

# Brood Care in Freshwater Crayfish and Relationship With the Offspring's Sensory Deficiencies

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**ABSTRACT** Prolonged brood care is one of the evolutionary clues for the successful colonization of freshwater habitats by freshwater crayfish (Astacida). By means of macrophotography, light microscopy, and scanning electron microscopy we investigated all phases of brood care in freshwater crayfish, with particular emphasis on the morphological structures involved. We selected the recently discovered parthenogenetic marbled crayfish (species identity not yet known) as a model organism due to its fast reproduction and high resistance to handling stress. In order to examine if there is a causal relationship between brood care and the developmental status of the offspring's sensory apparatus, we additionally investigated major sense organs of juvenile Stages 1–5 in comparison with those of the adults. Brood care in the marbled crayfish is characterized by initial and final “active” phases dominated by specific maternal or juvenile behavior and a medial “passive” phase based more on the action of temporarily developed structures rather than on behavior. The most remarkable feature of this period, which includes permanent carrying of the eggs and the first two juvenile stages under the mother's abdomen, is safeguarding of hatching by a telson thread that keeps the helpless newborn hatchlings linked to the egg cases on the maternal pleopods and thus prevents them from being lost. Further important transient structures are the recurved hooks on the first pereopods of Stage 1 and 2 juveniles that are used to firmly attach these nonfeeding stages to the mother's abdomen. In hatchlings all sense organs necessary for an independent life, such as eyes, olfactory aesthetascs, gustatory fringed setae, hydrodynamic receptor hairs, and statocysts are not developed or are underdeveloped, making brood care indispensable. Most of these sense organs appear in Stage 2 juveniles, but only from Stage 3, the first freelancing and feeding stage, are all sense organs well developed and operating, thus reducing brood care in this final period to temporary provisioning of shelter. Brooding of the eggs and postembryonic brood care are to some extent also found in other freshwater Decapoda like freshwater crabs and aeglid anomurans, but safeguarding of hatching is confined to the Astacida only. This sophisticated mode of passive brood care is unique in the animal kingdom and is apparently related to the sensory deficiencies of the first juvenile stage. *J. Morphol.* 262:566–582, 2004. © 2004 Wiley-Liss, Inc.

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Larval development in Crustacea is basically characterized by a prolonged sequence of planktonic

instars that finally molt into juveniles with an adult habitus. The Decapoda, a 10,000 species-large crustacean group of outstanding ecological and commercial importance, follow this ground pattern in principal, but abbreviation of larval development is also quite common (Gurney, 1942). The latter phenomenon occurs across different phylogenetic lines (Rabalais and Gore, 1985; Anger, 2001) and is particularly frequent in Arctic and Antarctic waters (Clarke, 1993), the deep sea (King and Butler, 1985), and freshwater environments, including caves (Juberthie-Jupeau, 1974; Anger, 1995; Reynolds, 2002). Abbreviation of larval development is characterized not only by changes in the size and state of development at which the larvae hatch but also by an increase of the egg size due to the extensive accumulation of nutrient reserves. The switch from small planktotrophic to larger lecithotrophic first larval stages certainly enhances the probability of larval survival in the above listed extreme environments.

Interestingly, abbreviation of larval development is often accompanied by an increase of parental care. In its broadest sense, parental care includes preparation of nests and burrows, production of heavily yolked eggs, care of the eggs, provisioning of the young, and care of the offspring after nutritional independence (Clutton-Brock, 1991). It significantly affects the ecological success and evolutionary potential of species by enhancing the survival and fitness of the offspring. Brood care is called “active” if specific parental activities are directed towards the brood and “passive” if such specific behavior is lacking (Dick et al., 1998). In the decapod Crustacea parental care is the rule but is usually confined to carrying, cleaning, and aeration of the eggs that are attached to ovigerous setae on the pleopods at the underside of the abdomen (Hazlett, 1983; Anger, 2001). Only the phylogenetically ancestral dendro-

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branchiate shrimps lack brood care, releasing their fertilized eggs directly into the water.

Extended parental care that includes care for the hatched juveniles is rare in the Decapoda and has evolved mainly in limnetic and terrestrial species (Thiel, 2000). The highest level of active brood care is probably shown by Jamaican bromeliad crabs, which raise their young in water-filled leaf axils of bromeliads. These crabs not only defend their small brooding pools against predators but also supply food for the young and manipulate the water quality by removal of detritus, oxygenation, and maintenance of a certain pH and calcium level (Diesel and Schuh, 1993; Thiel, 2000). The most advanced example of passive brood care is found in freshwater crayfish in which the first postembryonic stages remain fixed to their mother's abdomen by transient attachment structures, the telson thread and pereopodal hooks (Andrews, 1907; Holdich and Reeve, 1988; Scholtz, 1995). Although varying in detail, this unusual mode of parental care is shared by all crayfish families, the Astacidae, Cambaridae, and Parastacidae, and was therefore used as an apomorphic character to argue for the monophyletic origin of the Astacida and their singular invasion from marine environments into freshwater (Scholtz, 2002; Scholtz and Kawai, 2002).

It was the first aim of this study to investigate in detail all morphological features accounting for the close mother-hatchling interrelation in freshwater crayfish by applying modern microscopic techniques, including scanning electron microscopy (SEM). The two principal structures involved, the telson thread and pereopodal hooks, were detected in the 19th century (reviewed by Andrews, 1907), but descriptions of these structures are limited to a few species only and high-resolution microscopic techniques have only rarely been applied (Price and Payne, 1984; Scholtz, 1995; Scholtz and Kawai, 2002). As the experimental animal, we chose the Marmorcrebs, or marbled crayfish, a parthenogenetic Cambaridae of unknown geographic origin and species identity that appeared in the German aquarium trade in 1995. Since then it has been widely distributed among aquarists in Middle Europe. Morphological and molecular analyses revealed a close affinity to the North American genus *Procambarus*, particularly to *Procambarus fallax* (Scholtz et al., 2003). We selected this species due to its fast reproduction (Vogt et al., 2004), intense brood care, robustness, and high resistance to handling stress that is of great advantage for such an investigation.

Since crayfish are apparently the only Decapoda that secure their hatchlings by a kind of safety line, we were also interested in the biological background of that highly derived mode of brood care. Parental care during and after hatching would be dispensable if the offspring were born as complete individuals with well-developed and functioning locomotory apparatus, feeding appendages, and sense organs.

However, it has previously been assumed as a result of behavioral observations that the sense organs of crayfish might be underdeveloped at hatching and during the first postembryonic stages (Andrews, 1907; Baumann, 1932; Bieber, 1940), which would not allow an early independence from the mother. It was therefore the second aim of our study to test this hypothesis by investigating the visual, olfactory, gustatory, and hydrodynamic sense organs of hatchlings and Stage 2–5 juveniles in comparison to adults.

## MATERIALS AND METHODS

### Animals and Rearing Conditions

Ovigerous females of the marbled crayfish (species identity not yet known) were obtained in an early stage of incubation from Frank Steuerwald (Ostringen-Tiefenbach, Germany) and reared in our department. For documentation of egg attachment to the pleopods and brooding of the eggs, three specimens were used, having total lengths of 3.9, 4.3, and 4.7 cm and weighing 1.4, 1.5, and 2.6 g, respectively. The 3.9 and 4.7 cm females also served for investigation of hatching and the very close mother-hatchling interrelation. For this purpose, in vivo macrophotographs were taken in a small aquarium. Moreover, to analyze the hatchling's attachment structures and sense organs, more than 20 Stage 1 juveniles were removed from their mothers either individually or together with the pleopods and examined in their native or fixed states by means of microphotography, light microscopy, and SEM. For investigation of the sense organs in Stage 2–5 juveniles and in adults, the cephalothoraces or isolated eyestalks, first and second antennae, and first to third pereopods were sampled from six specimens of each juvenile stage (removed from the 4.7 cm female) and two adults of total lengths of 2.3 and 5.1 cm.

The entire period of brood care was followed in the 4.7 cm female that was kept in a 2 L container equipped with sand and pieces of a synthetic fiber net to provide shelter and attachment facilities. The temperature was 18–21°C and oxygen content of the un aerated water fluctuated between 1.5 and 2.7 mg/L. In the 30 days from oviposition to molting of the last Stage 2 juvenile into Stage 3, the mother and juveniles were not fed and no water was added. Thereafter, mother and young were fed daily ad libitum with TetraMin flakes and water was cleaned and supplemented when required. TetraMin flakes are not only a complete instant food but are also good markers for the uptake of food since their green, yellow, and red color is easily detectable in the digestive tract of the transparent juveniles. From time to time the Stage 3–5 juveniles were shaken off the mother and counted. Their developmental status was determined under the stereomicroscope, using total length, carapace length, and morphology of the first antenna as stage-specific markers. Brood care was regarded as terminated when the female was found free of juveniles at 3 successive days.

### Macrophotography, Microphotography, and Light Microscopy

Carriage of eggs and the intense association of mother and juveniles were documented with a Canon EOS D60 digital camera using a Tamron 90 mm macrolens and a Nikon F90X camera with a 105 mm macrolens (macrophotography). Embryonized eggs, hatchlings, and the subsequent juvenile stages were photographed with a Wild M420 stereomicroscope, either in their native state or after fixation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 (microphotography). Fresh preparations of the antennae and pereopods of the juveniles were investigated with a Leitz Aristoplan microscope (light microscopy).

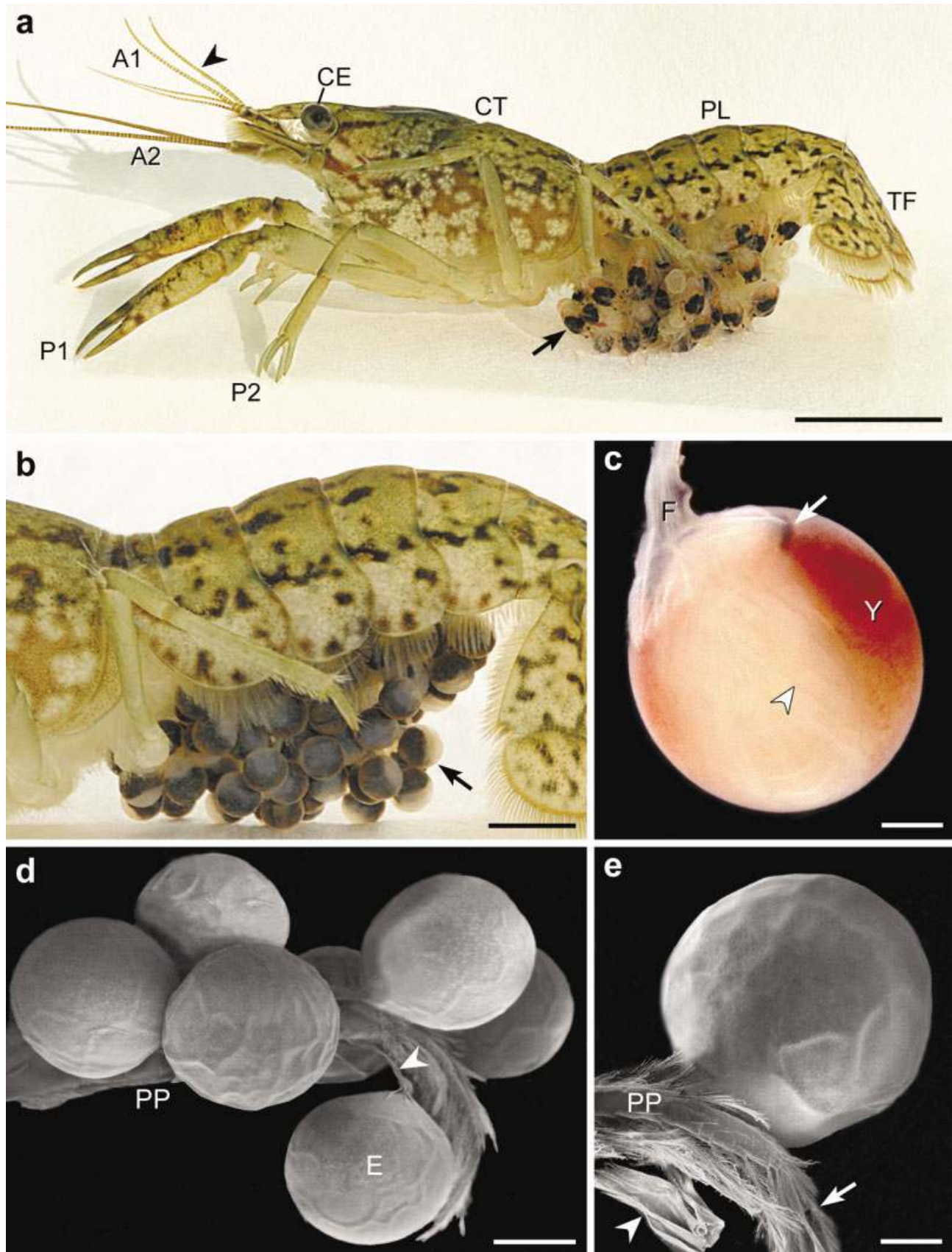


Figure 1

## Scanning Electron Microscopy

The eggs and hatchlings used for SEM and also the body parts of adults carrying major sense organs such as the eyestalks, first and second antennae, and first to third pereopods were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), repeatedly washed in cacodylate buffer, and postfixed for another 2 h with 1% osmium tetroxide. After washing in buffer and dehydration in a graded series of acetone (70–100%), the samples were dried in a Bal-Tec CPD 030 critical point dryer, sputtered with gold, and examined with a Philips 505 scanning electron microscope at the German Cancer Research Center (DKFZ) in Heidelberg.

## Film Material and Image Processing

The following film material was used: Kodak E100VS and Fujichrome 200 Sensia II for macrophotography, Kodak Elitechrome 200 for microphotography, Kodak EPY 64 T for light microscopy, and Agfa APX 100 (7 × 5.4 cm) for SEM. Pictures taken with the digital camera were directly loaded into the computer. Color slides obtained with the Nikon camera, the stereomicroscope, and the light microscope were directly scanned for digital processing, whereas the negatives for SEM were first printed on Agfa Brovira-Speed photopaper and then scanned. All photographs were digitally processed using Adobe PhotoShop 7.0. The background, scratches, and dirty spots on the objects were retouched.

## RESULTS

From the structural point of view, brood care in the marbled crayfish can be subdivided into two phases, an egg-carrying embryonic period and a juvenile-carrying postembryonic period (Fig. 1a,b). Under the harsh conditions of our experiment, the first phase lasted 17 days and the second 38 days, for a total of 55 days. Brooding females are not aggressive against their offspring and tolerate the juveniles on all parts of their body, including chelipeds and mouthparts (Fig. 7a). If disturbed, they try to hide and protect their brood by forward bending of the abdomen.

### Attachment of Eggs to Pleopods and Embryonic Development

From oviposition until hatching the eggs are carried under the mother's abdomen (Fig. 1b), where

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Fig. 1. Brooding in the marbled crayfish: attachment of eggs to pleopods and embryonic development. Macrophotography (a,b), microphotography (c), and SEM (d,e). **a:** General aspect of brooding female. Arrow denotes Stage 2 juveniles carried under the abdomen. A1, first antenna with aesthetascs bearing outer flagellum in upright position (arrowhead); A2, second antenna; CE, compound eye; CT, cephalothorax; P1, first pereopod; P2, second pereopod; PL, pleon; TF, tail fan. Scale bar = 1 cm. **b:** Abdomen of ovigerous female with clutches of eggs on pleopods. The eggs (arrow) are at about 50% of embryonic development. Scale bar = 3 mm. **c:** Embryonized egg at 90% development. Eyes (arrow), appendages (arrowhead), and yolk deposits (Y) of the embryo are well discernable. F, funiculus or egg stalk. Scale bar = 300  $\mu$ m. **d:** Group of eggs (E) adhered to distal part of pleopod (PP). Arrowhead denotes egg stalk. Scale bar = 600  $\mu$ m. **e:** Close-up of egg attached to pleopod. Arrow, terminal oosetae; arrowhead, broken egg stalk. Scale bar = 300  $\mu$ m.

the entire embryonic development is completed (Fig. 1c). Four pairs of pleopods on abdominal segments 2–5 serve as attachment sites. The eggs are adhered to the pleopods with a small stalk or funiculus (Fig. 1c–e), either to the protopods or, more frequently, to the exopods and endopods (Fig. 1d). The base of the funiculus includes bundles of branched oosetae arising from the pleopods. The egg stalk is continuous with the outer layer of the egg shell and comprises the same rigid material, the “cement,” that is secreted from the abdominal cement glands (sternal and pleopod tegumental glands) during oviposition. In the period of incubation the eggs are regularly fanned to provide the embryos with sufficient oxygen. At the beginning of incubation the eggs appear completely filled with yolk but later the yolk is gradually replaced by the developing embryo (Fig. 1b,c).

### Safeguarding of Hatching and Attachment of Stage 1 Juveniles to the Mother

At hatching, the egg capsule generally splits on the opposite side of the egg stalk over the back of the embryo and then the hatchling (Stage 1 juvenile) glides out backwards into the water (Fig. 2a,d). Freshly hatched juveniles have a spherical body form resembling the embryos (Fig. 2a). They soon become much larger than the eggs and achieve a more stretched form, probably by uptake of water (Fig. 2b). In their anterior body part, the cephalothorax, they contain extensive yellowish to brown yolk reserves (Fig. 2a–d). These yolk stores account for the curious front-heavy proportions of early hatchlings, with their first pair of walking legs being inserted posterior to the balance point of the body (Fig. 2a). Such a body form would be quite unfavorable for walking or swimming.

During and immediately after hatching the hatchling is largely immobile and helpless. However, in this precarious period of life it is safeguarded by a relatively long rope-like structure, the telson thread, that keeps the hatchling linked to the empty egg case (Fig. 2a–c). After a while the hatchling tries to get a hold on the maternal pleopods by approaching them with flapping movements of the abdomen and hooking in with its well-developed first pereopods. Sometimes several trials are necessary before the hatchling succeeds in firmly grasping either the oosetae or empty egg cases (Fig. 2c,d). For instance, on a 5-min sequence of digital photos of the hatching process, a newly hatched juvenile was found three times in a free-hanging position (Fig. 2c), each followed by upward movements and attempts to get a hold on the pleopods. This indicates that in vivo the telson thread indeed functions as a safety line that prevents the hatchlings from being lost.

By SEM the telson thread often appears as a twisted rope that includes larger pieces of tissue (Fig. 3a–c). At the hatchling's side it is tightly fixed to the posterior margin of the abdomen by 14 spines



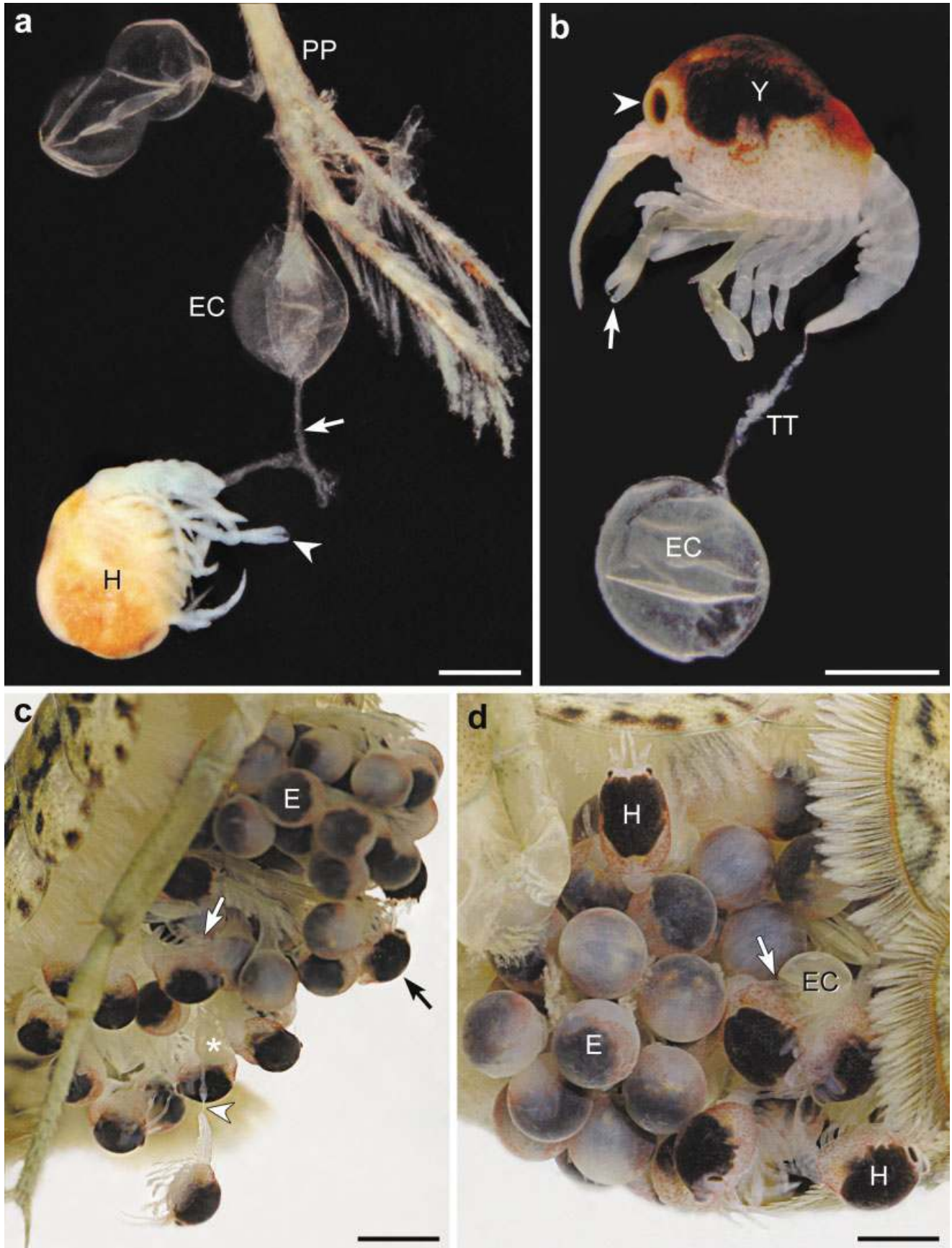


Figure 2

that penetrate into the thread (Fig. 3b). At the egg's side, attachment is accomplished by lateral contact of the thread's terminal segment with the inner surface of the egg case (Fig. 3c). The telson spines seem to release a secretion, as may be concluded from tiny filaments that are occasionally visible on the tips of the spines and interwoven in the first segment of the telson thread (Fig. 3b). In exuviae of Stage 1 juveniles the telson thread was found to be still attached to the telson but detached from the egg case. In that condition the thread appears more untwisted than in living specimens, making two different parts discernable: a short, narrow, and homogeneous string near the telson and a much longer, broader, and more heterogeneously composed segment farther posterior (Fig. 3d).

Aside from the telson thread, the hatchlings have prominent first pereiopods as further conspicuous characteristics. After hatching these well-developed appendages are stretched out to search for any structure to cling to (Fig. 2a). They have relatively large terminal chelae (Fig. 4a) that are equipped with prominent recurved hooks (Fig. 4b,c). These chelae are used to either hook into the funiculus or envelope of empty eggs (Fig. 4a,d) or hook onto the shaft of oosetae (Fig. 4e,f). The hatchlings are then doubly secured by the telson thread and the pereiopodal hooks. The telson thread can persist until molting to the next juvenile stage but often detaches from the egg case earlier.

The recurved hooks of the first pereiopods have sharp tips that can easily pierce the egg cases and egg stalks (Fig. 4a,d). The inner diameter of the hooks is 1.5–2 times larger than the average width of the oosetal shafts (Fig. 4c,f), and the hooks overlap when the chelae are closed (Fig. 4a,b). Moreover, the inner margins of the chelae are studded with anteriorly directed spines (Fig. 4c) that may interact with the lateral setules of the pappose oosetae, thus preventing the hooks from slipping out, particularly if the setules are agglutinated with cement (Fig. 4e,f). The chelae of the second and third pereiopods

are also equipped with terminal hooks, but these are smaller and less recurved than those of the first pereiopods (Figs. 4b, 5f). Such hooks may assist in attachment either by clinging to egg cases and pleopodal setae as well or by holding onto neighboring juveniles.

### Morphology of Visual, Olfactory, Gustatory, and Hydrodynamic Sense Organs in Hatchlings, Stage 2–5 Juveniles, and Adults

The sense organs of the hatchlings investigated were either underdeveloped, like the eyes, or not developed at all, like the olfactory aesthetascs, gustatory fringed setae, and hydrodynamic receptor hairs. Although the eyes of hatchlings appear well developed at first glance, since they are relatively large and pigmented (Fig. 2b), they are certainly not entirely functional because the partitioning into ommatidia, the functional optical units of compound eyes, is not yet finalized (cf. Fig. 5a,b). The first and second antennae of the hatchlings are glabrous, except for a few setal precursor buds at their apices (Fig. 5a), indicating that the hatchlings are devoid of aesthetascs and hydrodynamic receptor hairs. In adults the aesthetascs are located on the ventral side of the outer branch of the first antenna (Fig. 5d,e) and hydrodynamic receptor hairs are on the inner branch of the first antenna (Fig. 5c) and on the second antenna. The hatchlings also lack gustatory fringed setae (Fig. 5f) that in adults are found along the inner margins of the propodus and dactylus of the chelae of pereiopods 1–3 (Fig. 5g,h). In Stage 2 juveniles the ommatidia of the eyes, as well as the aesthetascs, hydrodynamic receptor hairs, and fringed setae appear fully developed but are still relatively few in number (Fig. 6d–h). In the following juvenile stages these sense organs are then enlarged and their functional units are multiplied (Fig. 7f–h).

Concerning the statocysts, we do not have much structural data, but their efficiency may be deduced from the behavior of the various juvenile stages. Stage 1 juveniles always lie on their side when removed from the mother. Although they can move all appendages quite rapidly, they can neither walk nor swim, perhaps because the statocyst is not developed yet or is malfunctioning. Stage 2 juveniles can walk and swim when removed from the maternal pleopods but still seem to have problems with orientation in space, since they often fall on their back and continue moving in that unnatural position. Stage 3 juveniles, in contrast, have a spatial orientation like the adults, and on squash preparations of the base of the first antenna, major structural components of the statocyst, like its basal disc with receptor hairs or the setal coverage of the statocyst's aperture, are visible. Therefore, the sense of orientation seems fully developed only from Stage 3 on.

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Fig. 2. Safeguarding of hatching in the marbled crayfish. Macrophotography (c,d) and microphotography (a,b). **a:** Newly hatched juvenile passively attached to a maternal pleopod (PP) via telson thread (arrow) and stalked egg case (EC). Note rolled-up embryonic habitus of the hatchling (H) with its first pereiopod (arrowhead) stretched out to grasp for structures suited to cling to. Scale bar = 1 mm. **b:** Older hatchling with more straightened body form still connected to the egg case by a telson thread (TT). This Stage 1 juvenile was removed from a pleopod where it was passively adhered to via telson thread and egg case and additionally hooked upon with its first pereiopods (arrow). Arrowhead, pigmented eye; Y, yolk. Scale bar = 1 mm. **c:** Free-hanging hatchling secured by the telson thread (arrowhead). Other hatchlings (arrows) have already attached on pleopodal structures. Asterisk, empty egg case; E, embryonized egg. Scale bar = 2 mm. **d:** Newly hatched juveniles among eggs in final stage of development under the mother's pleon. Arrow denotes hatchling emerging from the egg case. Scale bar = 1.5 mm.

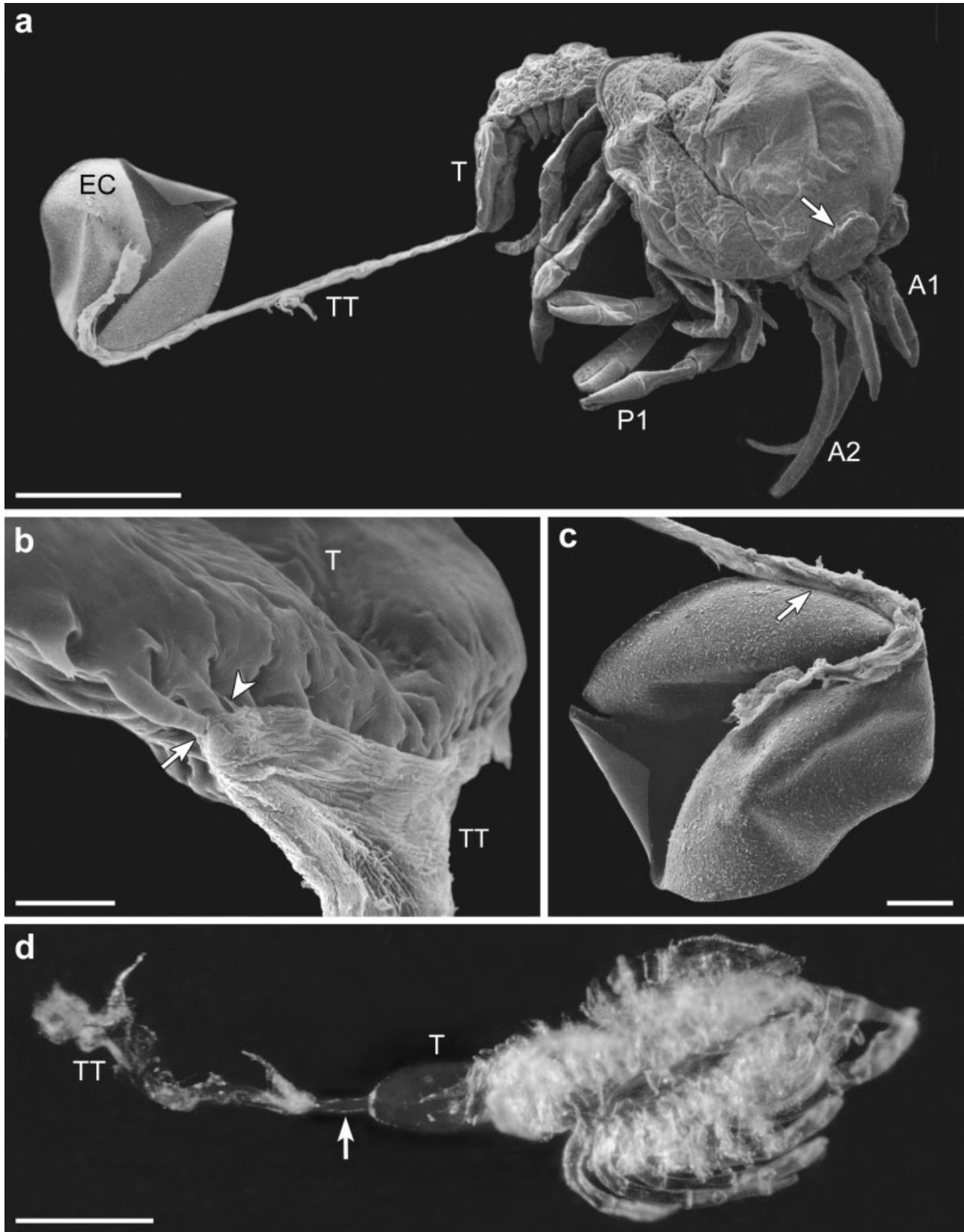


Fig. 3. Passive attachment of hatchlings to the mother in the marbled crayfish. SEM. **a:** Hatchling connected to empty egg case (EC) by a telson thread (TT). Arrow, eye; A1, first antenna; A2, second antenna; P1, first pereiopod; T, telson. Scale bar = 1 mm. **b:** Ventral view of posterior part of a hatchling's abdomen illustrating attachment of telson thread to spines (arrow) at margin of the telson. Arrowhead denotes filamentous material in telson thread. Scale bar = 20  $\mu$ m. **c:** Attachment of telson thread to inner surface of egg shell (arrow). Scale bar = 200  $\mu$ m. **d:** Exuvia of Stage 1 juvenile with attached telson thread. Note division of telson thread into two parts, a short and narrow unstructured string close to the telson (arrow) and a broader heterogeneous part farther posterior. Scale bar = 1 mm.

### Brood Care and Development in Stage 2-5 Juveniles

In order to investigate brood care in the various posthatchling stages of development it was first necessary to find clear-cut characters for each juvenile stage. Stage 2 juveniles can be distinguished from Stage 1 juveniles by their larger size (2.3 mm carapace length (CL) and 4.5–4.7 mm total length (TL) instead of 1.8 mm CL and 3.4–3.6 mm TL), stalked eyes, and much longer first and second antennae (cf. Figs. 6a–c and 2a–d). Stage 3 juveniles can easily be recognized by their tail fan, which is composed of telson and uropods (Fig. 7d), whereas the tail fan of Stage 2 juveniles consists only of the telson (Fig. 6b). Stage 3 juveniles have a CL of 2.7 mm and a TL of 5.7–6 mm and still exhibit yolk reserves, at least in the initial phase (Fig. 7d). Their first antenna has an outer branch composed of five to six articles, the two outermost equipped with aesthetascs, and an inner branch of six to eight articles studded with hydrodynamic receptor hairs (Fig. 7g). Variation in the number of articles is due to incomplete separation of the most proximal articles in each of the two branches. Stage 4 juveniles are characterized by a CL of 3.1 mm and a TL of 7.0–7.3 mm and normally have no yolk inclusions (Fig. 7e). The outer flagellum of their first antenna consists of seven articles, the outermost three with aesthetascs, and the inner flagellum consisting of eight articles. Stage 5 juveniles have a CL of 3.8 mm and a TL of 7.7–8.1 mm and possess a first antenna composed of an outer branch of eight articles, four of them equipped with aesthetascs, and an inner branch of 10 articles.

Stage 2 juveniles are permanently attached to the maternal pleopods (Figs. 1a, 6a), like the hatchlings, but are more mobile and can easily change position. For attachment, they also use their first pereopods, which have hooks resembling Stage 1 juveniles, but in Stage 2 these are less recurved (cf. Figs. 6d and 4b). Like hatchlings, Stage 2 juveniles are nonfeeding and thrive on their extensive yolk reserves (Fig. 6a–c). Later stages (Stages 3–5 juveniles) associate only temporarily with their mother for resting and shelter (Fig. 7b,c), since they regularly leave for feeding excursions. Therefore, at a particular time only a part of the offspring are found attached to the maternal pleopods, whereas the rest are rambling somewhere in the surroundings. Only occasionally are all of the juveniles under the mother's abdomen. Although Stage 3 juveniles still have extensive yolk reserves (Fig. 7b,d), they start feeding soon after molting to Stage 3, as deduced from the presence of TetraMin flakes in the stomach and intestine. Normally, the yolk reserves are exhausted after the first half of the Stage 3 period, but in exceptional cases small yolk remnants were found even in freshly molted Stage 4 juveniles. Interestingly, all egg cases disappeared from the maternal pleopods when the Stage 2 juveniles had molted into Stage 3, which

may suggest that this material serves as first food for the Stage 3 juveniles. When artificially removed from the pleopods, Stage 3 juveniles immediately try to mount their mother again to attach to the pleopods (Fig. 7a). Juvenile Stages 3–5 also cling to the maternal pleopods with the chelae of their first pereopods, but these are not equipped with recurved hooks as in Stage 1 and 2 juveniles. Instead, they have short, slightly curved, and sharp terminal spikes (Fig. 7f). Adults, in contrast, have blunt knobs at the tips of the propodus and dactylus of the cheliped (Fig. 5g).

Juvenile Stages 1 and 2 are passed through rather synchronously. In our experiment the first juvenile stage lasted 4–5 days and the second stage another 6–7 days. From Stage 3 on development became very heterogeneous. The fastest specimen needed 10 days in this stage or a total of 20 days from hatching to molting into Stage 4, whereas the slowest individual needed 38 days from hatching. The first molting into Stage 5, in contrast, was documented 32 days after hatching. These differences in the speed of development account for the conjoint occurrence of Stage 3, 4, and 5 juveniles under the mother's abdomen. In our experiment the close mother–juvenile association was disbanded after the last Stage 3 juveniles had molted into Stage 4. Termination of brood care was probably initiated by the mother by an increasing aggressiveness towards the offspring, since in the time concerned 5 out of 35 juveniles were cannibalized, although food was available in excess.

### DISCUSSION

From the behavioral point of view, brood care in the marbled crayfish, and in freshwater crayfish in general, can be subdivided into three phases, a first "active" period characterized by specific breeding behavior of the female, a subsequent "passive" period, based on the interaction of preformed and only temporarily functioning structural features rather than on behavior, and a final "active" period dominated by the behavior of the juveniles. The first phase includes prespawning and spawning, the second phase lasts from attachment of the eggs to the pleopods until molting to juvenile Stage 3, and the third phase covers the remaining time until dispersal of the mother–juvenile association.

### Spawning, Attachment of Eggs, and Embryonic Development

In the spawning period, females of freshwater crayfish pass through a remarkable behavioral sequence that can be subdivided into prespawning, spawning, and postspawning behavior (Mason, 1970). Prespawning behavior includes cleaning of the underside of the abdomen, including the pleopods, the later attachment sites of the eggs. Spawn-



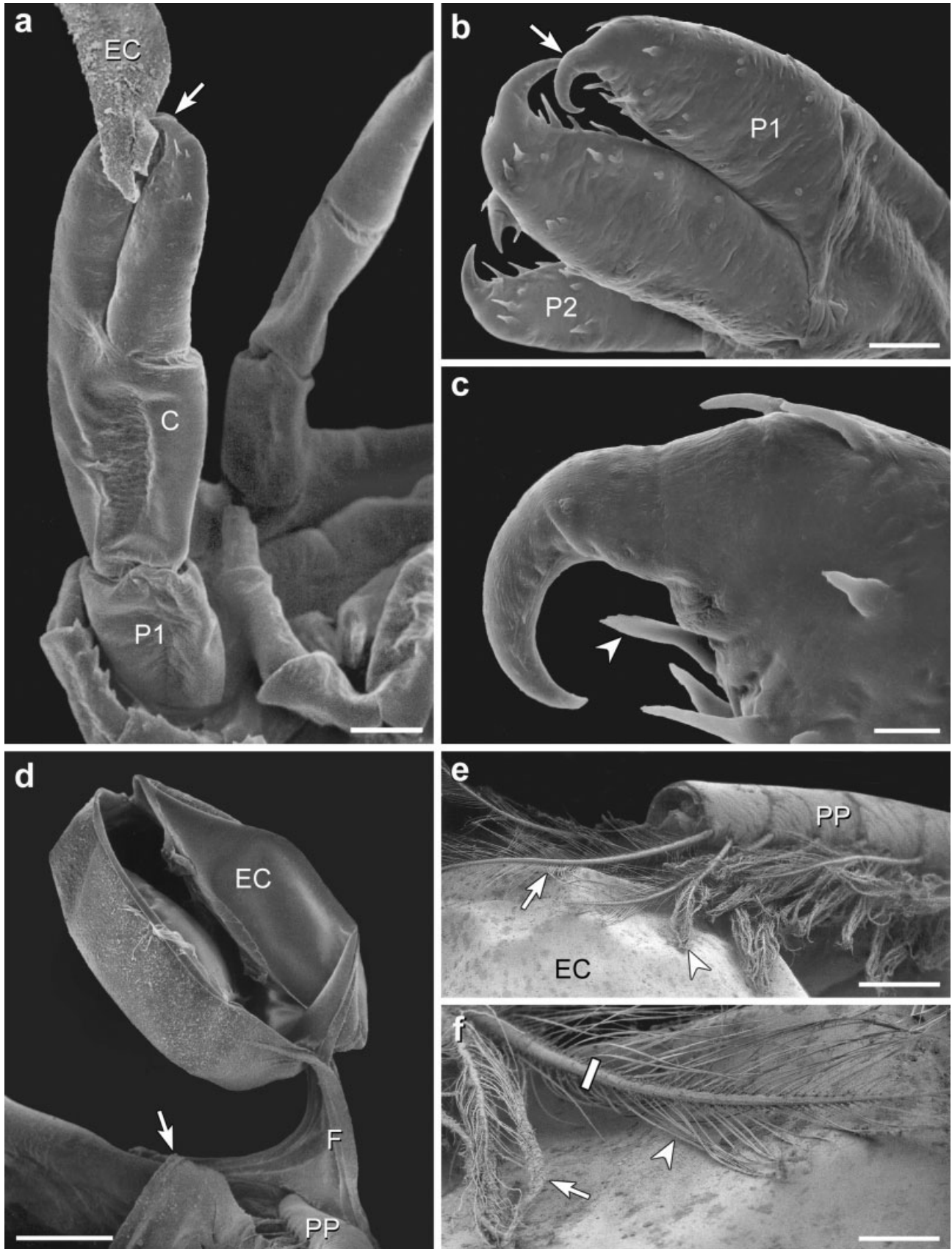


Figure 4

ing behavior is characterized by formation of a spawning pouch by bending the abdomen towards the underside of the cephalothorax. Lying on her back, the female first fills this pouch with a gelatinous secretion probably derived from different sources, e.g., the oviducts and cement glands (glair glands, pleopod tegumental glands) that are located in the sterna and pleura of the abdomen, the tail fan, and all rami of the pleopods. Then the eggs are released from the gonopores into this pouch and fertilized by sperm that is stored either in the annulus ventralis (Cambaridae) or in ventrally attached spermatophores (Astacidae and Parastacidae) (Andrews, 1904; Zehnder, 1934; Mason, 1970; Johnson and Talbot, 1987; Krol et al., 1992; Reynolds, 2002; Vogt, 2002). The marbled crayfish follows this general scheme (Seitz, 2001) with the exception that the eggs are not fertilized in this parthenogenetic species (Scholtz et al., 2003; Vogt et al., 2004).

By very specific rolling and turning movements the fertilized eggs are then attached to the pleopods (Andrews, 1904; Zehnder, 1934; Sukô, 1961; Mason, 1970), a postspawning process that in *Procambarus clarkii* lasts about 24 h (Sukô, 1961). This time period is necessary for proper attachment of the eggs to the pleopods and hardening of the "cement" that forms not only the egg stalk but also the outer layer of the egg shell (Thomas, 1991). As soon as the eggs are firmly attached to the pleopods the brooding behavior of the female is restricted to fanning and cleaning the eggs. The entire embryonic development that lasts 2–3 weeks in the marbled crayfish but more than a year in *Paranephrops zealandicus* (Whitmore and Huryn, 1999) is then completed in these attached eggs (Zehnder, 1934; Celada et al., 1987).

### Safeguarding of Hatching and Attachment of Juveniles to the Maternal Pleopods

At hatching, the egg capsule bursts on the opposite side of the egg stalk and the hatchling emerges

backwards, as observed in the marbled crayfish and other species (Andrews, 1907; Zehnder, 1934; Sukô, 1961). Hatching in decapod crustaceans is by no means fully understood, but appears to be the result of a combination of swelling and moderate mechanical action of the embryo (Davis, 1968, 1981). Literature data and our observations suggest that this is also the case in freshwater crayfish. In *Procambarus clarkii* the eggs enlarge from 2.5 to 3 mm in the 2 days before hatching (Sukô, 1961), and this phenomenon can only be explained by influx of water into the eggs. This expansion was accompanied by a decrease of the thickness of the three-layered egg shell from 1.8 to 1.4  $\mu\text{m}$  and paralleled by changes in its mechanical properties. Interestingly, Geier and Zwilling (1998) reported that during the same prehatching period the embryos of *Astacus astacus* synthesize high quantities of a hatching enzyme that belongs to the astacin family of zinc-metalloproteases. This enzyme may weaken the egg shell and detach its thin inner layer from the chorion layer, as observed by Sukô (1961). Action of the hatching enzyme on the egg shell may also enhance its permeability to water, as reported in the crab *Sesarma haematocheir* (Saigusa and Terajima, 2000). Water influx would then allow the embryo to enlarge by water uptake until the egg case disrupts with the help of flapping movements of the hatchling's abdomen (Andrews, 1904; Sukô, 1961). This interpretation would explain the fact that freshly hatched individuals are considerably larger than late embryos.

Hatching juveniles are soft, largely immobile, and unable to use their limbs. As a consequence, they would be easily dislodged from their mother by the water current if they were not roped to the egg case by the telson thread. This functional interpretation of the telson thread is very plausible and was therefore advocated by earlier authors, but in the marbled crayfish we were able to document this function for the first time in vivo. As in the other species investigated, the telson thread of the marbled crayfish was found to span from the inner surface of the egg case to the end of the abdomen, where it is tightly fixed to spines at the posterior margin of the telson. With respect to its formidable length, the telson thread of the marbled crayfish resembles more that of astacid than cambarid crayfish (Andrews, 1907; Price and Payne, 1984; Scholtz and Kawai, 2002). This structure was earlier believed to be derived from glandular secretions of the telson (Skorikow and Redikorzew, 1911; Baumann, 1932), the inner lining of the egg capsule (Hamr, 1992), or the exuvia of an embryonal molting that occurs concomitant with the hatching process (Andrews, 1907; Scholtz, 1995). Our results suggest that in the marbled crayfish the telson thread is composed of the inner lining of the egg capsule that is attached to the telson by a narrow string originating from a hardened secretion of telson glands.

Fig. 4. Active attachment of hatchlings to the mother in the marbled crayfish. SEM. **a:** Attachment of hatchling to part of egg case (EC) with terminal hooks (arrow) on first pereopod (P1). C, chela. Scale bar = 100  $\mu\text{m}$ . **b:** Chelae of first (P1) and second (P2) pereopods with terminal hooks. Arrow denotes particularly prominent recurved hooks on first pereopod. Scale bar = 50  $\mu\text{m}$ . **c:** Close-up of recurved hook and anteriorly directed cuticular spines (arrowhead) on propodus of first pereopod. Scale bar = 10  $\mu\text{m}$ . **d:** Empty egg case attached to pleopod (PP) by a funiculus (F). These structures are made up of "cement" that occasionally also covers other parts of the pleopods (arrow). Scale bar = 300  $\mu\text{m}$ . **e:** Broken pleopod with pappose oosetae lying on egg. The oosetae that are hooked upon by the chelae of the first pereopods have long lateral setules (arrow) that are often glued together by cement (arrowhead). Scale bar = 100  $\mu\text{m}$ . **f:** Close-up of oosetae with setules (arrowhead). Black and white bar indicates approximate inner diameter of recurved hooks of first pereopods to show how well hooks and oosetal shafts are matched. Arrow, setules covered with cement. Scale bar = 50  $\mu\text{m}$ .

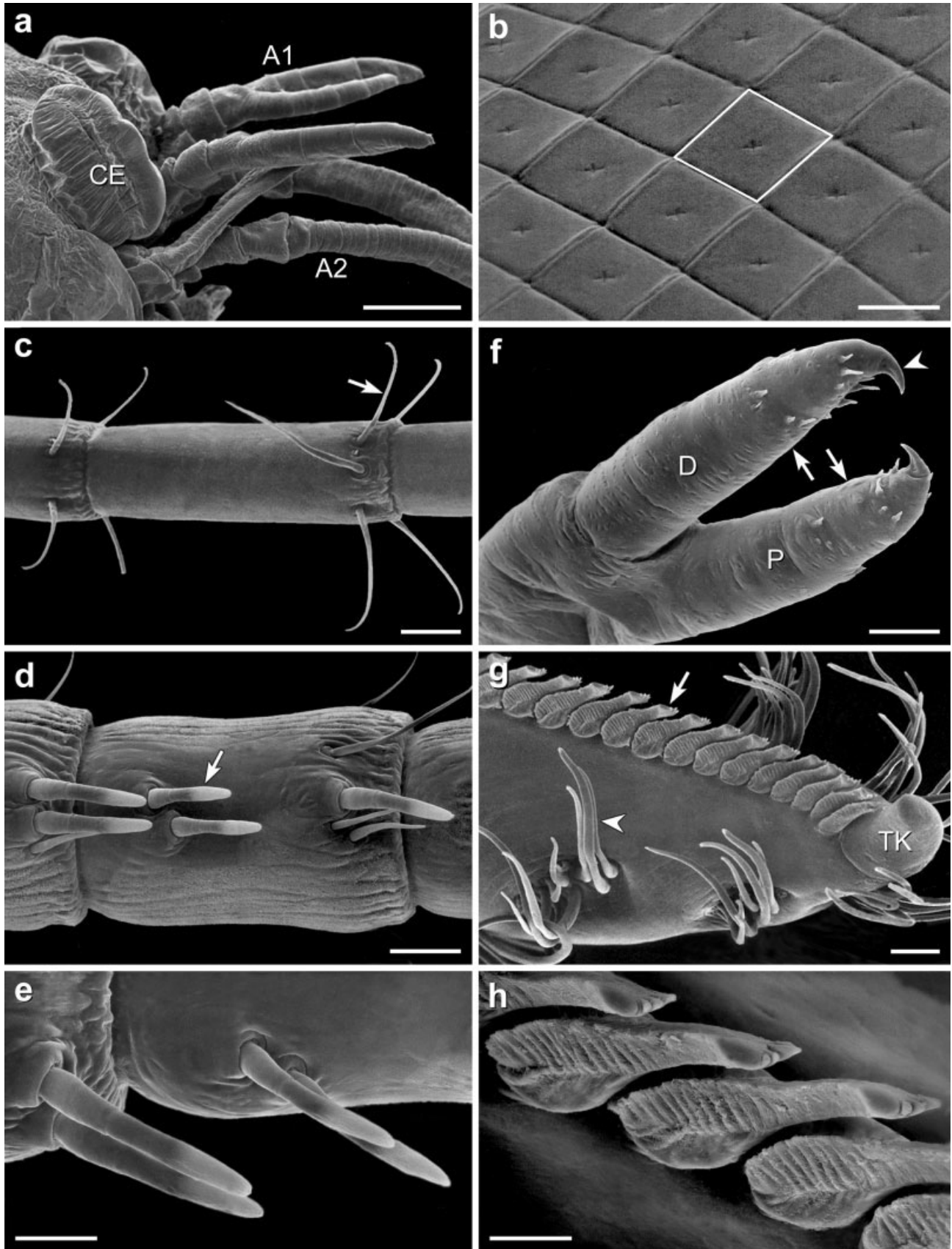


Figure 5

After some time hanging on the telson thread, the hatchlings of the marbled crayfish can move their chelae and abdomen, and by flapping of the abdomen they approach the mother's pleopods to grasp for pleopodal setae or empty egg cases. As usual in Cambaridae and Astacidae (Huxley, 1884; Andrews, 1907; Holdich and Reeve, 1988; Scholtz, 2002), they cling to the pleopods with recurved hooks on the chelae of their first pereopods. The young of the Parastacidae, in contrast, hold on with specialized hooks on the fourth and fifth pereopods (Rudolph and Rios, 1987; Scholtz, 2002). In the marbled crayfish prominent but less recurved hooks were also present on the tips of the second and third pereopods of the hatchlings, probably aiding attachment.

The chela of the first pereopod of hatchlings of the marbled crayfish (and of the Astacoidea in general) with its sharp recurved hooks, supporting anteriorly directed cuticular spines, and an overlapping closure mechanism is a biomechanical construction particularly suited for a firm grip (Nachtigall, 1974). The hooking mechanism of the Parastacoidea, composed of one hook only, without a counteracting second hook, appears to be less efficient. Frank Steuerwald (pers. commun.) indeed observed a much higher loss of juveniles in his cultured parastacid species than in the marbled crayfish. Like the telson thread, which disintegrates after some days, the pereopodal hooks of the crayfish hatchlings are transient postembryonic structures serving a sole function—the attachment of the hatchlings to their mother. In the marbled crayfish they are replaced by less recurved hooks in Stage 2 juveniles and then by relatively normal chelae after molting into the first freelancing juvenile Stage 3, which is another example of the plasticity and very variable use of the chelae in decapod crustaceans (Lee, 1995).

Aside from freshwater crayfish, only the postembryonic stages of the polar shrimp *Sclerocrangon ferox* were reported to have specific attachment structures to cling to their mother, namely, terminal hooks on the chelae of the fourth and fifth pereio-

pods (Wollebæk, 1906). In other marine and freshwater decapods that carry their young for a while the juveniles seem to grip the maternal pleopods with their normally developed chelipeds (Wear, 1967; Pace et al., 1976) as do the Stage 3–5 juveniles of the marbled crayfish.

During the attachment phase in crayfish, which comprises the first (Astacidae) or the first two postembryonic stages (Cambaridae and Parastacidae), the juveniles hang rather inactively for days on the mother's pleopods, initially doubly secured by the telson thread and the pereopodal hooks. When molting into Stage 2 the juveniles of the Cambaridae and Parastacidae remain linked to the hooked exuvia of the first stage for a short period of time by an anal thread that is composed of the inner lining of the hindgut (Andrews, 1907; Scholtz, 1995). However, it is doubtful whether this tiny structure has a securing function comparable to that of the telson thread. We have not observed such an anal thread in the marbled crayfish, perhaps because it persisted for only a very short period of time.

#### Development of Sense Organs in Juveniles

Investigation of the hatchlings of the marbled crayfish revealed that, of the major sense organs, only the compound eyes have emerged in this stage. However, partitioning into ommatidia is not yet completed. Olfactory aesthetascs, gustatory fringed setae, and hydrodynamic receptor hairs are not developed at all. The same seems to hold for the statocysts, as can be concluded from the unoriented behavior of the hatchlings (detailed information on the structures and functions of crayfish sense organs are found in Altner et al., 1983; Breithaupt and Tautz, 1990; Hafner and Tokarski, 1998; and Vogt, 2002). Comparable results with respect to the absence of aesthetascs and hydrodynamic receptor hairs in crayfish hatchlings were earlier obtained by means of light microscopy for the parastacids *Cherax destructor* and *Paranephrops zealandicus* (Sandeman and Sandeman, 1991, 1996), and with regard to the lack of statocysts for the astacid *Austropotamobius pallipes* (Thomas, 1973). Hence, practically all sense organs needed for orientation in the habitat and successful foraging or defense are absent or underdeveloped in crayfish hatchlings, which makes brood care necessary to ensure survival of the brood. In the aquarium, lost hatchlings can survive despite their locomotory and sensory deficiencies since their yolk reserves are sufficient enough to fuel metabolism until molting to freelancing Stage 3 juveniles (Baumann, 1932; Fioroni, 1969; our obs.). In nature, however, such life stages unable to swim or walk would be easy prey.

All major sense organs, with the exception of the statocysts, are structurally fully developed in Stage 2 juveniles, although their functional units are often present only in small numbers. These sense organs

Fig. 5. Comparison of major sense organs of hatchlings (a,f) and adults (b–e,g,h) of the marbled crayfish. SEM. **a**: Anterior cephalothorax of hatchling with incompletely developed compound eyes (CE) and glabrous first (A1) and second (A2) antennae. Scale bar = 200  $\mu\text{m}$ . **b**: Surface view of eye of adult characterized by rectangular ommatidia (marked by white lines). Scale bar = 50  $\mu\text{m}$ . **c**: Inner branch of first antenna of adult with whorls of hydrodynamic receptor hairs (arrow). Scale bar = 50  $\mu\text{m}$ . **d**: Ventral side of outer branch of first antenna of adult showing olfactory aesthetascs (arrow). Scale bar = 50  $\mu\text{m}$ . **e**: Lateral view of aesthetascs. Scale bar = 30  $\mu\text{m}$ . **f**: Chela of second pereopod of hatchling with terminal hooks (arrowhead) and smooth inner margins (arrows) of dactylus (D) and propodus (P). Scale bar = 50  $\mu\text{m}$ . **g**: Propodus of second pereopod of adult with numerous gustatory fringed setae (arrow) at inner margin. Instead of a terminal hook adults have a blunt terminal knob (TK). Arrowhead, groups of nongustatory sensory hairs. Scale bar = 50  $\mu\text{m}$ . **h**: Close-up of fringed setae. Scale bar = 20  $\mu\text{m}$ .



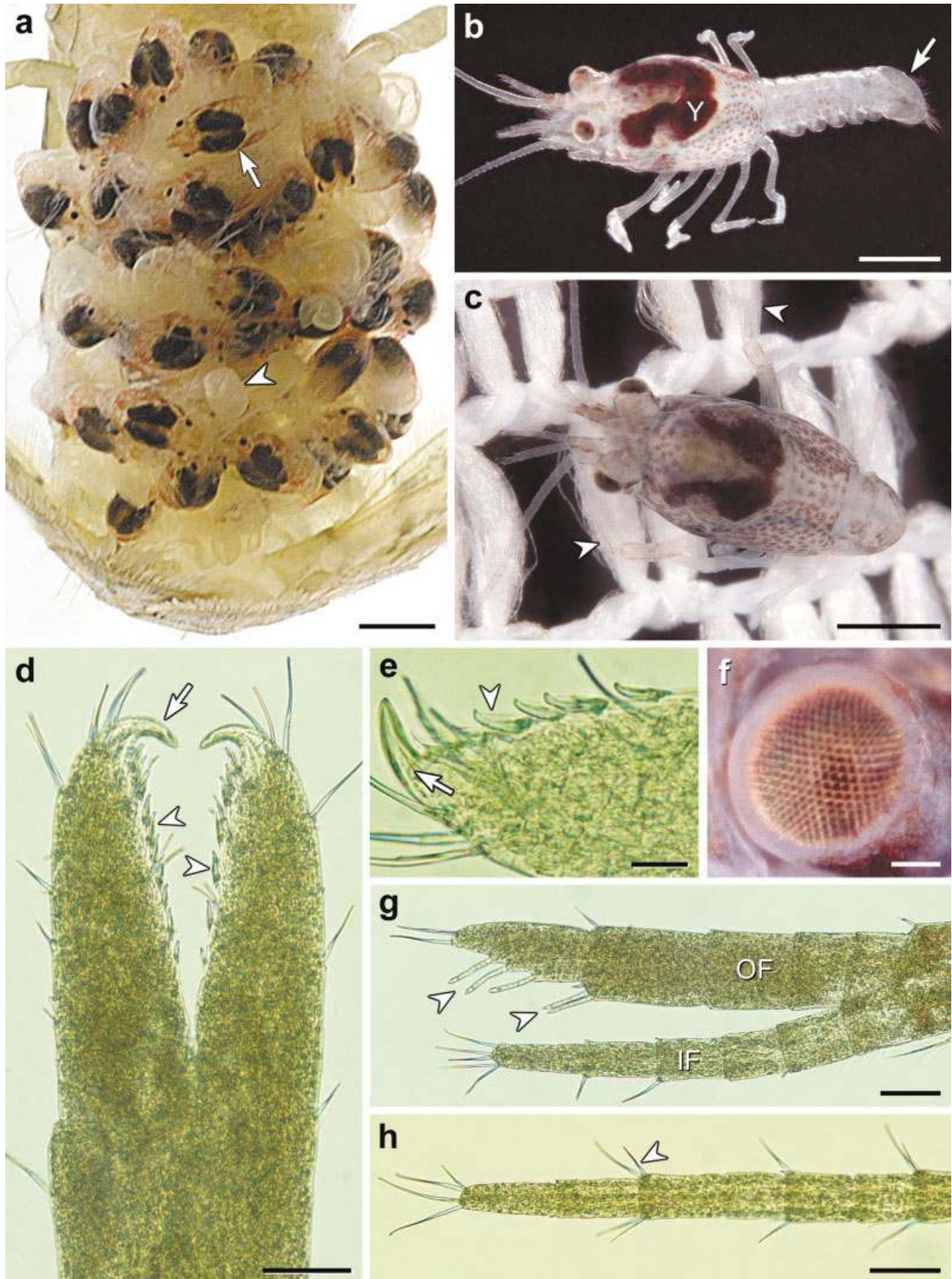


Figure 6

are not yet utilized to start an independent life, since Stage 2 juveniles are nonfeeding and are permanently adhered to the mother, but may serve to slowly adapt to the environment. Of particular interest are the aesthetascs, which in this stage may help the juveniles to recognize their mother via perception of a maternal pheromone. The existence of such a pheromone, secreted by brooding females to perpetuate the association between mother and juveniles, particularly the freelancing ones, was experimentally proved, but its biochemical identity is not resolved yet (Little, 1975; Gherardi, 2002). This pheromone is species-specific but not mother-specific.

From juvenile Stage 3 on all sense organs seem to be fully developed and functional. Hence, the juveniles can start to feed and explore the environment, having first contacts with aggressive nonsibling conspecifics and predators. Fortunately, they have enough yolk reserves in this life period to compensate for failures of foraging and also can return under their mother's abdomen to rest in a well-protected and secure place. This sheltering function of the mother can last for weeks, as in the marbled crayfish, and in extreme cases even for more than 4 months (Whitmore and Huryn, 1999). There is evidence from our observations of marbled crayfish and also from the literature (Gherardi, 2002; Reynolds, 2002) that brood care is terminated when the mother's behavior shifts back from brooding to aggressive behavior due to unknown triggers.

### Evolutionary Implications of Brood Care in Crayfish and Comparison With Other Freshwater Decapoda

All major groups of freshwater Decapoda, the atyid and palaemonid shrimps, aeglid anomurans, crayfish, and freshwater crabs, have some reproductive traits in common, namely, the reduction of egg

number compared to their marine relatives, the increase of egg size accompanied by extensive deposition of yolk, and the tendency to abbreviate larval development (Jalihal et al., 1993; Bond-Buckup et al., 1996; Sternberg et al., 1999; Shy et al., 2001; Reynolds, 2002). These features are interpreted as specific adaptations to riverine habitats or even as a preadaptation to the colonization of freshwater (Scholtz, 2002). The extensive yolk reserves ensure nutritional independence of the first postembryonic stages and facilitate rapid initial growth. The abbreviation of development, i.e., the hatching of larger, more advanced larval stages from the eggs, reduces the risk of being washed away or predated (Hancock, 1998). This aspect of the evolution of freshwaterization in decapods is nicely supported by the broad spectrum of reproductive features shown by the palaemonid shrimp genus *Macrobrachium*, which includes more than 70 species inhabiting the entire range of aquatic habitats from fully marine to freshwater. These species indeed show a gradual reduction of egg number, increase of egg size, and abbreviation of larval development from the estuaries to the headwaters (Jalihal et al., 1993).

The most successful groups of freshwater decapods with respect to species number, geographic range, morphological diversity, and ecotypes, the freshwater crayfish and crabs, have completely abolished planktonic larval stages. This reproductive strategy seems to be superior to the strategy of atyid and palaemonid shrimps that, with a few exceptions, release a free zoea-like stage into the plankton where larval development is continued by another three to six instars (Gurney, 1942). In the polyphyletic freshwater crabs (>950 species; Neil Cumberlidge, pers. commun.) the embryonic and larval period is entirely completed within the eggs, resulting in hatching of miniature adults (Sternberg et al., 1999). These juveniles are essentially independent after their first day of life (Pace et al., 1976) but often return to the mother for shelter in the next 2 weeks (Liu and Li, 2000). They can cling to their mother with their chelae but these chelae have no specialized attachment structures (Pace et al., 1976). The same is true for the ~60 species of Aegilidae, an anomuran group restricted to freshwater habitats of South America (Martin and Abele, 1988; Bond-Buckup and Buckup, 1994).

Freshwater crayfish, in contrast, are born in a more premature, defenseless, and dependent state and develop into freelancing stages with well-functioning locomotory and sensory organs only after the second or third postembryonal molt, but in this vulnerable life period they are well protected by their mother. One of the protection mechanisms, safeguarding of hatching by a telson thread, is unique in the animal kingdom. Aside from the evolution of the ability to adjust osmoregulation to freshwater in all life stages, including the hatchlings (Susanto and Charmantier, 2001), invention of safe-

Fig. 6. Brood care, attachment mechanisms, and sensory development of Stage 2 juveniles of the marbled crayfish. Macro-photography (a), microphotography (b,c,f), and light microscopy (d,e,g,h). **a:** Stage 2 juveniles (arrow) under the mother's abdomen (ventral view). Arrowhead, empty egg case. Scale bar = 2  $\mu\text{m}$ . **b:** Free-moving Stage 2 juvenile after removal from the maternal pleopods. Arrow denotes simple tail fan composed of the telson only. Y, yolk. Scale bar = 1  $\mu\text{m}$ . **c:** Stage 2 juvenile in typical attachment posture clinging with its first pereopods to a synthetic net (arrowheads). Scale bar = 1  $\mu\text{m}$ . **d:** First pereopod with recurved terminal hooks (arrow) and gustatory fringed setae (arrowheads). Scale bar = 100  $\mu\text{m}$ . **e:** Propodus of second pereopod with slightly curved terminal spike (arrow) and fringed setae (arrowhead). Scale bar = 40  $\mu\text{m}$ . **f:** Compound eye clearly subdivided into ommatidia. Scale bar = 100  $\mu\text{m}$ . **g:** First antenna with olfactory aesthetascs (arrowheads) on terminal articles of outer filament (OF). The inner filament (IF) is studded with a few hydrodynamic receptor hairs. Scale bar = 100  $\mu\text{m}$ . **h:** Terminal part of second antenna with hydrodynamic receptor hairs (arrowhead). Scale bar = 100  $\mu\text{m}$ .



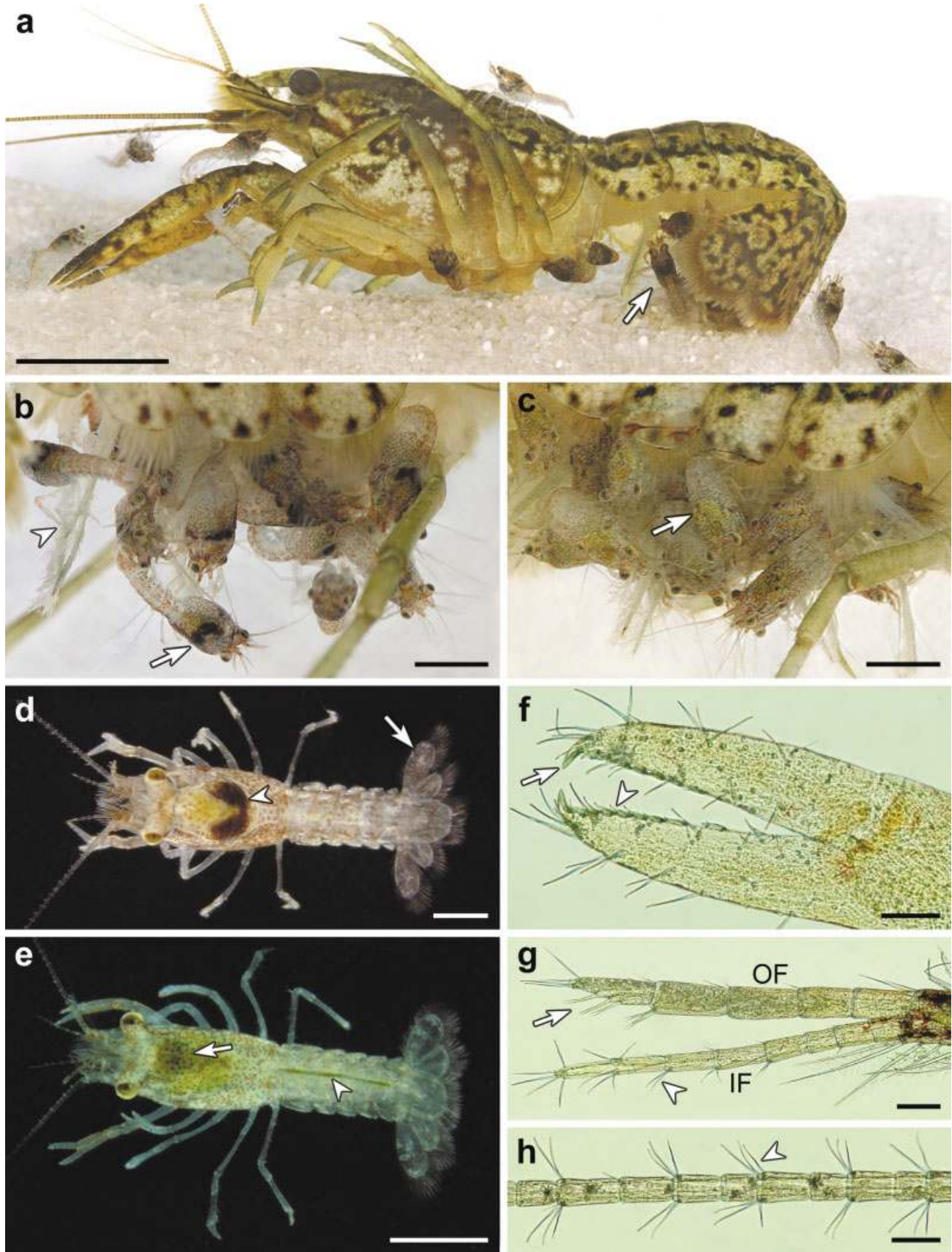


Figure 7

guarding of hatching may have been a major evolutionary clue for the worldwide colonization of most diverse freshwater habitats by the Astacida and their extensive radiation into more than 540 species.

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Fig. 7. Brood care, attachment structures, and sensory development in Stage 3 and Stage 4 juveniles of the marbled crayfish. Macrophotography (a–c), microphotography (d,e) and light microscopy (f–h). **a:** Stage 3 juveniles mounting their mother from all sides. The female shows typical brooding behavior tolerating juveniles on all parts of her body inclusive of the antennae, chelipeds, and mouthparts. Arrow denotes juvenile grasping a pleopod. Scale bar = 1 cm. **b:** Stage 3 juveniles under their mother's abdomen. Arrowhead denotes adherence of juvenile to pleopod, and arrow points at yolk reserves in cephalothorax of juvenile. Scale bar = 3 mm. **c:** Stage 3 and Stage 4 juveniles (arrow) under the mother's pleon. Scale bar = 3 mm. **d:** Freelancing early Stage 3 juvenile with typical tail fan composed of telson and uropods (arrow). Despite extensive yolk reserves (arrowhead), such juveniles feed. Scale bar = 1 mm. **e:** Freelancing Stage 4 juvenile with food in stomach (arrow) and intestine (arrowhead). Scale bar = 2 mm. **f:** First pereopod of Stage 3 juvenile with short and slightly curved terminal spikes (arrow) and numerous gustatory fringed setae (arrowhead). Scale bar = 100  $\mu$ m. **g:** First antenna with olfactory aesthetascs (arrow) on terminal articles of outer filament (OF) and abundant hydrodynamic receptor hairs (arrowhead) on inner filament (IF). Scale bar = 100  $\mu$ m. **h:** Subterminal segment of second antenna with hydrodynamic receptor hairs (arrowhead). Scale bar = 100  $\mu$ m.



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