 Aug
Hatched zoeas (%)
Type 2
D
SU: 13 Aug
N=2370
(26 Aug)
19 Aug
20 Aug
21 Aug
22 Aug
Type 3
E
SU:13 Aug
N=3240
675
680
(26 Aug)
21 Aug
22 Aug
23 Aug
24 Aug
F
SU:31 July
N=40 300
6400
26 700
(10 Aug)
4 Aug
5 Aug
6 Aug
7 Aug
8 Aug
9 Aug
Number of mature and immature zoeas (and unhatched embryos)
Type 4
G
SU:31 July
N=247
Incubation maintained
(18 Aug)
12 Aug
13 Aug
14 Aug
H
SU:31 July
N=316
All embryos slipped
7 Aug
8 Aug
9 Aug

Hatching synchrony of one female (Fig. 6D) deteriorated but occurred periodically for 4 days (19–22 August; type 2). None of the embryos hatched from this female. Hatching of another female (Fig. 6E) started at night on 20 August, and a few embryos hatched over the next 4 days. This female showed type 3 hatching; characterized by a loss of rhythmicity during the first 2 days that sometimes reappears in the last 2 days. In a third female (Fig. 6F), hatching started on 5 August and lasted for 4 days. Hatching was arrhythmic for the first 3 days (type 3), and a huge number of zoeas that hatched at midnight on 8 August were released by gentle-release behavior. Immature embryos also hatched.

Hatching of five other females was suppressed (type 4). These females carried their embryos for >10 days after surgery. The hatching of two of these females is shown in Fig. 6G,H. Very few mature zoeas were sporadically released and submerged in the beaker. It was not clear whether the pattern of hatching in these five females was arrhythmic or persistent. Most embryos that remained attached to the females died during the 2-week experiment or slipped from the female without hatching (Fig. 6H).

Experiment III-3: legions of the dorsal-half of the MT

Females were collected on 25 July and 10–12 August 2000, and the dorsal-half of the MT was cut on 2 August (seven females) and on 10–13 September (five females), respectively. Seven females had started the hatching program before surgery (Table 6). No females died after surgery. In all cases, the hatching pattern was classified as type 1. Zoeas were always liberated by vigorous-release behavior and almost all of the zoeas swam. The mean deviation (± S.D.) of hatching from the nocturnal high tide was 1.7±0.9 h, suggesting that the circatidal rhythm of these females is still maintained.

Experiment III-4: legions of the ventral half of the MT

Females were collected on 25 July and 10–12 August 2000, and the ventral half of the MT was cut on 16 August (10 females). One female died after surgery, and three females had already started the hatching program before incision (Table 6). Hatching of two females (Fig. 7A,B) was not different from that of the control group (type 1). However, one of the females (Fig. 7B) took 18 days to release her larvae.

Fig. 7. Pattern of hatching after lesions were made on the ventral half of the medulla terminalis (MT; experiment III-4). The effects of the lesions were classified into four types (see Table 5 for details of each type). Parts (A)–(H) represent the pattern of hatching in individual females; data represent either the percentage of hatched zoeas (A,B) or the number of hatched zoeas (and that of embryos dropped without hatching) (C–H). Filled bars represent the number of mature, swimming zoeas; hatched bars represent the number of mature, but non-swimming, zoeas; stippled bars represent the number of embryos dropped from the female without hatching. The 24 h L:D cycle is shown by the horizontal bar at the top of each graph (the filled section represents the dark phase). SU indicates the date when the surgery was performed. *N* is the total number of zoeas hatched. The asterisks indicate the date when the optic peduncles were inspected under the stereomicroscope. In (E–G), the arrowhead indicates the time when the incubation was stopped.

As shown in Fig. 7C, hatching synchrony of one female deteriorated and was periodic for 4 days (type 2). The pattern of eight other females was classified as type 4; five of these patterns are shown in Fig. 6D–H. A few zoeas hatched only sporadically from these females. Some females dropped their embryos without hatching, during 3–8 September, while others still carried their embryos almost one month after surgery. The pattern of hatching in three other females (data not shown) was similar to those in Fig. 6D–H.

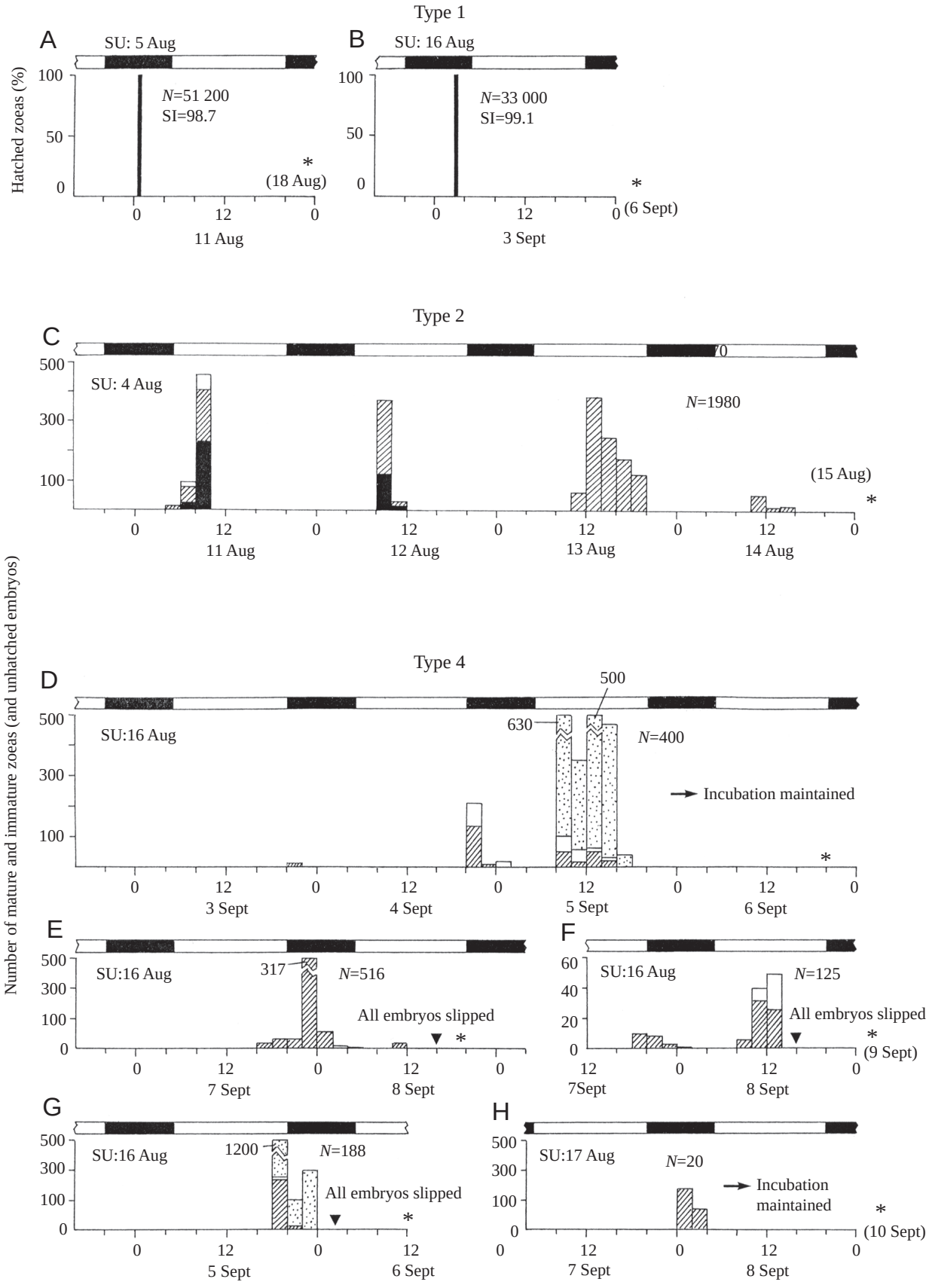
Hatching of embryos that had started the hatching program before surgery

The day of hatching is difficult to predict in *S. haematocheir*; the only indication that hatching is imminent is the brownish-green color of the embryos. Thus, some females had started the hatching program before the surgical cuts were made, and embryos of those females were all destined to hatch irrespective of surgery (Table 6). Hatching from the females in experiments II-1 (five females) and II-3 (four females) all occurred within 1.5 h, indicating that hatching and hatching synchrony were hardly affected by the removal of the optic peduncle up to the MI. Hatching in experiment III-3 (seven females) was also highly synchronous [period of hatching (*t*) ≤1.0 h], indicating that dorsal-half cuts of the MT do not affect either hatching synchrony or timing of hatching. Zoea larvae were liberated by vigorous-release behavior.

Table 6. *Hatching profiles of the females that had started the hatching program before surgery*

Experimental group	SI (*N*)			Period of hatching	Pattern of hatching
II-1	25<SI (5)			0.5≦*t*≦1.0	Highly synchronized
II-2	–			–	
II-3	25<SI (4)			0.5≦*t*≦1.5	Highly synchronized
III-1	–			–	
III-2	8.9 (1)	5.4 (1)	2.3 (1)	3.0≦*t*≦7.0	Deteriorated
III-3	25<SI (7)			0.5≦*t*≦1.0	Highly synchronized
III-4	25<SI (1)	5.6 (1)	Deteriorated (1)	0.5≦*t*≦8.0	Highly synchronized, deteriorated

SI, synchrony index; *t*, period of hatching (h).


Type 1
A
SU: 5 Aug
N=51 200
SI=98.7
(18 Aug)
11 Aug
B
SU: 16 Aug
N=33 000
SI=99.1
(6 Sept)
3 Sept
Hatched zoeas (%)
Type 2
C
SU: 4 Aug
N=1980
(15 Aug)
11 Aug
12 Aug
13 Aug
14 Aug
Number of mature and immature zoeas (and unhatched embryos)
Type 4
D
SU:16 Aug
500
630
N=400
Incubation maintained
3 Sept
4 Sept
5 Sept
6 Sept
E
SU:16 Aug
317
N=516
All embryos slipped
7 Sept
8 Sept
F
SU:16 Aug
N=125
All embryos slipped
(9 Sept)
7Sept
8 Sept
G
SU:16 Aug
1200
N=188
All embryos slipped
5 Sept
6 Sept
H
SU:17 Aug
N=20
Incubation maintained
(10 Sept)
7 Sept
8 Sept

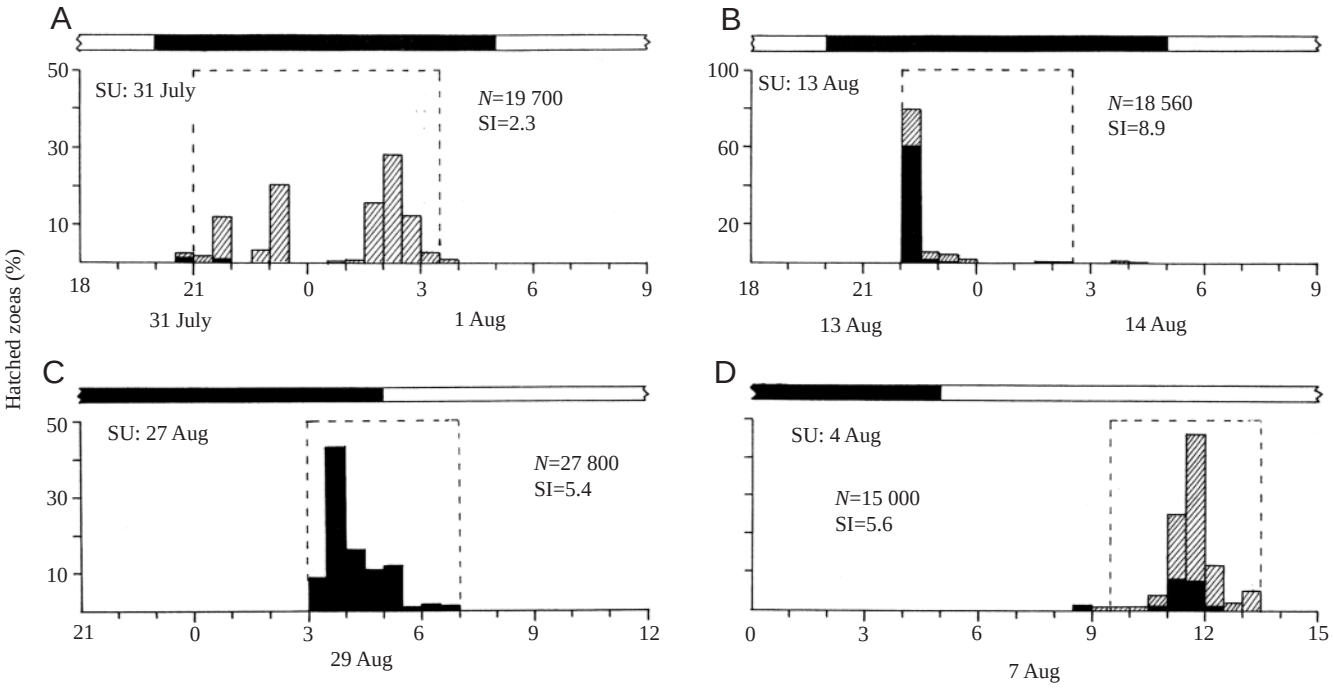


Fig. 8. Hatching profiles of four females (A–D) that had started the hatching program before surgery was performed. Filled bars represent the percentage of swimming zoeas; hatched bars represent the percentage of mature, but non-swimming, zoeas. The area surrounded by broken lines indicates 95% hatching. SU indicates the date when the surgery was performed. *N* is the total number of zoeas hatched.

In contrast, in experiment III-2 (three females), hatching synchrony of all females deteriorated. In one female (Fig. 8A), hatching lasted for one night. Almost all embryos were mature but could not swim. The SI was 2.3. In the second female (Fig. 8B), hatching was still synchronized among most zoeas, but the SI decreased to 8.9. In the third female (Fig. 8C), the SI was 5.4. The SI of these three females was similar to those of detached embryos (Table 1). Zoea larvae were released by gentle-release behavior. In experiment III-4 (three females), hatching synchrony of one female (data not shown) was maintained and zoeas were released by vigorous-release behavior. The SI of the second female was 5.6 (Fig. 8D) and hatching of the embryos attached to the female was delayed as much as 6 h from that of the embryo cluster detached on 5 August (data not shown). In the third female (data not shown), hatching lasted through the night. Females 2 and 3 liberated zoeas by gentle-release behavior.

Inspection of the success of surgical cuts under the stereomicroscope

After the experiments (at times indicated by asterisks in Figs 6, 7), both eyestalks were removed from the female, and the optic peduncles were carefully excised to determine the success of the surgery (Fig. 2H,I). This inspection was made for three experimental groups where surgical lesions were made to the MT (i.e. experiments III-2, III-3 and III-4; Table 5).

Experiment III-2 (11 females; data presented in Fig. 6)

Fig. 6A. The right peduncle was completely cut between MI and MT. The left peduncle was also cut in the lower half of the MT. *Fig. 6B.* The upper half of the MT in the right peduncle was cut at the middle region (see Fig. 3A), but the lower half seemed to be undamaged. In the left peduncle, the lower half of the MT was cut at the posterior region (Fig. 3A). *Fig. 6C.* No inspection. *Fig. 6D.* The right peduncle was almost cut at the middle region of the MT, leaving a portion of the upper half. Both MI and ME were lost in the left peduncle, but the anterior region of the MT seemed to be slightly damaged. *Fig. 6E.* In the right peduncle, the middle region of the MT was cut, leaving a portion of the upper half. In the left peduncle, the MT was cut at the middle region, but a portion of the lower half seemed to remain. *Fig. 6F.* The MT of the right peduncle was almost all cut at the middle region; only a portion of the upper half remained. The left peduncle was partially cut at the middle region; only the lower part was slightly damaged. *Fig. 6G.* In the right peduncle, the MT was completely cut at the middle region. The base of the optic peduncle (Fig. 3) was completely cut in the left peduncle. *Fig. 6H.* In the right peduncle, more than half of the MT was cut at the middle region; only a portion of the upper part remained. The base of the optic peduncle was completely cut in the left peduncle.

Experiment III-3 (five females; data presented in Table 5)

The impression of the cuts clearly remained on the dorsal half of the MT. *Female 1.* In the right peduncle, the dorsal half was cut up to the middle region of the MT, and a dark white lump appeared at the dorso-lateral region. In the left peduncle,

the dorsal half of the MT was largely cut up to the middle region. *Female 2*. In both optic peduncles, the dorsal half of the MT was cut up to the middle region. The optic peduncles of the other three females were not inspected.

Experiment III-4 (11 females; data presented in Table 5 and Fig. 7)

Fig. 7A. The MT of the right peduncle was partially damaged from the middle to the posterior region of the ventral half. The MT of the right peduncle was cut in the middle region of the ventral half. *Fig. 7B*. Damage towards the MT was not clearly visible in the right peduncle (the middle region of the ventral half may have been slightly damaged). The middle region of the ventral half was cut in the left peduncle. *Fig. 7C*. In the right peduncle, the ventral half of the MT was cut, but damage was limited to the frontal region of the MT (see Fig. 3B). A white spot (assemblage of neurosecretory cells) was seen from the opening of the tissue, and the bundle of axons reached upwards. In the left peduncle, only slight damage was seen in the ventral half of the MT, just posterior to the retina. *Fig. 7D*. In the right peduncle, the ventral half of the MT was cut in the middle to posterior region, and the whole peduncle was small in size. The left peduncle was completely cut at the base of the optic peduncle. *Fig. 7F*. In the right peduncle, the posterior region of the ventral half of the MT may have been a little damaged. No serious damage was seen in the MT, at least in appearance. The left peduncle was cut at the base of the optic peduncle. *Fig. 7G*. In the right peduncle, lesions were seen in the posterior region of the ventral half of the MT. In the left peduncle, the ventral half of the MT was cut. *Fig. 7H*. In both optic peduncles, the ventral half of the MT was slightly cut in the middle to posterior regions.

Inspection of the optic peduncles was also carried out in the females that had started the hatching program before surgery (Table 6). *Experiment III-2* (three females). *Fig. 8A*. In the right peduncle, the MT was completely cut in the posterior region. In the left peduncle, lesions were seen in the posterior region of the ventral half of the MT. *Fig. 8B*. The right peduncle was completely cut in the frontal to middle region of the MT. The left peduncle was also severely damaged in the middle region of the MT, but the ventral half of the MT appeared to partially remain without damage. *Fig. 8C*. No inspection. *Experiment III-3* (seven females). *Female 1*. The dorsal half of the MT was cut in the middle region in the right peduncle, but no serious damage was seen in the left peduncle. *Female 2*. The dorsal half of the MT was clearly cut in the middle to posterior regions in both optic peduncles. *Female 3*. The dorsal half of the MT was clearly cut in the middle to posterior region in both peduncles. *Females 4–7*. Lesions were much the same as in Females 2 and 3. *Experiment III-4* (three females). *Female 1* (SI>25). In the right peduncle, the ventral half of the MT was clearly cut in the middle to posterior regions. In the left peduncle, the ventral half of the MT was cut in the posterior region. *Female 2 (Fig. 8D)*. No clear damage was seen in either optic peduncle, at least in appearance. *Female 3*. No inspection.

Discussion

Hatching of *S. haematocheir* embryos shows a tidal rhythm, and the timing of hatching is controlled by an endogenous clock or pacemaker in the female. This study focused on the localization of the circatidal clock in the optic peduncle of the eyestalk in the female. The treatments and their effects on hatching and hatching synchrony are summarized in Table 7. ‘No effects’ indicates that neither hatching nor hatching synchrony were different from those of the control group (embryos attached to the female in Table 1 and Fig. 4A,B). In these females, hatched larvae were

Table 7. *Summary of the treatments and their effects on hatching*

Treatment (Expt no.)	Effects on hatching and hatching synchrony	Results
Expt I. Eyestalk ablation		
One eyestalk (I-1)	No effects	Table 2
Both eyestalks (I-2)	Suppression of hatching	Table 2
Expt II. Removal of the optic peduncle		
Removal of the compound eye–retina complex (II-1)	No effects	Table 3; Fig. 5
Removal up to the medulla externa (II-2)	No effects	Table 3; Fig. 5
Removal up to the medulla interna (II-3)	No effects (deterioration of hatching synchrony in two females)	Table 3; Fig. 5
Expt III. Lesions of the medulla terminalis		
Control (III-1)	No effects	Table 5
Transverse cut (III-2)	Suppression of hatching or deterioration of hatching synchrony	Table 5; Fig. 6
Dorsal-half cut (III-3)	No effects	Table 5
Lower-half cut (III-4)	Suppression of hatching or deterioration of hatching synchrony	Table 5; Fig. 7

See Table 6 for females that had started the hatching program before surgery.

liberated by vigorous-release behavior. Synchrony with the nocturnal high tide was also maintained in these females. Furthermore, hatching and hatching synchrony of five females in experiments III-2 and III-4 (Figs 6A–C, 7A,B) were much the same as those of the control group. Close correlation with the time of hatching to nocturnal high tide (see type 1 in Table 5) suggests that the circatidal rhythm of these females is also maintained.

It is clear from Table 7 that the transverse lower-half lesions of the MT caused severe effects on hatching and hatching synchrony. The same results were obtained from females that had started the hatching program before surgery (Table 6). However, these results do not indicate that the circatidal clock is abolished if the hatching synchrony deteriorates (e.g. Figs 6D–F, 7C). A central question arises as to the specific role of the MT in the maintenance of hatching synchrony. Another concern is the mechanism by which hatching and hatching synchrony are accomplished under the control of the circatidal pacemaker.

In this paper, I would first state that the timing of hatching is controlled by a single clock (i.e. a circatidal clock) or not by a circadian rhythm manifested in non-tidal conditions in the laboratory, in order to clearly show the endogenous circatidal rhythm that is the objective in this study. The role of the MT and enhancement of hatching by the circatidal clock are discussed below.

The internal pacemaker controlling hatching: a classic problem of one or two

The complexity of most circatidal rhythms is synchrony not only with the tidal cycle but also with the day/night cycle (e.g. Saigusa, 1981, 1982; Saigusa and Akiyama, 1995). Synchrony with both day/night and tidal cycles has long been interpreted in terms of an interaction between two internal clocks: i.e. the circadian and the circatidal clocks (Palmer, 1995). The circatidal rhythm of *S. haematocheir* is easily phase-shifted by a 24 h L:D cycle; one feature is that the magnitude of phase-shift clearly corresponds to that of the phase-shift of the L:D cycle (Saigusa, 1986, 1992a). It is clear that the 24 h L:D cycle is one of the entraining agents of the circatidal rhythm. However, this does not imply that the circatidal rhythm is governed by two kinds of internal clock. The two-clock hypothesis is only an ‘interpretation’ of the timing system proposed on the basis of manifestation of the activity patterns monitored in the laboratory. If this hypothesis is tested with elaborate experiments, we would notice that no positive evidence supports that two kinds of endogenous rhythm are present simultaneously in individual animals. A single clock is enough to explain synchrony with the nocturnal high tide. The property of *S. haematocheir* circatidal rhythm could be explained in terms of ‘oscillators’ (α and β oscillators): the ‘subsystem’ of the circatidal clock (Saigusa, 1986, 1988; see also Pittendrigh, 1960, 1981). In brief, the timing system of the endogenous circatidal rhythm is very similar to that of the circadian rhythm. The circatidal rhythm responds to tide-correlated cycles or moonlight cycles, as well as to 24 h L:D cycles, and, accordingly, the role and the action of each oscillator would be somewhat different from that of the circadian rhythm (for *S. haematocheir*, see Saigusa, 1988).

A variety of behavioral and physiological events show a circadian rhythm in the optic peduncle of the eyestalk in many crustaceans; e.g. retinal structure (Barlow et al., 1977, 1987; Arikawa et al., 1987) and electroretinogram (ERG) amplitude (Aréchiga and Wiersma, 1969; Bryceson, 1986; Aréchiga et al., 1993). Many events in the retina of *S. haematocheir* may also be modulated by the circatidal clock. But the circatidal rhythm of *S. haematocheir* was not affected by the removal of the optic peduncle from the compound eye–retina complex to the MI (Table 3). This may suggest that the circatidal pacemaker (α and β oscillators) that controls both hatching and hatching synchrony is not located in the region of the optic peduncle from the compound eye to the MI.

Role of the MT in the control of hatching and hatching synchrony

In intact females, highly synchronized hatching was followed by vigorous-release behavior. However, when the hatching synchrony deteriorated, zoea larvae were liberated by gentle-release behavior. So, one could speculate that, although all the embryos actually hatch synchronously, the vigorous-release behavior is lost as a result of lesions of the MT, causing deterioration of hatching synchrony. This hypothesis, however, cannot explain why the hatching is suppressed by lesions of the MT. In addition, if hatching is highly synchronous, zoea larvae should be released into the water even by gentle-release behavior, possibly within 1 h. So, lesions of the MT must have directly caused deterioration of hatching synchrony or suppression of hatching.

The morphology of the optic peduncle of *S. haematocheir* is much the same as that of other crabs; neurosecretory cells are located in four clusters, at the edge of the ME (Fig. 2F), in the X organ (Fig. 2J), between the ME and the MI, and on the dorsal region of the MT. Neurons are distributed all over the optic peduncle (Fig. 2J). They are localized and form a mass in the MT; one cluster in the frontal part (N1) and another cluster next to the X organ (N2) (Fig. 2K). The major bundle of axons of neurosecretory cells runs from the X organ at the posterior region of the ventral half of the MT to the dorsolateral region, towards the sinus gland (Enami, 1951; Andrew and Saleuddin, 1978; Andrew et al., 1978; Jaros, 1978). Furthermore, large blood vessels are present on the dorsal surface of the MT and the MI (Sandeman, 1967; Govind, 1992).

Removal of the MI and dorsal-half cuts of the MT (experiment III-3 in Table 5) may have caused damage to blood vessels, because lesions often caused a large amount of bleeding. Not only the sinus gland but also the cluster of neurosecretory cells at the dorsal region of the MT may have been damaged by this operation. Nevertheless, as hatching was not affected by this operation (Tables 5, 6), we could speculate that hatching is not controlled through the X organ–sinus gland system.

In both experiments III-2 and III-4, only three out of 14 females had started the hatching program before surgery was performed (Table 5). But in experiment III-3, seven out of 12 females started the hatching program before surgery. Hatching of five females occurred on the night after surgery, while hatching of two other females occurred one day after the operation. The possibility that dorsal-half cuts of the MT advance the date of hatching is suspected. Hatching is induced through a special developmental process (hatching program) that lasts 48–49.5 h (Saigusa, 1992b, 1993). This program would be initiated around the time of the nocturnal high tide two nights before hatching. It is not plausible that the interval of hatching program is reduced to several hours or one day. Females were randomly chosen for each experiment and, therefore, females that had already started the hatching program would have been chosen by chance.

Lesions made to the ventral half of the MT (experiment III-4) were observed from the middle to the posterior regions (Fig. 2J, white lines). Neurons are distributed in two areas in the MT. One cluster is localized from the frontal to the lateral region of the ventral half (N1) while the other is localized close to the X organ (N2) (Fig. 2J,K,L). The bundles of neuronal axons are tangled and occupy a large area of the MT. The surgery, especially the lesions made towards the ventral half of the MT, would have cut the bundle of neuronal axons tangled in the ventral half of the MT. It seems that most of the neuronal axons generate from the cluster N1 (see Fig. 2J). So, if the circatidal clock is present in the MT, N1 is the possible location. Suppression of hatching, or only sporadic hatching (Fig. 7D–H), may be caused by completely cutting through the axon bundles related to hatching. The periodic hatching (Fig. 7C) may be caused by incomplete incision of these axon bundles. These speculations suggest that ventral-half cuts would not damage the function of the circatidal pacemaker. In contrast, arrhythmic patterns (especially Fig. 6F) may be evidence to suggest that the clock neurons are actually located in the MT.

In *Limulus polyphemus*, the circadian pacemaker has also been suggested to be located in the brain (Barlow et al., 1977). This study did not report the effects of lesion of the brain. (Lesions of the brain severely affected locomotion, which made it difficult to monitor the hatching itself.) If the circatidal pacemaker is assumed to be located in the brain, neurons in the MT would only function to induce hatching. The light information (day/night and moonlight cycles; see Saigusa, 1988) would be transferred to the circatidal pacemaker via the retina or via extra-retinal photoreceptors (e.g. Hanna et al., 1988). If the pacemaker is located in the brain, lesions on the ventral half of the MT (Table 5) would have cut the bundles of neuronal axons from the circatidal pacemaker located in the brain to the neurons inducing hatching in the MT, causing deterioration or suppression of hatching. However, if hatching and hatching synchrony are generated via the X organ–sinus gland system, this possibility may be reasonably supported. However, the present study supports the possibility that hatching is induced via the neuronal pathway. It is difficult to answer why hatching is induced by neurons located in the MT. It seems reasonable to speculate that the circatidal pacemaker is located in the cluster of neurons located in the MT (possibly N1).

Enhancement of hatching synchrony

When the embryo cluster is detached from the female two or more nights before larval release and is maintained in the water with aeration, no embryos hatch (Fig. 4). The critical period of inducing hatching is 48–49.5 h before larval release (i.e. hatching of the embryos attached to the female), and this period corresponds to the time of high tide two nights before larval release (Saigusa, 1992b). So, I speculate that the embryos have a special 48-49.5 h developmental process of hatching called the ‘hatching program’, and that this program is triggered by the circatidal pacemaker (Saigusa, 1993). If the embryos are detached from the female after initiation of this program, they are sure to hatch (Fig. 4, middle and bottom panels). On the other hand, no embryos would hatch if they are detached before initiation of this program (Table 1).

Although hatching of the embryos attached to the female is highly synchronous, hatching synchrony of detached embryos deteriorated (Table 1; Fig. 4). So, the female must enhance the hatching synchrony by an, as yet unidentified, factor that finally determines the time of hatching. Hatching synchrony deteriorated in some females that had already started the hatching program before surgery (Table 6; experiments III-2 and III-4). In these females, the hatching-synchrony-enhancing stimuli may have been lost as a result of the surgery, and the pattern of hatching (Fig. 8) may have been similar to that of detached embryos (Fig. 4). On the other hand, upper-half cuts of the MT (experiment III-3) did not affect the hatching synchrony (Table 6). These results suggest that hatching synchrony is governed by the same pacemaker that induces hatching.

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