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Setogenesis and characterization of the new moult substages in the freshwater shrimp *Palaemon argentinus* (Nobili, 1901) (Caridea: Palaemonidae)

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ABSTRACT

The moult cycle of crustaceans is continuous and during different stages of this cycle, physiological, biochemical and morphological changes occur. Therefore, understanding the different stages of the moult cycle in the target species becomes critical for a wide range of biological studies. Here we describe the natural cycle changes in the freshwater shrimp *Palaemon argentinus* and identify two new substages of post-moult, B₁ and B₂, that are substages occurring before the intermoult, a stage widely used in crustacean studies. Furthermore, we present a more detailed description of stages already known, describing modifications of the structures and its presence or absence in each stage, in conjunction with explanatory pictures. We also indicate the duration for each stage of the cycle, thus expanding our knowledge of the moult cycle and setogenesis for *P. argentinus*.

Keywords

Moult cycle, Palaemonidae, post-moult, shrimp, stages.

INTRODUCTION

Crustaceans, like other arthropods, are protected by a carapace, the exoskeleton, and this structure is linked to epithelial tissues of these animals; in order to grow, they need to replace it with a larger one (Chang and Mykles, 2011). This process is known as ecdysis or moulting, and develops in a continuous cycle, which basically involves five stages: intermoult (C), early postmoult (A), late post-moult (B), pre-moult (D), and ecdysis (E) (Drach, 1939). The number of substages, however, varies among crustacean species.

Through time, various methods were employed to identify the moult stages of crustaceans, among them the observation of the layers of the exoskeleton (Drach and Tchernigovtzeff, 1969), measuring gastroliths (Shechter et al., 2008) as well as hardness and texture of the exoskeleton (Almeida-Neto and Freire, 2007). However, these evaluations may not always be used alone, and it is essential to complement them with detailed information about the formation of new setae, termed setogenesis (Drach and Tchernigovtzeff, 1969; Almeida-Neto and Freire, 2007). Setogenesis was described by Drach and Tchernigovtzeff (1969) for Palaemon serratus (Pennant, 1777). This technique is based on the development of structures of setae and appendages along the moult cycle (Aiken and Waddy, 1987; Chan et al., 1988; Díaz et al., 1998). The setae consist of animal epicuticle structures similar to elongated hairs, and their development occurs concomitantly with integument modifications (Felgenhauer *et al.*, 1989; Garm, 2004).

Usually the setae are hinged and can function as mechanoreceptors and chemoreceptors, allowing contact between the living tissue (epidermis) and the external environment, or serving as mechanical effectors. Many terms have been used for these structures, including setae, sensilla, bristle or even "hairs". For crustaceans, the most frequently used term is setae (Felgenhauer *et al.*, 1989; Garm, 2004). Setogenesis is used to identify the moult stages in many decapods, as it is a minimally invasive technique, even after several analyses (Chan *et al.*, 1988).

Identifying the moult stage of the studied animal is important for a variety of studies. During the moult cycle, animals undergo major morphological, biochemical and physiological changes (Mykles, 2011). The body increases in the post-moult due to water uptake (Drach, 1939). The accumulation of lipid, carbohydrate and protein reserves during the pre-moult (Chang, 1995) and the hormonal variation between the different stages (Mykles, 2011) are some examples of factors that may influence the results obtained in a wide range of studies that use crustaceans.

The description of setogenesis has been used widely in a variety of studies on crustaceans (Sousa and Petriella, 2006; Sugumar *et al.*, 2013; Foguesatto *et al.*, 2017). However, the lack of images, detailed descriptions, and differences in the nomenclature of the observed structures may create confusion. Moreover, many species are lacking a description of setogenesis and may have substages not yet described, as is the case of the species studied here.

Palaemon argentinus (Nobili, 1901), a freshwater shrimp that reaches a maximum size of 32 mm (females) and 29 mm (males) (Bond-Buckup and Buckup, 1989) can be maintained in the laboratory and offers a great variety of study opportunities, such as: reproductive biology (Schuldt and Capítulo, 1985), osmoregulation (Lignot et al., 1999; Ituarte et al., 2016), morphology, histology of the digestive tract (Sousa and Petriella, 2006), growth (Montagna, 2011), parasitism (Neves et al., 2004), moult cycle in the natural environment (Díaz et al., 1998; Felix and Petriella, 2003), behavioral ecology (Gancedo and Ituarte, 2017), and recently also the regulation of cell volume during the moult cycle (Foguesatto et al., 2017). Setogenesis in P. argentinus has been described only for juveniles (Díaz et al., 1998); some substages of the moult cycle of this species, however, have not yet been elucidated. Therefore, the present study aimed to disclose the new substages of adult P. argentinus, with a detailed description of the setogenesis of all moult cycle stages.

MATERIAL AND METHODS

Shrimps were collected from the riverbanks of the São Gonçalo river, Rio Grande, state of Rio Grande do Sul, Brazil (32°07'13.8"S 52°35'38.9"N). Specimens were kept in the Federal University of Rio Grande in 150 liter tanks of freshwater constantly aerated, and the bottom filled with gravel. The tanks were acclimated at room temperature (~23°C), with a 12/12 h light/dark photoperiod and fed once a day with fish food (Alcon Basic[®]). For a description of setogenesis, the study by Drach and Tchernigovtzeff (1969) was used as reference; the appendages chosen for observation were uropods (Fig. 1A). A total of 30 adult shrimps, males and non-ovigerous females, measuring between 25-32mm and weighing between 110 to 315mg, were removed from the tank and placed in a 50ml beaker with water from its aquarium. In the laboratory, these individuals were placed individually in a Petri dish with a small amount of water and observed under the light microscope with a 10x magnification. The analysis consisted of the identification and modification of the structures of setae and uropods. To determine the duration of each moult stage, shrimps were kept in separate containers until they completed the moult cycle; values are presented as mean and standard deviation of total animals. A light microscope (Primo Star, Zeiss) was used to perform the analysis and images were taken with a camera (ERc5s, AxioCam).

RESULTS

Five moult stages (early post-moult - A, late postmoult - B, intermoult - C, pre-moult - D, and ecdysis - E) were identified and five pre-moult substages $(D_0, D_1, D_1, D_1, D_1, D_1, D_2)$ were described, including two new late post-moult substages (B₁ and B₂).

Intermoult

The following structures of the setae were identified: setal cone, septum, setal node and setal base as well as other uropod components: epidermis and setal matrix (Fig. 1B). These structures are present in the intermoult stage (C) and will be modified during the other stages. The intermoult lasted 3–6 days (4.8 ± 1.02 , n = 20).

In the intermoult, setal structures were well developed. The setal cone is present in all the setae and exhibit a septum; the setal nodes are dense, the epidermis is opaque and connected to the setal base; setal matrix is the same color as the epidermis being distinguishable by an edge (known as the epidermal line in the pre-moult). The classification of the intermoult should be done cautiously in this species, because the epidermis near the base may be similar to the gap formed by the apolysis in pre-moult D_0 . A magnification of 20x is recommended for a more detailed observation.

Although *P. argentinus* is not highly calcified, there is a decrease in rigidity of the exoskeleton between intermoult/pre-moult and post-moult. In addition,

after sacrificing the animal during intermoult for experimental analysis, one can easily separate the exoskeleton of the abdominal tissue, while in the post-moult (A) the tissue is more attached to the exoskeleton; in the pre-moult D_2 , the exoskeleton is more rigid but presents a higher adherence to tissue.

Pre-moult

The pre-moult stage (D) lasted between 7–13 days $(9.4 \pm 2, n = 30)$. Within the pre-moult, five substages were identified. The substage D_0 (early pre-moult) was identified by separation of the epidermis and the cuticle (Fig. 2A). In substage D_1 , new setae start to develop, appearing between the gap formed by the apolysis, and reaching the base of the old setae; the setal matrix begins to show folds that will form the setal axes (a place where new setae originate) (Fig. 2B). In substage D_1 , the invagination of the epidermis around the setal axis begins (Fig. 2C) but is not yet as evident as in the following stages.

In D_1^{m} the invaginations that start in the previous substage, make up the setal axis, leaving the setal matrix more evident (Fig. 2D). The new setae are inserted in setal cone of the old exoskeleton, reaching about half the length of the setal cone. This stage can last from one to two days and can easily be confused with $D_{2,}$ however, the pigmentation of the epicuticle occurs in D_2 . The setal axis is more evident due to pigmentation (Fig. 2E). From this stage, *P. argentinus* reaches the moult in less than 24 hours.

During ecdysis (E) of all crustaceans, the animal sheds off the old exoskeleton. In *P. argentinus* it is possible to observe the detachment of new setae from the old exoskeleton (Fig. 2F), with their respective structures (setal cone, septum, setal base and setal node). The moult in this species is a very brief process, occurring in a few minutes.

Post-moult

Soon after the ecdysis, *P. argentinus* has a new cuticle, softer and flexible, and the uropods do not yet display all the features present in the intermoult. This stage is called early post-moult (A) with a duration of about 24 hours (24 ± 1.3 , n = 30). The new setae do not have setal cone and are filled up to the end of the setae with vesicular inclusions (Fig. 3), which is an important feature, which allows us to differentiate early post-



Figure 1. Posterior region of *Palaemon argentinus*. a, Circle indicates the observed region of the uropods (U) and setae (S) for determining the moult stages. b, Microphotography in intermoult. Abbreviations: s.m, setal matrix; s.n, setal node; s.b, setal base.



Figure 2. Uropod microphotography of *Palaemon argentinus*, pre-moult substages. a, D_0 : formation of the epidermal line with the distance of the epidermis toward the setal matrix indicates apolysis. b, D_1 : new setae crossing the gap and reaching the setal base of the old setae. c, D_1 : epidermis invagination around the setal axis. d, D_1 : new setae inserted in the setal cone of old setae; setal axes more visible. e, D_2 : last pre-moult stage; setal axis pigmented. f, Ecdysis, shedding of the new setae from the setae of the old exoskeleton. Abbreviations: e.l, epidermal line; s.m, setal matrix; \leftrightarrow apolysis; n.s, new setae; s.b, setal base; e, epidermis; s.a, setal axis.

moult from the other stages. Furthermore, the setal nodes are a bit dense and the setal matrix is opaque and has a granular appearance, and is positioned close to the setal base (Fig. 4A).

The late post-moult (B) showed a total duration of between 3–4 days (n = 20). Two late post-moult substages were identified (B_1 and B_2), which had not yet been described for *P. argentinus*. Our results showed that the substage B_1 , starting on the second day after ecdysis and having a duration of approximately 24 hours (24 ± 1.3 , n = 20), can be identified by the regression of vesicular inclusions that retreat towards the setal base, towards levels where the setal cone will be formed. In addition, qualitatively, shrimp at this stage have larger and darker setal nodes, just as the setal base is larger compared to the animals of early post-moulting. Additionally, the setal matrix loses its granular appearance (Fig. 4B).

The other substage identified in this study was designated as $B_{2,}$ and lasts 24–48 hours (35 ± 11.07, n = 20). In B_{2} the setal node appeared as round dark bodies. The setal base and all cone is formed, and the formation of the septum begins. Since these features are also observed in the intermoult stage, this

substage can be easily confused with the intermoult stage. We observed, however, that the setal cone still exhibits vesicular inclusions and the septum is yellowish and lobe-shaped (Fig. 4C), thereby differentiating the B_2 substage from the intermoult stage (Fig. 4D).



Figure 3. Uropod microphotography of Palaemon argentinus. Early post-moult (A). Abbreviation: vesicular inclusions (v.i).



Figure 4. Uropod microphotography of *Palaemon argentinus*. a, Early post-moult (A): vesicular inclusions fill the setae, setal nodes that are a bit dense. b, substage B_1 : of vesicular inclusions to levels where setal cone will be formed. c, Substage B_2 : beginning of the formation of septum, setal base formed, and the presence of vesicular inclusions in setal cone. d, Intermoult: absence of vesicular inclusions; s.n, setal nodes; s.b, setal base.

DISCUSSION

The intermoult of P. argentinus lasted 3-6 days, similar to the values reported by Díaz et al. (1998) for the same species (4–6 days). This relatively short intermoult period can be related to the average temperature maintained in both studies; $20 \pm 2^{\circ}C$ (Díaz et al., 1998) and 23°C for the present study. Montagna (2011) reported for this species an optimum temperature of 25°C and detected an increased body size of P. argentinus in relation to a shorter intermoult. This rapid growth rate in optimum temperatures may be associated with increased food consumption (Wyban et al., 1995), which was widely available for the species under study. Moreover, according to Díaz et al. (2003), a 13h/11h light/dark photoperiod favors the moult frequency, and our laboratory conditions were similar to this photoperiod.

In the present study, the pre-moult stage (D) presented a duration slightly less (7–13) than that reported by Díaz *et al.* (1998: 13–16 days for the same species). The substage D_0 (early pre-moult) was identified by separation of the epidermis and the cuticle, also referred to as apolysis by Drach and Tchernigovtzeff (1969). During this process the epidermis moves away from the base of the setae towards the setal matrix, forming a gap between the setal base and epidermal line (see Fig. 2A). Vijayan *et al.* (1997) referred to this gap as the "amber-colored zone", while Promwikorn *et al.* (2004) named it the "clear zone". We prefer to use the term "gap", since this region has no coloring in *P. argentinus*. Furthermore, the setal matrix becomes slightly wavy.

In substage D1, the new setae start to develop and the setal matrix begins to show folds that will form the setal axes. According to Drach and Tchernigovtzeff (1969) the depth of these folds may also serve to characterize the pre-moult substages. These "folds" are also referred to as "setal axis" by Aiken and Waddy (1987), "double-channel" (Aiken and Waddy, 1987; Díaz *et al.*, 1998) or "barbules/adornments" as the epicuticle becomes pigmented in D₂ (Drach and Tchernigovtzeff, 1969; Aiken and Waddy, 1987; Chan *et al.*, 1988; Díaz *et al.*, 1998). We decided to use the term "setal axis", referring to the place from where the new setae originate towards the base of the old setae.

The main change observed in the late pre-moult (D_2) is the epicuticle pigmentation. This characteristic has been well established by Drach and Tchernigovtzeff

(1969). Depending on the species studied, substage D_3 can still be observed, for example in *Litopenaeus vannamei* (Boone, 1931) (Chan *et al.*, 1988; Almeida-Neto and Freire, 2007) or even conjoined substages D_2 - D_3 such as in *Fenneropenaeus indicus* (see Vijayan *et al.*, 1997). We believe that for *P. argentinus*, D_2 is equivalent to the substage D_3 or D_4 of other species, since no other alteration was observed until ecdysis (see Fig. 2F).

Our description of the early post-moult stage agrees with the classification established by Drach and Tchernigovtzeff (1969) and observed in *P. serratus* (see Felgenhauer *et al.*, 1989) and *L. vannamei* (see Chan *et al.*, 1988). The vesicular inclusions are also named as "cellular element" in that same stage in *Macrobrachium olfersii* (see McNamara *et al.*, 1980) or "cellular matrix" in *H. araneus* (see Anger, 1983).

In the early post-moult stage (A) vesicular inclusions fill the setae (see Fig. 3) and in the late pre-moult stage (B₁) these vesicular inclusions retreat towards the setal base and the setal cone and septum is not formed (see Fig. 4A). This description is similar to that for *C. crangon*, where in the A₂ stage the same pattern of regression of the vesicular inclusions (denominated by the authors as "matrix") was observed (Hunter and Uglow, 1998). In addition, the setal nodes (or cuticular nodes) of *C. crangon* are larger and darker due to continuous secretion of the endocuticle (Hunter and Uglow, 1998) as well in *P. argentinus*. These characteristics indicate that the substage B₁ in *P. argentinus* is equivalent to the substage A₂ in *C. crangon*.

In the present study, the substage B_2 starts with the formation of the setal cone that houses the vesicular inclusions. In *Penaeus merguiensis* (De Man, 1888) the formation of the cone begins during the late postmoult (B), with the constriction of the matrix (vesicular inclusion) within the setae (Longmuir, 1983). When compared to early post-moult, the vesicular inclusions are more homogeneous and fill only half the setae. Moreover, the setal base is filled by the epidermal matrix (see Fig. 4C). Probably this filling confers the lobed and yellowish aspect that we observed in the setal base in *P. argentinus*.

Our results revealed a total post-moult (A-B) duration of 4–5 days, slightly higher than previously reported for the same species (Díaz *et al.*, 1998: 2–3 days). This difference may be due to the discovery of the two new substages ($B_{1,2}$). This post-moult duration is not surprising, since during this stage important morphological changes occur, such as the calcification of the new cuticle, which causes an increase in the integument thickness (Drach, 1939; Promwikorn *et al.*, 2004).

Based on the descriptions presented herein, we suggest the inclusion of substages B_1 and B_2 for the late post-moult stage for *P. argentinus*. This distinction becomes important, since significant physiological changes may occur between the late post-moult and intermoult stages.

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