Moult cycle and setal development of the Atlantic ditch shrimp *Palaemon varians* Leach, 1814

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ABSTRACT

The Atlantic ditch shrimp *Palaemon varians* Leach, 1814 is a common estuarine and brackish water species on Northern Atlantic coasts. *P. varians* is an appealing model organism for studying arthropod developmental processes, such as moulting (ecdysis). Detailed morphological information on its moult cycle is still lacking, hence we have characterised the changes in the setal features corresponding to the moult stages of *P. varians* grown under laboratory conditions. The stages of the moult cycle were differentiated and described using microscopic analysis of the setae in the uropods of *P. varians* based on Drach's classification system. Moult stages were defined as early and late post-moult (A and B), inter-moult (C), early-, mid- and late pre-moult (D_0, D_1 and D_2), as well as ecdysis stage (E), the actual shedding of the exuvia. Average moult cycle duration was 8.7 days, where pre-moult accounted for the longest duration of 4.4 days on average. This study provides a morphological reference for determining the moult stage of *P. varians* without the use of invasive techniques, and thus it is well suited for repetitive observations of an individual to track the entire moulting process.

KEYWORDS: Crustacea, moulting, ecdysis, arthropod, uropod, setae, exoskeleton

INTRODUCTION

Arthropods such as crustaceans need to shed their exoskeleton periodically in order to grow in a process called moulting. Moulting is a complex and tightly regulated event, governed primarily by the two main group of hormones, the ecdysteroids and sesquiterpenoids. Ecdysteroids and sesquiterpenoids play a crucial role in coordinating the various stages of moulting from the preparatory events leading to and after ecdysis (Cheong *et al*., 2015; Qu *et al*., 2018; Truman & Riddiford, 2019, Campli *et al*., 2024). In decapod crustaceans, the progression of each of these stages is initiated and coordinated by ecdysteroids synthesized in the Y-organ which is negatively regulated by the molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH), synthesized in the X-organ/sinus gland complex in the eyestalk ganglia (Skinner, 1985; Mykles, 2011, Hopkins, 2012; Webster *et al*., 2012). During the premoult stage, hemolymph ecdysteroid titers increase, the old exoskeleton is degraded, calcium and minerals is reabsorbed, and the new exoskeleton is synthesized (McCarthy & Skinner, 1977; Skinner, 1985; Skinner *et al*., 1992; Hopkins & Das, 2015;). The decrease in hemolymph ecdysteroid titers triggers a stereotyped movement and muscle contraction referred to as the ecdysis behaviour (White & Ewer, 2014) ultimately leading to the exit of the animal from its exuvia through sutural exit gapes (Daley & Drage, 2016). Successful emergence of the animal prompts post-ecdysial events such cuticle tanning and sclerotization. Given the intricate interplay of hormonal, biochemical, and behavioral processes during moulting, it is crucial to accurately determine the moult stage of an animal in an experimental design. Identifying the moult stage is essential for studying the temporal aspects of hormonal regulation, gene expression patterns, and specific physiological responses related to moulting (Mykles & Chang, 2020).

The moult cycle of Crustacea is divided into the four main stages, post-moult, intermoult, pre-moult, and ecdysis or exuviation, the actual shedding of the old cuticle (Drach, 1939; Spindler *et al*., 1974). Each moult stage can be determined in various ways such as measuring hemolymph ecdysteroid titer (Anger & Spindler, 1987; Snyder & Chang, 1991; Martin-Creuzburg *et al*., 2007; Styrishave *et al*., 2008; Mykles, 2011; Techa & Chung, 2015), gastrolith size (Shechter *et al*., 2008; Gramitto, 1998), and the structural changes of the exoskeleton and various epithelial structures (Drach & Tchernigovtzeff 1967; reviewed in Gorissen & Sandeman, 2022). Among these, one of the most widely used approaches to determine the moult stage of an animal is by examining the structural changes in its setae. Setogenesis, the formation of new setae, was initially described for moult staging of the brachyuran crab *Cancer pagurus* by Drach (1939) and *Palaemon serratus* by Drach & Tchernigovtzeff (1967). Drach's moult stage classification, which describes the main moulting stages and their corresponding substages, includes the post-moult stage, divided into the early (stage A) and late post-moult (stage B), intermoult (stage C), pre-moult (stage D and substages referred to as D_n), and ecdysis (stage E). Since then, Drach's staging system has been widely adapted to other crustacean species such as euphasiids (Buchholz, F. 1982; Buchholz, F. 1991; Buchholz & Buchholz, 2010), brine shrimps (Criel & Walgraeve, 1989), crayfish (Stevenson 1968; Mills & Lake, 1975), lobsters (Aiken 1973; Lyle & Macdonald, 1983; Musgrove 2000; Marco 2012), stomatopods (Reaka 1975), barnacles (Davis *et al*., 1973), amphipods (Graf 1986), crabs (Sugumar *et al*., 2013) and various shrimp species (Freeman & Bartell 1975, Robertson *et al*., 1987; Corteel *et al*., 2011; Foguesatto *et al*., 2017). In order to use Drach's classification system, it is first necessary to characterize the morphologically recognizable stages of the moult cycle, which may then be used as a reference system

for other studies of the species of interest. However, challenges can arise when there is a lack of images and detailed descriptions of setal structures for target taxa. One such species for which descriptions on the moult cycle and setogenesis are lacking is *Palaemon varians*.

The Atlantic ditch shrimp *Palaemon varians* Leach, 1814 is a common estuarine and brackish water species found on Northern Atlantic coasts. The species, which was formerly known as *Palaemonetes varians* (De Graves & Ashelby, 2013) is eurythermal and displays an abbreviated life cycle consisting of at least four larval instars (Oliphant & Thatje, 2021). *P. varians* is an appealing candidate for studying arthropod developmental processes, such as moulting, owing to its well understood ontogenetic series, amenability to breeding in the laboratory, and wide tolerance of aquarium conditions. Hence, we have determined the moult cycle and the duration of each stage of *P. varians* grown under laboratory conditions and provided specific anatomical description of the changes in its setae for each of the stage and substages using a simple and non-invasive technique.

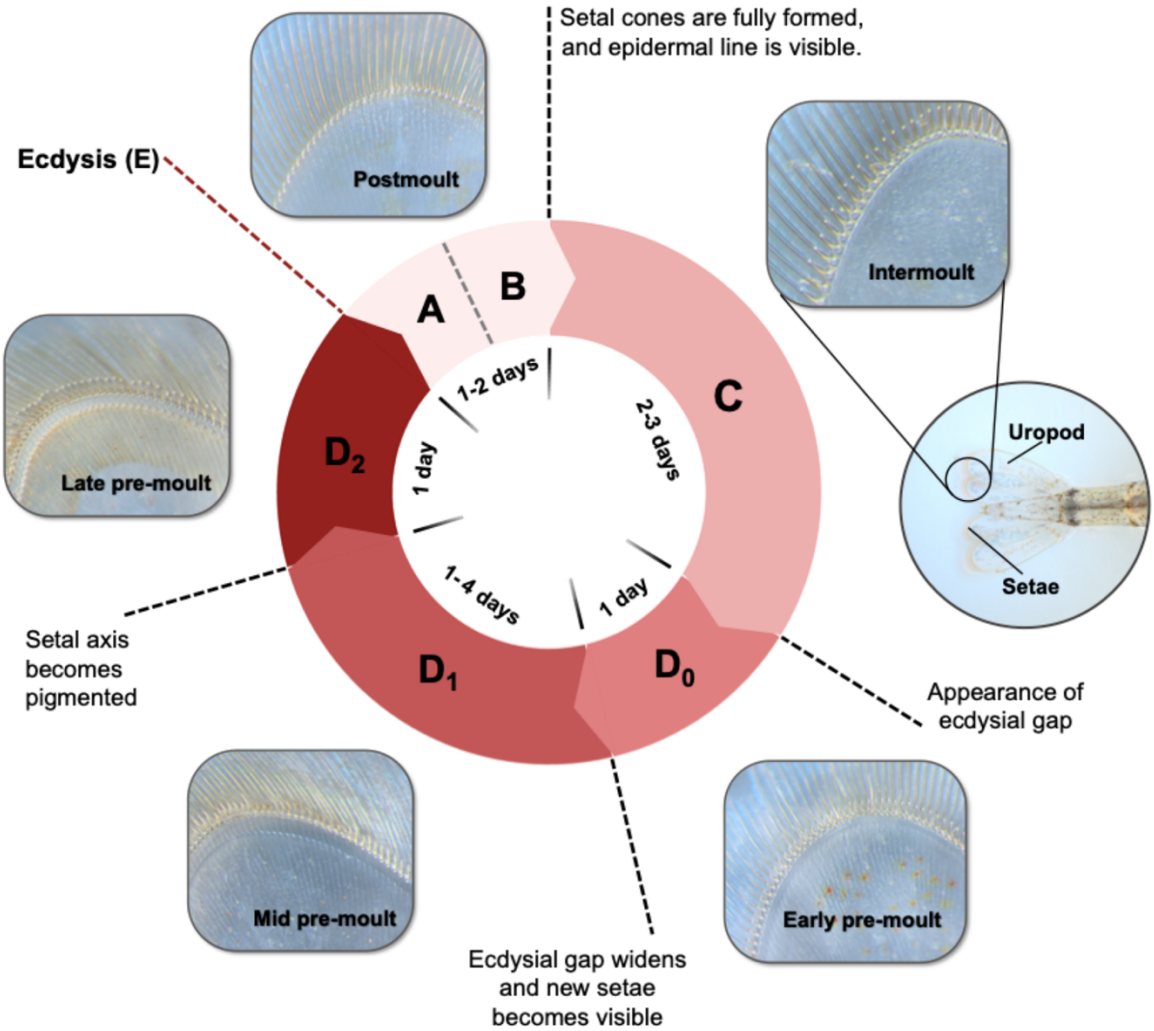

Figure 1. Overview of the moult cycle of *Palaemon varians*

Schematic diagram of the moult cycle of *Palaemon varians* and the duration in days of each stage and substage namely the postmoult stage which is divided into early (Stage A) and late post-moult (Stage B), intermoult (Stage C), pre-moult divided into early (Stage D_0), mid (Stage D_1), and late pre-moult (Stage D_2). Ecdysis stage (E) refers to the actual exit of the shrimp from its exuvia which takes place 1 to 2 days after late pre-moult (D_2).

MATERIALS AND METHODS

The shrimps used in this study were reared in an aquarium research laboratory of the Institute of Earth Sciences, University of Lausanne. Tanks containing shrimp were maintained with a water temperature of 21°C and salinity with a specific gravity of 1.019. Biofiltration was used to stabilise a large 300 litre tank for the adult population, while regular water changes were required for the small tanks that hosted juveniles. This kept total ammonia-N below 0.5 mg l-1, nitrite-N below 0.15 mg l-1, and nitrates as close to zero as possible. All tanks received artificial sunlight for 8 h per day. Breeding saltwater shrimp in captivity has some challenges and the protocol for the breeding program of this species was optimised as follows: Ovigerous (berried) females were isolated in a nursery tank until the eggs hatched, at which point the adult female was immediately removed and placed back in the main tank. Newly hatched larvae were kept isolated in the nursery (12 l) tank that was aerated using an air pump but had no filtration, as the larvae would otherwise be sucked in to the filter. They were fed every day with *Artemia nauplii* daily for 3 weeks and were then weaned onto a commercial pelleted feed. Once they grew to 1 to 1.5 cm in length, they were moved to another small juvenile tank (20 l) with filtration, and when they reached 2 cm or greater in length, they could be introduced to the main adult population tank (300 l). Mixing life stages caused cannibalistic predation so they were kept isolated until of appropriate size.

Moult stages were differentiated and characterised by observing the setae of uropods of the shrimp based on previous studies by Drach & Tchernigovtzeff (1967) and Foguesatto *et al.,* (2019). A total of 30 adult shrimps, males and non-ovigerous females, measuring between 2.5 and 4.5 cm were used for the setal observation.

Ovigerous females were not included in the study since their moult cycle gets arrested during intermoult and they undergo moulting a day after oviposition. During each of the observation periods, shrimps were kept in a separate tank (21°C, 1.019 specific gravity, 20 l, no filtration, air pump) and were followed individually each day for the duration of at least one entire moult cycle. Following the methodology of Stevenson *et al*., (1968), animals were observed by wrapping the shrimp in a wet piece of paper cloth or tissue paper with its tail protruding and placed in a petri plate for observation. The setae of uropods were observed using an Olympus SZX10 stereomicroscope using an observed magnification of 0.63 to 6.3x and were imaged using an SC50 5-megapixel colour 118 camera (Olympus Life Science Solutions) with Preciv Image Analysis Software (version 1.2; Evident Corporation). The terminology for anatomical structures used to describe setogenesis and moult stages herein follows that of Foguesatto *et al*., (2019).

RESULTS

Post-moult (Stages A and B)

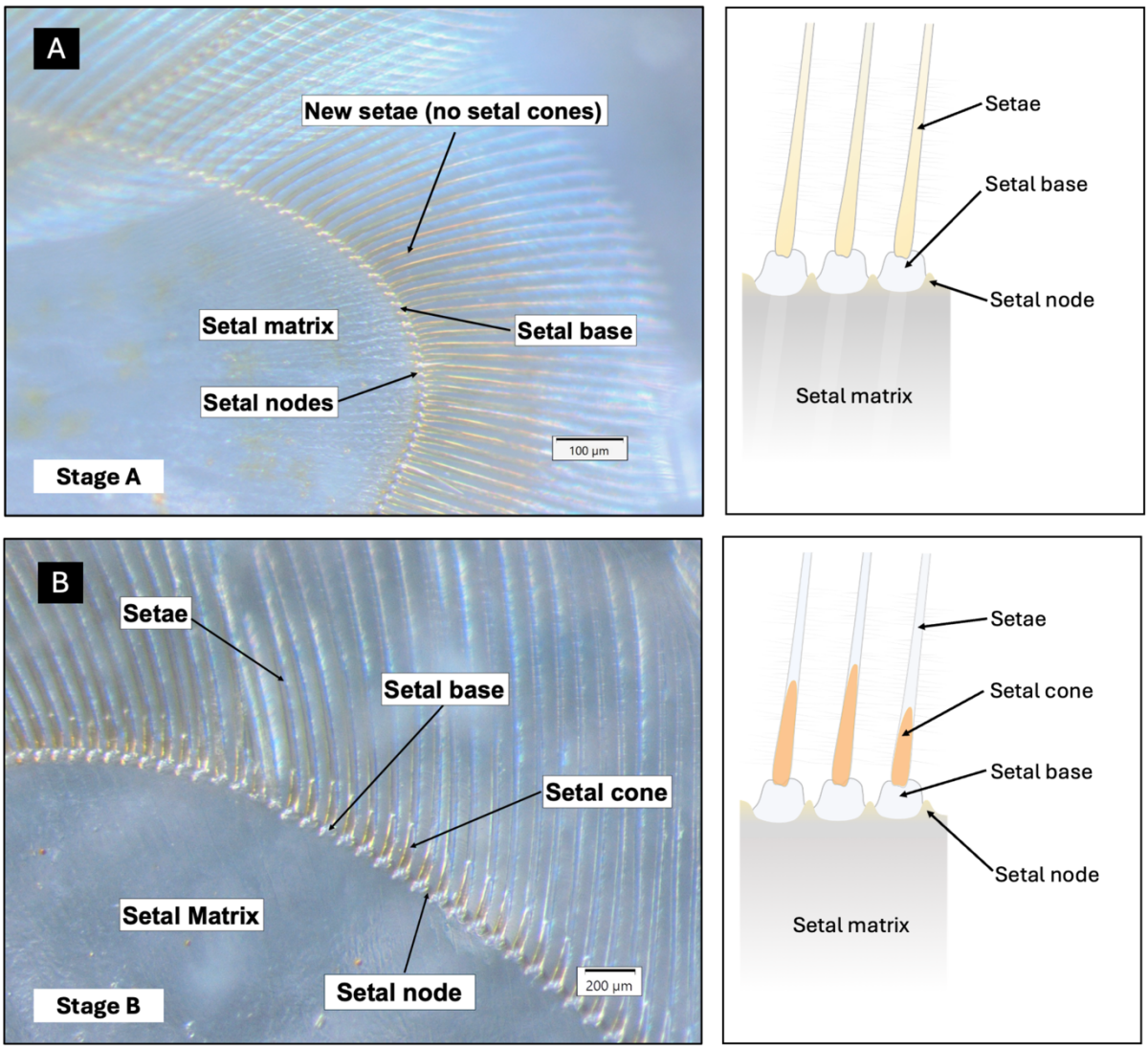

Figure 2. Microscope image (left) and simplified illustration (right) of the uropod setae of *Palaemon varians* during early post-moult **(A)** and late post-moult **(B)**

After ecdysis, the cuticle of the uropod setae of *P. varians* has a pale appearance with no visible internal features. During early post-moult (Stage A), the setae do not have setal cones and the setal bases are transparent and with slightly rounded appearance. The setal matrix reaches the base of the setae with no visible epidermal line (Fig. 2A). Most individuals reach intermoult after 24 hours except for the larger ones (size > 4 cm) which can take up to 48 hours. During late post-moult stage (Stage B) the setal

bases have a more rounded appearance and the setal matrix still reaches the setal base with no visible epidermal line in between. Setal cones have not fully formed in all of the setae (Fig. 2B). Post-moult stage has a duration of 1 to 2 days (mean = 1.13 ± 0.35, *N* = 30).

Intermoult (Stage C)

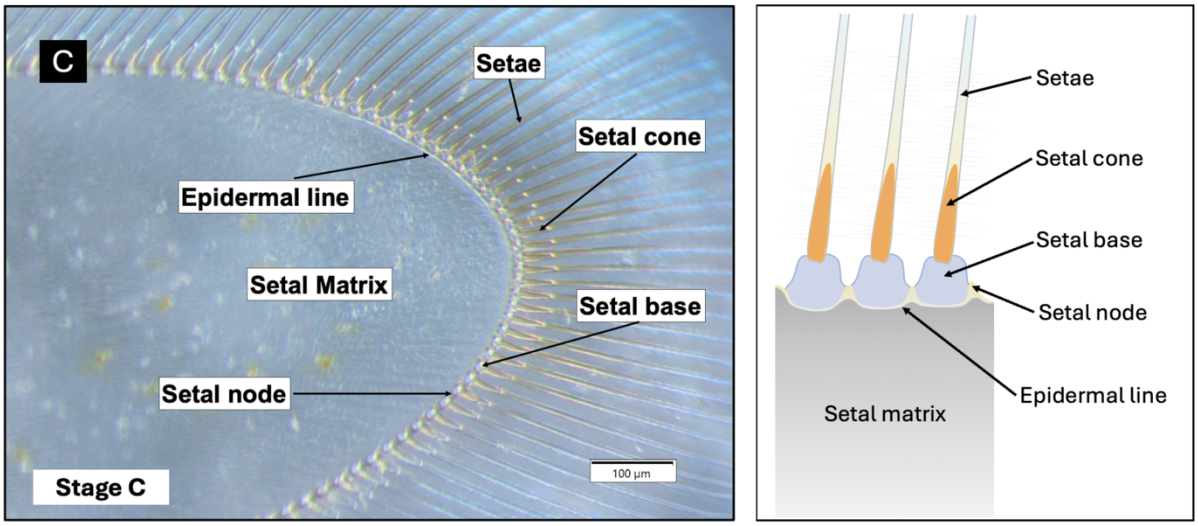

Figure 3. Microscope image (left) and simplified illustration (right) of the uropod setae of *Palaemon varians* during intermoult (C)

During intermoult, the setal structures of the uropods are well developed. The setal cone is present and visible at the base of all the setae and the epidermal line parallel to the base of the setae is visible. The epidermal line during intermoult closely follows the contour of the base of the setae and no gap is observed. Faint parallel lines can also be observed in the setal matrix where the setal axis will eventually form during the pre-moult stage (Fig. 3). The total duration of the intermoult stage is around 2 to 3 days (mean = 2.8 ± 0.38, *N* = 30). This is shorter than the observed intermoult period for *P. argentinus*, with a mean of 4.8 days from the study of Foguesatto *et al.,* (2019) and 4 to 6 days from Diaz *et al.,* (1998).

Pre-moult (Stage D)

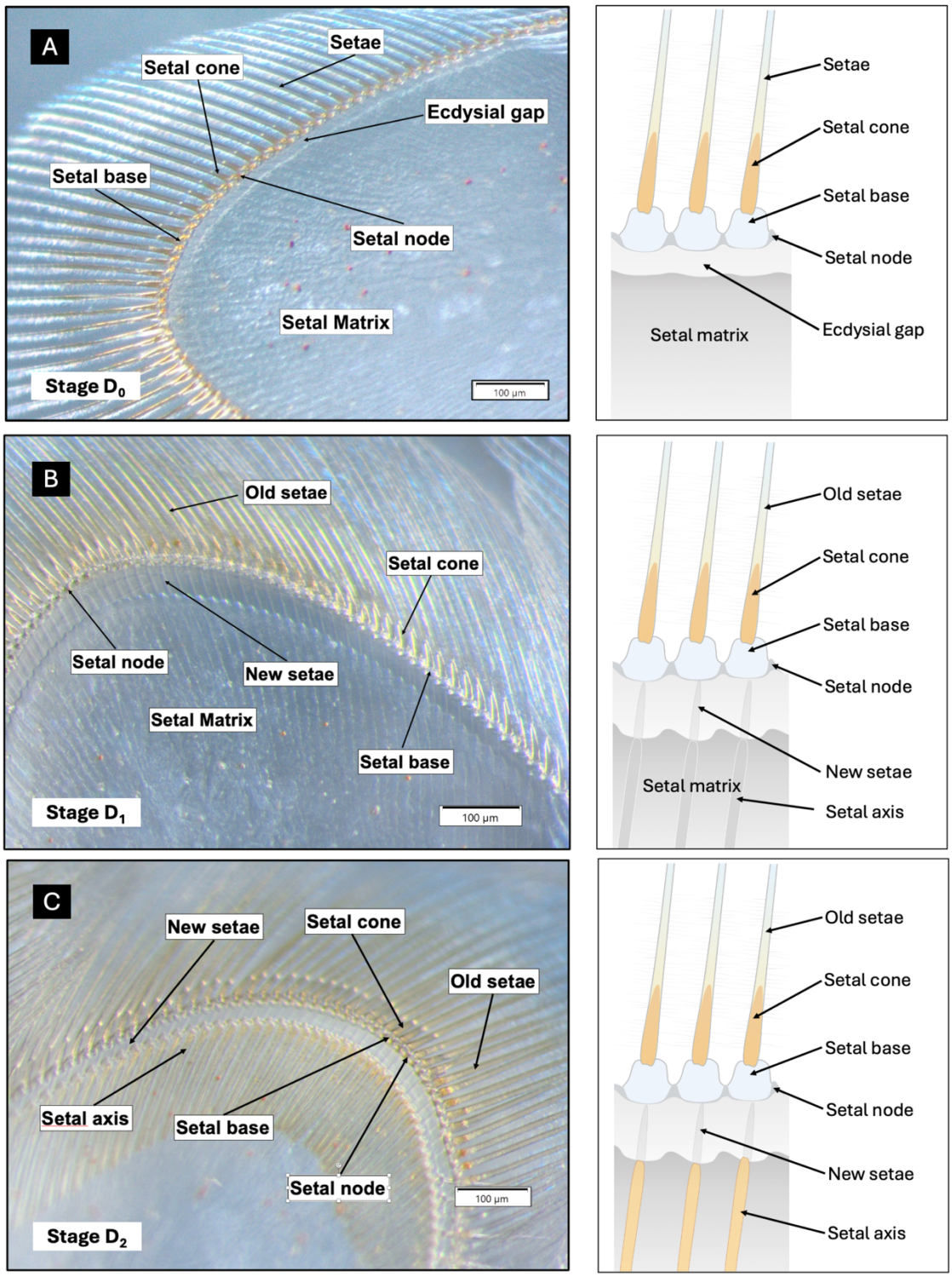

Figure 4. Microscope image (left) and simplified illustration (right) of the uropod setae of *Palaemon varians* during early-, mid- and late pre-moult respectively (A, B and C).

The early pre-moult phase (D_0) is marked by the appearance of an ecdysial gap, created by the retraction of the epidermal line away from the setal nodes as the epidermis detaches from the old exoskeleton (apolysis) (Fig. 4A). Within the setal matrix, the epidermis around the setal axes is not yet invaginated and the tips of the new setae can already be observed although it only becomes evidently visible in the next stages. During mid-premoult (D_1), the new setae within the ecdysial gap formed by apolysis and the invagination of the epidermis around the setal axes become visible (Fig. 4B). The progression of mid pre-moult towards the late pre-moult stage is characterised by the invagination of the epidermis around the setal axes, the widening of the ecdysial gap, and with the new setae and the setal axes being more visible. Finally, the late pre-moult phase (D_2), is marked by the pigmentation of the setal axes, which also exhibit a fully formed tube-like appearance (Fig. 4C). Late pre-moult phase usually lasts for 24 to 48 hours until ecdysis. Total duration of pre-moult stage is 3 to 7 days (mean = 4.4 ± 1.09, N = 30).

Ecdysis (Stage E)

This stage consists of the act of exuviation, with the animal shedding its old exoskeleton. In this species, moulting occurs rapidly in just a few seconds to minutes. The process of moulting and the behaviour immediately prior to and following exuviation is thrillingly described by (Jefferies, 1964), which was confirmed by our observations. Discarded exuviae show that the ecdysial suture opens between the carapace of the cephalothorax and the abdomen along the dorsal and lateral margins. The cephalothorax and the antennae are gradually withdrawn with an upward (dorsal) motion, after which the posterior body leaves the exuviae with a rapid flick of the abdomen (Jefferies, 1964).

DISCUSSION

The overall duration of the moult cycle of *P. varians* is 6 to 12 days (Mean = 8.7 ± 1.4, *N* = 30) with smaller individuals moulting faster than bigger ones (Pearson correlation coefficient r = 0.93, *P* = 1.3e-13). This moult cycle duration is close to that previously reported for several penaeid shrimps such as *Penaeus setiferus* and *Penaeus stylirostris,* 11.5 and 13.6 days, respectively (Robertson *et al*., 1987), *Penaeus monodon and Penaeus vannamei*, 12.3 and 10.9 days, respectively (Corteel *et al*., 2011) and relatively shorter than the duration reported for *Palaemon argentinus* (19 days) (Foguesatto *et al*., 2019). In addition to size differences, several extrinsic factors such as temperature, salinity, nutrition, and water chemistry may influence moulting duration (Lemos & Weissman, 2021). Hence, even within the same species, moulting duration exhibits variability between separate studies, as previously observed in *Palaemon argentinus* (Diaz *et al*., 1998; Foguesatto *et al*., 2019) and *Penaeus monodon* (Chan *et al*., 1988; Corteel *et al*., 2011). Even though the relative duration of the moult cycle of *P. varians* is shorter than *P. argentinus,* the defining structural changes of its setae during the moulting process are almost the same. Similarly, no species nor sex specific structural changes were observed during the moult cycle stages between *P. setiferus* and *P. stylirostris* except for the duration of the moult stages (Robertson *et al*., 1987).

During early premoult (stage D_0), formation of a gap caused by the retraction of the epidermis from the cuticle is observed. This is the main defining characteristic of the beginning of premoult similar to the observation in other shrimp species (Robertson *et al*., 1987; Chan *et al*., 1988; Corteel *et al*., 2011; Diaz *et al*., 1998; Foguesatto *et al*., 2019). However, in other species the premoult stage takes a much

longer time, making it much easier to detect and to assign specific substages. In *P. argentinus* the stage D_0 is characterized only by the formation of apolysis. In *P. varians*, the emergence of the new setae can already be seen but it is not yet as evident as in the next stages. This is similar to the moult staging for *P. setiferus* and *P. stylirostris.* Hence instead of having a separate D_0 and D_1, the moult stage was referred to as D_0 to D_1, both categorized as early premoult (Robertson *et al*., 1987). Despite having this overlapping characteristic between D_0 and D_1, the D_1 stage in *P. varians* exhibits more pronounced characteristics, including a wider ecdysial gap, the invagination of the epidermis around the setal axis, and the new setae being more protruded and visible. This observation is similar to the progression of D_0 to D_1 observed in *Palaemonetes pugio*, where towards late D_0 to D_1 the retraction of the epidermis widens, and the invaginations further develop (Freeman and Bartell, 1974). However, in contrast to *P. varians* where the emergence of the new setae already starts towards late D_0, this is only observed towards the end of D_1 in *P. pugio* and is only visible in the last stage of premoult (stage D_2). During stage D_2, the defining characteristic that is observed in *P. varians* is the pigmentation of the setal axes and the setal axes exhibiting a tubular appearance similar to *P. argentinus*. In contrast, there is no reported pigmentation of the setal axes or setal-forming regions as referred to by Freeman and Bartell (1974) in the D_2 stage of *P. pugio*. No further changes were observed during stage D_2 until ecdysis that would justify adding stages D_3 or D_4, as observed in other shrimp species (Chan *et al.*, 1988; Hunter & Uglow, 1998; Foguesatto *et al*., 2019). During the post-moult stage, the main defining feature in *P. varians* is the absence of setal cones, which is similar to other shrimp species (Robertson., et al 1987; Chan et al., 1988; Corteel et al., 2011; Diaz et al., 1998; Foguesatto et al., 2019). However, the vesicular inclusions characteristic of the post-

moult stage in *P. argentinus* were not observed in *P. varians*. There are also no definitive criteria that is observed between early and late post-moult except for the formation of setal cones.

In the present study, the moult cycle of *P. varians* was determined without the use of invasive techniques such as cutting off appendages, since these are known to influence moulting rate in crustaceans (Skinner 1985). We were able to characterize early and late post-moult (Stages A and B), inter-moult (Stage C), and early, mid and late pre-moult (Stages D_0, D_1 and D_2) using the setal changes in the uropods of *P. varians*. Discrepancies and differences in the moult stage characteristics of *P. varians* in comparison to other shrimp species and across decapoda are observed such as during the mid pre-moult and transition of late post-moult to intermoult. Our findings are consistent with the general trend of moult staging criteria across decapoda where intermoult (stage C) is characterized by presence of setal cones and absence of apolysis, early premoult (D_0) is characterized by formation of apolysis, mid-premoult (D_1) by setal invagination and pigmentation of the setal axis in late pre-moult (D_2). While there are no consistent criteria to define early and late post-moult, the absence of setal cones appear to be widely observed in various shrimp and crayfish species (Gorissen & Sandeman, 2022).

Apolysis and exuviation are processes that are conserved in Arthropoda, and the presence and absence of apolysis and the widening of the ecdysial gap in the epithelial structures can serve as potential moult stage criteria in addition to other structural changes that may be extended to other crustaceans. The present study, in addition to the work of various researchers on decapod crustaceans, have provided a rich source of morphological reference and established a nomenclature for each of the

moult stages using various epithelial structures (reviewed in Gorissen & Sandeman, 2022). Uncovering the availability of references and assessing the similarities in the moult cycle and moulting traits of decapod crustaceans to other arthropod groups remains as a future work. Current initiatives such as moultDB (www.moultdb.org), a database of moulting traits and genes, is currently in progress to collate available references across extant and extinct arthropods. Lastly, it remains as a challenge to clarify and evaluate how comparable are the moult stages in crustaceans and its corresponding nomenclature to other arthropod taxa using both morphological and genomic approaches, enabling more studies on comparative and evo-devo of moulting across Arthropoda.

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