"First Report of Developmental Changes Inside the Eggs of the Chinese Freshwater Crab, Sinopotamon yangtsekiense Bott, 1967, with Comments on Its Evolutionary Significance"

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First report of amniotic eggs in a true freshwater crab, *Sinopotamon yangtsekiense* (Potamoidea, Potamidae), with comments on its evolutionary significance

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ABSTRACT

The fertilized eggs and the egg-yolk mass of true freshwater crabs are relatively bigger and richer compared to the eggs of most marine crabs. The embryos of true freshwater crabs can develop either under water or out of water. The breeding characters show an important significance for the crab evolution. This paper focused on embryonic development of the eggs of *Sinopotamon yangtsekiense*. The eggs of *S. yangtsekiense* are surrounded by an outer membrane, and there is a second membrane that forms a fluid-filled sac around the developing embryo. A small fluid-filled transparent sac (the amnion) and an “egg nauplius” can be clearly seen on one side of the egg after spawning 13 days. After 19 days, the fluid-filled amniotic sac had enlarged further until it covered about one-half of the egg surface, with the developing embryo visible on one side of the sac. After 35 days, the amniotic sac has now expanded over the yolk mass until it wraps around the entire egg. After 65 days, the white-colored embryo has grown and has recognizable larval features, In this case, the complete developmental process inside the egg case took 77 days at which point the egg-juvenile crab hatched into a free-living hatchling crab.

Key Words*:Sinopotamon yangtsekiense*, true freshwater crab, embryonic development, amniotic egg, evolution

INTRODUCTION

Most of the more than 6,800 species of brachyuran crabs (Decapoda, Crustacea) are marine species and produce large numbers of small eggs (Ng et al., 2008). In contrast, the more than 1,280 species of true freshwater crabs are highly adapted to life in fresh water and complete their life cycle entirely independently of seawater (Yeo et al., 2008; Cumberlidge et al., 2009). Although some species of marine crabs have successfully invaded the land, most of these species still need to return to the sea in order to reproduce (Anger et al., 1990). The fertilized eggs and the egg-yolk mass of true freshwater crabs are relatively bigger and richer compared to the eggs of most marine crabs, and freshwater crab eggs remain attached to their mother's abdominal pleopods until embryonic development is complete, lying protected in the brood pouch formed by the greatly enlarged abdomen (Cumberlidge, 1999). Some species of true freshwater crabs live their entire life cycle in water, while many other species divide their time between water and land, leaving their rivers and streams when the females are carrying eggs (Liu & Li, 2000). There are even a few freshwater crabs (such as the tree hole crab, *Globonautes macropus* from Liberia) that live most of their lives out of water and are functional land crabs that rarely (if ever) enter rivers and streams (Cumberlidge, 1999).

The eggs and embryos of most aquatic organisms require immersion in water for normal development, but it would appear that the embryos of true freshwater crabs can develop either under water or out of water, and immersion is not an important factor. In other words, freshwater crab eggs have overcome the reliance on water for embryonic development seen in their marine crab relatives. Whatever the ecological preferences of the species, the eggs of all true freshwater crabs worldwide show direct development whereby all free-living larval stages typical of marine crabs are lacking, and the fertilized eggs attached to the female's pleopods mature to produce young crabs (Sternberg & Cumberlidge, 2001).

The present work focuses on embryonic development of the eggs of *Sinopotamon yangtsekiense*, a freshwater crab from the Yangtze River basin in China (Dai, 1999). This species lives in deep water for most of the year, and either moves into more shallow water or migrates onto nearby land during the breeding season. This species was therefore selected as a model organism to investigate embryonic development in freshwater crab eggs when out of water. The developing eggs of *S. yangtsekiense* are frequently not submerged for much of the 70 days or so that it takes them to mature to the point when they release the juvenile crabs. The changes that take place in the developing eggs over time are described and the theoretical significance of this independence from water in terms of freshwater crab evolution is considered.

METHODS

Freshwater crabs (*Sinopotamon yangtsekiense*) were collected from the Qiantang River, in Zhejiang, China, between July and September 2002. The crabs were maintained in the laboratory in aquaria (0.50×0.30×0.35 m3) supplied with fresh water at a temperature of 25±1°C. The fresh water was leached with a water purifier (MF-1 Filter) and replaced every two days. Crabs were fed every evening with mealworms (Tenebrio moliton) and each aquarium was partially covered with black strawboard to create a shaded area over part of the tank. Three pairs of crabs were initially cultured in each aquarium, until the female crabs spawned, at which point they were separated and cultured in individual aquaria. Ovigerous crabs were observed every day from spawning until hatching, and 2 to 4 eggs were removed each day at 8:00 am and fixed in 70% ethanol for analysis. Embryos were carefully peeled away from the egg membrane under a stereomicroscope, and photographed using a digital camera fitted to the microscope.

RESULTS

Newly laid fertilized eggs removed from the pleopods of female *Sinopotamon yangtsekiense* are relatively large, round, and uniformly orange/yellow in color. The eggs of *S. yangtsekiense* are surrounded by an outer membrane that envelopes the entire egg (the yolk mass and embryo), and there is a second membrane that forms a fluid-filled sac around the developing embryo. This latter membrane is referred to here as the amnion (following the terminology used for insect eggs). The newly laid eggs of *S. yangtsekiense* appear to consist almost entirely of yolk, but after 13 days, a small fluid-filled transparent sac (the amnion) can be clearly seen on one side the egg capsule, with the rest of the egg comprising dark yellow yolk (Fig. A). After 13 days the amniotic sac contains an embryo that is in the ‘egg nauplius’ larval stage (Fig. B). The embryo developing within this fluid-filled amniotic sac is visible as a small white mass sitting on top of the yolk. After 19 days, the fluid-filled amniotic sac had enlarged further until it covered about one-half of the egg surface, with the developing embryo visible on one side of the sac (Figs. C, D, E). After 35 days, the amniotic sac has now expanded over the yolk mass until it wraps around the entire egg. Thirty days later (after 65 days) the white-colored embryo has grown and has recognizable larval features (such as compound eyes), the amniotic space (between the embryo and the sac) has become narrow, and the yolk has changed color from bright orange to light brown (Fig. F). In this case, the complete developmental process inside the egg case took 77 days at which point the egg-juvenile crab hatched into a free-living hatchling crab.

DISCUSSION

It is clear from these studies that the fertilized eggs of freshwater crabs do not depend on immersion in water for embryonic development to proceed normally. All of the true freshwater crabs complete their entire life cycle in fresh water habitats and these crabs never enter the sea at any stage of their life (in fact, they actively avoid salt water environments) (Cumberlidge, 2008). This option is possible in true freshwater crabs because of modifications of their reproductive strategy, the direct development of their eggs to produce juvenile crabs. This strategy replaces that used by marine crabs (whereby eggs hatch in seawater and release a free-living larval stage) with one whereby eggs can hatch either in fresh water or out of water, and mature to release a miniature hatchling crab (rather than a free-living larva). In true freshwater crabs, all of the normal brachyuran larval stages are passed though during the lengthy embryonic development within the egg case, with the result that young hatchling crabs emerge directly out of the egg. Another notable feature of this reproductive strategy is that true freshwater crabs produce small numbers of large, yolky eggs attached to the female’s pleopods that develop over several weeks to produce the first juvenile (hatchling) crabs, which in turn remain in the abdominal brood pouch for several more weeks (Cumberlidge, 1999). Independence from aquatic environments is of tremendous evolutionary significance to these animals because it has allowed them to colonize not only the inland fresh waters, but also the more terrestrial parts of their habitats, in places where competition from their aquatic congeners was reduced.

It is safe to assume that the ancestors of true freshwater crabs were marine crabs that acquired the ability to osmoregulate and penetrate brackish and fresh water environments. Without adaptations of their reproductive system these ancestral crabs would still need seawater for the development of their eggs and larvae. The need to breed in seawater is still seen in those species of land crabs (Gecarcinidae, Sesarmidae) that live either in fresh water or on land when they migrate back to the ocean during the breeding season (Melo et al., 2006). The evolution of amniotic eggs and direct development in the true freshwater crabs freed them from the requirement to return to the sea, and allowed them to complete their entire life cycle in fresh water. Today's true freshwater crabs never enter the sea at any stage of their life, and all of them actively avoid salt water environments worldwide. Significantly, this reproductive strategy replaced the strategy used by marine crabs (whereby eggs hatch in seawater and release a free-living larval stage) with one where the eggs develop either in fresh water or on land and mature to release a miniature hatchling crab (rather than a free-living larva). In true freshwater crabs, all of the normal brachyuran larval stages are passed though during the lengthy embryonic development within the egg case, with the result that young hatchling crabs emerge directly out of the egg case. Another notable feature of this reproductive strategy is that true freshwater crabs produce small numbers of large, yolky eggs attached to the female's pleopods that develop over several weeks to release juvenile (hatchling) crabs, which in turn remain in the abdominal brood pouch for several more weeks (Cumberlidge, 1999).

The novel evolutionary adaptation that made direct development possible in freshwater crabs was the switch from small eggs with little yolk and a thin outer membrane, to large eggs with a massive yolk supply that had a tough outer membrane resistant to dehydration. The large-diameter egg, with an extra large yolk mass provides enough nutrition for the entire phase of enclosed larval development, because in marine crab eggs with less yolk, only the early free-living larval stages need nutrition from the egg, while the later larval stages can feed themselves. This increase in egg size in the freshwater crabs also meant that the number of eggs was reduced from many thousands to a few hundred. Another important adaptation of the eggs of freshwater crabs for independence from water was the focus of the present study on the changes in the egg membranes during embryonic development. Here we have shown that developing freshwater crab eggs have two membranes, an outer one that surrounds both the yolk mass and embryo, and an inner one (the amnion) that surrounds the embryo and forms a fluid-filled cavity (the amniotic cavity) for the developing embryo. This represents another novel adaptation because the inner amniotic membrane around the embryo provides an aquatic environment for the developing embryo inside the egg case throughout development. These adaptations meant that embryonic development in these species was possible without any contact with external environmental water (whether salty or fresh) that was a key evolutionary innovation that contributed to their success in colonizing fresh waters and eventually the land.

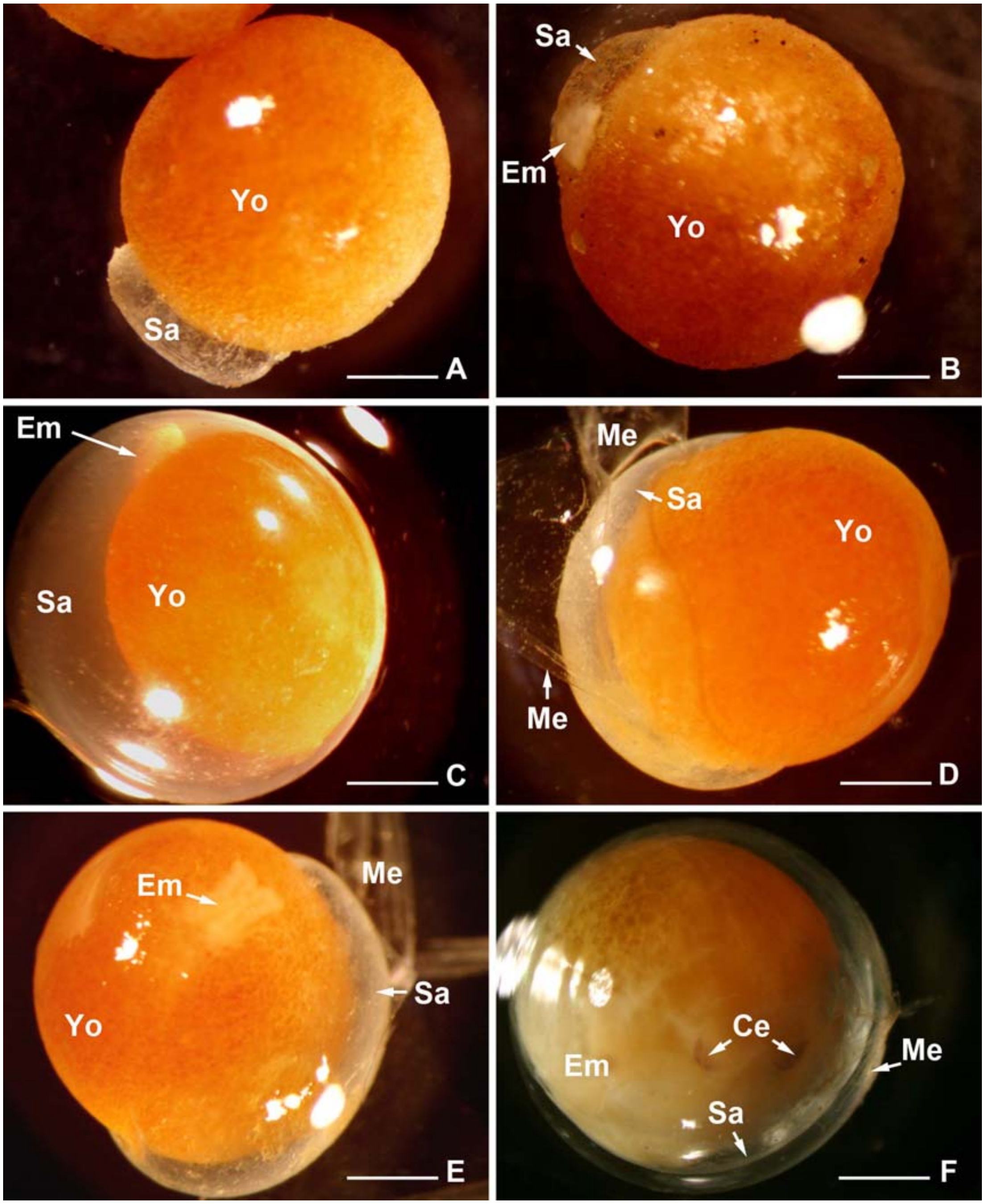

Fig. 1. The embryo of the potamid freshwater crab, *Sinopotamon yangtsekiense*. A-B the egg after 13days; C-E, the egg after 19 days; F, the egg after 65 days; Yo = yolk; Sa = sac; Em = embryo; Me = embryonic membrane; Ce = compound eye; scale bar = 1mm.

The amnion of freshwater crab eggs is comparable to the amnion of the amniote vertebrates (reptiles, birds and mammals), which is the innermost of four membranous sacs that surround and protect the developing embryo and bathe it in amniotic fluid. This reproductive adaptation provides an aquatic environment for the embryo even when in very dry habitats, and represents an important evolutionary innovation that has contributed to the success of these vertebrates in colonizing the land (Stewart, 1997). While not homologous to the amnion of the amniote vertebrates, the amniotic membrane of freshwater crabs eggs is functionally analogous in that it provides a protective fluid-filled sac around the developing embryo, and in both groups the embryo is nourished by a large yolk mass.

The presence of direct development by amniotic eggs in a semi-terrestrial species of potamid freshwater crab has no doubt contributed to its ability to survive for long periods out of water even during the breeding season. This is because the amnion of freshwater crab eggs creates an aquatic micro-environment for the developing embryo, even when the adult crab, with her eggs attached to her feathery pleopods, is out of the water. This adaptation represents an important evolutionary innovation that broke their dependency on water (both fresh water and salt water) to complete their life cycle. This has undoubtedly contributed greatly to the success of these crabs in colonizing the inland waters of the continents as well as allowing their expansion into more terrestrial habitats. Significantly, this adaptation also removed the need for these crabs to return to seawater to complete their life cycle that is the case for many of the brachyuran crabs (e.g., species of Sesarmidae, Grapsidae, Portunidae, and Xanthidae) that are found in fresh water ecosystems today. Similarly, the eggs and larvae of land crabs (Gecarcinidae) need salt water to develop and this restricts these species to the coastal areas of the world and is the reason that these crabs are never found inland far from the sea living alongside true freshwater crabs.

The amniotic eggs of freshwater crabs were most likely an adaptation that followed after the ancestral freshwater crabs had developed the osmoregulatory abilities to survive in low salt environments and had moved from sea water into fresh water. It is possible that the early ancestors of freshwater crabs still needed to return to the sea to breed, as do species of Eriocheir, Sesarma and Varuna (Varunidae) today. Direct development in freshwater crabs with amniotic eggs may have evolved after these brachyurans had colonized fresh waters near the coast, but once available it would have released them from the need to return to the sea, thereby allowing them to radiate into all available niches in inland fresh waters worldwide, including associated terrestrial habitats. The evolution of the freshwater crab amniotic egg severed the need for aquatic egg development and probably first evolved for reproduction in fresh water. The fact that this adaptation also meant that freshwater crabs were independent of any kind of water for their reproductive success probably opened the door for their subsequent conquest of drier and more terrestrial environments.

Interestingly, amniotic eggs are also found in insects whereby the eggs are surrounded by two extraembryonic membranes, an outer one (the serosa) that surrounds the entire egg, and an inner one (the amnion) that surrounds the developing embryo and forms a fluid-filled sac (Schmidt-Ott, 2000). It would appear that amniotic eggs are a necessary prerequisite for reproduction on land in many animal groups, and besides the reptiles, birds, and mammals, amniotic eggs are also found in terrestrial insects (Uchifune & Machida, 2005). The present work is the first time such an adaptation has been described for brachyuran crustaceans, and this discovery has implications for a possible common evolutionary origin of these two arthropod groups (Friedrich & Tautz, 1995). Indeed, some authors have included the Hexapoda (insects) and the Crustacea together in the Pancrustacea based on evidence from morphology, physiology, developmental biology, ecology and molecular biology (Zrzavy & Stys, 1997; Giribet et al., 2001). Further work on the genetics of freshwater crabs and other terrestrial arthropods is sure to cast more light on this interesting question.

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