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Metamorphosis in Balanomorphan, Pedunculated, and Parasitic Barnacles: A Video-Based Analysis

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Synopsis

Cypris metamorphosis was followed using video microscopy in four species of cirripeds representing the suspension-feeding pedunculated and sessile Thoracica and the parasitic Rhizocephala. Cirripede metamorphosis involves one or more highly complex molts that mark the change from a free cypris larva to an attached suspension feeder (Thoracica) or an endoparasite (Rhizocephala). The cyprids and juveniles are so different in morphology that they are functionally incompatible. The drastic reorganization of the body implicated in the process can therefore only commence after the cyprid has irreversibly cemented itself to a substratum. In both *Megabalanus rosa* and *Lepas*, the settled cyprid first passes through a quiescent period of tissue reorganization, in which the body is raised into a position vertical to the substratum. In *Lepas*, this is followed by extension of the peduncle. In both *Lepas* and *M. rosa*, the juvenile must free itself from the cypris cuticle by an active process before it can extend the cirri for suspension feeding. In *M. rosa*, the juvenile performs intensely pulsating movements that result in shedding of the cypris carapace ~8 h after settlement. *Lepas* sp. sheds the cypris cuticle ~2 days after settlement due to contractile movements of the peduncle. In *Lepas anserifera*, the juvenile actively breaks through the cypris carapace, which can thereafter remain for several days without impeding cirral feeding. Formation of the shell plates begins after 1-2 days under the cypris carapace in *Lepas*. In *M. rosa*, the free juvenile retains its very thin cuticle and flexible shape for some time, and shell plates do not appear until sometime after shedding of the cypris cuticles. In *Sacculina carcini*, the cypris settles at the base of a seta on the host crab and remains quiescent and aligned at an angle of ~60° to the crab’s cuticle. The metamorphosis involves two molts, resulting in the formation of an elongated kentrogon stage with a hollow injection stylet. Due to the orientation of the cyprid, the stylet points directly towards the base of the crab’s seta. Approximately 60 h after settlement the unsegmented vermigon stage, preformed in the kentrogon, passes down through the hollow stylet and into the crab’s hemocoel in a process lasting only 30 s. In *S. carcini*, the carapace can remain around the metamorphosing individual without impeding the process.

Introduction

About 150 years ago, Darwin (1851, 1853) chose cirripedas as his model organisms for many of the same reasons that scientists today study this group. Cirripedes start as free-swimming larvae, but the adults are sessile and exhibit a wide variety of life styles ranging from intertidal suspension feeders (acorn barnacles), over many epibiotic forms to some of the most advanced parasites (Rhizocephala) known in the Metazoa. The suspension-feeding forms are unique among crustaceans in being clad in shell plates that are not molted during growth, while the parasitic forms pass through an endoparasitic stage that is so reduced that they cannot be recognized by structure as crustaceans, or even as arthropods (Anderson 1994). The intertidal barnacles are highly...
important members of their habitats and the most prominent foulers of man-made structures in the sea. Rhizocephalan cirripedes are parasitic castrators of other crustaceans and hence potentially important regulators of crab populations, many of which are ecologically and commercially important (Høeg et al. 2005). While adult barnacles’ morphology, ecology, and evolution have been subjected to many studies, remarkably little attention has been given to the profound metamorphosis into a juvenile that follows settlement of the cypris larva, or of how this process deviates between the widely different life forms found in the cirripedes (Høeg and Møller 2006).

Following settlement, the metamorphosing cyprid faces a multitude of challenges. They must avoid desiccation (intertidal forms) or being groomed away by the host (parasites) (Ritchie and Høeg 1981; Walker 1995). The suspension-feeding forms must first extricate themselves from the cypris cuticle before they can commence feeding, while the parasitic must gain access to the crab host through its protective body armor. Finally, the entire metamorphosis must be completed successfully before the finite amount of energy contained in the settled cypris has been exhausted (Lucas et al. 1979). In the Thoracica, there are no accurate descriptions of metamorphosis in any of the many pedunculated forms. For sessile (acorn) barnacles, the exploration of the surface before cementation upon it was studied by Lagersson and Høeg (2002) and Maruzzo et al. (2011). Walley (1969) gave a detailed histological study of metamorphosis, but did not use laboratory-maintained animals and so could neither provide a detailed time line of events nor accurately describe processes that are completed within a few minutes or hours. Glenner and Høeg (1993, 1998) and Takenaka et al. (1993) focused on specific organs only, rather than the overall course of metamorphosis. Maruzzo et al. (forthcoming) conducted the only accurate study of the metamorphosis of the model species Balanus amphitrite. For parasitic barnacles (Rhizocephala), Glenner and Høeg (1995), Glenner et al. (2000) and Glenner (2001) were the first to observe the actual invasion of the host crab (in Loxothylacus panopei). The vast majority of rhizocephalan literature concerns Saccculina carcini, in which metamorphosis has not been studied in detail since Delage’s (1884) paper and the actual invasion of the host remains unobserved (Høeg 1987). Here we use laboratory experiments and video microscopy to study metamorphosis of the cyprids in S. carcini (Rhizocephala), in two species of Lepas (anserifera and sp., Thoracica Pedunculata) and in the acorn barnacle Megabalanus rosa (Thoracica Balanomorpha). We describe the timing of the overall series of events and focus on similarities and differences among these widely different cirripede species in relation to their respective modes of life.

**Materials and methods**

**Lepas**

Newly settled cyprids of *L. anserifera* and a *Lepas* sp. were collected on the surface of spherical floating buoys in the He-Ping-Dao, northeastern coast of Taiwan during December 2009 and January 2010. In the laboratory, 20 cyprids (10 individuals per species) were kept individually in covered petri dishes (3 cm diameter), filled with autoclaved seawater (salinity 33‰, without supplying any plankton for food). They were kept in constant temperature at 24°C and the seawater was changed daily. The cyprids were regularly monitored and photographed at a magnification of 30× at intervals of 6 h. The specimens were monitored for 5–6 days, when the cyprids had fully metamorphosed into juveniles and commenced suspension feeding. The times indicated in Fig. 1 are the times after collection, when the cyprids were already slightly advanced in metamorphosis.

**Megabalanus rosa**

Settlement-competent cyprids were kindly provided by Dr Yasuyuki Nogata (Environmental Research Laboratory of Central Research Institute of Electric Power Industry, Japan) and cultured as in Yoshimura et al. (2006). Videos of *M. rosa* cyprids were recorded under a Nikon SMZ800 dissection microscope equipped with a Shimadzu CCD video camera sequentially connected to a Victor SR-S990 time-lapse recorder and a Toshiba AK-G200 HDD recorder. The recordings presented here were made of a cyprid attached to the side of a transparent tight-sealed plastic chamber (Advantec, tight lid type, 50 × 11 mm and filled with 0.2 μm-filtered, natural sea water) kept at room temperature (25°C).

**Saccculina carcini**

Larvae of *S. carcini* were raised to cyprids and their sex determined (Walker 1985; Høeg 1987). Female cyprids were exposed to settlement on crabs (*Carcinus maenas*) as described by Høeg (1984) and
Glenner and Werner (1998). Crabs were exposed to cyprids in aquaria in 16°C noncirculating seawater for periods between 6 and 20 h and then isolated and screened for settled larvae. A total of 162 settled cyprids were removed from the crabs, together with a tiny piece of crab cuticle, using the tip of a scalpel or a fine needle. These live preparations were mounted individually in small plastic wells or (for video recording) on slides with depressions 30-mm wide and 1-mm deep, and sealed with a large coverslip against desiccation; these were incubated at 16–17°C. Ten specimens yielded no perceptible development or died during incubation; the remaining 152 specimens developed kentrogons with stylets. Development in 121 of these was followed until the stylet had also been evaginated from the kentrogon. Selected specimens were continuously inspected visually or recorded on sVHS tape through a Olympus IX70 inverted microscope from the time of stylet formation and until the parasite had been injected. Selected video sequences were transferred to digital format and further processed. Final editing of all videos and inclusion of text and still pictures was carried out using PowerDirector v10©. To provide accurate estimates of developmental events, we used timings taken from the most accurate experiments (6 h exposure to crabs), and not from the full data set.

Results

Lepas sp. (Figs. 1A–J, 4A–D)
The cyprids are initially attached by a small amount of cement beneath the antennules, which are almost completely retracted. The body is free from the substratum and aligned at a very acute angle to the surface. Soon after attachment (Fig. 1A), the tissues begin to separate slightly from the cypris carapace, indicative of the ongoing metamorphic molt. During the following 48 h (Figs. 1B–C and 4A–B) the body raises to a completely upright position with its long axis angled 90° to the surface as in the adult barnacle. After 1–2 days (Figs. 1C–D and 4C) the peduncle begins to form in the anterior half of the body as a whitish, opaque mass of tissue and the shell plates begin to appear beneath the cyprid carapace, which still encloses the entire metamorphosing specimen.

After 3 days, the peduncle extends to its full length within ~12 h. As a result, the ‘cypris’ carapace come to lie around the apical part of the body, the capitulum, from whence it soon falls away. The extension must involve some swelling of tissue, since the peduncle in Fig. 1F has a much larger volume than when still enclosed in the cyprid (Fig. 1E). In adults, the newly formed peduncle is very flexible (Fig. 1I and J) and can alternately contract or extend to a very slender state (compare. Fig. 1G with Fig. 1F and H). The compound eyes of the cyprid remain attached to the juvenile for some time after shedding of the carapace, but are lost after 3 days (Figs. 1H and 4D). Shedding of the carapace clears the way for extending the thoracopodal cirri, which are first seen after 3–4 days. From this time, the juvenile essentially has the shape and armature of five plates as in adult specimens.

Lepas anserifera (Figs. 1K–S, 4A–D)
Metamorphic events and their timing are essentially as in Lepas sp. in terms of changes in orientation, shell-plate formation, and formation and extension of the peduncle. But in L. anserifera, the elimination of the cyprid carapace proceeds very differently. The carapace remains at its original position close to the substratum and with the compound eyes still attached (Fig. 4C2). When the peduncle extends the juvenile pushes against the thorax and thoracopods of the cyprid, so they become separated from the carapace which still stays at the basal position of the peduncle. The carapace splits into two halves along the weak middorsal hinge and finally falls away after 4 days. Such differences in the elimination of the carapace between L. anserifera and Lepas sp. are consistent in all replicates used in the experiment. It follows that in L. anserifera the cirri can be extended while the carapace still remains around the basal part of the juvenile. The capability for extending, bending, and contracting the peduncle is readily seen (compare Fig. 1P with Fig. 1Q).

Megabalanus rosa (Figs. 2A–F, 4E–J)
Maruzzo et al. (forthcoming) made accurate video observations on the metamorphosis in the balanomorph B. amphitrite, and their observations, timing, and phases are fully comparable to that described here for M. rosa. The cmentation process in M. rosa is presently under close study (Okano et al., manuscript in preparation). Here, we focus exclusively on the metamorphic events that take place within 6–10 h (variable for each specimen) after cmentation at the end of exploration of the surface. From the attachment and until sometime after shedding of the cyprid
Fig. 1 Metamorphosis in *Lepas*. (A–J) *Lepas* sp. (A) cyprid cemented by the tips of its antennules; the black spot in the ‘cypris’ body is the compound eye; (B–E) the whole cypris body is raised around the attachment point and there is incipient formation of the shell plate [asterisk, arrowhead, and arrow in (C)]; (F) the peduncle extended and the shedding of the ‘cypris’ cuticle is visible; (G) early juvenile with the peduncle in a contracted state; (H–J), juvenile with cirri extended for feeding and showing increasingly developed shell plates. (K–S) *Lepas anserifera*. (K–N) cyprid cemented by the tips of its antennules incipient formation of the shell plate is evident [asterisk and arrow in (L)]; (O–Q) the peduncle is extended and the cypris carapace (ca) remains around the base of the specimen; (R–S) the cypris thorax (th) is shed, and the juvenile has its cirri extended for feeding; the shell plates are better developed.
carapace, the metamorphosing individual is attached only by the tiny mass of cement secreted at the tip of the antennules (Fig. 2A, arrowhead).

About 20 min from the onset of cementation, the characteristic and complex movements involved in this process have almost ceased. At this time the antennular muscles contract, pulling the cypris carapace down towards the substratum (Fig. 2A). This marks the start of a long (externally quiescent) phase, in which the body remains still and tightly applied to, and aligned parallel with, the surface. During this phase, the tissues undergo drastic reorganization as previously described from histological preparations (Walley 1969; Takenake et al. 1993; Glenner and Høeg 1998), but on the live and comparatively thick cyprids we could not follow this in detail (Fig. 2A–B). However, the initial part of video clip 1 (fast time-lapse recording) clearly shows how pigmented cells move around in the metamorphosing specimen. In addition, the integument of the prospective juvenile begins to separate from the cypris cuticle, indicative of the ongoing metamorphic molt. The externally quiescent phase of tissue reorganization continues for almost 7 h.

About 7.5 h (range 6–10 h from all specimens examined) from the onset of cementation, the body suddenly starts twitching and gradually the whole body elevates itself around the small attachment point (Fig. 4E–G). The twitching frequency increases as the body angle increases until it reaches a vertical position after ~30 min. (Fig. 2C and D; video clip 1 in Supplementary Data). The twitching and raising movements cause the whole individual, including the loosely attached carapace, to pivot around the attachment point. Inside the cypris carapace, the juvenile performs pumping movements, which are possible because its cuticle is still very thin and flexible. Soon all these rather violent movements result in the juvenile escaping from the cuticles of the cyprid (Figs. 2E and 4H). The antennules and compound eyes of the cyprid can become separated some time before the juvenile finally slips out of the cypris carapace (Figs. 2E and 4H–I, video clip 1). The free juvenile has numerous cuticular hairs extending from its surface, and it retains its very flexible shape and continues with the pulsating, pivoting movements for some time after its escape from the cypris carapace (Figs. 2F and 4J). After a few hours the base of the free juvenile becomes tightly applied to the surface and its cuticle hardens as it assumes the typical volcanic shape of a juvenile barnacle.

The thoracopods (cirri) of the juvenile start beating already before ecdysis, but remain for some time retracted inside the juvenile, even after the carapace is eliminated. Conceivably, the cuticle of the cirri is still too soft to sustain feeding, and the beating principally ventilates the mantle cavity.

The shell plates are not visible immediately after ecdysis. As the juvenile begins to achieve a fixed shaped, the shell plates appear, first as primordial of strongly sclerotized cuticle but later becoming increasingly calcified (Glenner and Høeg 1993).
At this stage the thoracopods begin to extend as a basket of cirri, and the juvenile commences its life as a suspension-feeding acorn barnacle protected by an armor of calcified shell plates.

**Sacculina carcini** (Fig. 3 and 4K–O)

As in all other cirripedes, attachment of the cyprid takes place by means of a cement gland (Høeg 1987). It can occur almost anywhere on the surface of the crab, but typically is on the arthrodial cuticle at the base of a plumose seta.

The cyprid carapace can, and often does, remain around the metamorphosing specimen during the entire metamorphosis without, in any way, impeding the metamorphosis or the injection of the parasite into the host crab. The cyprid also remains oriented at the acute angle to the crab cuticle, which it assumed at cementation (Fig. 4K–L). But otherwise, metamorphosis in *S. carcini* is considerably more complex than in the thoracican barnacles.

Except for the actual invasion of the host, the succeeding events were brilliantly described by Delage (1884) in one of the first experimental studies on marine larvae. The metamorphosis involves a total of two molts, which can be followed rather easily on live specimens due to their small size and transparency. The first molt forms the kentrogon instar, which performs the actual invasion of the host. The kentrogon is an elongated-cylindrical body, entirely devoid of appendages and situated in the anterior half of the spent cypris body. The volume of the kentrogon is much smaller than the cypris, and thus a considerable amount of degenerating tissue remains outside in the spent cypris body, as is also described by Høeg (1985) for the rhizocephalan *Lernaeodiscus porcellanae*. Anteriorly, the cuticle of the kentrogon links up with the base of the cypris antennules, which anchors the entire specimen to the crab.

The second molt is more complicated. (Figs. 3, 4L and M). It involves formation of the injection stylet, which is slightly curved and hollow; it extends throughout most of the length of the kentrogon’s body. Anteriorly, the pointed tip of the stylet enters the base of one of the two cypris antennules, where it has a minute, subterminally situated opening, barely visible under the light microscope (Delage 1884; Høeg 1987; Glenner and Høeg 1994). Posteriorly, the lumen of the stylet opens like a funnel into the kentrogon’s body.

The stylet is fully formed ~48–56 h after settlement (20°C; 30 observations) and is shown in Fig. 3. The kentrogon of *S. carcini* contains no muscles, and the force responsible for penetration of the
The stylet remains unknown (Høeg 1987). The actual penetration normally takes place 57–65 h after settlement (13 observations). Penetration lasts \( \frac{9}{24} - 11 \) min (four observations) and is shown in real time (video clip 2). The stylet passes down through one of the antennules, which acts as a guide tube directing it to the base of the crab’s seta. There the stylet pierces the thin arthrodial cuticle and continues down the setal canal until it reaches the blood space underneath the integument (Fig. 4N). This is not seen in our videos and figures, because the attached specimens were manipulated off the crab and mounted for \textit{in vitro}
monitoring under the microscope. In some specimens, left in situ, we observed how the stylet initially bent into an arc under the force required for penetration, but eventually the arthrodial cuticle gave in and the stylet rapidly passed further on. The subterminal position of the pore in the stylet assures against its being clogged or damaged during penetration.

After a short delay of 10–120 s after penetration of the stylet, the internal parasite (vermigon) is injected into the crab in a process lasting only 10–30 s (four observations; Fig. 4O). Glenner et al. (2000) and Glenner (2001) provided morphological details of the vermigon and its formation in the rhizoccephalan Loxothylacus panopaei. Here, the vermigon and its injection from the kentrogon is shown live for the first time in S. carcini (video clip 3). In S. carcini, the vermigon is variably slug-shaped or amoeboid shaped and is enclosed in a very thin and flexible cuticle. This thin cuticle allows the extreme change of form needed to pass down the 1-µm wide lumen of the stylet, where the vermigon cells seem to migrate almost single file. At the stylet’s tip the vermigon is initially almost explosively “spouted” out into the blood space where it immediately expands in size (video clip 3). Following injection, the vermigon remains inactive in the hemolymph of the host, until it eventually gets disconnected from the tip of the stylet and is able to migrate through the blood space. However, this could not be followed on our specimens incubated in vitro. Eventually, the vermigon reaches the anterior part of the abdomen of the crab, where it grows internally to finally emerge as a virgin female externa (Høeg 1995).

**Discussion**

In cirripedes, the metamorphosis from cypris to juvenile requires one or more complex molts, in which critical events must proceed in exactly the right sequence (Fig. 4). Among the species examined here, this metamorphosis proceeds differently, not only between the parasite S. carcini and the suspension-feeding thoracican species, but also among the latter, even at the generic level. In all species, the juvenile is so different from the free cyprid that any significant morphological transformation must be delayed until after cementation to the substratum. The morphology of the free cyprid is so complex that it would be incapable of either swimming or exploring the substratum if it commenced metamorphosis to any significant degree (Høeg 1985). Only a very slight separation of the epidermis from the cyprid cuticle, visible only by TEM, may herald the coming metamorphic molt (Høeg 1987). Following cementation, the metamorphosis must be fast because the attached specimens face various external dangers such as desiccation or predation, and the metamorphic events are therefore highly related to the ecology of the species. Rhizoccephalan cyprids, such as those of S. carcini, risk being groomed away by the crab before they enter the blood stream. Successful invasion means that the host becomes sterilized (Ritchie and Høeg 1981). For intertidal barnacles such as M. rosa, the settled cyprids risk predation, mechanical removal by wave action, and being killed by heat and desiccation during low tides or by bulldozing from mobile molluscs (Chan and Williams 2003). Cyprids settle during a high tide period and increase their chance of survival if they reach the better protected juvenile stage before the onset of the next low tide. As a result, the whole metamorphosis of M. rosa is completed in about 6–10 h (approximate the period between high and low tides on the lower intertidal shore). Opposed to Megabalanus, Lepas is adapted to a pelagic life, being attached to the immersed sides of floating objects, where they risk neither predation nor stress from heat or desiccation. In accordance, Lepas have a slow metamorphosis lasting up to 3–4 days.

All metamorphosing barnacle cyprids operate with a finite amount of energy (stored as oil cells) until the juvenile can commence feeding either as suspension feeders or as parasites (Lucas et al. 1979; Thiyagarajan et al. 2002a, b, 2003). The amount of lipid reserve in cyprids can affect the time length and success of metamorphosis and subsequent settlement process. When the lipid reserve in cyprids of Semibalanus balanoides are used up, the larvae lose the competence to metamorphose successfully (Lucas et al. 1979). Delayed metamorphosis in Amphibalanus amphitrite resulted in reduced metamorphic growth rate (Pechenik et al. 1993) and settlement success (Satuito et al. 1996). In the rhizoccephalan L. porcellanae Høeg and Ritchie (1987) observed a sharp decline in settlement rate as soon as 5 days after the nauplius–cyprid molt, presumably because energy reserves are rapidly depleted in these very small larvae. In our study, metamorphosis lasts longest in Lepas, which also has the largest cyprids and therefore contains greater amount of lipid reserve.
In both *Lepas* and *Megabalanus*, the raising of the body is a prerequisite to successful shedding of the cyprid shell. In *Lepas* sp. and *M. rosa*, extension of the cirri depends upon shedding of the cypris cuticle. If this process fails, or is long delayed, the animal may run out of energy before it can commence suspension feeding. In contrast, juveniles of *L. anserifera* can extend their cirri while the carapace remains around the base of the peduncle (Fig. 1O), and this ability to initiate early feeding may confer an advantage on this species. In *S. carcini* the cypris carapace can remain in place without impeding the invasion of the host (Fig. 3). In this species, it seems that the correct position and alignment of the cypris is the most critical factor, because the stylet can only penetrate if it hits the narrow area of arthrodel membrane encircling the seta. We suggest that the cypris may actively use the orientation of the seta to align itself correctly. Moreover, settling in the “lee” of a seta may also afford the cyprid some protection against being groomed away by the crab or accidentally lost.

In *Lepas*, the emerging peduncle has a much larger volume than when forming inside the cypris carapace. We therefore suggest that the process is due to some kind of tissue swelling. In both *Lepas* species, the development of the peduncle is critical to the subsequent extension of the cirri. In *Lepas* sp. the peduncular movements assist in shedding all the cypris cuticles (Fig. 1F), while *L. anserifera* forces the peduncle through the carapace, which remains in its basal position for a long time (Fig. 1O). The shell plates are important in protecting the developing juvenile against external damage. In *Lepas* they become visible already beneath the carapace of the settled cypris, while in *Megabalanus* and other balanomorphans these plates do not appear until sometime after the cyprid cuticle has been shed (Glenner and Høeg 1993). One reason may be that the plates in *Lepas* are preceded by cuticular primordia while in most balanomorphans they are calcified from first appearance (Glenner et al. 1995).

**Conclusions**

In all species examined, the metamorphosis is a very complex process that cannot commence until after attachment and then proceeds under constrictions imposed by the environment and by the limited energy available before the juvenile starts feeding. Aside from being a molt, metamorphosis differs extensively among species, all of which seemed highly specialized for their particular environment. The variation in metamorphosis observed between the species of *Lepas* indicates that this critical process is constantly being modified during cirripede evolution. It is in contrast to the almost stereotypical morphology of cirripede cyprids, which mainly deviate from each other only in ultrastructural details of the sensory organs (Hoeg et al. 2004).

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**Supplementary Data**

Supplementary Data are available at *ICB* online.

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