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The effects of pre-digested protein sources on the performance of early–mid stage *Panulirus ornatus* phyllosoma

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ABSTRACT

Phyllosoma of the tropical spiny rock lobster, *Panulirus ornatus*, possess a rudimentary digestive system with a limited capacity to digest large protein molecules. As such, to foster the successful aquaculture of this species, research into dietary requirements should place a focus on feed ingredients aligned with digestive capacity. Thus, the aim of the present study was to assess the effects of two protein pre-digestion treatments: acid denaturation and enzyme hydrolysis, on a regular fishmeal ingredient in a novel formulated diet for early–mid stage *P. ornatus* phyllosoma (Stages III–VIII). Three *iso*-nitrogenous, *iso*-lipidic and *iso*-energetic diets were formulated with 100% of protein originating from intact fishmeal (IFM), acid-denatured fishmeal (DFM) or enzyme hydrolysed fishmeal (HFM) and fed to early–mid stage phyllosoma for a period of 35-days. Growth performance metrics were all significantly higher in phyllosoma receiving the HFM treatment compared to the DFM and IFM treatments. Phyllosoma fed the HFM diet also had the most advanced development stages, with a significantly greater proportion of individuals reaching Stage VII (2). No significant differences were detectable in either the protein-bound or FAA composition of phyllosoma across all treatments, suggesting that the superior growth performance of the HFM fed phyllosoma was the result of an increased abundance of intermediate, shorter chain dietary peptides. The present study suggests that enzyme hydrolysed fishmeal is a superior protein ingredient for artificial diets and most closely resembles the requisite dietary protein format for *P. ornatus* phyllosoma.

1. Introduction

The lucrative economic potential of Palinurid spiny rock lobster aquaculture has motivated many scientific endeavours to cultivate these crustaceans in captivity to meet demand that exceeds supply from the wild (Francis et al., 2014; Kittaka, 1997; Matsuda and Takenouchi, 2006; Nelson et al., 2004; Wu et al., 2012). However, hatchery closure of the life cycle is severely impeded by the obscurity of key nutritional requirements during the complex pelagic larval stage (Cox and Johnston, 2003; Jeffs, 2007). Of all spiny rock lobsters, the tropical species, *Panulirus ornatus*, presents a propitious candidate for aquaculture, possessing a comparatively short larval phase and fast growth rate (Francis et al., 2014; Smith et al., 2009a).

Previous attempts to culture *P. ornatus* phyllosoma have relied on live and fresh diets such as enriched *Artemia*, oceanic zooplankton and mussel gonad (Perera et al., 2008; Wu et al., 2012). Notably, survival from newly hatched phyllosoma to puerulus under these feeding regimes is typically very low, and hence unviable from a commercial perspective (Francis et al., 2014). The development of formulated feeds for larval crustaceans presents many advantages including nutrient optimisation, consistent quality, and generally, a lower cost (Andres et al., 2011; Kovalenko et al., 2002; Ohs et al., 1998). However, poor digestibility and subsequent difficulties in nutrient assimilation are often present as crucial obstacles in the successful replacement of live feeds with formulated feeds (Arredondo-Figueroa et al., 2013; Cahu et al., 1999; Jones et al., 1993). Live feeds contain a natural abundance of digestive enzymes which may contribute to the digestive capacity of the larvae (Kumlu, 1999). On the other hand, these digestive enzymes are typically not present within formulated feeds, resulting in assimilation difficulties for developing larvae (Jones et al., 1997; Lauff and Hofer, 1984; Rønnestad et al., 2013; Walford and Lam, 1993). This is especially problematic for spiny rock lobster phyllosoma, which possess only a rudimentary gut system with low digestive enzyme levels (Johnston, 2006; Kumlu, 1999; Lipcius and Eggleston, 2000).

Inefficient dietary protein assimilation presents a key difficulty in the use of formulated feeds for larval culture of crustacean species (Crear et al., 2000; Le Moullac et al., 1996; Perera et al., 2005; Tolomei et al., 2003).

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As the most expensive component of formulated diets (Perera et al., 2005), proteins are integral for the growth, repair and maintenance of all cells, as well as the supply of amino acids, which are essential constituents of all life forms (Kaushik and Seiliez, 2010). Proteins are large nutrients that must be hydrolysed in the digestive tract by proteases into more readily assimilated nutrients such as free amino acids (FAAs). It is only the small molecular weight peptides and free amino acids that can be assimilated into the digestive glands and be effectively utilised by the larvae to promote growth (Hovde et al., 2005; Kaushik and Seiliez, 2010; Kvale et al., 2002; Perera et al., 2005). In lobsters, the efficiency of such assimilation is determined by the quality and quantity of dietary protein, the development stage of the larvae, and the digestibility of the protein source (Jensen et al., 2013; Ward et al., 2003). Judicious selection of the protein component is therefore integral to the efficacy of feeds formulated for phyllosoma culture.

Pre-digested protein has been used in formulated diets of various larval fish species to facilitate and maximise digestibility in underdeveloped gut systems (Johannsdottir et al., 2014; Kolkovski and Tandler, 2000; Kvale et al., 2002; Nankervis and Southgate, 2009; Srichanun et al., 2014). It has been shown that diets containing predigested protein increase survival and growth (Lindner et al., 1995; Nankervis and Southgate, 2009; Savoie, 2006), enhance attraction (Lian et al., 2008), reduce deformities (Johannsdottir et al., 2014; Zambonino Infante et al., 1997) and expedite gut development (Cahu et al., 1999) in larval fish. However, to the best of our knowledge, the use of pre-digested protein has not been tested for the culture of planktonic larval crustaceans.

Acid denaturation and enzyme hydrolysis are two practical methods of protein pre-digestion (Nankervis and Southgate, 2009). Acid denaturation disrupts the normal alpha-helix and beta sheets in a protein and uncoils it into a random shape, losing the quaternary, tertiary and secondary structure (Buxbaum, 2007; Jaenicke and Rudolph, 1990). The uncoiling of proteins into polypeptide chains increases the available sites for proteolytic enzymes to bind, which in turn increases protein digestibility (Buxbaum, 2007; Lauff and Hofer, 1984). As the acid denaturation reactions do not break the peptide bonds, the amino acid sequence remains the same (Fink et al 1994, Uversky and Yuji, 2009). Alternatively, enzyme hydrolysis of protein cleaves peptide bonds, resulting in compounds of reduced molecular size (Geirsdottir et al., 2011; Jaenicke and Rudolph, 1990; Uversky and Yuji, 2009). Smaller peptide chains and FAAs are generally considered easier to absorb and thus increase the level of amino acids available for protein accretion (Buxbaum, 2007; Kaushik and Seiliez, 2010; Kvale et al., 2002).

The development of an optimal diet for *P. ornatus* phyllosoma relies upon the fulfilment of a number of criteria, including sourcing a protein ingredient that can be effectively utilised by phyllosoma. To augment previous work on this topic, this study sought to test the efficacy of a readily digestible protein component for inclusion in formulated larval diets. Three dietary protein treatments were compared: untreated fishmeal, acid-denatured fishmeal and enzyme hydrolysed fishmeal, with the superior treatment being determined by analyses of phyllosoma growth, development, nutritional composition and dietary gut retention. Since the digestive ability of spiny rock lobster phyllosoma is expected to improve with development and alter dietary requirements (Jeffs, 2007), this investigation focused exclusively on the early–mid larval stages (III–VIII).

2. Materials and methods

2.1. Animals and husbandry

P. ornatus phyllosoma were hatched from broodstock held in the Tropical Mariculture Facility at the Australian Institute of Marine Science (AIMS, Townsville, Australia, lat. 16° 17.728′S, long. 145° 27.121′E). Fifteen replicate U-shaped tanks of 25 L capacity were each stocked with phyllosoma at a density of 8 individuals L^{-1} (200 $tank^{-1}$). Each dietary treatment was randomly assigned 5 replicate tanks. Tanks were supplied with ultrafiltered, UV-treated seawater (salinity: 34 ppt) and set at a flow rate of 1.8 L min^{-1}. Water temperature remained at 26 ± 0.5 °C and the photoperiod was set at 12 h light:12 h dark. Phyllosoma were 32 days post-hatch at the commencement of the trial. Development stages were determined using the morphological features described by Smith et al. (2009b). Based on a subsample of 150 individuals, the relative proportion of development stage for the phyllosoma in each tank at the beginning of the trial was 64% 3 (3), 35% 4 (1) and 1% 4 (2) [development stage (instar number)], with an average initial weight of 2.5 ± 0.1 mg. Throughout the experimental duration, moults and mortalities were removed and recorded during daily tank maintenance.

2.2. Dietary treatments

Prior to the experiment, all phyllosoma were fed *Artemia* nauplii before weaning onto a moist, sodium alginate bound, formulated diet (formulation details not provided due to a commercial-in-confidence agreement) as per the method described by Conlan et al. (2014). From the beginning of the experiment, phyllosoma were fed one of the three experimental diets at a ration of 1.2 g $tank^{-1}$ twice daily. Phyllosoma were fed to excess, as confirmed by observations of leftover quantities of remaining food and full hepatopancreas. The three experimental diets were formulated to be *iso*-nitrogenous, *iso*-lipidic and *iso*-energetic, differing only by the pre-digestion treatment of the fishmeal ingredient (Peruvian anchovy meal, Ridley Aqua-feed, Australia), which served as the protein component of the diets. Based on the suggestions of Francis et al. (2014), protein constituted 10% (wet weight) of the total diet composition. The three experimental diets included a control diet of standard, intact fishmeal (IFM), an acid denatured fishmeal diet (DFM) and an enzyme hydrolysed fishmeal diet (HFM). For the IFM diet, the fishmeal was freeze-dried, sieved to 250 μm, pulverised and sieved to <106 μm, before being added to the total formulation. To make the DFM diet, the fishmeal was denatured using a modified method of Nankervis and Southgate (2009). Briefly, the fishmeal was suspended in reverse osmosis water (300 mg mL^{-1}) and heated to 28 ± 1 °C for 30 min. The suspension was constantly mixed using an IKA-Werke mixer (GmbH & Co. KG, Germany) and pH adjusted to 3.0 with 2 M HCl. After 30 min the pH was adjusted to 8.0 with 2 M NaOH to end the denaturing process. The denatured suspension was then processed as per the IFM and added to the diet. For the HFM diet, the fishmeal was hydrolysed using a modified method of Kolkovski and Tandler (2000). This followed the same protocol as stated above; however, after the addition of HCL, *Streptomyces griseus* pronase (Boehringer-Mannheim Biochemicals, Germany) was added at a rate of 0.1 mg g $fishmeal^{-1}$. After 24 h, the solution was heated to 80 °C in a water bath for 10 min to denature the digestive enzymes and stop all enzymatic activity. The pH was then adjusted to 8.0 with 2 M NaOH to

Table 1
Proximate composition of the IFM, DFM and HFM experimental diets (wet weight).

		Proximate composition (mg g $sample^{-1}$)	
Diet	IFM	DFM	HFM
Moisture	756 ± 0.0	755 ± 0.0	753 ± 0.0
Protein	105 ± 0.0	105 ± 0.0	106 ± 0.1
Lipid	69.7 ± 0.1	68.1 ± 0.0	66.8 ± 0.0
Ash	25.9 ± 0.0	34.5 ± 0.0	34.9 ± 0.0
NFE[a]	43.7 ± 0.1	37.8 ± 0.0	39.3 ± 0.1
Energy kJ/g[b]	5.2 ± 0.0	5.2 ± 0.0	5.1 ± 0.0

Values are presented as means ± SEM, n = 5. IFM = Intact fishmeal, DFM = Denatured fishmeal and HFM = Hydrolysed fishmeal.
[a] NFE: nitrogen free extract — calculated by difference.
[b] Calculated on the basis of 23.6, 39.5 and 17.2 kJ g^{-1} of protein, lipid and carbohydrate, respectively.