[REVIEW]

Hatching and Its Control by the Circatidal Clock in Marine Crustaceans

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The newly oviposited eggs of most decapod crustaceans are encased by transparent layers, clustered on the ovigerous hairs of the female by a stalk (the funiculus), and ventilated by movements of the pleopods beneath the abdomen of the female (Herrick, 1909; Yonge, 1937, 1946, 1955; Cheung, 1966; Fisher and Clark, 1983). With the completion of embryonic development, hatching occurs and the larvae are released into the water by the fanning movements of the female (Burkenroad, 1947). In intertidal and estuarine crabs, hatching occurs at the time of high tide at their habitat (Saigusa and Hidaka, 1978; DeCoursey, 1979; Bergin, 1981; Saigusa, 1981, 1982; Forward *et al.*, 1982; De Vries and Forward, 1989, 1991a).

Each ovigerous female carries a number of embryos (*e.g.*, about 10,000-50,000 for an estuarine terrestrial crab, *Sesarma haematocheir*), and releases all of the larvae at the same time with the fanning motion. Hatching occurs just before the release; *i.e.*, the stimulus of hatching induces the release behavior (Saigusa, 1992a). For the release of all of the larvae into the water at one time, the hatching must be highly synchronized among the embryos. In addition, since the larval release is carried out at the time of high tide, the hatching must also be synchronized with the high tide.

The mechanisms of hatching in crustaceans have been the subject of controversy for many years (Yonge, 1937, 1946; Burkenroad, 1947; Clegg, 1964; Davis, 1968, 1981; Hall and MacDonald, 1975; Trotman *et al.*, 1980; De Vries and Forward, 1991b). The process of embryonic envelope formation and the attachment of the embryos to the maternal ovigerous hairs in decapod crustaceans have also been subjects of much discussion (*e.g.*, Herrick, 1895; Andrews, 1906; Yonge, 1937, 1946, 1955; Mawson and Yonge, 1938; Linder 1960; Suko, 1961; Cheung, 1966; Fisher and Clark, 1983; Goudeau and Lachaise, 1980, 1983; Goudeau *et al.*, 1987; Hinsch, 1990; Talbot and Demers, 1993). Several experiments have demonstrated that the timing of hatching follows a so-called 'biological clock' (Foward and Lohmann, 1983; Saigusa, 1986, 1992b, 1997; Saigusa and Kawagoye, 1997; De Vries and Forward, 1989, 1991a). The control of hatching by endogenous clocks of the embryos and/or the mother is an exciting new subject in the field of crustacean behavior and reproduction.

This review presents the currently available information on the structure of the embryonic envelope and the attachment of embryos to the ovigerous hairs, the mechanisms of hatching, and the control of hatching by the circatidal clock in intertidal and estuarine crabs. Hatching in a variety of animals is well known to be induced by hatching enzymes, and the relevant information on hatching enzymes in marine crustaceans is discussed. The differences in hatching between crustaceans and other animals are also described. Much attention has recently been focused on the results of embryo exchange experiments, which have revealed extensive new information on the role of endogenous clocks in the control of hatching. On the basis of those findings, a possible mechanism of the control of hatching by the circatidal clock is proposed.

1. Structure of the embryonic envelope and formation of the funiculus in crustaceans

A female intertidal or estuarine crab has four pairs of abdominal appendages, each of which bears one plumose and one non-plumose (ovigerous) seta (Fig. 1A). Many fine hairs (ovigerous hairs) are arranged in whorls along the non-plumose seta (Fig. 1B). As shown in Figure 1C, D, fertilized eggs are attached to these hairs by a funiculus and incubated by the female until hatching (*i.e.*, breakage of the embryonic envelope) occurs.

The embryonic envelope formation and attachment to ovigerous hairs in decapod crustaceans were first described by Yonge (1937, 1946) in the lobster *Homarus vulgaris*. Yonge noted that the embryonic envelope of crustaceans consists basically of two layers; an inner layer of 'chitin' secreted by the walls of the oviduct (oviducal epithelium), and the outer layer of 'cuticle' secreted by the cement glands (tegumental glands) in the pleopods of the female (Yonge, 1937). This 'cement' has properties identical to those of the superficial layer of the integument or epicuticle formed by similar tegumental glands throughout the body in the iso-electric point,

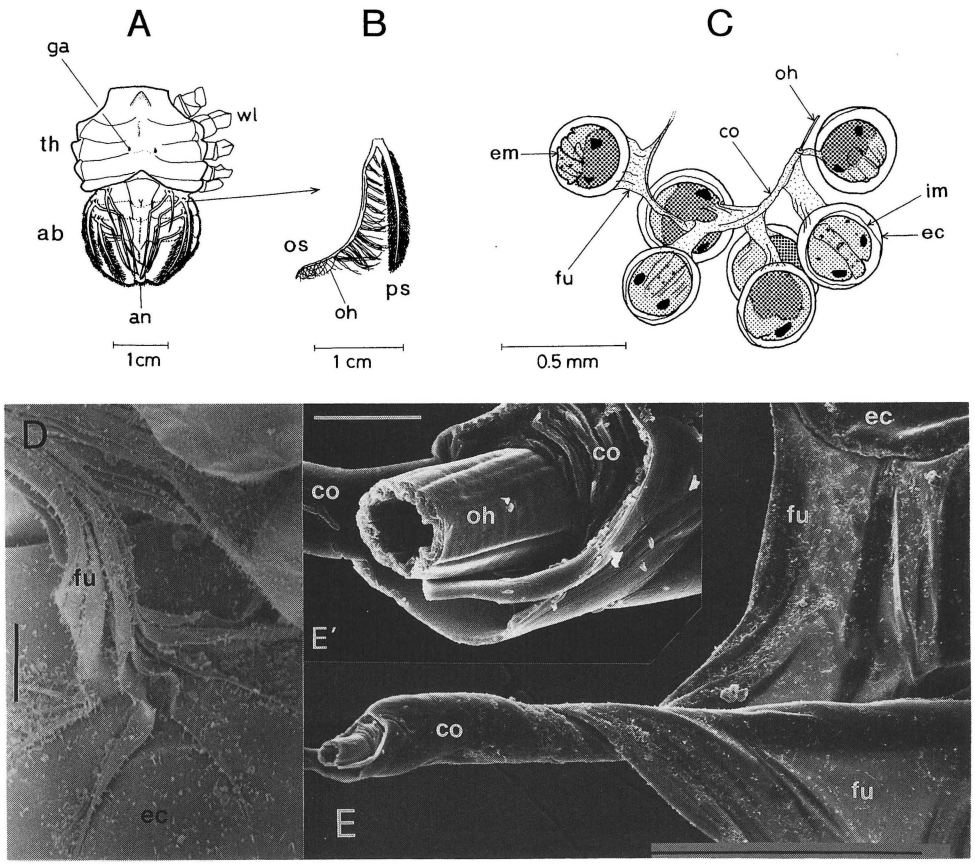


Fig. 1 The setae and attachment of embryos in an estuarine terrestrial crab, *Sesarma haematochier*. A. Thorax (th) and abdomen (ab) of a female. ga: genital aperture, wl: walking leg, an: anus. B. The second abdominal appendage showing plumose (ps) and ovigerous setae (os). oh: ovigerous hairs arranged in whorls on the ovigerous seta. C. Embryos attached to an ovigerous hair (oh) by a funiculus (fu). The specimen was fixed with 5% formalin and observed in 50% EtOH. em: embryo, co: coat wrapping an ovigerous hair, ec: embryonic capsule, im: inner membrane. The term 'inner membrane' is that of Yonge (1937). D. Funiculus (fu) connecting embryonic capsule (ec) and the coat investing an ovigerous hair. Bar = 10 μm. E. The coat (co) wrapping an ovigerous hair. Bar = 100 μm. E'. Magnification of the portion where the ovigerous hair (oh) penetrates the coat. Bar = 10 μm. (After Saigusa, 1994).

staining and chemical reactions, and in the presence of lipid (Yonge, 1937, 1946). The same two-layer structure was observed in the embryonic envelope of other crustaceans, *e.g.*, Anostraca (Mawson and Yonge, 1938) and Caridia (Yonge, 1955). Stephens (1952) reported a correlation between the developmental state of the cement glands and the degree of oocyte development in the uropods of the crayfish *Cambarus*. This correlation may indirectly support Yonge's hypothesis.

A contrasting theory was presented by Cheung (1966) who studied the embryonic envelope of the shore crab *Carcinus maenas*. As Yonge (1937) indicated, the embryonic envelope consists of two layers observable under a light microscope; *i.e.*, the thick outer layer and the inner thin layer. Cheung (1966) indicated that the outer layer of *C. maenas* consisted of three sub-layers called 'trichromatic membranes' that are histochemically distinct from each other and not be similar to the epicuticle. As shown in Figure 2A, the outermost sub-layer (layer 1) is derived from the vitelline membrane; the second (layer 2) is formed through solidification of a fluid produced from the egg itself upon ovulation; and the third (layer 3) is formed by a highly 'periodic acid–Schiff reaction (PAS)' positive substance produced from the fertilized egg. With the progress of embryonic development, one or two chitinous layers are added to the inside of the trichromatic membrane (layers 4 and 5, in Fig. 2A). Since these two layers appear after the trichromatic membrane has been formed, they cannot have been formed by epithelial cells in the oviduct. Cheung thus concluded that all the layers of the embryonic envelope are formed by the ovum as a consequence of fertilization. Moreover, no pleopodal 'cement glands' associated with oviposition were found, and Cheung thus proposed that the function of these glands was not related to the envelope formation.

On the fine structure, the embryonic envelope of a terrestrial crab, *Sesarma*, consists of three distinctive layers and two inner layers (Fig. 3A, B). The outer three layers correspond to the trichromatic membranes observed by Cheung (1966) under the light microscope, although they did not correspond exactly to each sub-layer. The outermost layer (L_1) is further divided into two sub-layers. Underneath the L_1 layer, the thickest middle layer (L_2) is found. This layer has a uniform structure, and is delineated with an electron-dense over-

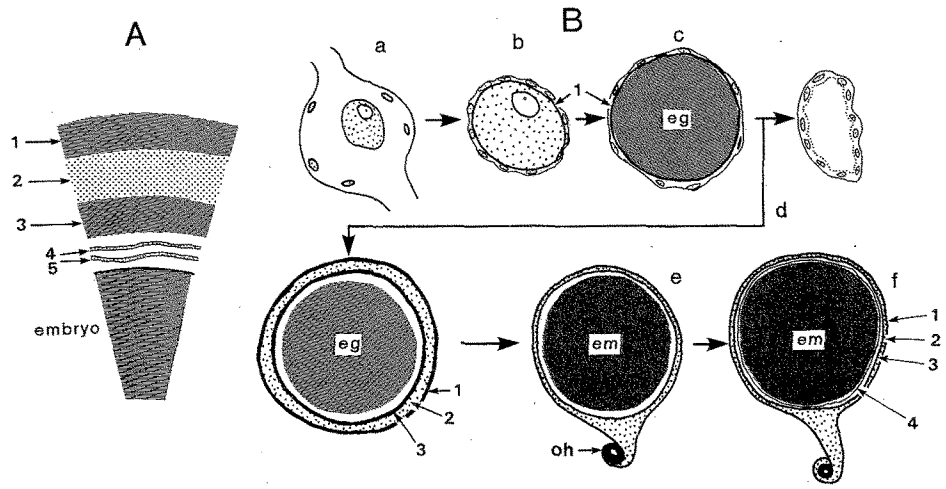


Fig. 2 Structure and formation of the egg capsule in an intertidal crab, *Carcinus maenas*. A. A hypothetical structure of the embryonic envelope. Layers 1, 2 and 3 show the outer envelope, called the 'trichromatic membrane'. Layers 4 and 5 are chitinous membranes that are formed internal to the trichromatic membrane as the embryonic development proceeds. B. A possible mechanism of the envelope formation and embryo-attachment to an ovigerous hair. Stages of egg maturation and embryonic development are shown in a–f. a: early oocyte in immature ovary, b: follicular nuclei surrounding oocyte, c: mature egg in ovary, d: empty follicle and newly oviposited, fertilized egg, e: formation of funiculus, f: embryo in which development is in progress. (Re-drawn from Cheung, 1966).

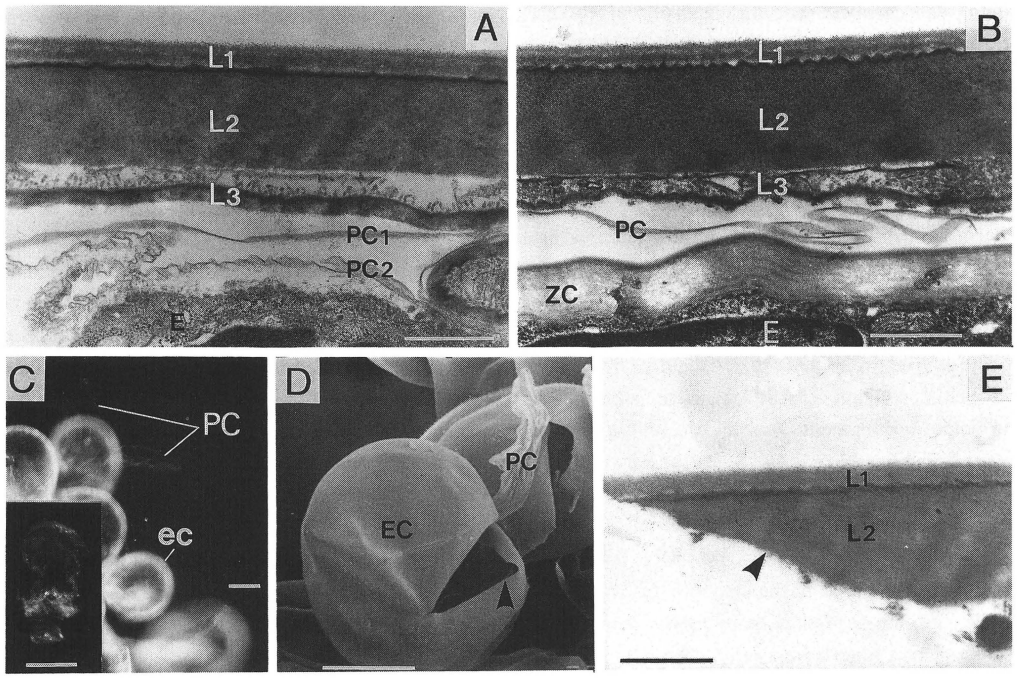


Fig. 3 Embryonic envelope before and after hatching in *Sesarma haematocheir*. A. Fine structure of the envelope 10 days before hatching. L_1, L_2, and L_3 show the outer, middle, and inner layers, respectively. In additon to these layers, two very thin layers (PC_1 and PC_2) are observed between the envelope and epidermal cells of the embryo (E). These layers are not present at the earlier stage of development, and appear to have been detached from the embryonic exoskeleton. Thus, they could be the so-called prezoeal cuticle. Bar = 1 μm. B. The envelope 7 h before hatching. Structural changes are not found on the outer (L_1) and middle (L_2) layers at all, but the inner layer (L_3) becomes thinner than that in A. One of the prezoeal cuticles (probably PC_2) was missing in this section. The embryo (E) is covered with a thick zoeal cuticle (ZC). Bar = 1 μm. C. A light micrograph of the empty egg capsules (ec) remained on the ovigerous setae after hatching and larval release. Prezoeal cuticles (PC) protrude from the embryonic capsule upon the liberation of hatched zoea. Inset: prezoeal cuticle cast off by a zoea. The upper half of this cuticle invested the dorsal side of thorax, and the lower half covered the ventral side of the thorax and appendages. Bar = 0.5 mm. D. A scanning electron micrograph showing broken embryonic capsules associated with hatching. PC: prezoeal cuticle protruding with the liberation of hatched larvae. Bar = 100 μm. E. A section of the broken edge of the embryonic envelope after zoeas hatched out. The arrowhead indicates no sign of dissolution of the outer and middle layers. Bar = 1 μm. (Saigusa, original).

lay, but is firmly attached to the outermost L_1 layer except at the sites where the outermost layer and the funiculi meet. There is a thin and electron-dense layer (L_3: inner layer) generally inside L_2; this inner layer is often observed as separated from L_2, and fine granular materials sometimes fill the space between it and L_2. The outer surface of L_3 is also delineated with a thin membraneous structure. This structure was also observed in an intertidal crab, *Hemigrapsus sanguineus* (unpublished data). Goudeau and Lachaise (1983) reported that the embryonic envelope of the shore crab *Carcinus maenas* is the same as that seen in Figure 3A, B. This structure may thus be common among the egg capsules of intertidal and estuarine crabs.

As shown in Figure 3A, B, the outer layer (L_1) is further divided into two sub-layers. Goudeau and Lachaise (1980) demonstrated that the *Carcinus maenas* oocyte is enclosed by two superimposed vitelline mem-

branes, and they suggested that the outer layer is formed by these two membranes after the material of the inside sub-layer (they called '1b') undergoes remarkable structural modifications. They found that a new material appears inside these sub-layers after the oviposition, and successively forms other inner layers of the envelope. These sub-layers are also observed in the embryos of the lobsters *Homarus americanus* and *H. gammarus* (Goudeau *et al.*, 1987). An ovulated oocyte is wrapped with a single coat (envelope) composed of these two sub-layers, both of which appear to originate in follicle cells, and this envelope becomes sticky after the oocyte passes through the gonopore and is exposed to seawater. When the oocyte is fertilized after passing through the gonopore, a second envelope (L_2 in Fig. 3A, B) forms between envelope 1 (L_1 in Fig. 3A, B) and the fertilized egg, after a complex cortical reaction (Talbot and Goudeau, 1988). Following the ovulation of fertilized eggs, the female beats the pleopods vigorously, which probably causes envelope 1 to stretch and form the funiculus. The funiculus of *Homarus* folds around an ovigerous hair, forming many turns. For the innermost coiling turn, the funiculus wall adheres tightly to the hair surface, although no adhesive substance is detectable. The beating of the pleopods probably causes this coiling structure (Goudeau *et al.*, 1987).

Ultrastructural studies of the envelope formation in marine decapod crustaceans (Goudeau and Lachaise, 1983; Goudeau *et al.*, 1987; Talbot and Goudeau, 1988) supported Cheung's (1966) hypothesis that the funiculus is also formed from a coat laid down in the ovary. In addition, their ultrastructural studies did not find that the pleopod tegumental gland secretions form either the funiculus or a new envelope wrapping the egg, as was suggested by Yonge and his coworkers (Yonge, 1937, 1946, 1955; Mawson and Yonge, 1938). It was revealed that pleopod tegumental glands are not related to the formation of the embryonic envelope. Embryos of marine crabs are further covered with two thin layers (PC_1 and PC_2). These layers are not found in the newly oviposited embryos; they appear in the embryos as the embryonic development proceeds (Goudeau and Lachaise, 1980). Thus, they would be deposited by the embryos during their development. These two layers would clearly correspond to the inner thin layers (layers 4 and 5 in Fig. 2A) described by Cheung (1966). On the other hand, the 'inner layer' described by Yonge (1937), that swells just before hatching (Fig. 4), may not be these layers, but layer L_3 on the fine structure (Fig. 3A, B). This problem has not been settled.

Fisher and Clark (1983) also studied the embryonic envelope formation with an estuarine shrimp, *Palaemon macrodactylus*, and observed that the envelope is formed by material originating within the pleopods, and that the same material makes up the funiculus. A precursor substance is stored in packets along the basipodites of the female pleopods. These packets (mucilage packets) are found prior to ecdysis, and coin-

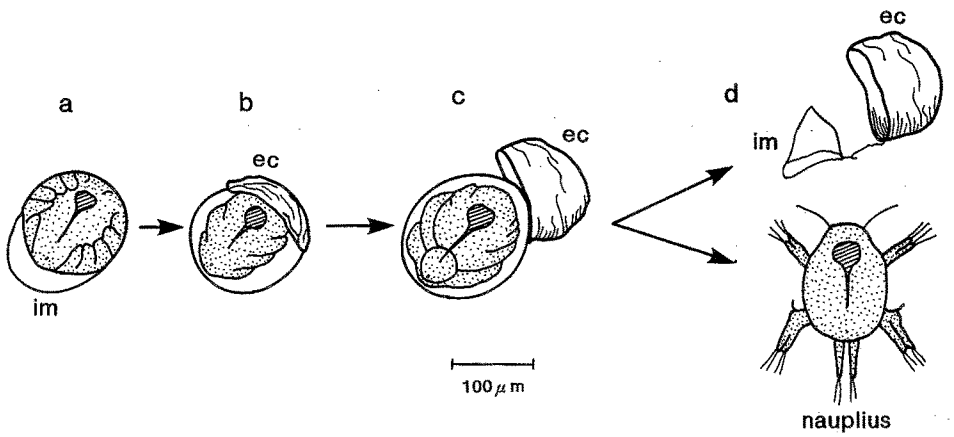


Fig. 4 Hatching of a freshwater copepod, *Cyclops viridis*. a. Initiation of hatching in which the inner membrane (im) is bulging out. b. Breakage of the egg capsule. c. Emergence of the embryo still covered with the inner membrane. d. Rupture of the inner membrane and swimming of the nauplius. (Re-drawn from Marshall and Orr, 1954).

cide with the appearance of adhesive material on the external surfaces of the pleopods. These observations do not support the hypotheses proposed by Cheung (1966) and Goudeau and Lachaise (1980, 1983) in that fertilization is not required for the attachment of the embryos to ovigerous hairs, and that at the least the surface layer of the embryonic envelope is produced by material secreted from female pleopods, not from the eggs themselves.

Regarding the embryo attachment system of intertidal and estuarine crabs, there is a finding not previously reported; *i.e.,* the funiculus is connected to the ovigerous hairs not directly, but indirectly. The attachment is accomplished with the '*investment coat*' that I named (Saigusa, 1994). The investment coat is composed of the same materials that make up the funiculus and the outer surface of the embryonic envelope (*i.e.,* L_1 in Fig. 3A, B). In terrestrial crabs, ovulation is obviously associated with a large quantity of mucus fluid that becomes sticky within 30 min (unpublished observation). This observation supports the hypothesis that the embryo attachment system of crabs is at least partially responsible for the materials secreted by the female. An acceptable general theory of the embryo attachment system in decapod crustaceans is necessary, and merits further investigation.

2. Hatching mechanisms in crustaceans

It has long been known that hatching is induced by the digestion of the embryonic envelope in a variety of animals; *e.g.,* insects (Slifer, 1937), sea urchins (Ishida, 1936; Barrett and Edwards, 1976), ascidians (Berrill, 1929; Hoshi and Numakunai, 1981), fishes (Ishida, 1944; Yamagami, 1972; Hagenmaier, 1974; Yamamoto *et al.,* 1979), amphibians (Carroll and Hedrick, 1974; Katagiri, 1975), and mammals (Perona and Wassarman, 1986; Sawada *et al.,* 1990). The digestion of the envelope is generally due to the proteinases secreted by the embryos, *i.e.,* hatching enzymes. A number of studies have tried to purify and characterize these enzymes (for reviews, see Barrett and Edwards, 1976; Yamagami, 1988; Yamagami *et al.,* 1992), and more elaborate purifications and characterizations have recently succeeded in sea urchin blastula (Lepage and Gache, 1989; Roe and Lennarz, 1990; Nomura *et al.,* 1991) and the embryos of a teleost (Yasumasu *et al.,* 1989a, b).

In contrast, many studies dealing with the hatching of crustaceans suggest a different mechanism: the embryonic envelope is not dissolved by an enzyme, but is ruptured by an increasing internal pressure due to osmotic effects (Yonge, 1937, 1946; Burkenroad, 1947; Marshall and Orr, 1954; Davis, 1959, 1964; Clegg, 1964; Anderson and Rossiter, 1969; Hall and MacDonald, 1975; Trotman *et al.,* 1980) or due to the swelling of the embryo itself (Saigusa, 1992a). For example, the hatching of copepods takes place according to the same general plan (Marshall and Orr, 1954). The embryos are wrapped by two kinds of layer; a rigid outer layer, and a very thin inner layer, and these layers are not distinguished until the hatching occurs. At the end of the embryonic development, the embryo changes color; this is the first sign of hatching. At the time of hatching, first the outer layer cracks, and then the inner layer swells (Fig. 4). The swelling of the inner layer occurs very quickly in *Cyclops* and gradually in *Calanus*. The inner layer then ruptures and the nauplius hatches. The breakage of the embryonic envelope was accounted for by the hypothesis that a sudden increase of excretion by the embryo leads to an increased volume of environmental water as a result of osmotic effects (Marshall and Orr, 1954). A similar hatching mechanism has been reported in *Haplostomella*, a parasite of the ascidians (Anderson and Rossitor, 1969), and in the fresh-water copepods (Davis, 1959). The question remains, however, as to what kind of substance induces the swelling of the inner membrane.

In the brine shrimp *Artemia salina*, the embryo is encased with two thin layers and a thick capsule (the egg-shell), and at hatching the outer layer and capsule break and the nauplius emerges from the capsule, still covered by the inner layer (Nakanishi *et al.,* 1962; Morris, 1971; Trotman *et al.,* 1980). With incubation, the cysts (*i.e.,* the embryos enclosed by the capsule) begin to produce glycerol from trehalose (Clegg, 1962). This substance is highly concentrated in the nauplius emerging from the capsule, and then rapidly appears in the incubation medium (Fig. 5). Clegg (1964) thus speculated that glycerol must be stored in the extra-embryonic space inside the bulging inner layer, and would increase the osmotic pressure, breaking the thick outer capsule. As shown in Figure 5, the production of the glycerol was closely related to the external osmotic pressure, which would enable the embryos to hatch at the same time with different concentrations of NaCl. Clegg's

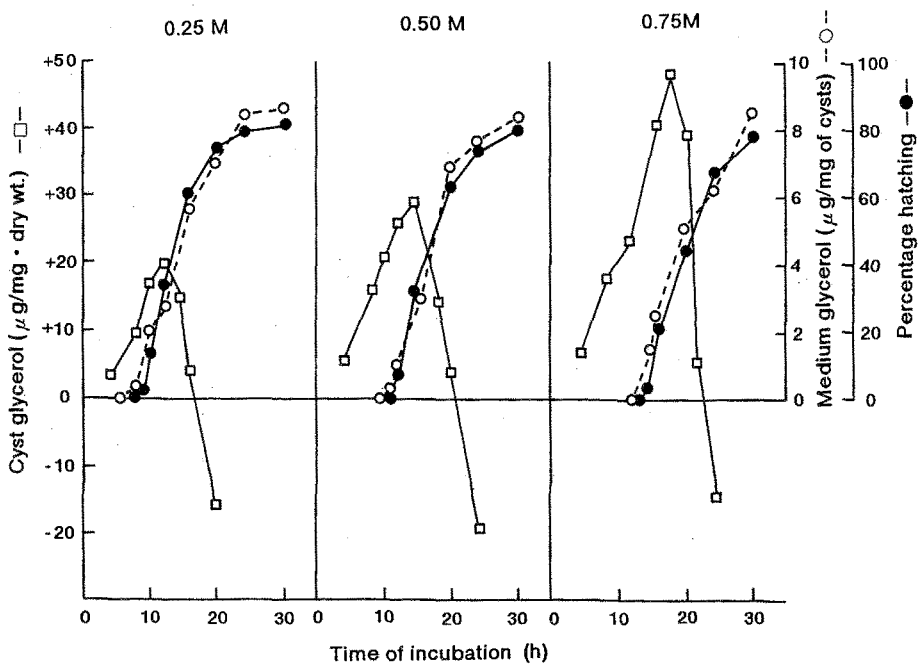


Fig. 5 Synthesis of glycerol in *Artemia salina* cysts and release outside the embryos. Time course of the concentration of glycerol in the cysts (－□－), the level of glycerol in the medium within the egg capsule (－－○－－), and percentage of emergence of nauplii (－●－), in relation to different concentrations of NaCl in the incubation medium. (Re-drawn from Clegg, 1964).

hypothesis was supported by Hall and MacDonald (1975).

In contrast, the hatching of decapod crustaceans is not associated with the swelling of the thin inner layer (*e.g.*, Davis, 1964, 1965). In these animals, for example, *Homarus* and *Palaemonetes*, no fluid-filled spaces appear between the embryo and the inner layer prior to the bursting of the outer layer, and Davis (1968) felt that the pressure for bursting is derived from the swelling of the embryo itself, as a result of an osmotic effect.

In the terrestrial crab *S. haematocheir*, fertilized eggs are at first dark brown (200 × 280 μm in diameter) because of the accumulation of yolk. When the hatching approaches, yolk rapidly diminishes and the color of the embryos turns brownish green. At this stage, well-formed compound eyes, chromatophores, and globules of orange yolk are clearly visible through the transparent embryonic envelope under a stereomicroscope. The size of the embryos averaged 340 × 320 μm in diameter. Occasional movements of the body and heartbeat are observable. Swelling, if any, was slight as hatching was imminent. The abdomen of the embryos curled ventrally before the hatching, and no fluid-filled space could not be observed in any embryo. Hatching occurred with the sudden rupture of the embryonic envelope; no motion of the body was observed at that time (Saigusa, 1992a).

Yang (1977) observed embryonic development and hatching of nauplii in a copepod, *Paracalanus crassirostris*, and he also found that the outer layer was ruptured without bulging out of the inner thin layer. This observation suggests that swelling of the inner layer is induced immediately *as a result of* the breakage of the outer layer. If so, the hatching of crustaceans would not be explained in terms of an increasing pressure inside the inner layer.

3. Hatching of decapod crustaceans and larval release in synchrony with environmental cycles

In Crustacea, when the embryonic development is complete, the embryonic envelope breaks, and the lar-

vae hatch. These larvae are immediately liberated into the water with a special fanning motion of the female's abdomen. Larval release is generally a short-timed event in many decapod crustaceans. In such estuarine terrestrial crabs as *Sesarma haematocheir, Sesarma intermedium,* and *Sesarma dehaani* (Saigusa and Hidaka, 1978; Saigusa, 1981), the release is complete in only 3 – 5 sec with vigorous fanning behavior. For the species releasing zoeas under the water, including an estuarine semi-terrestrial crab *Sesarma erythrodactylum* (Saigusa, 1997) and the seacoast semi-terrestrial crab *Sesarma pictum* (Saigusa, 1992b), the fanning behavior of the abdomen last a few minutes. The larval release of the intertidal crab *Hemigrapsus sanguineus* requires more time; 15 min to as long as a few hours (unpublished observation).

The timing of larval release is correlated with such environmental cycles as day-night, tidal, and lunar cycles. In lobsters (Ennis, 1973; Branford, 1978; Moller and Branford, 1979), for example, zoeas hatch at the same time each night. The larval release of most estuarine crabs, such as the fiddler crab *Uca* (Bergin, 1981; DeCoursey, 1979) and *Sesarma* (Saigusa, 1981, 1982, 1997; De Vries and Forward, 1989) is synchronized with nocturnal high tides. In contrast, as shown in Figure 6, the larval release of the seacoast semi-terrestrial crab *Sesarma pictum* is synchronized with both high tides (Saigusa, 1992b). A sublittoral crab, *Neopanope sayi*, also shows a bimodal tidal rhythm (De Vries and Forward, 1989). The larval release of an intertidal crab, *Hemigrapsus sanguineus*, is correlated not only with the 12.4-h interval of tides, but also with the semidiurnal inequality of tide height (Saigusa and Kawagoye, 1997). The influence of the tidal cycle at the habitat thus appears to strongly affect the pattern of the larval release (Saigusa, 1997).

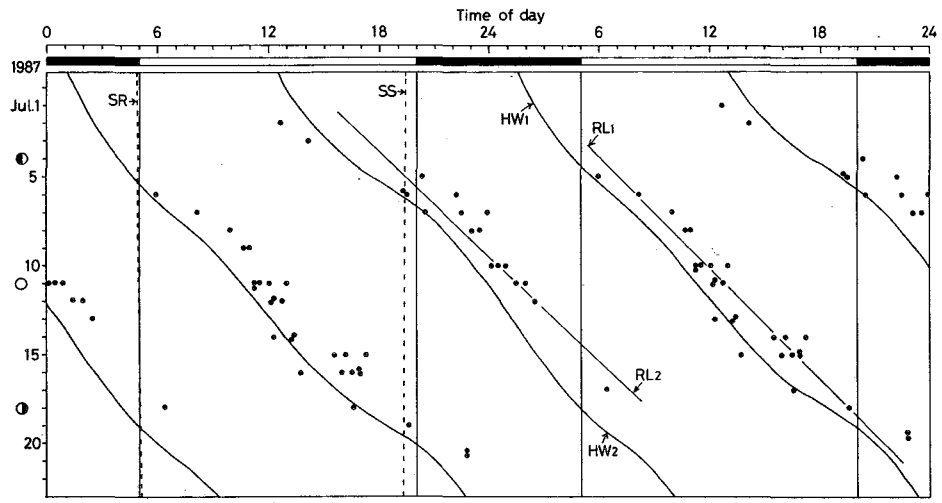


Fig. 6 Circatidal rhythm with a bimodal phase in the seacoast semi-terrestrial crab *Sesarma pictum*. Forty-six females were collected at the habitat on 30 June, 1987, and the larval release of each crab was monitored under a 24-h light-dark regime (LD 15:9) in the laboratory without any tidal influence. Black dots indicate the time of day of larval release by these females. Vertical lines indicate the times of light-off and light-on in the chamber, respectively (light-on at 5:00, light-off at 20:00). For comparison, environmental cycles in the field are characterized by the times of sunset (SS) and sunrise (SR), by the curves connecting the predicted time of day of high tides at the habitat (HW_1 and HW_2), and by the phase of the moon (○: full moon, ◐ and ◑: the first and last quarters of the moon). The entire record is duplicated on the right and displaced upwards one day, so that each day's data can be matched with those of the following day. Diagonal lines (RL_1 and RL_2) are least-square regression lines fitted to each phase of the circatidal rhythm. (Saigusa, original).

4. Hatching controlled by the circatidal clock and environmental cues for entrainment

The larval release of decapod crustaceans occurs just after hatching. In *S. haematocheir*, ovigerous females come out to the riverside just after sunset, and wait for the time of high tide there. The hatching of this species occurs when the females walk about the riverside; all of the zoeas hatch synchronously from each batch of embryos, possibly within 5 – 30 min in the field. As soon as the hatching occurs, the females enter the water to release the larvae. This suggests that the larval release behavior is induced by the stimuli of hatching, and that what is actually controlled by the circatidal clock is the timing of hatching, and not that of the larval release behavior (Saigusa, 1992a).

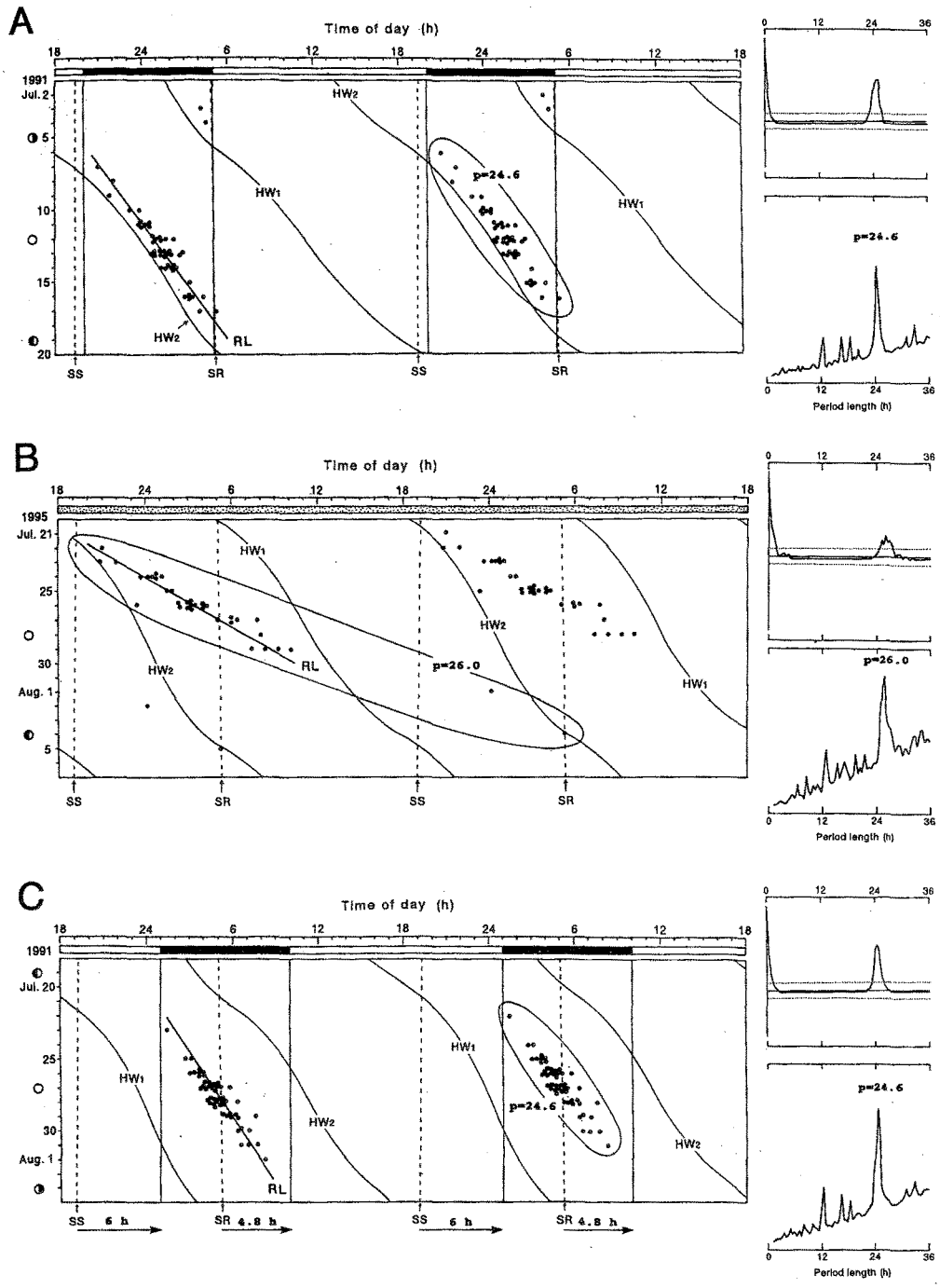
To determine whether the timing of hatching is controlled endogenously in each female, ovigerous females of an estuarine semi-terrestrial crab, *Sesarma erythrodactylum*, were collected from the field, and the larval release activity was monitored under different light regimes in the laboratory. Figure 7A–C summarizes the results of these experiments. When the crabs (47 females) were maintained in photoperiods the phase of which was similar to that in the field (light-on at 5:00; light-off at 20:00), the population showed the tidal rhythm of larval release in synchrony with the times of high tide at night (Fig. 7A). In contrast, when an other 35 females were maintained in a constant, very dim light (LL) condition after collection, the release activity showed a free-running rhythm with a pattern slightly (1.4 h) different from that of the tidal cycle (Fig. 7B). This suggests that the timing of release is controlled by an endogenous clock in each female. Since this rhythm was clearly synchronized with the tidal cycle even in the non-tidal condition in the laboratory (Fig. 7A), the rhythm shown in Figure 7B should be regarded as the free-run of a tidal rhythm, and not that of a daily rhythm.

The rhythm was also phase-shifted by 24-h cyclic light, the phase of which was changed; the magnitude of the phase-shift corresponded to that of the light cycle between the field and laboratory (Fig. 7C; see also Saigusa, 1986, 1992b). The 24-h light-dark (LD) cycle thus is the zeitgeber (environmental cue) of the circatidal rhythms of many species living in intertidal and estuarine environments. In the field, however, these rhythms are not likely to be entrained solely by the 24-h LD cycle. Tidally correlated factors such as the periodic fluctuations of water turbulence on the shore should be considered. Entrainment of the circatidal rhythm by use of simulated tidal factors in addition to 24-h LD cycles has been tried many times, but only an experiment with a simulated 24.5-h moonlight cycle has succeeded (Saigusa, 1988). Even in that experiment, entrainment required more than one month, which suggests that the 24-h LD cycle may be a dominant zeitgeber not only in the circadian rhythms but also circatidal rhythms.

5. Is the timing of hatching controlled by the embryo, or by the female?

In a number of crustaceans, embryos are incubated by the female until hatching occurs. This phenomenon complicates the control of the rhythm of hatching and larval release. Indeed, as described in the preceding section, it is clear that the timing of hatching is under the circatidal clock, but the question of how it is controlled by the clock of the embryo, or that of the female, or both remains unanswered.

Previous studies have obtained conflicting results. Pandian (1970) felt that a clock in the embryo itself sets the time of hatching for the lobster *Homarus gammarus*. Ennis (1973) also suggested an endogenous factor controlling the time of hatching in this species, but was inconclusive about whether it is in the embryo or in the female. More direct evidence has been derived from experiments in which a portion of embryos were detached from an ovigerous female, and the time of hatching of those detached embryos was compared with that of embryos attached to the female. In some crustaceans, the embryos complete their development and hatch as normal larvae even when they are separated from the female and aerated thereafter in water. Branford (1978) reported that detached embryos of *Homarus* hatched rhythmically in a 24-h LD cycle, but arrhythmically under constant light (LL) or constant dark (DD) conditions. However, when the ovigerous female was maintained in the DD conditions, hatching showed a marked 24-h period. Branford (1978) thus concluded that any endogenous component of the rhythmic hatching is located in the female. Detached embryos also hatched in an estuarine crab, *Rhithropanopeus* (Forward and Lohmann, 1983). The embryos removed from the female within two days of larval release hatched at a time similar to the hatching of female-attached embryos, but this hatch-


A
Time of day (h)
1991
Jul. 2
HW2
HW1
p=24.6
RL
SS
SR
Period length (h)
B
Time of day (h)
1995
Jul. 21
Aug. 1
HW1
HW2
RL
p=26.0
SS
SR
Period length (h)
C
Time of day (h)
1991
Jul. 20
Aug. 1
HW1
HW2
RL
p=24.6
SS 6 h
SR 4.8 h
Period length (h)

ing synchrony deteriorated with longer removal times. From these results, Forward and Lohmann (1983) suggested that the timing of hatching is controlled by the embryo itself and that the role of the female is to synchronize the embryonic development.

De Vries and Forward (1991a) collected three species of ovigerous crabs at different habitats in relation to tidal influence, and compared the hatching time of female-attached embryos with that of detached embryos. In *Neopanope sayi*, the mean hatching time of detached embryos had a good correlation with that of female-attached embryos, but in other crabs *Uca pugilator* and *Sesarma cinereum*, the hatching time was delayed about 2 h from that of attached embryos. The authors of the study suggested that hatching time is controlled by the embryos in *N. sayi*, but that females signal the embryos to stimulate hatching in the other two species.

A) Hatching of the embryos detached from the female

Hatching of detached embryos has also been investigated in *S. haematocheir* (Saigusa, 1992c). This study obtained results very different from any of those of previous studies. As shown in Figure 8a, a batch of embryos incubated by a female hatched within an extremely brief period. The time required for the completion of hatching of all zoeas cannot be determined exactly, because the embryos are attached in a mass, but it was about 30 min in this female.

Embryos detached from the female hatched on the same night as those attached to the female (Fig. 8b). The hatching of the detached embryos was not as synchronous as that of the attached embryos; it was delayed and extends for 5 – 6 h. Similarly, the embryos detached one day earlier all hatched on the same night as the larval release; the time of hatching was delayed and the simultaneity of hatching was deteriorated to the same extent as that of the embryos detached on the same day of larval release. Both experiments (Fig. 8a, b) were carried out under the same temperature, indicating that these results, *i.e.*, desynchronization and delay of hatching in detached embryos, are not due to water temperature; they could be due to the absence of some cue from the female (Saigusa, 1992c).

In contrast to these results, the embryos detached more than two days before larval release did not hatch at all, although they remained alive, if aerated. (When such embryos are placed in a beaker for a week or longer, hatching occurs sporadically and premature larvae appear). These results suggest that the induction of hatching and the appearance of swimming larvae may be dependent on the interval between detachment and natural (female-attached) hatching. To test this hypothesis, a cluster of embryos was detached at various times of the day before the larval release by the female. As shown in Figure 9a, b, a drastic change in hatching suc-

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Fig. 7 Clock-controlled circatidal rhythm of larval release in an estuarine semi-terrestrial crab, *Sesarma erythrodactylum*, and the phase-shift of the rhythm by a 24-h light-dark (LD) cycle. (After Saigusa, 1997).

A. Daily timing of larval release monitored under the conditions of a 24-h LD cycle (LD 15:9) and no tidal influence. The phase of the light cycle was similar to that in the field (light-off at 20:00, light-on at 5:00). Date of collection: 2 July, 1991. Forty-seven crabs were used (black dots indicate the time of day of larval release by these females). Vertical lines indicate the times of light-off and light-on in the experimental room, respectively. Symbols are the same as in Figure 6. A diagonal line (RL) is the least-square regression line fitted to the data of larval release. The right two panels show the period length (hour) of the rhythm (enclosed with an ellipse) estimated by correlogram (upper diagram), and periodogram (lower diagram). Dotted line indicates 95% confidence limit. The entire record is duplicated on the right.

B. Time of day of larval release monitored under a regime of continuous light (LL: 0.5 – 1.0 lux) and no tidal influence in the laboratory. Date of collection: 21 July, 1995. Thirty-five specimens were used. The entire record is duplicated on the right. Estimated period length is shown on the right panels.

C. The phase-shift of the circatidal rhythm under the light cycle (LD 15:9) the phase of which was changed by 5 – 6 h with respect to the natural light cycle is shown by vertical lines (lights-off at 1:00, lights-on at 10:00). Note that the phase of the rhythm is delayed corresponding to the magnitude of the phase shift of the light cycle (compare with A). Estimated period length is shown on the right panels. The entire record is duplicated on the right.

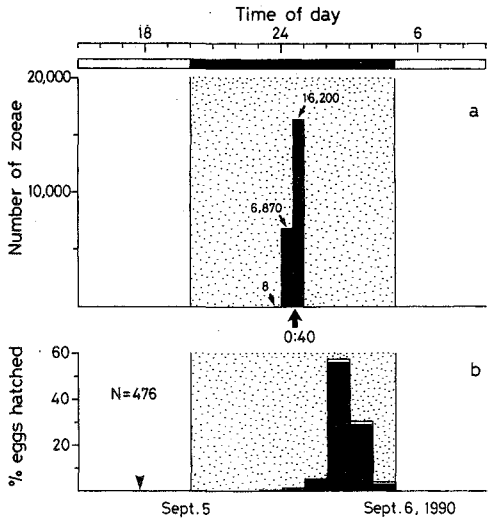


Fig. 8 Comparison of the hatching of embryos attached to a female with the hatching of embryos detached from that female. a. Hatching profile of the female-attached embryos. The ovigerous female was confined in a plastic cage with many holes, and this cage was suspended from the rime of a glass beaker containing 0.7 l of diluted seawater, so that the bottom of the cage was immersed about 1 cm deep in water. Newly-hatched zoeas escaped when the female soaked herself in the water. The cage was placed in a new beaker every 30 min, and the number of swimming zoeas was counted (small arrows). In this experiment, the female exhibited release behavior at 0:40, shown by the upward arrow. The time required for the hatching of all zoeas would be about 30 min in this female. b. Distribution of hatching in the separated embryos. Detachment occurred at 17:45 (downward arrow head). N: total number of emerged larvae. Dotted area shows the dark period of the 24-h LD cycle. (After Saigusa, 1992c).

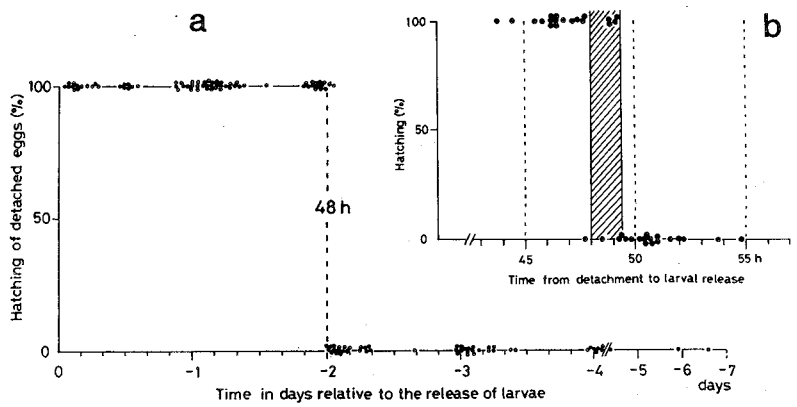


Fig. 9 Relation between the success of hatching in detached embryo clusters and the time of separation of those embryos from the females. a. Hatching profile of embryo clusters detached from a total of 110 females. Black dots represent the percentage of larvae hatched from each cluster of embryos. b. A closer examination of hatching from embryos detached 10 h before, and 10 h after, a critical interval (shadowing), *i.e.*, 48−49.5 h before larval release by their females. For further details, see text. (After Saigusa, 1992c).

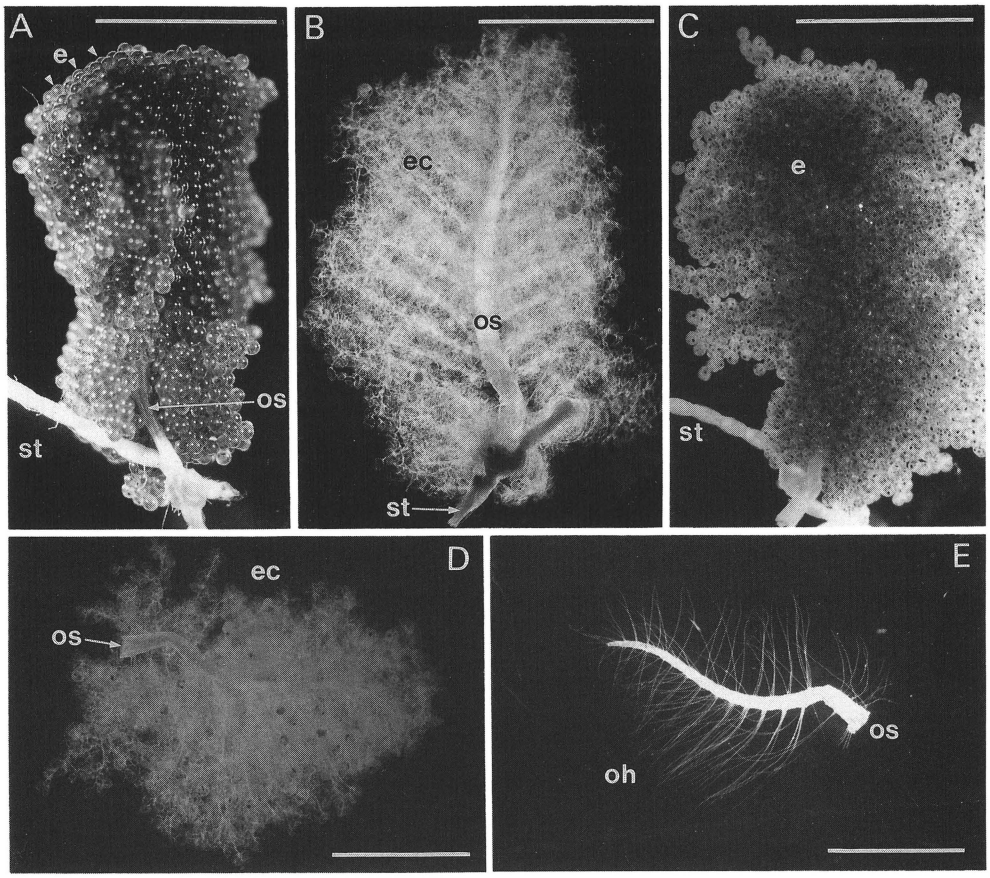
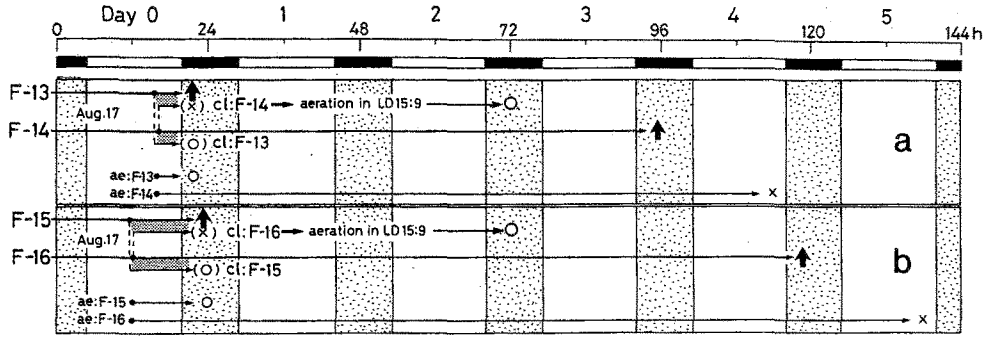


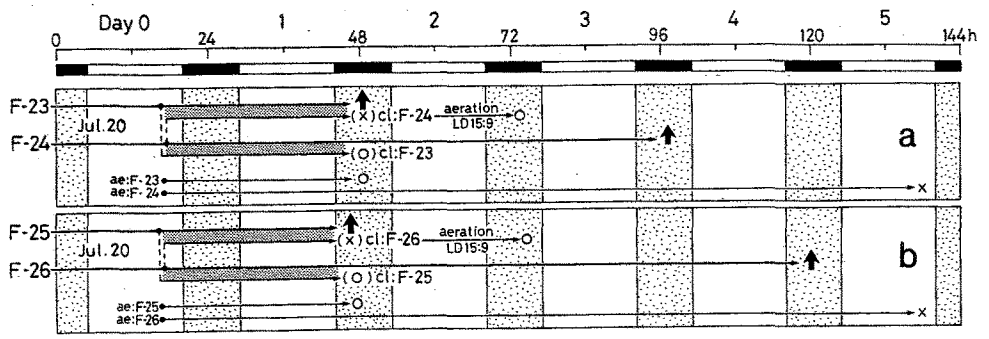
Fig.10 Embryos of *Sesarma haematochier* and their hatching. A. A cluster of embryos detached from a female and the cut base of an ovigerous seta which is tied with a fine silk thread. e: embryos, os: ovigerous seta, st: silk thread making a knot at the cut base of the ovigerous seta. Bar = 1 cm. B. The transplanted cluster in which all larvae hatched at the same time as the hatching of embryos attached to the female. ec: empty egg cases, os: ovigerous seta, st: silk thread. Bar = 1 cm. C. An embryo cluster removed from the host female just after the larval release of that female. These embryos did not hatch at all, but the cluster lost elasticity (compare with the cluster shown in A). When such a cluster was washed by water several times and aerated in a beaker, the embryos hatched after one night (*Pattern I-2*, Fig. 11) or after two nights (*Pattern I-1*, Fig. 11). e: embryos, st: silk thread. Bar = 1 cm. D. An embryo cluster removed from the female's abdomen just after hatching occurred; all the larvae were liberated by the female. os: cut base of the ovigerous seta. Bar = 1 cm. E. Ovigerous seta (os) cut on the morning after larval release. Ovigerous hairs (oh) were already cleaned off completely. Bar = 0.5 cm. (Saigusa, original).

cess occurred within a critical interval: 48 – 49.5 h (the shadowed interval). This suggests that the embryos have a distinct program for hatching, which I call the '*hatching process* (or *hatching program*)', and that this process starts at 48 – 49.5 h (*i.e.*, at the time of high tide two nights) before the larval release. This process is irreversible, and the initiation would be under the circatidal clock of the embryo (for further details, see Saigusa, 1992c).

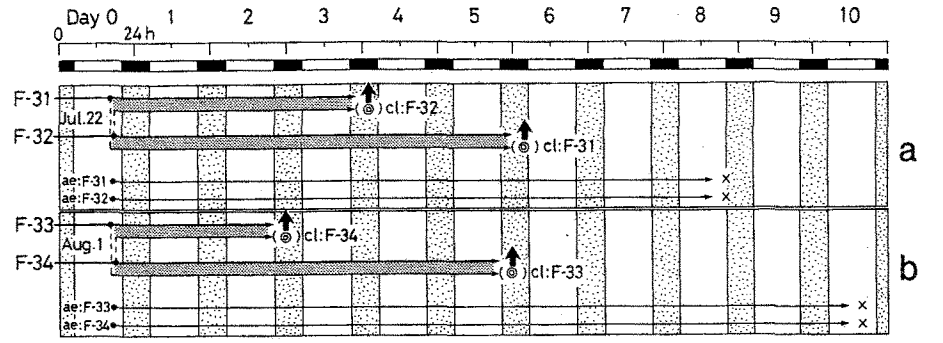
Pattern I (1)



Pattern I (2)



Pattern II



B) Exchange of the embryos between two females

The question of whether the timing of crustacean hatching is controlled by the embryo or by the female has also been investigated in the female. The method used for this experiment was the exchange of one cluster of embryos between two females (Saigusa, 1993). The transplanted embryos (Fig. 10A) survived on the host female and hatched successfully. The hatching of these embryos was clearly divided into two patterns depending upon the number of nights intervening between the exchange and the hatching in both or either of the females.

Pattern I (1) (top panel of Fig. 11). This pattern of hatching appeared when *one* of the paired females released larvae *on the first night after the embryo exchange.* In the upper diagram (a), the embryo exchange was carried out between female F-13 and F-14. Hatching from F-13 occurred 5.5 h after. The cluster transplanted to F-14 (*i.e.,* cl: F-13) and the embryos detached and aerated (ae: F-13) also hatched on the same night as the larval release of the donor female (open circles between Day 0 and Day 1). In contrast, another cluster of embryos (cl: F-14) remained in the host female (F-13) without hatching (× with a parenthesis; see also Fig. 10C). This cluster (cl: F-14) was removed from the female just after the larval release and aerated in the beaker. Hatching occurred two nights after (open circle between Day 2 and Day 3) (see also Fig. 10B). In this experiment, therefore, the hatching of the aerated cluster (cl: F-14) occurred one night earlier than that of the female-attached embryos (F-14). Another experiment (b) in which an embryo exchange was made between F-15 and F-16 shows the same results.

Pattern I (2) (middle panel of Fig. 11). These two experiments summarize the results in which the *first* larval release occurred *on the second night after the embryo exchange.* For example, as shown in the lower panel

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Fig.11 Induction of hatching in the transplanted cluster of embryos. (After Saigusa, 1993).

Pattern I (1): Results of two experiments in which one of the paired females released larvae on the first night of embryo exchange. a. Embryo exchange between F-13 and F-14 in the afternoon (16:10 – 16:30) on Day 0 (17 August). Dotted area indicates the time when the detached embryos were transplanted to the host female. The larval release (upward heavy arrow) of F-13 occurred on the night when the embryos were exchanged. The embryo cluster transplanted to this female (cl: F-14) was immediately removed (see Fig. 10C) and aerated in a beaker. × indicates that the cluster did not hatch. The hatching of these embryos occurred two nights later (open circle). In contrast, the larval release of F-14 occurred 4 nights after the embryo exchange. The embryo cluster transplanted to F-13 (cl: F-14) was removed from the host female immediately after larval release. The embryos all soon hatched on the same night. ae: F-13 and ae: F-14 indicate control embryos that were aerated without embryo exchange. b. Embryo exchange between F-15 and F-16 at noon on Day 0 (17 August). The larval release of F-15 occurred on the night of the embryo exchange. The transplanted cluster (cl: F-16) hatched two nights after, while the hatching of the female-attached embryos (F-16) required two more nights (*i.e.,* until the night on Day 4). ae: F-15 and ae: F-16 indicate control embryos.

Pattern I (2): Results of two experiments in which one of the paired females released larvae on the second night after the embryo exchange. a. Embryo exchange between F-23 and F-24 at 16:30 – 17:00 on 20 July. Larval release: F-23, at 00:20 on 22 July (early morning on Day 2); F-24, at 01:10 on 24 July (early morning on Day 4). b. Embryo exchange between F-25 and F-26 at 17:10 – 17:40 on 20 July. Larval release: F-25, at 22:30 on 21 July (late evening on Day 1); F-26, at 23:50 on 24 July (late evening on Day 4). The hatching of transplanted clusters (cl: F-24 and cl: F-26; open circles without parenthesis) occurred one night after they were removed from the host females. Compare the dates of their hatching with those of hatching in the female-attached embryos: *i.e.,* F-24 and F-26 (upward heavy arrows).

Pattern II: Hatching in the transplanted embryos in synchrony with the hatching of female-attached embryos. a. Embryo exchange between F-31 and F-32. Detachment and exchange of clusters: 16:30 – 17:00 on 22 July. Larval release: F-31, at 02:10 on 26 July; F-32, at 03:20 on 28 July. b. embryo exchange between F-33 and F-34 at 16:25 – 16:45 on 1 August. Larval release: F-33, at 23:35 on 3 August; F-34, at 23:50 on 6 August. The transplanted clusters *all* hatched in synchrony with the hatching of the female-attached embryos (double open circles, see Fig. 10B).

(b), the cluster of embryos (cl: F-25) transplanted to the host female (F-26) occurred on the same night as the larval release (open circle with a parenthesis). The control embryos (ae: F-25) hatched on the same night. In contrast, another cluster (cl: F-26) did not hatch at all in the host female (F-25) releasing larvae (indicated by ×). This cluster was immediately removed from this female, and aerated thereafter. Its hatching occurred on the next night (*i.e.*, in the early morning on Day 3; open circle). The hatching of this cluster (cl: F-26) was, therefore, induced two nights earlier than that of the female-attached embryos (F-26). These results indicate that the embryos under the hatching program could induce the hatching of transplanted embryos in which the hatching program had not been in progress. Another experiment (a) shows the same results as in b.

Pattern II (bottom panel of Fig. 11). As shown in Figure 9, embryos detached from the female two nights or more before the larval release do not hatch, but the hatching of such embryos can be induced if they are incubated by the female (*Pattern I*). A further question, when these unhatched embryos are transplanted to the female, is whether their hatching occurs in synchrony with that of the female-attached embryos. These two experiments (a and b) summarize the results in which larval release occurred three nights or more after the embryo exchange. In the lower experiment (b), the larval release of one of the paired females (F-33) occurred three nights after the embryo exchange, and that of another female (F-34) was six nights after. The hatching of the transplanted clusters (cl: F-33 and cl: F-34) occurred in synchrony with that of the host females (F-34 and F-33), respectively (Fig. 10B). Another experiment (a) also obtained the same results.

The results of these embryo exchange experiments (Fig. 11) indicate that the induction of hatching is controlled by the female. Each embryo also has an endogenous rhythm of hatching (for further details, see Saigusa, 1993). In contrast, the hatching synchrony is markedly deteriorated and the time of hatching is delayed in embryos isolated from the female (Fig. 8b). Thus, it also appears that the female has some mechanism not only for enhancing hatching synchrony, but also for setting the time of hatching.

C) Control of hatching by the circatidal clocks of female and embryo; a possible mechanism

As described above, the hatching of intertidal and estuarine crabs occurs in synchrony with the times of high tide at their habitat, and shows a tidal rhythm (Bergin, 1981; Saigusa, 1981, 1982, 1988, 1992a, 1997; Saigusa and Kawagoye, 1997; Forward *et al.*, 1982; De Vries and Forward, 1989, 1991a). The free-running rhythm observed in constant conditions indicates that the time of larval release is controlled by an endogenous clock, and larval release immediately follows hatching; therefore, what is controlled by the clock is the timing of hatching, and not that of the release behavior (Saigusa, 1992a). A possible mechanism of clock-controlled timing of hatching suggested by the results of the above-described embryo detachment (Saigusa, 1992b) and of embryo exchange (Saigusa, 1993) is as follows.

As seen in Figure 9, the hatching of *Sesarma haematocheir* does not immediately follow the completion of embryonic development. The embryos have a special developmental program (the 'hatching process') of 48 – 49.5 h in each embryo, and hatching occurs after this process. With the completion of the normal developmental process, the embryos await the stimuli that initiate the hatching process. *Hatching-process inducing factor (HPIF)* is produced by the female (stippled area in Fig. 12); they have not been yet identified. Once these signals are received by the embryos, the hatching process starts, *under the endogenous clock* within each embryo, from the time of high tide at night.

As shown in Figure 12, the female's endogenous pacemaker is expressed by a sine curve with a 24.8-h period. Similarly, the embryo's endogenous pacemaker for hatching is expressed by a recutangular wave with a 24.8-h period. In the field, both pacemakers should be synchronized with the time of nocturnal high tide. When the embryos were detached from the female, hatching peak was deteriorated among the newly hatched zoeas (Fig. 8b). Thus, the hatching process may not be highly synchronized among these embryos.

The hatching process is irreversible. Once HPIF are received, the start of the hatching process should be determined by the endogenous clock within each embryo, and hatching would follow 48 – 49.5 h thereafter; *i.e.*, at the time of high tides two nights later, whether the embryos are attached to the female or are detached. What time is required for the start of the hatching process after HPIS are transmitted to the embryos? The embryo exchange experiments indicated that the embryos can hatch if they are in contact with female-attached

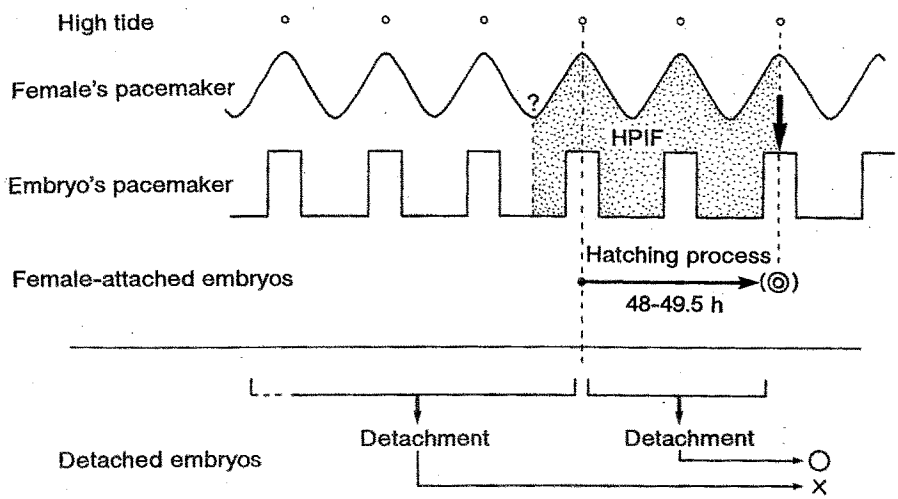

Fig.12 Proposed mechanism of induction of hatching and synchronization of hatching with high tide at night. Endogenous pacemakers related to hatching are shown with a sign curve (female) and a rectangle (embryos). They are indicated at a 24.8-h interval here, but there might be a 12.4-h interval. The small circle above the female's pacemaker indicates the time of the nocturnal high tide. Stippled area (HPIF): factor(s) that induce the hatching process (hatching program) in each embryo; they have not yet been specified. The heavy downward arrow represents stimuli to enhance the hatching synchrony among embryos. The success rate of hatching in detached embryos is shown under the horizontal line. (After Saigusa, 1993).

embryos in which the hatching process is in progress for more than 5 – 6 h. It can be therefore postulated that the embryos must be in contact with the stimuli for at least 5 – 6 h for the hatching process to start. In the intertidal crab *Hemigrapsus sanguineus*, the stimuli are probably transmitted to the embryos when the habitat is exposed to the air, *i.e.*, at the time of low tide (Saigusa and Kawagoye, 1997). If the stimuli from the female are insufficient to start the hatching process of all embryos, some embryos must wait for one night. This would result in the split hatching peaks that are never observed in female-attached embryos (for further details, see Saigusa, 1993).

As shown in the lower diagram of Figure 12, if embryos were detached from the female before the hatching process, they would not hatch at all. In contrast, if they were detached while the hatching process was in progress, they would hatch on the same night as the larval release. The hatching from detached embryos was deteriorated and delayed compared with the hatching peak of female-attached embryos (see Fig. 8). Since highly synchronized hatching has been observed for female-attached embryos, the females appear to have some mechanism (a downward arrow in Fig. 12) for enhancing hatching synchrony when they are waiting for the time of hatching on land. The hatching synchrony of the intertidal crab *H. sanguineus* is not as high as that of the terrestrial crabs (Saigusa and Kawagoye, 1997). The hatching of this species occurs in the water, and such a mechanism may, therefore, not be developed in intertidal and sublittoral crustaceans.

6. Active factors released at the time of hatching

A) OHSS (ovigerous-hair stripping substance)

Some of the embryo exchange experiments (Saigusa, 1993) exhibited a curious phenomenon not usually seen in intact females, either in the field or the laboratory. In the experiment shown in Figure 13a, one of the paired females (F-37) released larvae on the night of embryo exchange. The embryo cluster transplanted to

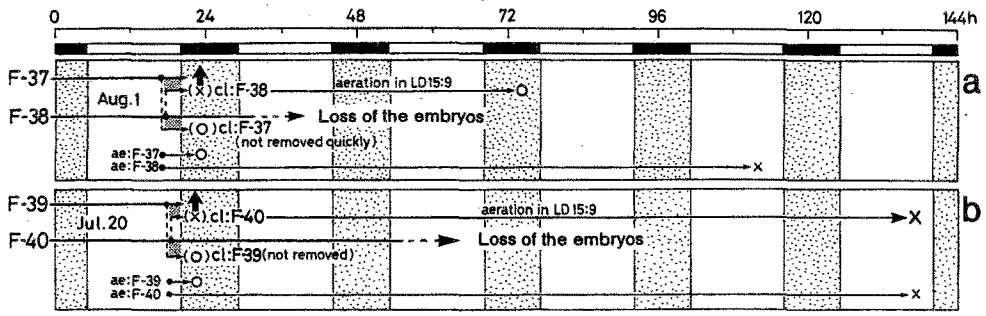

Fig.13 Loss of female-attached embryos without hatching. a. Embryo exchange between F-37 and F-38 at 16:10 – 16:25 on 1 August. Larval release: F-37, at 23:35 on 1 August. Hatching of the transplanted cluster (cl: F-38) that had been removed from the host female occurred two nights later (open circle), but the female-attached embryos (F-38) were all lost without hatching within a few days. b. Embryo exchange between F-39 and F-40 at 17:50 – 18:20 on 20 July. Larval release: F-39, at 22:30 on 20 July. The transplanted cluster (cl: F-39) was not removed until the next morning. The female (F-40) lost all her embryos without hatching. The transplanted embryos (cl: F-40) were removed just after the larval release of F-39 and aerated thereafter, but hatching was not induced. This cluster was in contact with the female-attached clusters for only about 4.5 h, not enough time to induce the hatching process in the transplanted embryos. In contrast, in a, the detached cluster was held in the host female for 7 h, which would be sufficient time to induce the hatching process. (After Saigusa, 1993).

this female (cl: F-38) was immediately removed and transferred to aeration where it hatched two nights later. In contrast, the reciprocally transplanted cluster (cl: F-37) was not removed before hatching started, and was left with the female-attached embryos for more than 1 h until most of the embryos had hatched in the female's abdomen. All of the embryos attached to the female (F-38) dropped to the bottom of the beaker within a few days. None of the embryos hatched. Thus, if a transplanted cluster is not removed before many embryos hatch, the host female's own attached embryos drop within a few days. This suggests that some active factor is released with hatching, which would cause a disruption of the female's incubation.

Further experiments (Saigusa, 1994) indicated that when ovigerous females were kept in 'hatch water' (*i.e.*, the filtered medium into which larvae had been released), they liberated their embryos without hatching. Detachment occurs when the ovigerous hairs slip out of the investment 'coat' that binds to the embryos through the funiculus (Fig. 1D, E). In normal females (*i.e.*, those not treated with hatch water), broken embryonic cases and funiculi remain for a time after hatching with the 'coat' on the ovigerous hairs (Fig. 10D), but they are gone by the morning after hatching (Fig. 10E). The secretion of this factor may therefore participate in cleaning the ovigerous hairs of old investment coats, funiculi, and broken egg capsule after larval release, thus preparing for the attachment of the next clutch of embryos. This active fator, which I call *ovigerous-hair stripping substance (OHSS)*, is released by the embryos, and not by the female (Saigusa, 1995).

As shown in Table 1, hatch waters from four species of *Sesarma* were applied to the embryos of six species of crab. Although the response to OHSS differed among species, all species were affected by all of the types of hatch water, suggesting that the OHSS occurs widely in intertidal and estuarine crabs. Indeed, the OHSS acts on the coat wrapping ovigerous hairs to slough off the remnants after the larval release, but one might speculate that it is also directly involved in the hatching mechanism, because it is released at the time of hatching. However, there is no evidence that this factor is a hatching enzyme of the sort known in many groups of animals (Saigusa *et al.*, submitted).

B) Proteinase

Hatching in crustaceans is clearly different from other animals in that it is caused by the rupture of the embryonic envelope (Davis, 1968, 1981). As mentioned above, many investigators have suggested that this is

Table 1 Reciprocal tests on the effect of OHSS. (After Saigusa, 1995).

Source of hatch water	Source of cluster of embryos	Percentage of stripped hairs ($\bar{X} \pm SD$) *	
		Hatch water	10 ppt SW**
Sesarma dehaani	*S. haematocheir*	81.9± 9.2(12)	3.6±4.1(12)
S. erythrodactylum	*S. erythrodactylum*	84.5±13.1 (6)	5.2±5.7 (6)
	S. pictum	89.3± 6.3 (4)	2.2±3.8 (6)
	S. haematocheir	61.7±18.6 (6)	3.7±5.1 (6)
S. haematocheir	*S. bidens*	99.2± 1.3 (4)	8.4±5.5 (4)
	Gaetice depressus	49.7± 8.9 (6)	1.9±2.1 (6)
S. pictum	*Hemigrapsus sanguineus*	41.7±22.5 (6)	1.6±1.0 (6)
	S. erythrodactylum	94.1± 3.7 (6)	3.7±3.5 (6)
	S. haematocheir	60.8±12.1 (4)	2.6±2.6 (4)
	S. pictum	64.1±11.5 (6)	5.0±6.3 (6)

*Number of setal segments tested is in parentheses.
**Diluted sea water (control).

due to an increase of internal pressure caused by osmotic effects. Recently, De Vries and Forward (1991b) found that the embryos of intertidal and estuarine crabs release a proteinase at the time of hatching. In other experiments, however, the embryonic capsules after hatching showed no sign of dissolution (Saigusa, 1992a). Crab hatch water contains a proteinase, and its proteolytic activity is eluted in the fractions different from those of the OHSS on gel filtration chromatography (Saigusa, 1996). Our studies on the fine structure showed that the embryonic envelope is certainly digested prior to hatching. However, digestion was observed only on a very thin layer inside the capsule; the main component of the capsule was not dissloved at all (Saigusa *et al.*, submitted). Thus, whether this proteinase can be called a 'hatching enzyme' of crustaceans is not yet known. In addition, we do not yet know at what site in the embryos these active factors (OHSS and proteinase) are synthesized during development, or at what point in the hatching process they are secreted (possibly under the circatidal clock of each embryo).

7. Summary and conclusions

Fertilized eggs of most decapod crustaceans are encased by the envelope, clustered on the ovigerous hairs of the female by the funiculus, and ventilated by the female. The embryonic envelope formation and attachment to ovigerous hairs have been the subject of controversy for many years. Recent investigations on the fine structure have indicated that the embryonic envelope consists of three layers in the mature embryos; *i.e.*, the outermost layer, the thickest middle layer, and the innermost very thin layer. Further two thin layers enclose the embryo. They would be so-called 'embryonic cuticles' that have been deposited by the embryos during development. In contrast, the process of the formation of the outermost layer and the attachment of the embryos to the maternal ovigerous hairs have not been throughly settled. A number of investigations have indicated that the embryos secrete a proteinase upon hatching. These proteinases dissolve a portion of the embryonic envelope, thus causing hatching of embryo. But hatching of crustaceans occurs upon the rupture of the embryonic envelope; the envelope after hatching shows no sign of dissolution.

The timing of hatching in decapod crustaceans is under the control of a biological clock. Physiological mechanisms of clock-controlled hatching would be an exciting new subject in the field of crustacean behavior and reproduction. Embryo exchange experiments have indicated that the control of hatching by endogenous clocks of both the embryos and the mother. On the basis of the results of these experiments, this review presented a possible hatching mechanism controlled by the circatidal clock in an estuarine terrestrial crab (Fig. 14).

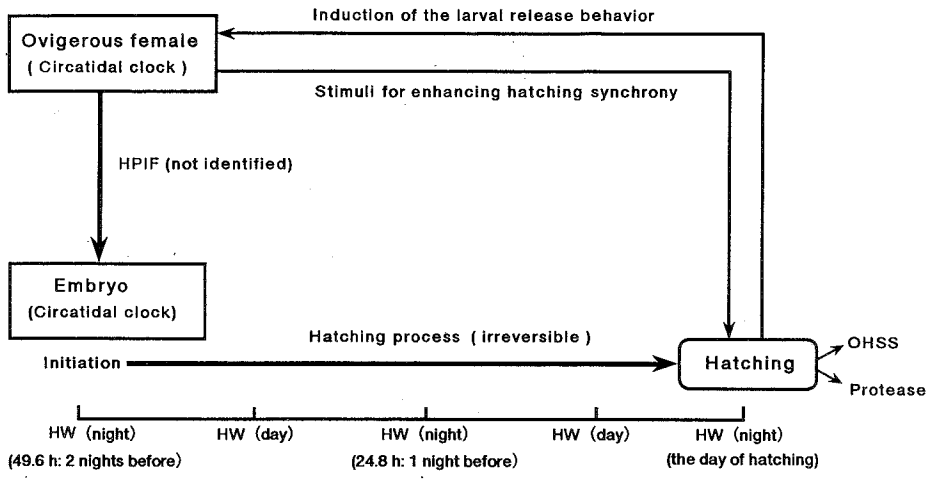

Fig.14 Summary of hatching in the estuarine terrestrial crab *Sesarma haematocheir*, which was described in this review. OHSS and the proteinase are released outside the embryonic envelope upon hatching, but the exact time when they are secreted from each embryo is not yet known. Females release a factor to induce the hatching process in each embryo (HPIF) (stippled area shown in Fig. 12), but this factor has not been yet identified. Females would send the embryos some factor or motion to stimulate hatching, which could enhance hatching synchrony among embryos and could set the time of their hatching at the high tide. (Saigusa, original).

In this hypothesis, embryos have a special program for hatching (the hatching process) in addition to the normal developmental process. This program is initiated under the endogenous clock in each embryo, when the embryos are subjected to *hatching-process inducing stimuli (HPIS)* for at least 5－6 h. The hatching process is irreversible, and hatching occurs after 48－49.5 h in synchrony with the nocturnal high tide. Female-attached embryos hatch synchronously, whereas detached embryos also hatch, but their hatching synchrony is deteriorated and their hatch peak is delayed. Thus, it appears that some stimuli not only enhancing hatching synchrony but also setting the hatch time would be transmitted from the female upon hatching. When the hatching has occurred, the female immediately enters the water to release the larvae. The stimuli of hatching thus induces the larval release behavior of the female.

Hatching is associated with the release of at least two kinds of active substance: *OHSS (ovigerous-hair stripping substance)* and proteinase. The roles that these factors play in the hatching mechanism of marine crustaceans are not yet known.

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References

Anderson, D. T. and G. T. Rossiter (1969) Hatching and larval development of *Haplostomella australiensis* Gotto (Copepoda, Fam. Ascidicolidae), a parasite of the ascidian *Styela etheridgii* Herdman. *Proc. Linn. Soc. N. S. W.*, **93**, 464－475.

Andrews, E. A. (1906) Egg-laying of crayfish. *Am. Nat.*, **40**, 343－356.

Barrett, D. and B. F. Edwards (1976) Hatching enzyme of the sea urchin *Strongylocentrotus purpuratus*. *Methods Enzymol.*, **45**, 354－373.

Bergin, M.E. (1981) Hatching rhythms in *Uca pugilator* (Decapoda: Brachyura). *Mar. Biol.*, **63**, 151－158.

Berrill, N. J. (1929) Studies in tunicate development. Part I. General physiology of development of simple ascidians. *Phil. Tran. R. Soc. Lond., Ser. B*, **218**, 37－78.

Branford, J. R. (1978) The influence of daylength, temperature and season on the hatching rhythm of *Homarus gammarus*. *J. Mar. Biol. Ass. U. K.*, **58**, 639–658.

Burkenroad, M. D. (1947) Reproductive activities of decapod Crustacea. *Am. Nat.*, **81**, 392–398.

Carroll, E. J. Jr. and J. L. Hedrick (1974) Hatching in the toad *Xenopus laevis*: morphological events and evidence for a hatching enzyme. *Dev. Biol.*, **38**, 1–13.

Cheung, T. S. (1966) The development of egg-membranes and egg attachment in the shore crab, *Carcinus maenas*, and some related decapods. *J. Mar. Biol. Ass. U. K.*, **46**, 373–400.

Clegg, J. S. (1962) Free glycerol in dormant cysts of the brine shrimp *Artemia salina*, and its disappearance during development. *Biol. Bull.*, **123**, 295–301.

Clegg, J. S. (1964) The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. *J. Exp. Biol.*, **41**, 879–892.

Davis, C. C. (1959) Osmotic hatching in the eggs of some fresh-water copepods. *Biol. Bull.*, **116**, 15–29.

Davis, C. C. (1964) A study of the hatching process in aquatic invertebrates. XIII. Events of eclosion in the American lobster, *Homarus americanus* Milne-Edwards (Astacura, Homaridae). *Am. Mid. Nat.*, **72**, 203–210.

Davis, C. C. (1965) A study of the hatching process in aquatic invertebrates. XX. The blue crab, *Callinectes sapidus* Rathbun. XXI. The nemertean, *Caricinonemertes carcinophila* (Kölliker). *Chesapeake Sci.*, **6**, 201–208.

Davis, C. C. (1968) Mechanisms of hatching in aquatic invertebrate eggs. *Oceanogr. Mar. Biol. Annu. Rev.*, **6**, 325–376.

Davis, C. C. (1981) Mechanisms of hatching in aquatic invertebrate eggs. II. *Oceanogr. Mar. Biol. Annu. Rev.*, **19**, 95–123.

DeCoursey, P. J. (1979) Egg-hatching rhythms in three species of fiddler crabs. *In* E. Naylor and R. G. Hartnoll (eds.), *Cyclic Phenomena in Marine Plants and Animals*, pp. 399–406. Pergamon Press, Oxford.

De Vries, M. C. and R. B. Forward, Jr. (1989) Rhythms in larval release of the sublittoral crab *Neopanope sayi* and the supralittoral crab *Sesarma cinereum* (Decapoda: Brachyura). *Mar. Biol.*, **100**, 241–248.

De Vries, M. C. and R. B. Forward, Jr. (1991a) Control of egg-hatching time in crabs from different tidal heights. *J. Crust. Biol.*, **11**, 29–39.

De Vries, M. C. and R. B. Forward, Jr. (1991b) Mechanisms of crustacean egg hatching: evidence for enzyme release by crab embryos. *Mar. Biol.*, **110**, 281–291.

Ennis, G. P. (1973) Endogenous rhythmicity associated with larval hatching in the lobster *Homarus gammarus*. *J. Mar. Biol. Ass. U. K.*, **53**, 531–538.

Fisher, W. S. and W. H. Clark, Jr. (1983) Eggs of *Palaemon macrodactylus*. I. Attachment to the pleopods and formation of the outer investment coat. *Biol. Bull.*, **164**, 189–200.

Forward, R. B. Jr. and K. J. Lohmann (1983) Control of egg hatching in the crab *Rhithropanopeus harrisii* (Gould). *Biol. Bull.*, **165**, 154–166.

Forward, R. B. Jr., K. Lohmann and T. W. Cronin (1982) Rhythms in larval release by an estuarine crab (*Rhithropanopeus harrisii*). *Biol. Bull.*, **163**, 287–300.

Goudeau, M. and F. Lachaise (1980) Fine structure and secretion of the capsule enclosing the embryo in a crab (*Carcinus maenas* (L.)). *Tiss. Cell*, **12**, 287–308.

Goudeau, M. and F. Lachaise (1983) Structure of the egg funiculus and deposition of embryonic envelopes in a crab. *Tiss. Cell*, **15**, 47–62.

Goudeau, M., P. Talbot and R. Harper (1987) Mechanism of egg attachment stalk formation in the lobster, *Homarus*. *Gamete Res.*, **18**, 279–289.

Hagenmaier, H. E. (1974) The hatching process in fish embryos. IV. The enzymological properties of a highly purified enzyme (chorionase) from the hatching fluid of the rainbow trout, *Salmo gairdneri* Rich. *Comp. Biochem. Physiol.*, **49B**, 313–324.

Hall, R. E. and L. J. MacDonald (1975) Hatching of the anostracan branchipod *Chirocephalus diaphanus* Prevóst. I. Osmotic processes and the possible role of glycerol. *Hydrobiologia*, **46**, 369–375.

Herrick, F. H. (1895) The American lobster—a study of its habits and development. *Bull. U. S. Fish Comm.*, **1895**, 1–252, 54 pls.

Herrick, F.H. (1909) Natural history of the American lobster. *Bull. U. S. Bureau Fish.*, **1909**, 149–408, 15 pls.

Hinsch, G. W. (1990) Arthropoda—Crustacea. *In* K. G. Adiyodi and R. G. Adiyodi (eds.), *Reproductive Biology of Invertebrates, Vol. 4, Part B: Fertilization, Development, and Parential Care*, pp. 121–155. John Wiley & Sons, New York.

Hoshi, M. and T. Numakunai (1981) Hatching enzyme of the solitary ascidian, *Halocynthia roretzi*. *Acta Embryol. Morphol. Exp.*, **2**, 163–169.

Ishida, J. (1936) An enzyme dissolving the fertilization membrane of sea-urchin eggs. *Annot. Zool. Jpn.*, **15**, 453–459.

Ishida, J. (1944) Hatching enzyme in the fresh-water fish, *Oryzias latipes*. *Annot. Zool. Jpn.*, **22**, 137–154.

Katagiri, C. (1975) Properties of the hatching enzyme from frog embryos. *J. Exp. Zool.*, **193**, 109–118.

Lepage, T. and C. Gache (1989) Purification and chracterization of the sea urchin embryo hatching enzyme. *J. Biol. Chem.*, **264**, 4787–4793.

Linder, H. J. (1960) Studies on the fresh water fairly shrimp *Chirocephalopsis bundyi* (Forbes). II. Histochemistry of egg-shell formation. *J. Morphol.*, **107**, 259–284.

Marshall, S. M. and A. P. Orr (1954) Hatching in *Calanus finmarchicus* and some other copepods. *J. Mar. Biol. Ass. U. K.*, **33**, 393–401.

Mawson, M. L. and C. M. Yonge (1938) The origin and nature of the egg membranes in *Chirocephalus diaphanus*. *Q. J. Micrsc. Sci.*, **80**, 553–565.

Moller, T. H. and J. R. Branford (1979) A circadian hatching rhythm in *Nephrops norvegicus* (Crustacea: Decapoda). *In* E. Naylor and R. G. Hartnoll (eds.), *Cyclic Phenomena in Marine Plants and Animals*, pp. 391–397. Pergamon Press, Oxford.

Morris, J. E. (1971) Hydration, its reversibility, and the beginning of development in the brine shrimp, *Artemia salina*. *Comp. Biochem. Physiol.*, **39A**, 843–857.

Nakanishi, Y. H., T. Iwasaki, T. Okigaki and H. Kato (1962) Cytological studies of *Artemia salina*. I. Embryonic development without cell multiplication after the blastula stage in encysted dry eggs. *Annot. Zool. Jpn.*, **35**, 223–228.

Nomura, K., H. Tanaka, Y. Kikkawa, M. Yamaguchi and N. Suzuki (1991) The specificity of sea urchin hatching enzyme (envelysin) places it in the mammalian matrix metalloproteinase family. *Biochemistry*, **30**, 6115–6123.

Pandian, T. J. (1970) Ecophysiological studies on the developing eggs and embryos of the European lobster *Homarus gammarus*. *Mar. Biol.*, **5**, 154–167.

Perona, R. M. and P. M. Wassarman (1986) Mouse blastocysts hatch *in vitro* by using a trypsin-like proteinase associated with cells of mural trophectoderm. *Dev. Biol.*, **114**, 42–52.

Roe, J. L. and W. J. Lennarz (1990) Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. *J. Biol. Chem.*, **265**, 8704–8711.

Saigusa, M. (1981) Adaptive significance of a semilunar rhythm in the terrestrial crab *Sesarma*. *Biol. Bull.*, **160**, 311–321.

Saigusa, M. (1982) Larval release rhythm coinciding with solar day and tidal cycles in the terrestrial crab *Sesarma*. *Biol. Bull.*, **162**, 371–386.

Saigusa, M. (1986) The circa-tidal rhythm of larval release in the incubating crab Sesarma. *J. Comp. Physiol.*, **A159**, 21–31.

Saigusa, M. (1988) Entrainment of tidal and semilunar rhythms by artificial moonlight cycles. *Biol. Bull.*, **174**, 126–138.

Saigusa, M. (1992a) Observations on egg hatching in the estuarine crab *Sesarma haematocheir*. *Pacif. Sci.*, **46**, 484–494.

Saigusa, M. (1992b) Phase shift of a tidal rhythm by light-dark cycles in the semi-terrestrial crab *Sesarma pictum*. *Biol. Bull.*, **182**, 257–264.

Saigusa, M. (1992c) Control of hatching in an estuarine terrestrial crab. I. Hatching of embryos detached from the female and emergence of mature larvae. *Biol. Bull.*, **183**, 401–408.

Saigusa, M. (1993) Control of hatching in an estuarine terrestrial crab. II. Exchange of a cluster of embryos between two females. *Biol. Bull.*, **184**, 186–202.

Saigusa, M. (1994) A substance inducing the loss of premature embryos from ovigerous crabs. *Biol. Bull.*, **186**, 81–89.

Saigusa, M. (1995) Bioassay and preliminary characterization of ovigerous-hair stripping substance (OHSS) in hatch water of crab larvae. *Biol. Bull.*, **189**, 175–184.

Saigusa, M. (1996) Two kinds of active factor in crab hatch water: ovigerous-hair stripping substance (OHSS) and proteinase. *Biol. Bull.*, **191**, 234–240.

Saigusa, M. (1997) The circatidal clock of an estuarine semi-terrestrial crab, *Sesarma erythrodactylum*. *Benthos Res.*, **52**. (in press).

Saigusa, M. and T. Hidaka (1978) Semilunar rhythm in zoea-release activity in the land crabs *Sesarma*. *Oecologia*, **37**, 163–176.

Saigusa, M., and O. Kawagoye (1997) Circatidal rhythm of an intertidal crab, *Hemigrapsus sanguineus*: synchrony with unequal tide height and involvement of a light-response mechanism. *Mar. Biol.* (in press).

Sawada, H., K. Yamazaki and M. Hoshi (1990) Trypsin-like hatching protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. *J. Exp. Zool.*, **254**, 83–87.

Slifer, E. H. (1937) The origin and fate of the membranes surrounding the grasshopper egg; together with some experiments on the source of the hatching enzyme. *Q. J. Micrsc. Sci.*, **79**, 493–506, 3 pls.

Stephens, G. C. (1952) The control of cement gland development in the crayfish, *Cambarus*. *Biol. Bull.*, **103**, 242–258.

Suko, T. (1961) Studies on the development of the crayfish. VII. The hatching and the hatched young. *Sci. Rep. Saitama Univ., Ser. B,* **4**, 37–42.

Talbot, P. and D. Demers (1993) Tegumental glands of Crustacea. *In* M. N. Horst and J. A. Freeman (eds.), *The Crustacean Integument,* pp. 151–191. CRC Press, Boca Raton, Florida.

Talbot, P. and M. Goudeau (1988) A complex cortical reaction leads to formation of the fertilization envelope in the lobster, *Homarus. Gamete Res.,* **19**, 1–18.

Trotman, C. N. A., B. C. Mansfield and W. P. Tate (1980) Inhibition of emergence, hatching, and protein biosynthesis in embryonic *Artemia salina. Dev. Biol.,* **80**, 167–174.

Yamagami, K (1972) Isolation of choriolytic enzyme (hatching enzyme) of the teleost, *Oryzias latipes. Dev. Biol.,* **29**, 343–348.

Yamagami, K. (1988) Mechanisms of hatching in fish. *In* W. S. Hoar and D. J. Randall (eds.), *Fish Physiology, Vol. 11A,* pp. 447–499. Academic Press, Sandiego, California.

Yamagami, K., T. S. Hamazaki, S. Yasumasu, K. Masuda and I. Iuchi (1992) Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. *Int. Rev. Cytol.,* **136**, 51–92.

Yamamoto, M., I. Iuchi and K. Yamagami (1979) Ultrastructual changes of the teleostean hatching gland cell during natural and electrically induced precocious secretion. *Dev. Biol.,* **68**, 162–174.

Yang, C.-M. (1977) The egg development of *Paracalanus crassirostris* Dahl, 1894 (Copepoda, Calanoida). *Crustaceana,* **33**, 33–38.

Yasumasu, S., I. Iuchi and K. Yamagami. (1989a) Purification and partial characterization of high choriolytic enzyme (HCE), a component of the hatching enzyme of the teleost, *Oryzias latipes. J. Biochem.,* **105**, 204–211.

Yasumasu, S., I. Iuchi and K. Yamagami (1989b) Isolation and some properties of low choriolytic enzyme (LCE), a component of the hatching enzyme of the teleost, *Oryzias latipes. J. Biochem.,* **105**, 212–218.

Yonge, C. M. (1937) The nature and significance of the membranes surrounding the developing eggs of *Homarus vulgaris* and other Decapoda. *Proc. Zool. Soc. Lond., Ser. A,* **107**, 499–517, 1 pl.

Yonge, C. M. (1946) Permeability and properties of the membranes surrounding the developing egg of *Homarus vulgaris. J. Mar. Biol. Ass. U. K.,* **26**, 432–438.

Yonge, C. M. (1955) Egg attachment in *Crangon vulgaris* and other Caridia. *Proc. R. Soc. Edin., B,* **65**, 369–400.