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Reproductive cycle and ovarian development of the marine ornamental shrimp *Stenopus hispidus* in captivity

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ABSTRACT

The reproductive cycle and ovarian development of the marine ornamental shrimp *Stenopus hispidus* were described starting from adult mating pairs under laboratorial conditions. The pairs were individually kept in aquaria under constant conditions of salinity (35‰), temperature (26 ± 0.5 °C) and photoperiod (12L:12D). Molting, mating, spawning and hatching were recorded during three consecutive reproductive cycles. Females at different stages of ovarian development were anesthetized and their ovaries were removed, photographed, fixed and processed for histological examination. Mating occurred after female molting. Mean incubation time was 22.8 ± 2.1 days and mean intermolt periods were 25.5 ± 2.3 days for females and 26 ± 4.1 days for males. The ovaries varied in size and color during each reproductive cycle, which could be clearly seen through the transparent carapace. Spent ovary (translucent), lasted for 3 days after egg spawning; developing ovary (white), lasted 7 days; developed ovary (light green), between 5 and 8 days and advanced ovary (dark green) lasts 10 to 13 days. Microscopically, the ovarian stages differed in proportion of follicular cells, oocytes and oogonia. Follicular cells were abundant and mature oocytes were absent at the spent stage, while the number and size of secondary oocytes increased gradually thereafter. The present study indicates that *S. hispidus* can undergo multiple reproductive cycles under culture conditions. The changes in the macroscopic appearance of the ovary are strongly associated with the reproductive cycle.

1. Introduction

Stenopus hispidus (Decapoda, Stenopodidea), commonly known as "banded coral shrimp," "cleaner shrimp" or "boxer shrimp," is one of the most popular decapods in the marine aquarium trade due to its bright coloration and hardiness (Limbaugh et al., 1961; Johnson, 1977; Calado et al., 2003a). It occurs in the Indo-Pacific region, Red Sea and Western Atlantic Ocean (Holthuis, 1946, 1993), where stocks are heavily collected to satisfy demand. In the natural environment, adults of *S. hispidus* are typically found in mating pairs at coral reefs (Limbaugh et al., 1961; Johnson, 1977; Colin, 1978). The males and females form stable pairs for a long period, remaining together for at least two consecutive reproductive cycles, during which males follow and protect their female mates (Johnson, 1969). The couples inhabit a restricted area and exhibit strong agonistic behavior against conspecifics (Limbaugh et al., 1961; Young, 1979; Fletcher et al., 1995).

In the past years, efforts have been directed to reduce the fishing pressure on marine ornamental stocks and to promote their sustainable use (Corbin, 2001; Calado et al., 2003b). Many attempts have been made to develop technologies for the large-scale culture of a few decapod species including *S. hispidus*, but with little success (Fletcher et al., 1995; Palmtag and Holt, 2001; Lin et al., 2002). It may be due to the lack of biological information on this species. Studies on reproductive cycles and ovarian development provide information for the establishment of standards applicable to the management of natural stocks (Quintero and Gracia, 1998) and for the culture of commercially important shrimps (Tan-Fermin, 1991). In decapod crustaceans, ovarian development is accompanied by changes in color and size during the reproductive cycle (Adiyodi and Subramonian, 1983; Arculeo et al., 1995; Cavalli et al., 1997). These changes in color, which are easily visualized in some species, result from differences in the content of carotenoids during oogenesis, which play an important role during embryogenesis (Goodwin, 1951; Dall et al., 1995; Liñán-Cabello et al., 2002).

The ovarian development has been investigated based on macroscopic changes in color and size to determine the onset of physiological sexual maturity in natural decapod populations (López et al., 1997; Pinheiro and Fransozo, 1998; López-Greco and Rodríguez, 1999; Santos and Negreiros-Fransozo, 1999; Swiney and Shirley,

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2001; Flores et al., 2002; Castiglioni and Negreiros-Fransozo, 2006). The oogenesis process and the ovarian cycle have also been described microscopically, mainly for species of economic importance belonging to the Penaeoidea (Rodríguez, 1981; Tan-Fermin and Pudadera, 1989; Quinitio and Millamena, 1992; Quintero and Gracia, 1998; Palacios et al., 1999; Sakaji et al., 2000; Sakaji, 2001; Dumont and D'Incao, 2004), Caridea (Moraes, 1995; Mossolin and Bueno, 2002), Astacidea (Ando and Makioka, 1998; Silva-Castiglioni et al., 2006; Vazquez et al., 2008) and Brachyura (Wenner et al., 1987; Minagawa, et al., 1993; Ando and Makioka, 1999; Castiglioni et al., 2007; Rotllant et al., 2007). There is no available information on these subjects for shrimps of Stenopodidea. Therefore, this study aims to describe macro and microscopically the ovarian development of *S. hispidus* and characterize the reproductive cycle of females held in captivity.

2. Materials and methods

Stenopus hispidus were caught in Salvador (Bahia State), Brazil, by licensed fishermen of marine ornamental species. Adult shrimps with similar sizes were used in the experiment. In the laboratory, females were distinguished by the presence of the ovary, while males were distinguished by the presence of median spines on the abdominal sternites (Johnson, 1977). Due to their aggressive behavior, shrimps were separated into mating pairs of similar size, and each pair was placed in one of 12 aquaria (0.45 m × 0.20 m × 0.30 m) under constant aeration, salinity (35‰), temperature (26 ± 0.5 °C) and photoperiod (12L:12D). The aquaria were provided with beach sand as substrate and live rock for shelter.

The shrimps were maintained in a water-recirculation system adapted from Calado et al. (2007) provided with a digital thermostat (±0.5 °C accuracy), protein skimmer, UV lamp (30 w) and activated carbon (500 g, replaced monthly). In this system, the chemical communication (pheromones) was not prevented. Newly hatched larvae were caught with a light trap installed in each aquarium (Fig. 1).

The shrimps were fed in excess each afternoon with Tetra Color® diet composition, pieces of shrimp muscle, squid and shellfish. The leftover food at the following morning was siphoned out. The following parameters were checked every 15 days: Salinity, with a hand refractometer; ammonia, nitrites, pH and alkalinity, with titration test kits (Tropic Marin®). The seawater in each aquarium was partially exchanged every month with 80 l of filtered and UV-irradiated seawater. Each shrimp was considered to be acclimated after two molts.

The mean ± SD of carapace length of the shrimps, defined as the distance from the orbital angle to the posterior margin of the carapace, was 11.9 ± 0.3 mm. Each mating pair was followed for three consecutive reproductive cycles to estimate the mean duration (in days) of a reproductive cycle. The ovarian development was evaluated macroscopically by comparing the different colors of the ovary with a standard color chart (Pantone®). Each aquarium was examined daily for the presence of exuviae from each shrimp, eggs on female pleopods and newly hatched larvae. Exuviae were left in the aquaria to allow shrimps to ingest them. The number ± SD of newly hatched larvae from each female in each development cycle was counted, and data were compared among reproductive cycles using one-way ANOVA; the significance level was set at $\alpha = 0.05$ (Zar, 1996).

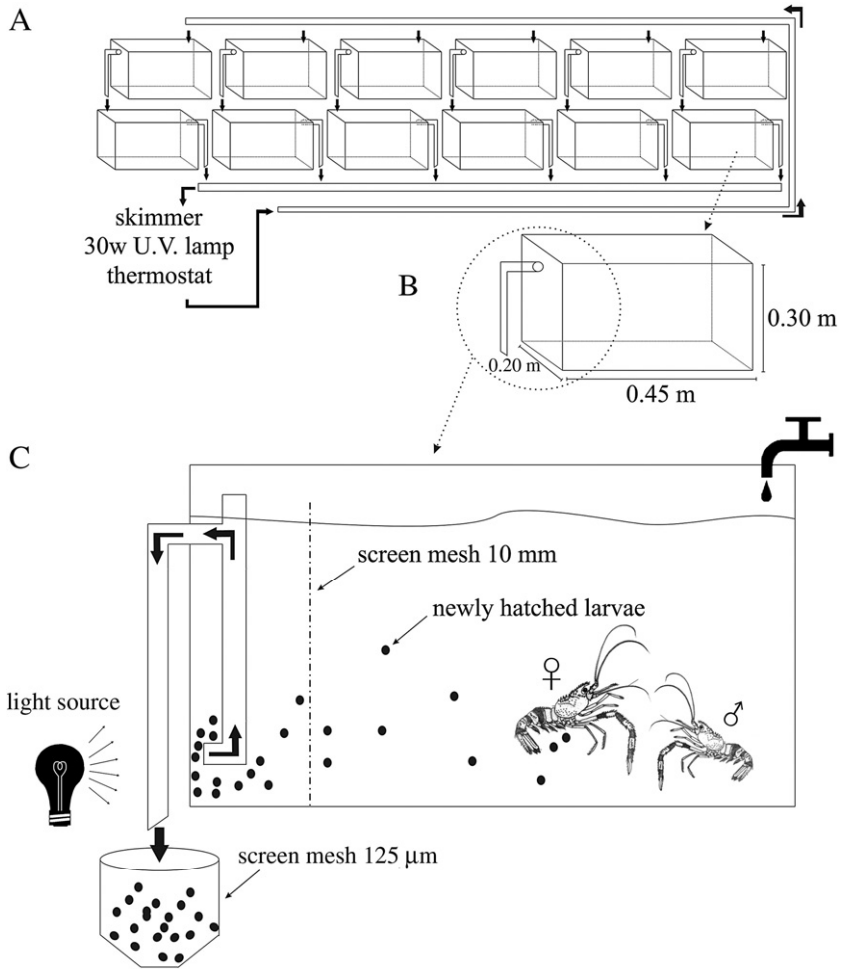

Fig. 1. Water-recirculation system for the maintenance of 12 adult mating pairs of *Stenopus hispidus*. A) General scheme of the system; B) detail showing the aquarium dimensions and overflow; and C) detail of the light trap for collecting newly hatched larvae, installed in each aquarium. Black arrows indicate water flow.

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After three consecutive reproductive events, three females were sacrificed per each ovarian stage. The shrimps were anesthetized in cold seawater, and their gonads were removed, photographed and processed for light microscopy. Tissues were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.3) for 24 h at room temperature, and embedded in glycol methacrylate (historesin) according to routine procedures; 5-μm-thick sections were stained with hematoxylin-eosin (HE) and toluidine blue. Analysis was performed under a Zeiss Axioskop 2 microscope (Carl Zeiss), equipped with a digital image system and measurement software.

To evaluate agreement between macroscopic ovarian appearance and histological findings, the largest diameter ± SD of 30 oogonia, primary oocytes and secondary oocytes were measured for each female (three female per ovarian stage). Data were log-transformed prior to analysis to meet parametric assumptions. Differences in secondary oocytes diameter among ovarian stages were tested using one-way ANOVA followed by Tukey's post-hoc test; the significance level was set at $\alpha = 0.05$ (Zar, 1996).

3. Results

During the 90-day experimental period, the twelve mating pairs underwent three mating events and had a survival of 100%. The mean embryo incubation period was 22.8 ± 2.1 days ($n = 36$). Females molted within 72 h after larval hatching, and mating was observed within the following 12 ± 3 h. Egg spawning occurred almost immediately after mating, except for two cases showing a delay of about 6 h. The mean intermolt period was 25.5 ± 2.3 days for females ($n = 36$) and 26 ± 4.1 days for males ($n = 35$) (Fig. 2). Females molted an average of 8.7 ± 2.6 days earlier than males. Females spawned at least twice and a third spawning occurred in 83.34% of the females. The mean number of newly hatched larvae was 1025 ± 166.5 ($n = 12$ females) for the first, 987.3 ± 139.4 ($n = 12$ females) for the second, and 1066 ± 109.1 ($n = 10$ females) for the third reproductive event, respectively. No significant differences were found in the number of hatched larvae among the three reproductive cycles (ANOVA, $F = 2.97$; $p = 0.06$).

The ovaries varied in size and color over each reproductive event, which could be clearly seen through the transparent carapace (Fig. 3, left). Four stages of ovarian development were distinguished by naked eye, which were confirmed by microscopic observations (secondary oocytes diameter significantly differed among stages, ANOVA, $F = 68.1$; $p = 0.00$) (Fig. 3, right). The four distinguished stages were described as follows:

- *Spent ovary*: Translucent (non-available in Pantone chart), small and thin. The gonad was difficult to recognize macroscopically and extended from the anterior region of the carapace to the beginning of the third abdominal somite. This stage lasted for 3 days after egg spawning (Fig. 4). Only oogonia and primary oocytes (mean diameter 76.3 ± 20 μm) were found in the central proliferation zone. Post-spawning was indicated by the presence of many disorganized follicular cells surrounding oocytes, empty follicles and some secondary oocytes undergoing resorption (Fig. 3A).
- *Developing ovary*: Milky-white coloration (Pantone 5875C). The gonad was larger than at the previous stage, occupying an area from the anterior region of the carapace to the end of the third abdominal somite. This appearance was maintained for 7 days and lasted 10 days after spawning (Fig. 4). Oogonia and primary oocytes (mean diameter 58.2 ± 20 μm) were observed. Advanced oocytes in early vitellogenesis (mean diameter 330.9 ± 49 μm) with yolk granules in the cytoplasm were also present (Fig. 3B).
- *Developed ovary*: Light green coloration (Pantone 556C). The gonad was much larger than at previous stages, occupying an area from the anterior region of the carapace to the end of the third abdominal somite. This appearance was maintained for 5 to 8 days (between 10 and 18 days after spawning) (Fig. 4). Oogonia and primary oocytes (mean diameter 69.5 ± 21 μm) were observed. Advanced oocytes in early vitellogenesis (mean diameter 353.5 ± 93 μm) with cytoplasmic yolk granules were larger than at the previous stage (Tukey, $p = 0.02$) (Fig. 3C).
- *Advanced ovary*: It showed a dark green coloration (Pantone 562C). At this stage the ovary reached its largest size and occupied an area from the anterior region of the carapace to the end of the third abdominal somite. It maintains this appearance for 10 to 13 days remaining in this stage until the next ecdysis and mating (Fig. 4). Oogonia and primary oocytes (mean diameter 78 ± 19 μm) were observed centrally. There were numerous mature oocytes (mean diameter 514.9 ± 85 μm) containing a large amount of yolk. The mature oocytes diameter was significantly higher than in all previous stages (Tukey, $p = 0.00$) (Fig. 3D).

4. Discussion

The analysis of the *S. hispidus* ovarian development showed that the macroscopic classification is closely related to its development and cellular organization. The gradual increase in the size of the ovarian cells has been attributed to the deposit of lipid in the ovaries during vitellogenesis (Quackenbush, 1991; Lubzens et al., 1995; Spaziani and Hinsch, 1997). As in *Artemesia longinaris*, the translucent carapace of *S. hispidus* allows direct observations, thus facilitating broodstock management at the laboratory and reducing the stress induced from handling (Dumont and D'Incao, 2004). In species such as *Penaeus monodon* and *Farfantepaenaus brasiliensis*, management procedures are difficult because the stage of female maturation is only assessed by full dissection of the gonad (Tan-Fermin, 1991; Quintero and Gracia,

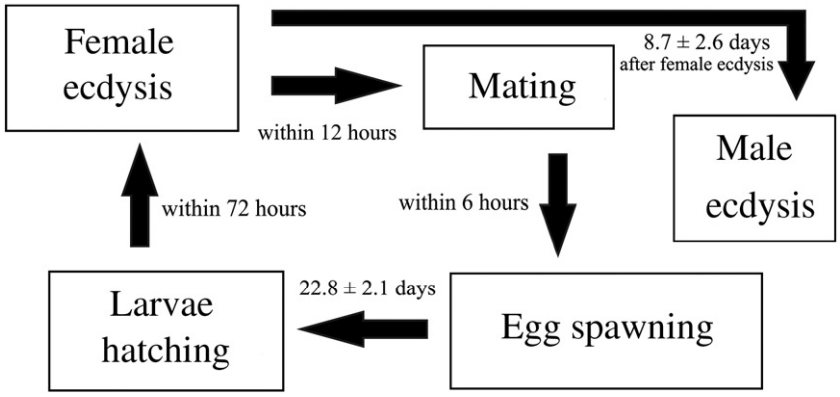

Fig. 2. Reproductive cycle of *Stenopus hispidus* under laboratory conditions. The scheme shows the relationship among ecdysis, copulation, spawning and larvae hatching, based on the observation of 12 mating pairs during 90 days after three consecutive reproductive cycles.

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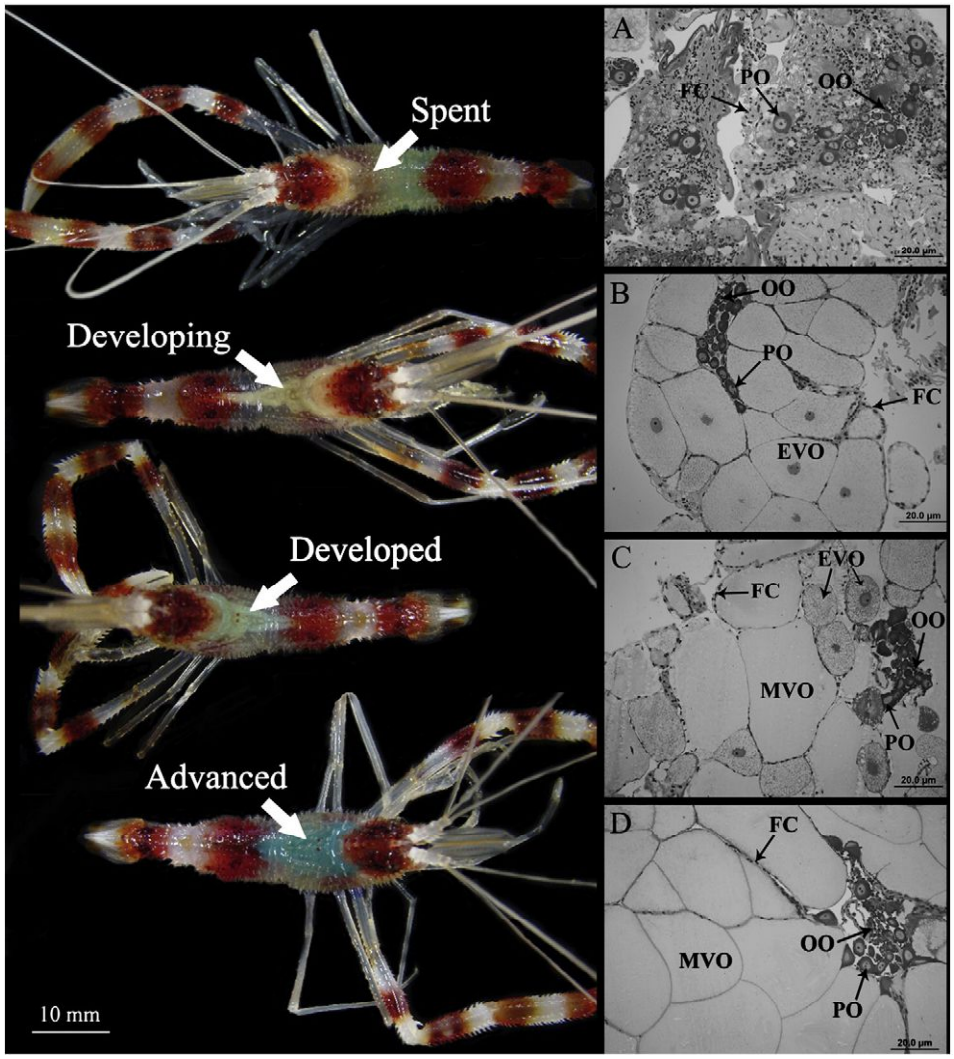

Fig. 3. *Stenopus hispidus*. Stages of ovarian development identified over one reproductive cycle under laboratory conditions. Left: Stages identified macroscopically; right: Stages identified microscopically (toluidine blue staining). A) Spent ovary; B) developing ovary; C) developed ovary; and D) advanced ovary. EVO = early vitellogenesis oocyte (secondary oocyte), FC = follicular cells, MVO = mature vitellogenesis oocyte (secondary oocyte), OO = oogonia, PO = primary oocyte.

1998). Up to five stages of ovarian maturation have been identified visually in penaeid shrimps (Vogt et al., 1989; Castille and Lawrence, 1991; Tan-Fermin, 1991; Medina et al., 1996). Subsequent studies indicated that some of these stages could be grouped due to similarities in oocyte diameter and its chemical composition (Quintero and Gracia, 1998; Dumont and D'Incao, 2004). In the present work, the four stages identified macroscopically showed a progressive increase in cell size over the ovarian cycle (Fig. 3).

The accurate measurement of the oocytes allows the validation of the macroscopic differentiation of the ovarian maturation stages, which is very useful when comparing the reproductive potential between natural and cultured populations. This comparison was already made for *P. monodon* (Yano, 1987), *Marsupenaeus japonicus* (Menasveta et al., 1993) and *Melicertus kerathurus* (Medina et al., 1996); in all cases broodstock fertility was lower in farms than in the wild, which was mainly attributed to the nutritional quality of food fed to females and to maintenance temperature.

In the present study, females of *S. hispidus* carried large egg masses and the number of larvae with positive phototropism was at least 800 per female. De Castro and Jory (1983) reported that females of this

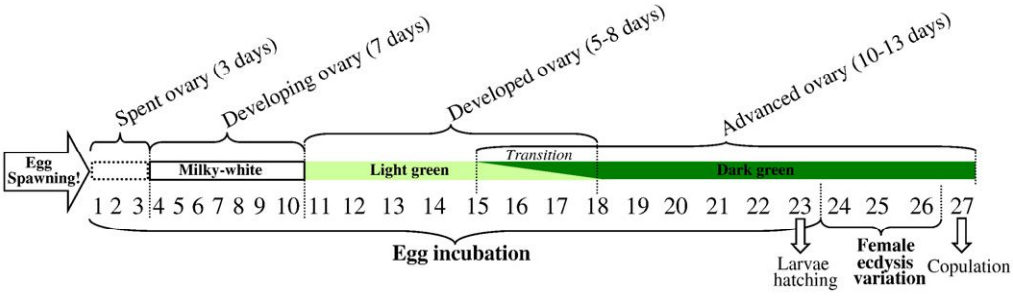

Fig. 4. Ovarian development of *Stenopus hispidus* under laboratory conditions. The scheme shows the color modification of ovaries along one reproductive cycle, based on the observation of 12 mating pairs during 90 days after three consecutive reproductive cycles.

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same species caught in the wild and maintained in captivity produced less than 850 larvae each. These authors assumed that larval production declines dramatically over time under laboratory conditions, but this was not observed under the conditions of the present study. When examining the importance of the diet on the reproductive performance of the ornamental shrimp *Stenopus scutellatus*, Lin and Shi (2002), recommended feed the females with a varied diet for obtaining more and better quality larvae.

Besides nutritional requirements, maintenance temperature and its variation are important factors influencing the reproductive cycle of *S. hispidus* held in captivity. In this study the female intermolt period was 25.5 days at stable temperature (26 ± 0.5 °C), while Zhang et al. (1997) estimated 22.5 days at temperatures between 26 and 31 °C.

The reproductive receptivity of females associated with the molting process is a common pattern among Crustacea and, in some penaeidean and caridean shrimps, the females are receptive for only a short period after ecdysis. (Salmon, 1983; Correa and Thiel, 2003). In the present study, mating always occurred within 12 h after female ecdysis, while Zhang et al. (1998) reported that *S. hispidus* females were receptive for up to 36 h after ecdysis, with the reproductive success being greater within the first 24 h.

The asynchronous molting between crustacean males and females suggests that sperm transfer requires that the male be in intermolt period or may enable the male to protect the female from predators and other males of the same species, which could be attracted by pheromones released into the water, as observed in crabs of the genus *Callinectes* (Gleeson, 1991).

The embryonic development is also influenced by temperature. In this study, the egg incubation time of *S. hispidus* at 26 °C ± 0.5 was longer than those reported for the same species under different temperature conditions: It was 16 days at 28 °C (Young, 1979) and 16 days at 25–29 °C (De Castro and Jory 1983). However, a longer egg incubation period may not have a negative effect on *S. hispidus* culture. Zhang et al. (1998), who conducted reproductive experiments with this species, observed that temperature variation had a severe negative effect on the number and size of eggs, and reported higher values of fecundity, dry weight and egg diameter for shrimps maintained under temperature variations of 1.5–2 °C than for those maintained under temperature variations of 4–6 °C.

The results provide a foundation for captive reproduction of this species. Further research is needed to overcome bottlenecks associated with the complex and extended larval cycle of the species to close the life cycle. Aquaculture production of the marine ornamental shrimp *S. hispidus* in captivity could reduce fishing pressure on wild stocks.

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