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*Research Article*

# Influence of Moult Cycles on Digestive Enzyme Activities during Early Larval Stages of *Panulirus ornatus*

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The tropical spiny lobster, *Panulirus ornatus*, has a complex life cycle characterised by a series of moults that occur throughout pelagic larval stages. Significant morphological, physiological, and biochemical changes commonly coincide with moulting and can have dietary implications when culturing this species. Digestive enzyme activities respond to nutritional requirements and have provided useful insight into nutrient use dynamics associated with first-feeding in *P. ornatus*. Beyond first-feeding, however, information on digestive enzyme activities in *P. ornatus* is scarce. Greater knowledge of fluctuations in digestive enzyme activities during moult cycles should facilitate better formulation of feeds and more efficient feeding regimens. As an initial step towards this goal, the present study evaluated the influence of moult cycles on digestive enzyme activities during early larval stages of *P. ornatus*. The investigation focused exclusively on early larval stages (stages I–III) when delivery of appropriate feeds and nutrition can dramatically affect subsequent growth and survival.

## 1. Introduction

The tropical spiny lobster, *Panulirus ornatus*, has a complex life cycle characterised by a series of moults that occur throughout pelagic larval stages. Each moult cycle is divided into four recurrent phases: proecdysis (pre-moult), which begins the process of withdrawing the epidermis from the cuticle; ecdysis, whereby the cuticle is shed; metecdysis (post-moult), which incorporates the absorption of water and hardening of a new cuticle; and anecdysis (inter-moult), the main period of tissue growth[1]. These processes commonly coincide with significant morphological, physiological, and biochemical changes [2, 3] that can have dietary implications when culturing this species [4, 5]. Elucidation of nutritional requirements for moulting is critical to optimise hatchery feeds and feeding regimens for *P. ornatus* because malnourished larvae commonly experience delayed and incomplete moulting [6, 7].

Digestive enzyme activities respond to nutritional requirements [8] and have provided useful insight into nutrient use dynamics associated with first-feeding in *P. ornatus* [9–11]. Beyond first-feeding, however, information on digestive enzyme activities in *P. ornatus* is scarce. Although studies have demonstrated the presence of enzyme activities in *P. ornatus* at varying larval stages [12], results were primarily concerned with ontogenetic trends and provided no indication of how moult cycles might influence these trends. Greater knowledge of fluctuations in digestive enzyme activities during moult cycles should facilitate better formulation of feeds and more efficient feeding regimens [8]. As an initial step towards this goal, the present study evaluated the influence of moult cycles on digestive enzyme activities during early larval stages of *P. ornatus*. Investigation focused exclusively on the early larval stages (stages I–III) when delivery of appropriate feeds and nutrition can dramatically affect subsequent growth and survival [7, 12].

## 2. Materials and Methods

The larvae used in our study were obtained from the Australian Institute of Marine Science (AIMS) as part of a broader research project [13]. Spawning induction and maintenance of berried females followed the methods of Genodepa et al. [9, 11]. Active and photopositive larvae (stage I) were collected within 12 h of hatching and volumetrically dispensed into 100 L upwelling conical culture vessels at a density of 30 larvae $L^{-1}$. Husbandry adhered to the standard rearing protocol for *P. ornatus* at AIMS that Smith et al. [4] describe in detail. As a source of food, *Artemia nauplii* (GSL strain: 430–520 $\mu$m) were used since they are readily consumed [4] and are routinely used in the hatchery culture of *P. ornatus* [5, 13]. Preparation of *Artemia* nauplii followed the method of Genodepa et al. [9], with nauplii being introduced to culture vessels daily to achieve a density of 1.5 nauplii $mL^{-1}$. To ensure nutritional consistency, uneaten *Artemia* was passively flushed from the culture vessel two hours before refeeding.

Early larval stages of *P. ornatus* developed on a typical 6-7-day moult cycle [2, 3, 12]. Samples ($n = 4$), each comprising multiple larvae (20–40 $sample^{-1}$), were collected from culture vessels shortly after hatch and then every second day between 1 and 15 days post-hatch. Samples of stage I larvae were collected on 0, 1, 3, and 5 days post-hatch. Larvae that had completed ecdysis by 6.5 days post-hatch were transferred to separate culture vessels before collecting samples of stage II larvae on 7, 9, and 11 days post-hatch. Larvae that had completed ecdysis by 12.5 days post-hatch were again transferred to separate culture vessels before collecting samples of stage III larvae on 13 and 15 days post-hatch. Sample weights were determined using an Orion Cahn® C-33 microbalance before storage at −70°C until assayed for enzyme activities.

Fluorescence-based assay techniques were used to detect $\alpha$-amylase, non-specific esterase, and trypsin-like protease activities in larval samples following the rationale and procedures of Genodepa et al. [9–11]. In brief, assays of $\alpha$-amylase were performed using an EnzCheck® Ultra Amylase Assay Kit (Molecular Probes™: E33651), assays of non-specific esterase were performed using 4-methylumbelliferyl butyrate (Sigma-Aldrich: 19362) as a fluorogenic substrate, and assays of trypsin-like protease were performed using an EnzCheck® Protease Assay Kit (Molecular Probes™: E6638). For each assay, larval samples were initially diluted (2% w/v) with cold (4°C) deionised water and homogenised. The suspensions were centrifuged and the resulting supernatants were diluted (to 1%) with assay-specific buffers. Fluorescence was measured with a Wallac VICTOR™ MultiLabel Counter. Enzyme activities in each sample were expressed as total ($mU$ $larva^{-1}$) activities.

Statistical analyses followed the approach of Genodepa et al. [9–11]. Generalised additive models (GAMs) were used to evaluate the influence of moult cycles on enzyme activities. Total activities were modelled, independently, for each enzyme assayed as a function of time (days) post-hatch. Statistical computing was performed with *R* programming (version: 4.2.1) and the GAMs, with a Gaussian error distribution, were fit using the inbuilt generalised cross-validation tool in the *mgcv* package to determine the optimal shape of the smooth functions [14]. Pre- and post-moult activities were compared with Tukey's range tests of the observed means.

## 3. Results and Discussion

During early larval stages of *P. ornatus*, activities of $\alpha$-amylase, non-specific esterase, and trypsin-like protease were significantly influenced by moult cycles (Figure 1). Enzyme activities followed a sigmoidal pattern, where the modelled minima corresponded to post-moult phases and the modelled maxima corresponded to either inter-moult or pre-moult phases. This pattern likely reflects episodic feeding, which in later development stages ceases prior to shedding the chitinous cuticle (i.e., pre-moult phase) and only resumes once the new cuticle hardens (i.e., late post-moult phase) [15]. Since digestive enzyme activities in stage I larvae of *P. ornatus* respond quickly (within 8 h) to an absence of exogenous substrates [11], an inability to feed immediately after ecdysis logically explains why post-moult activities were consistently, and significantly, lower than pre-moult activities (Table 1). While an absence or reduced availability of food can elicit a similar enzymatic response [9, 11], the sigmoidal pattern observed here was unequivocally associated with the moult cycle as both the type and density of food remained constant.

Early larval stages of *P. ornatus* are presumed to utilise either carbohydrate or lipid as the dominant metabolic substrate, allocating protein to tissue growth rather than metabolism [16]. The difference in timing for peak activities of $\alpha$-amylase and non-specific esterase (Figure 1) suggests a shift from carbohydrate-oriented metabolism during inter-moult phases to a lipid-oriented metabolism during pre-moult phases [11]. This supposition largely agrees with biochemical composition data which show early larval stages of *P. ornatus* accumulate lipid throughout the inter-moult phases and expend lipid while transitioning from pre- to post-moult phases [3]. With lipids also crucial in moulting processes as precursors for structural components and bioactive compounds (e.g., hormones) used in signal transmission [1], the peak in non-specific esterase activities during the pre-moult phase would reflect both metabolic requirements and the rearrangement of substrates to meet anabolic requirements for ecdysis [10].

When considering the ontogenetic trend of enzyme activities over multiple moult cycles, early larval stages of *P. ornatus* exhibited an inverse relationship between non-specific esterase and trypsin-like protease activities (Figure 1; Table 1); trypsin-like protease activities tended to decrease while non-specific esterase activities tended to increase after hatch. These trends coincide with declining growth and increasing relative lipid content in successive early larval stages of *P. ornatus* [17]. They were also decoupled from the sigmoidal pattern associated with the moult cycle. While the ontogenetic trends are potentially reflective of enzymatic

Table 1: Total ($mU$ larva$^{-1}$) activities of digestive enzymes in early larval stages (stages I–III) of Panulirus ornatus during the pre- and post-moult phases of the moult cycle. Activities presented as mean ± standard deviation; common superscripts denote means which are not statistically different ($P \geq 0.05$) for a given enzyme based on Tukey's range test.

| Total activities | Stage I | Stage II | | Stage III |
|---|---|---|---|---|
| | Pre-moult 5 days post-hatch | Post-moult 7 days post-hatch | Pre-moult 11 days post-hatch | Post-moult 13 days post-hatch |
| $\alpha$-Amylase | 2476.7 ± 703.8$^{a}$ | 1255.1 ± 238.3$^{b}$ | 3492.0 ± 447.9$^{a}$ | 1144.9 ± 455.8$^{b}$ |
| Non-specific esterase | 197.1 ± 10.1$^{a}$ | 104.2 ± 9.2$^{b}$ | 339.7 ± 31.3$^{c}$ | 195.5 ± 17.5$^{a}$ |
| Trypsin-like protease* | 138.5 ± 39.2$^{a}$ | 39.3 ± 15.6$^{bc}$ | 82.6 ± 15.7$^{b}$ | 32.2 ± 14.1$^{c}$ |

*Activities as $U$ larva$^{-1}$.

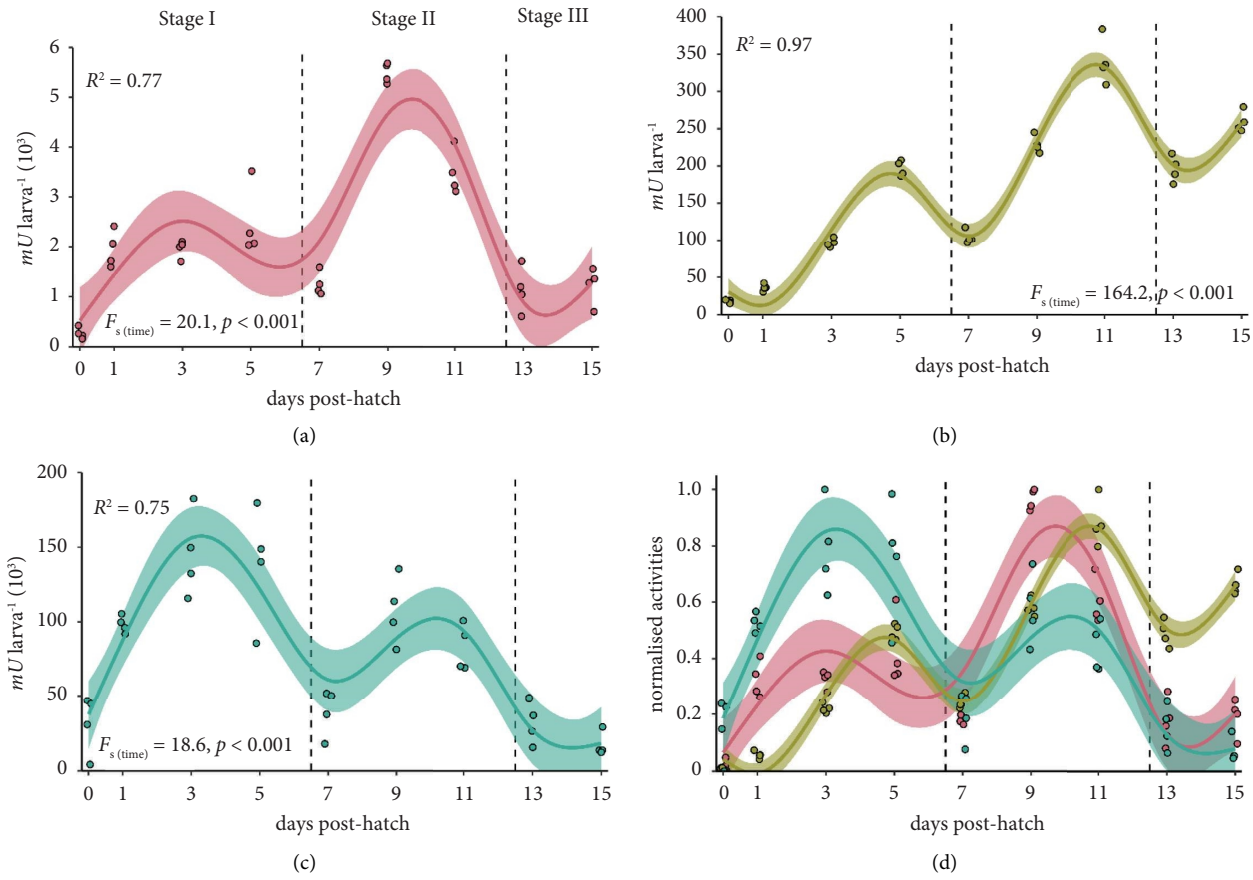


Figure 1: Observed and fitted values (±95% confidence intervals) for the generalised additive models illustrating fluctuations in total ($mU$ larva$^{-1}$) activities of (a) $\alpha$-amylase, (b) non-specific esterase, and (c) trypsin-like protease in early larval stages (stages I–III) of *Panulirus ornatus* during moult cycles. (d) Fluctuations in (a)–(c) visualised in relative terms with unity-based normalised data. Dashed vertical lines denote when sampled larvae had completed ecdysis.

acclimation to exogenous nutrition and depletion of maternally provisioned substrates [11], they also potentially reflect changing nutritional requirements [8, 12]. By stage III, the ability of larvae to feed and process diets has increased substantially [4], with our results confirming a concurrent shift in enzyme profiles. Although the transition from stage III to IV is commonly regarded to coincide with a major shift in the capture and consumption profiles of *P. ornatus* [4, 12], our results support the practice of gradually replacing *Artemia nauplii* with more lipid-rich feeds from as early as stage II (e.g., [3]).

In conclusion, moult cycles had an overarching influence on digestive enzyme activities in early larval stages of *P. ornatus*. During each moult cycle, total activities of $\alpha$-amylase, non-specific esterase, and trypsin-like protease followed a sigmoidal pattern that implied a shift from carbohydrate- to lipid-oriented metabolism during ecdysis. Moreover, reduced activities during the post-moult phase suggest that ecdysis coincides with reduced ingestive and/or digestive capabilities. Based on these results, adequate lipid nutrition during inter-moult phases appears critical. Increased lipid utilisation during pre-moult phases

corroborates hypotheses that a threshold concentration of certain lipid classes is necessary for moulting [3, 6, 7].

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

## Disclosure

First author's present address: Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo 5021, Philippines. This study was part of an AIMS@JCU collaborative mariculture research program involving the Australian Institute of Marine Science (AIMS) and James Cook University (JCU).

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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