

Influence of lipid-enriched diets on the reproductive performance of marine ornamental 'hinge-beak' shrimp *Rhynchocinetes durbanensis* (Caridea: Rhynchocinetidae)

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Funding information

Science and Engineering Research Board, Grant/Award Number: ECR/2015/000213

Abstract

The present study was designed to investigate the efficiency of lipid-enriched brood stock diets on the reproductive performance, such as fecundity, egg volume, fatty acid profile of newly extruded (stage 1) embryos and the starvation threshold of the newly hatched larvae of the marine ornamental 'hinge-beak' shrimp *Rhynchocinetes durbanensis* Gordon. Three dietary treatments (50, 100 and 150 g/kg lipids) were formulated to understand their influence on the reproduction of *R. durbanensis* under captive condition and compared with the wild-caught shrimps carrying embryos in the abdomen. The reproductive parameters varied significantly between the treatments (captive and wild). Further, the fatty acid profile revealed that essential fatty acid levels of newly extruded embryos in the wild collected shrimps were almost like that of embryos produced in 100 and 150 g/kg of lipid-enriched diets. The results of linear discriminant analysis (LDA) suggest that the fatty acid profile of embryos has confirmed the separation of four centroids indicating comprehensive differences among the embryos of captive-reared and wild-caught shrimps. Hence, it is recommended that the commercial diets formulated with essential nutrients would play a major role in enhancing the reproductive performance of marine ornamental shrimps.

KEYWORDS

brood stock maturation, commercial diet, essential fatty acids, marine shrimp, reproductive performance

1 | INTRODUCTION

Export of marine ornamental shrimps has become one of the important sources of income to the coastal communities of Indo-Pacific region. Almost, 90% of trade organisms are still being collected from the coral reefs. Increased exploitation of these higher priced resources has led to strict regulations being placed on their trade as well as focus towards the sustainable use for conservation (Calado, 2008; Rhyne et al., 2009; Wabnitz et al., 2003). Captive culture of ornamental shrimps is being considered as a sustainable alternative to

minimize the collection of wild specimens, as well as highly profitable economic activity (Calado et al., 2003). To achieve commercial scale production of some marine ornamental decapod species, many high-quality larvae must be produced through potential brooders at fixed intervals (Calado et al., 2007). Additionally, usage of optimal maturation diet should fulfil the dietary requirements of brooding animals (Wouters et al., 2001). Nutritional requirements of growing larvae, juveniles and adult stages can be fulfilled by the external food source; however, the developing embryos must solely depend on the brood stock nutrition (Leal et al., 2012).

In order to ascertain the suitability of maturation diets, quantitative and qualitative approaches are necessary to overcome the bottlenecks in the production of high-quality embryos and larvae. Offering common brood stock diets for different species of marine ornamental shrimps will not fulfil the fatty acid requirements of newly hatched larvae under captive conditions (Calado et al., 2010). Many studies have hitherto focused on the frozen brood stock diets to evaluate the reproductive performance of marine ornamental shrimps including newly hatched/enriched *Artemia* nauplii, *Artemia* adult biomass, clams, mussels, krill, shrimp, squid, polychaetes and commercial mixed diets (Calado et al., 2009, 2010; Lin & Shi, 2002; Lin & Zhang, 2001; Rhyne & Lin, 2004; Simoes et al., 1998). Frozen and fresh foods have the rapid decaying ability when fed and lead to contamination of the water medium (Sheen & Wu, 1999). Also, the frozen diets do not fulfil the nutritional requirements of brood stocks by producing elevated values in the fatty acid profiles of newly hatched larvae (Calado et al., 2010). Therefore, developing an alternate commercial brood stock diet is much needed to achieve sustainable production in the marine ornamental shrimp culture industry. Development of commercial diets is more appropriate to replace the fresh/frozen food due to longer shelf life, stable cost, constant availability of controlled nutrients, ease of use with low risk of contamination. Further, essential supplements such as vitamins, hormones and therapeutics can be added easily (Wouters et al., 2002).

The composition of suitable commercial diets must always consider macro- and micro-nutrients (lipids, proteins, carbohydrates, vitamins, minerals and carotenoids) before incorporating different ingredients (Calado, 2008). Surprisingly, most of the marine invertebrates are unable to elongate and desaturate essential fatty acids (EFAs) for the dietary requirements (Arts et al., 2009; Dalsgaard et al., 2003). Therefore, addition of essential fatty acids must be provided through an external source in the form of diet (Dalsgaard et al., 2003) to promote normal growth and maturation (Coman et al., 2007; Naessens et al., 1997). For instance, the essential fatty acids such as linoleic acid (LA: 18:2n-6) and alpha-linoleic acid (ALA: 18:3n-3) act as a starting point for the synthesis of highly unsaturated fatty acids (HUFAs) such as docosahexaenoic acid (DHA: 22:6n-3), eicosapentaenoic acid (EPA: 20:5n-3) and arachidonic acid (ARA: 20:4n-6) which play a vital role as metabolic energy reserves in brooding females to trigger the embryogenesis (Arts et al., 2009; Lee et al., 2006).

Previous studies have demonstrated that the fatty acid compositions of egg mass produced by marine invertebrates are directly attributable to the fatty acid composition of their diets (Imbs & Grigorchuk, 2019; Leal et al., 2012; Martinez-Pita et al., 2005). Further, biochemical profile of the newly extruded embryos from the wild could be used as a starting point in the formulation of appropriate brood stock diets (Calado et al., 2009; Rosa et al., 2005). Because, it helps to understand the utilization of energy reserves in females during gonadal maturation (Rosa et al., 2003). Therefore, developing a commercial diet added with required essential ingredients (Chen, 1998; Teshima, 1998) can be

treated as a baseline to enhance the brood stock maturation in marine ornamental shrimps.

The present study focused on one such marine ornamental 'hinge-beak' shrimp *R. durbanensis* from Gulf of Mannar (Prakash & Ajith Kumar, 2013). Recently, it has been revealed that *R. durbanensis* has been highly targeted and supplied to the marine aquarium trade within India (Prakash et al., 2017). Since marine ornamental trade in Gulf of Mannar is not well organized or managed to educate the fishermen about catch targets (i.e. maximum sustainable yield [MSY] or total allowable catch [TAC]), it is necessary to ensure the sustainability of the natural stocks (Bolker et al., 2002; Prakash et al., 2017). This has urged the importance of understanding the culture potential of the *R. durbanensis* as well as to standardize the commercial diet that can enhance the brood stock maturation and produce healthier eggs and larvae. Earlier, Prakash et al. (2015) have produced a snapshot on the sexual dimorphism, male morphotypes and mating behaviour of *R. durbanensis*. Nevertheless, they did not include the details on the brood stock maturation or the culture potential of this species. Another study has insisted the improvement in the breeding and rearing of marine ornamentals with special focus on shrimps (Ajith Kumar et al., 2015). Therefore, the main objective of this study is to provide first-of-its-kind information on whether the commercially lipid-enriched diets will enhance the reproductive performance of a marine ornamental 'hinge-beak' shrimp *Rhynchocinetes durbanensis* under captive conditions.

2 | MATERIALS AND METHODS

2.1 | Collection and maintenance of shrimps

The individuals of *Rhynchocinetes durbanensis* ($n = 50$) were collected from the crevices and rocks of the subtidal waters at Tuticorin, Tamil Nadu, by adopting SCUBA diving (Prakash & Ajith Kumar, 2013). The collected shrimps were packed immediately in air filled polyethylene bags and transferred to the experimental culture facility at Centre for Climate Change Studies, Sathyabama Institute of Science and Technology, Chennai. The shrimps were acclimatized for 4–5 h (Pers. Comm. R. Calado), and 18 individuals (9 females and 9 males) were selected to develop brood stocks. Selection of brood stocks was based on the morphotypes (i.e. Only intermedium males were selected to pair with the females (Figure 1) (Prakash et al., 2015 gave detailed information on *R. durbanensis* male morphotypes). The average total length (TL) of each shrimp was measured from the anterior tip of the rostrum to the posterior end of the telson (male: 5.5–6.2 cm TL, female: 5.8–5.9 cm TL). They were introduced as a pair in each rectangular glass tank (0.60 m long $\times$ 0.30 m wide $\times$ 0.30 m height) with a total capacity of 54 L filtered sea water connected to a re-circulating maturation system for ornamental decapods as described by Calado et al. (2007). All the tanks were illuminated from above with fluorescent white light (2000–3000 Lux) (Philips) by maintaining a photoperiod of 12L: 12D for initial acclimation. Salinity was maintained at 32–34‰, temperature 27–29°C, dissolved oxygen 4–5 ml/L and pH 8.0 to 8.1 respectively.

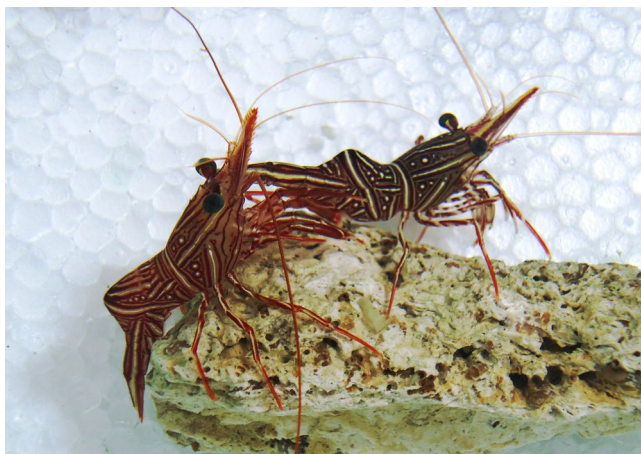


FIGURE 1 A pair of *Rhynchocinetes durbanensis* Gordon (intermedius male and female) under captive conditions

TABLE 1 Composition of ingredients used for making 50, 100 and 150 (g/kg) dietary lipids

Ingredient	Dry matter	CP	CF	CHO	Ash
Fish meal ^a	930	630	37.8	190	72.2
Rice bran ^a	950	170	18.5	610	151.5
Squid ^a	960	720	42.8	130	67.2
Groundnut oil cake ^a	940	314	30.8	485	110.2
Corn flour ^a	950	70	9.8	740	130.2
Soya meal ^a	940	455	12.4	368	104.6
Spirulina ^a	960	491	28.4	345	95.6
Shrimp ^a	940	550	36.9	400	33.1
Cod liver oil ^b	990	–	980	–	–
Vit-Min mix ^d	980	–	–	–	–
Gelatin ^c	990	–	–	–	–

Abbreviations: CF, Crude fat; CHO, Carbohydrates; CP, Crude protein.

^aProcured from local markets and landing centres.

^bSeven seas.

^cHi Media Laboratories.

^dComposition of vitamin-mineral mix EMIX PLUS, India: Vitamin A, vitamin D3, vitamin B2, vitamin E, vitamin K, vitamin B6, vitamin B12, calcium pantothenate, nicotinamide, choline chloride and trace elements: Mn, I, Fe, Zn, Cu, Co, Ca, L-lysine, DL-methionine, selenium, satwari (*Lactobacillus* and yeast culture).

Ammonia, nitrite and nitrate concentrations were monitored daily and maintained below the detectable levels in all the aquaria. The shrimps were introduced with an ad libitum supply of commercially prepared pellets so that it will get adapted and do not starve during the experiment. Excess food particles and faecal wastes were checked daily and removed from the brooder tanks and water exchange of 10%–20% was also performed. In addition, few females with orange coloured eggs (stage 1) were immediately flash frozen in liquid nitrogen after collection in the field in order to estimate the tissue proximate composition.

2.2 | Diet formulation and lipid enrichment

Three isonitrogenous experimental diets (550 g/kg crude protein) were formulated with different lipid concentrations (50, 100 and 150 g/kg) using commercially available ingredients such as fish meal, rice bran, squid meal, groundnut oil cake, corn flour, soya meal, spirulina powder, shrimp meal, cod liver oil (Seven Seas), Vitamin-Mineral mix (EMIX PLUS) and gelatin (Hi Media Laboratories) (Table 1). Feed with 550 g/kg of protein was chosen based on the result of the proximate composition of extruded embryos of wild collected shrimps. The dry ingredients were blended to make a homogenous mixture. Then, the mixer was sprinkled with enough water to form dough and cooked in steam for 15 min. After cooling, the dough was separated into three parts, fish oil of three different concentrations (50, 100 and 150 g/kg), vitamin and mineral mixture were added. Pellets (2 mm size) were prepared using a hand pelletizer and dried in the oven at 40°C for 24 to 48 h. Pellets were packed in airtight polythene boxes and kept aside for feeding the shrimps during the experiment. The shrimps were fed on a dry-weight basis three times a day (07:00, 13:00 and 19:00 h) with a combined total of 10%–15% of shrimp biomass.

2.3 | Larval production and starvation threshold

Embryonic development was monitored based on the colour of the eggs and the release of larvae was checked daily in the morning, prior to the routine task of feeding and water exchange. In order to ensure that only nutrients provided by the formulated diets were being mobilized for gonadal maturation, first two embryo batches and their corresponding larvae produced after pairing of shrimps were discarded. After initiating the experiment, reproductive performance of each pair was evaluated by counting the number of larvae produced in three consecutive batches ($n = 3$ shrimps per treatment = 3 larval batches/treatment $\times$ 3 treatments = 9 larval batches). Similarly, 3 of the wild-caught ovigerous females identified to stage 4 embryos (presence of eye spot) were acclimatized and allowed to spawn in the laboratory. Finally, the number of larvae produced by these shrimps was counted and compared. Survival of newly hatched larvae was examined by exposing the larvae to six different starvation periods (24, 36, 48, 60, 72, 84 and 96 h) by maintaining in a separate container (50 ml each). One hundred larvae from each batch were randomly selected and exposed to different starvation conditions without food as suggested by Calado et al. (2009). Water exchange in the larval container was done 100% using UV-treated sea water with the temperature maintained at 27 to 29°C.

2.4 | Reproductive performance of ovigerous females

To evaluate the reproductive performance of ovigerous females fed with different diets, we observed the following parameters: wet

body weight of adult females, wet weight of egg clutches and single egg, fecundity, relative fecundity (egg weight/wet weight of the spawned female) and volume of the newly extruded embryo. Egg clutch (wet weight) was removed from the abdomen carefully and weighed to the nearest of 0.01 mg after the excess water has been removed using repeated blotting (Cavalli et al., 1999). Only, stage 1 embryos were selected for the study, as during the incubation process part of the embryos can be naturally lost. Samples were taken in triplicates ($n = 3$) for each diet treatment, and consecutively, three wild collected shrimps were also analysed for comparison.

2.5 | Fatty acid analysis

For fatty acid analysis, extruded embryos were gently collected from the abdomen using forceps and stored at -80°C prior to the fatty acid analysis. 50 mg freeze dried samples were added to 1 ml of 1.2 M NaOH in 50% (v/v) of methanol. The solutions were heated at 100°C in water bath for 30 min for saponification. The saponified samples were then allowed to cool at room temperature for 30 min and subjected to methylation by adding 2 ml of a 54% (v/v) of 6N HCl with 46% (v/v) aqueous methanol and heated at 80°C for 10 min. After cooling, fatty acids were extracted with 1.2 ml of 50% (v/v) diethyl ether prepared with hexane followed by phase separation for 10 min. Finally, the supernatant was mixed with 3 ml of 0.3 M NaOH for 5 min. FAMES were cleansed with anhydrous sodium sulphate and then transferred to gas chromatography sample vials prior to analysis. The FAMES were separated by GC (HP 6890N, Agilent Tech.), using ultra-2 capillary columns ($25\text{ m} \times 2\text{ mm} \times 0.33\text{ }\mu\text{m}$ film thickness). Initially, the temperature was maintained at 170°C and temperature of ramp 1 and 2 were ranged between $5\text{--}260^{\circ}\text{C}$ and $4\text{--}310^{\circ}\text{C}$ respectively. The split injector ratio was 100:1 using hydrogen as a carrier gas and nitrogen as a makeup gas with a flow rate of 30 ml/min each. FAMES profiles were detected by a flame ionization detector (FID) and identified by comparing the commercial Eucary database with MIS software package (MIS v3.8) (Marudhupandi et al., 2016).

2.6 | Statistical analysis

Multiple regression analysis was performed to understand the significant differences between the starvation threshold of *R. durbanensis* newly hatched larvae fed with different dietary treatments. In addition, one-way ANOVA on the reproductive performances of *R. durbanensis* were also analysed using SPSS software package version 16.0, USA (Norusis, 2009). Further, raw data sets were initially tested for the assumption of normality and homogeneity of variances (one-way ANOVA). Lastly, the data were further explored and displayed using Linear Discriminant Analysis (LDA) using Paleontological Statistics (PAST) software package version 3.11 (Hammer, 2015). This analysis was robust to identify the number of

trends in the dataset that maximally discriminated among fatty acid profiles of the experimental groups (feeding treatments and wild-caught shrimp). The treatments were represented as centroids with 95% confidence level.

2.7 | Ethical statement

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines section, have been adhered to. Ethical clearance was not sought as the studied animals are not under the threatened category of IUCN Red List 2020 or Wildlife Protection Act, 1972. In addition, the studied animals were not covered under the provisions of the Prevention of Cruelty to Animals Act, 1960 and Breeding of and Experiments on Animals (Control & Supervision) Rules of 1998, 2001 and 2006 framed and enforced by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the act.

3 | RESULTS

3.1 | Proximate composition of ingredients and experimental diets

Proximate compositions of the commercial ingredients are given in Table 1. The various proportions of ingredients used in the preparations of three treatments 50, 100 and 150 g/kg dietary lipid concentrations and the proximate composition of these diets are given in Table 2. Ash (%) and gross energy (GE) values of 50, 100 and 150 g/kg experimental diets were 8.51, 10.94 and 10.69 and 20.48, 21.15 and 20.65 MJ/kg (Table 2). Survival of brooder shrimps was not affected by the experimental diets, as no mortalities were recorded during the study.

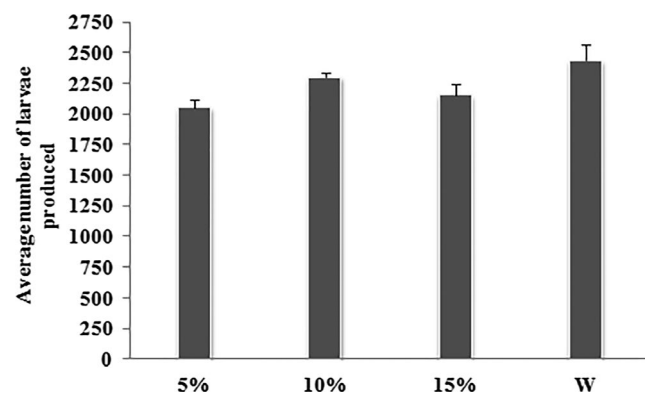
3.2 | Reproductive performance of *Rhynchocinetes durbanensis*

Wet body weight (WBW) of the adult females varied significantly among all the dietary treatments as well as from the wild-caught shrimp (2.46–2.63 gm). The weight of the egg clutch (wet weight in gm) showed significant differences among the treatments compared to the wild spawned embryos. However, there is no significant difference in the fecundity and relative fecundity (no. of eggs/gm WBW female) of brood stocks fed with 50 (mean $\pm$ SD: $1,953 \pm 78$ and 773 ± 28.6), 100 ($2,126 \pm 84$ and 848 ± 84.12) and 150 g/kg ($2,101 \pm 134$ and 797.6 ± 31.3) dietary lipid concentrations. Similarly, brooders spawned in the wild ($2,218 \pm 183$ and 859 ± 137.8) (Figure 2) showed no significant differences in the fecundity and relative fecundity ($F_{(3,8)} = 2.22$; $p = 0.16$ and $F_{(3,8)} = 0.72$; $p = 0.57$) when compared to the dietary treatments (Table 3).

TABLE 2 Formulation and proximate composition of dietary lipids contents (g/kg)

Ingredients	Dietary lipid contents (g/kg)		
	50	100	150
Fish meal	170	160	160
Rice bran	80	50	5
Squid	350	360	370
Groundnut oil cake	30	40	30
Corn flour	60	20	5
Soya meal	80	80	80
Spirulina	90	80	80
Shrimp	80	90	80
Cod liver oil	10	70	140
Vitamin-Mineral mix	30	30	30
Gelatin	20	20	20
Analysed composition (g/kg)			
Dry matter	935	882.3	808
Crude protein	557.1	553.6	543.3
Crude fat	52.3	101.7	150.5
Carbohydrates	305.5	235.3	199.3
Ash	85.1	109.4	106.9
GE (MJ/kg) ^a	20.48	21.15	20.65

^aGross energy was calculated according to Vergara et al. (1999): (23.6 MJ/kg × % protein + 39.8 MJ/kg × % lipid + 17.2 MJ/kg × % carbohydrate)/100.

**FIGURE 2** Average number (±SD) of larvae produced by *Rhynchocinetes durbanensis*, fed with different experimental diets (50, 100 and 150 g/kg lipids), compared with embryos spawned in the wild (W) ($n = 3$)

The volume of the eggs (mm^3) from the wild shrimps (0.026 ± 0.001) and 100 g/kg dietary experimental group was almost similar (0.026 ± 0.001), compared to the 50 (0.023 ± 0.001) and 150 g/kg (0.024 ± 0.001) ($F_{(3, 8)} = 1.96$; $p = 0.19$) of dietary experimental groups. Likewise, total length of the newly hatched larvae significantly varied among wild, 100 and 150 g/kg of experimental groups, but not in 50 g/kg experimental group ($F_{(3, 8)} = 5.30$; $p = 0.03$) (Table 3).

3.3 | Starvation threshold of larvae

There were no variations in the survival of larvae between the dietary treatments and the larvae obtained from the wild-caught shrimp for the first 24 h of starvation period. However, poor survival was observed after the 60 and 72 h in 50 g/kg dietary lipid concentration while compared to 100 and 150 g/kg dietary experimental groups and the larvae of wild shrimps spawned in the laboratory. However, the multiple regression analysis of different treatments (50: $R^2 = 0.936$, $t = 28.01$, $p < 0.001$; 100: $R^2 = 0.961$, $t = 33.47$, $p < 0.001$; 150: $R^2 = 0.975$, $t = 40.46$, $p < 0.001$) and the larvae of wild collected shrimps ($R^2 = 0.900$, $t = 22.94$, $p < 0.001$) showed significant decrease in their survival during the starvation periods of 96 h (Figure 3).

3.4 | Fatty acid analysis

Fatty acid compositions analysed for the formulated diets of 50, 100 and 150 g/kg constitute 48.9 ± 5.2 , 96.6 ± 2.4 and 145 ± 2.9 g/kg of total fatty acids. The saturated fatty acids, MUFA, PUFA and HUFA contents showed an increasing trend in the formulated diets (Table 4) due to the increased supplements of lipids.

Likewise, the one-way ANOVA of saturated fatty acid contents (palmitic acid-16:0; margaric acid-17:0; stearic acid-18:0) of the newly extruded embryos of the experimental groups fed with 50, 100 and 150 g/kg showed an increasing trend like 8.0 ± 0.9 , 12.0 ± 1.0 and 17.3 ± 0.8 g/kg respectively. Whereas, the saturated fatty acids of extruded embryos from wild collected shrimps was 14.0 ± 0.08 g/kg that lies between the 100 and 150 g/kg of the experimental diet. The MUFA (oleic acid-18:1 n-9) did not vary significantly among the dietary experimental groups and the wild collected shrimps. The PUFA (linoleic acid-18:2 n-6; alpha-linoleic-18:3 n-3; moroctic acid-18:4 n-3) and HUFA (arachidonic acid-20:4 n-6; eicosapentanoic acid-20:5 n-3; docosahexanoic acid-22:6 n-3) contents showed significant variation among the treatment groups (Table 5). In particular, the n-3 and n-6 fatty acid content of extruded embryos fed with 150 g/kg diet were higher (21.2 ± 2.2 and 14.8 ± 1.8) compared to the extruded embryos in the wild (18.6 ± 2.6 and 13.5 ± 1.5).

In addition, the three eigenvalues based on linear discriminant analysis (LDA) obtained from the fatty acid profiles were 188.8, 15.7 and 3.5 with the first component showing a variation of 90.81%. Higher eigenvalues represent distinct variation among the treatments. Plots of discriminant axis 1 and 2 are positive and negative correlation of fatty acid profile among different treatments and wild samples (Figure 4). Separation of four centroids in LDA were mainly due to the differences in the magnitude of middle and higher-level harmonics suggesting most of the discrimination were related to the finer variation in the values of individual fatty acids. Variation along axis 1 was mainly due to the higher concentration of fatty acids such as 18:1n-9, 18:3n-9, 18:2n-6 and 20:5n-3 in

Parameters	Treatments	Mean $\pm$ SD	F	df	p
Adult female (total length in cm)	Wild	5.73 $\pm$ 0.20 ^a	0.57	3,8	0.66
	5	5.80 $\pm$ 0.20 ^a			
	10	5.86 $\pm$ 0.15 ^a			
	15	5.9 $\pm$ 0.1 ^a			
Adult female wet body weight without egg clutch (gm)	Wild	2.46 $\pm$ 0.24 ^a	0.47	3,8	0.71
	5	2.51 $\pm$ 0.16 ^a			
	10	2.51 $\pm$ 0.16 ^a			
	15	2.63 $\pm$ 0.15 ^a			
Wet weight of egg clutch (mg)	Wild	0.40 $\pm$ 0.05 ^b	4.43	3,8	0.04 [*]
	5	0.30 $\pm$ 0.02 ^a			
	10	0.350 $\pm$ 0.02 ^{a,b}			
	15	0.37 $\pm$ 0.02 ^b			
Fecundity (nos)	Wild	2218.3 $\pm$ 183.4 ^b	2.22	3,8	0.16
	5	1953.6 $\pm$ 78.23 ^a			
	10	2126.6 $\pm$ 84.57 ^{a,b}			
	15	2101.0 $\pm$ 134.65 ^{a,b}			
Relative fecundity (nos)	Wild	859.0 $\pm$ 137.8 ^a	0.72	3,8	0.57
	5	773.0 $\pm$ 28.6 ^a			
	10	848.0 $\pm$ 84.1 ^a			
	15	797.0 $\pm$ 31.3 ^a			
Egg volume (mm ³)	Wild	0.026 $\pm$ 0.0 ^a	1.96	3,8	0.19
	5	0.023 $\pm$ 0.0 ^a			
	10	0.025 $\pm$ 0.0 ^a			
	15	0.026 $\pm$ 0.0 ^a			
Wet weight of single egg (μ g)	Wild	0.20 $\pm$ 0.03 ^a	1.29	3,8	0.34
	5	0.17 $\pm$ 0.0 ^a			
	10	0.19 $\pm$ 0.01 ^a			
	15	0.19 $\pm$ 0.00 ^a			
Egg ash (%)	Wild	1.85 $\pm$ 0.07 ^a	0.98	3,8	0.44
	5	1.78 $\pm$ 0.03 ^a			
	10	1.82 $\pm$ 0.03 ^a			
	15	1.85 $\pm$ 0.05 ^a			
Total length of newly hatched larvae (mm)	Wild	2.22 $\pm$ 0.04 ^c	5.30	3,8	0.03 [*]
	5	2.11 $\pm$ 0.03 ^a			
	10	2.19 $\pm$ 0.01 ^{b,c}			
	15	2.15 $\pm$ 0.05 ^{b,c}			

* $p < 0.05$; alphabet superscripts indicates significant levels based on Duncan's Multiple Range Test (DMRT).

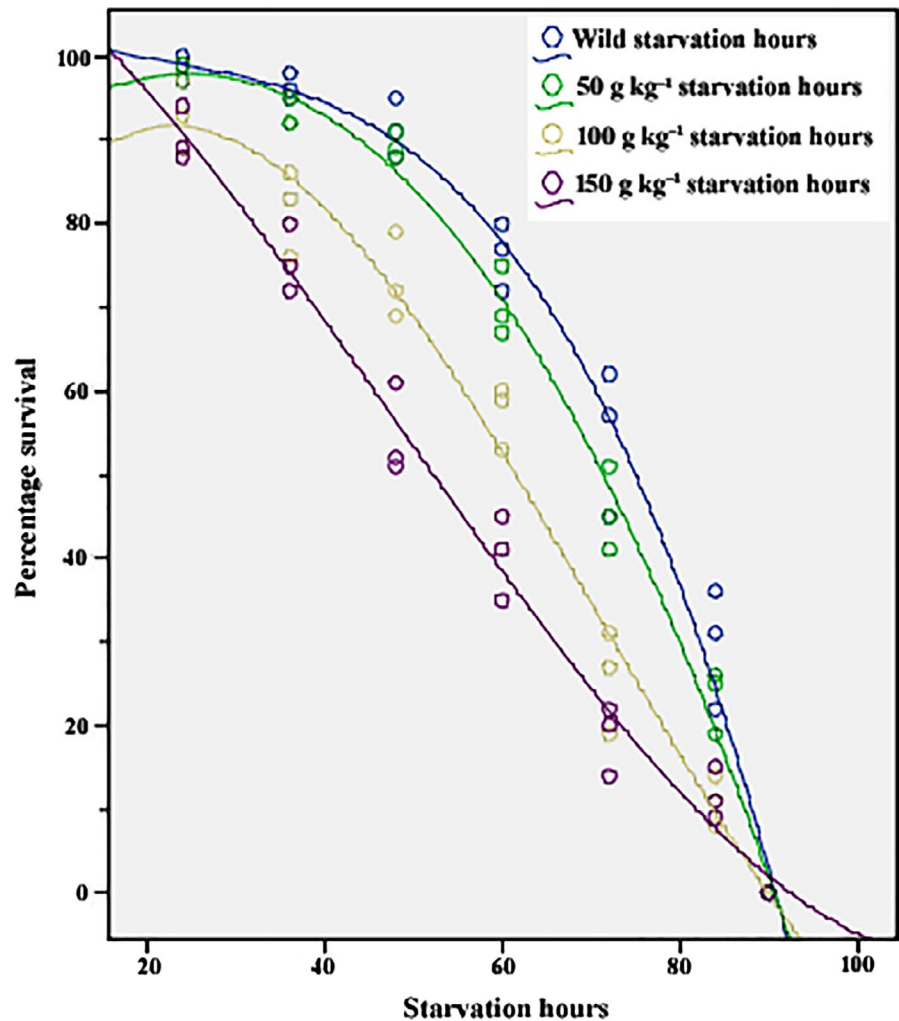
TABLE 3 One-way ANOVA of reproductive performance of *Rhynchocinetes durbanensis* fed brood stock diets of 50, 100 and 150 g/kg dietary lipid concentrations and the wild collected shrimps expressed as Mean $\pm$ SD (standard deviation) and SE (standard errors). Only stage 1 embryos were used for the analysis (see Materials and methods)

the extruded embryos of wild collected shrimps. Similarly, higher concentrations of fatty acids such as 16:00, 22:6n-3, 18:00 and 20:4n-6 in 150 g/kg were due to the increased supplementation of lipids in the maturation diet and remaining other fatty acids were expressed at low levels (Figure 4). Hence, it was suggested that the brood stock maturation diet should always possess 100–150 g/kg (10%–15%) of lipids to influence reproductive performance of ornamental shrimps, which is similar to the fatty acid contents of extruded embryos of wild-caught shrimps.

4 | DISCUSSION

The present study highlights the importance of lipid-enriched commercial diets in *Rhynchocinetes durbanensis* reproductive performance. Our results strongly suggest that the lipid-enriched diets of 100–150 g/kg would be appropriate in enhancing the fecundity, egg clutch weight, embryo volume and survival of newly hatched larvae though there is no statistical significance between the wild spawned shrimp. Further, the growth rate and survival of *R. durbanensis*

FIGURE 3 Multiple regression (cubic) analysis of starvation of newly hatched larvae of *Rhynchocinetes durbanensis* fed different diets (50, 100 and 150 g/kg) and wild larvae. The percentage survival rate of newly hatched larvae of different diets showed significant decrease to the increase starvation period (24, 36, 48, 60, 72, 84 and 96 h)



pairs were not affected by the diets, showing that the commercial diets would be suitable to induce maturation in marine ornamental shrimps in captivity (Calado et al., 2010). Likewise, the brood stock performance displayed marked differences, proving that the diets met the essential energy requirements of sexually active females in the case of gonochoric *R. durbanensis* shrimps (Prakash et al., 2015) as the dietary energy will always be directed towards the gonadal maturation. The incubation period of *R. durbanensis* ranged from 10 to 12 days as usual and did not show much variation during the experiment as observed in other ornamental *Lysmata* species (14–15 days) (Calado et al., 2010). It is assumed that under optimum physico-chemical conditions, developing embryos catabolizes their endogenous energy reserve at a constant rate (Pandian, 1994; Wear, 1974). This highlights that the brooders did not suffer from physiological stress during the experiments, and all the females produced healthy larvae in successive batches, which exhibited strong swimming ability in the present study.

The 100% survival of larvae was observed in all the treatments for the first 24 h post hatch in *R. durbanensis* larvae indicating that the food is not pivotal to complement the energy requirements at that stage. However, the drastic decrease in the survival rate between 60 and 72 h in 50 g/kg experimental groups was observed

compared to 100 and 150 g/kg experimental groups, suggesting the importance of dietary lipids as internal energy reserves representing facultative secondary lecithotrophy behaviour (Calado, Dionísio, et al., 2007; Zhang et al., 1998a, b). According to Anger (2001), the lecithotropic behaviour mainly depends on the parental energy investment in the reproduction of crustacean decapods. In the present study, energy requirements of *R. durbanensis* larvae could have been sustained from the endogenous reserves of brooding females provided by the lipid-enriched diets. In addition, the newly hatched larvae from the shrimps collected from the wild survived better, because the yolk reserves could be superior than the experimental groups as observed in the *Lysmata* larvae culture trials (Calado et al., 2009). Nevertheless, we did not monitor changes in the energy reserves of larvae during starvation threshold, which must be treated with caution in the future study. In addition, the lack of ability to moult from zoea I to zoea II in our *R. durbanensis* larvae agrees with the results of Calado (2008) and Calado et al., (2010) indicating the lack of larger yolk reserves in this species. This suggests the importance of feeding *R. durbanensis* larvae within 24 h of post hatch.

The influence of lipid-enriched diets on *R. durbanensis* had a significant effect on the reproductive parameters in the *R. durbanensis* including fecundity, egg clutch size, embryo volume and total

Fatty acid	Carbon chain	50	100	150
Palmitic acid	16:0	12.6 ± 0.7	13.9 ± 1.5	17.0 ± 0.7
Margaric acid	17:0	1.9 ± 0.02	4.7 ± 0.7	7.4 ± 0.6
Stearic acid	18:0	6.8 ± 1.1	10.7 ± 0.68	17.4 ± 1.7
Oleic acid	18:1 n-9	3.2 ± 0.32	4.2 ± 0.51	8.5 ± 0.65
Linoleic acid	18:2 n-6	3.4 ± 0.37	6.6 ± 0.97	11.0 ± 1.1
Alpha linolenic acid	18:3 n-3	1.8 ± 0.15	7.7 ± 1.8	8.1 ± 0.2
Moroctic acid	18:4 n-3	3.8 ± 0.5	10.4 ± 0.85	13.5 ± 0.6
Arachidonic acid	20:4 n-6	3.6 ± 0.5	8.6 ± 0.6	11.9 ± 0.8
EPA ^a	20:5 n-3	4.6 ± 0.45	12.0 ± 1.1	18.7 ± 1.1
DHA ^b	22:6 n-3	7 ± 0.8	17.7 ± 0.7	31.4 ± 1.9
	Σ SFA ^c	21.3 ± 2.1	29.3 ± 2.8	41.7 ± 3.0
	Σ MUFA ^d	3.3 ± 0.32	4.2 ± 0.5	8.5 ± 0.65
	Σ PUFA ^e	9.1 ± 1.0	24.7 ± 3.7	32.7 ± 2.0
	Σ HUFA ^f	15.2 ± 1.7	38.4 ± 2.4	62 ± 3.9
	n-3	17.2 ± 1.9	47.9 ± 4.5	71.7 ± 4.0
	n-6	6.9 ± 0.8	15.2 ± 1.5	22.9 ± 1.9
	DHA/EPA	1.52 ± 1.78	1.47 ± 0.63	1.67 ± 1.7
	Total fatty acids	48.9 ± 5.2	96.6 ± 2.4	145 ± 2.9

^aEicosapentanoic acid.

^bDocosahexanoic acid.

^cSFA - saturated fatty acids: 16:0, 17:0, 18:0.

^dMUFA - mono unsaturated fatty acids: 18:1 n-9.

^ePUFA - poly unsaturated fatty acids: 18:2 n-6, 18:3 n-3, 18:4 n-3.

^fHUFA - highly unsaturated fatty acids: 20:4 n-6, 20:5 n-3, 22:6 n-3.

TABLE 4 Fatty acid profiles of experimental diets to feed *Rhynchocinetes durbanensis* brood stocks using 50, 100 and 150 g/kg dietary lipid concentrations. Values are means of triplicate samples (mean ± SD)

length of the newly hatched larvae. All the above parameters were comparable to that of the reproductive performance of the wild collected shrimps. The species tested in this study has shown itself to be a continuous breeder in a cycle of 10–12 days: that is Females moult immediately after larval release; receptive females mated with males, extruded embryos and initiated a new embryo incubation cycle, simultaneously observed ovaries to mature (Bauer, 2004). As suggested by Clarke (1982), the lipid reserves stored in the hepatopancreas would be enough (lipid-enriched diets as employed in the present study) to rapidly initiate the ovarian maturation cycle. This has directly reflected in the fecundity of *R. durbanensis* fed with lipid-enriched diets, particularly 100 and 150 g/kg have an average of 2,126 and 2,101 eggs, which were almost equal to the fecundity of wild collected shrimps (avg. 2,218 eggs). However, the fecundity of *R. durbanensis* from the previous study, which were fed with commercial frozen diets had reported an average of only 564 ± 151 eggs (Calado et al., 2010). This indicates that the commercial diets provided in this study would be more appropriate and yield better results compared to the frozen diets provided by Calado et al. (2010). Another study by Tziouveli et al. (2011) also supports the previous hypothesis suggesting that the partial or complete replacement by artificial diets for shrimp maturation has significantly increased the larval production. Similarly, the size of the egg (in terms of volume, mm³ and wet weight, µg) was also evident from the present study

as the experimental groups 100 and 150 g/kg lipid-enriched diets displayed almost equal values compared to the egg size of the wild-caught shrimps. As the egg size is always correlated with the lipid content, maternal investment in the caridean shrimps (Clarke, 1993; Wehrtmann & Graeve, 1998).

With regard to the individual essential fatty acids, it serves as a potential source of energy reserve during embryogenesis and larval development (Clarke et al., 1990) and perhaps influences the early larval development. First, the saturated fatty acid (SFA) and the monounsaturated fatty acid (MUFA) values of extruded embryos of experimental groups, in particular, the 100 and 150 g/kg of lipid-enriched diets were significantly lower than the SFA and MUFA values of the wild shrimps, which are commonly used to stabilize the energy (Calado et al., 2005). This hypothesis supports the poor survival rate of larvae in the experimental groups after 24 h compared to the larvae spawned by wild-caught shrimps. Since SFAs can be synthesized internally or from the desaturation of MUFA and HUFA (Sargent, 1995), it has been less preferred than the other essential fatty acids. However, both SFA and MUFA were treated as a main source of substrate to stabilize energy and utilized by the newly hatched larvae (Morais et al., 2002). Second, the polyunsaturated fatty acids (PUFA) of the extruded embryos display more or less equal values in all the experimental groups (except moroctic acid, which is 2.5 times more in 150 g/kg

TABLE 5 One-way ANOVA of fatty acids profiles (dependent variables) of *Rhynchocinetes durbanensis* spawned embryos fed with 50, 100 and 150 g/kg dietary lipid and the embryos of wild collected shrimps

Dependent variables	Treatments	Mean $\pm$ SD	F	df	p
PA	Wild	4.5 $\pm$ 0.38 ^b	39.31	3,8	0.000*
	50	1.9 $\pm$ 0.20 ^a			
	100	4.1 $\pm$ 0.20 ^b			
	150	5.8 $\pm$ 0.20 ^c			
MA	Wild	0.40 $\pm$ 0.34 ^b	13.24	3,8	0.002
	50	0.22 $\pm$ 0.43 ^a			
	100	0.43 $\pm$ 0.43 ^b			
	150	0.61 $\pm$ 0.51 ^c			
SA	Wild	9.13 $\pm$ 0.09 ^c	68.19	3,8	0.000*
	50	5.93 $\pm$ 0.29 ^a			
	100	7.5 $\pm$ 0.35 ^b			
	150	10.9 $\pm$ 0.20 ^d			
OA	Wild	2.53 $\pm$ 0.41 ^c	20.16	3,8	0.000*
	50	0.20 $\pm$ 0.05 ^a			
	100	0.93 $\pm$ 0.03 ^a			
	150	1.7 $\pm$ 0.21 ^b			
LA	Wild	8.15 $\pm$ 0.28 ^b	53.02	3,8	0.000*
	50	2.26 $\pm$ 0.35 ^a			
	100	7.53 $\pm$ 0.44 ^b			
	150	8.27 $\pm$ 0.48 ^b			
ALA	Wild	12.07 $\pm$ 0.58 ^c	28.67	3,8	0.000*
	50	6.73 $\pm$ 0.49 ^a			
	100	9.5 $\pm$ 0.28 ^b			
	150	11.6 $\pm$ 0.40 ^c			
MOA	Wild	0.28 $\pm$ 0.03 ^{a,b}	30.59	3,8	0.000*
	50	0.17 $\pm$ 0.02 ^a			
	100	0.38 $\pm$ 0.04 ^{b,c}			
	150	0.71 $\pm$ 0.05 ^d			
ARA	Wild	5.73 $\pm$ 0.38 ^{b,c}	14.08	3,8	0.001*
	50	4.2 $\pm$ 0.46 ^a			
	100	6.63 $\pm$ 0.34 ^b			
	150	11.6 $\pm$ 0.44 ^c			
EPA	Wild	2.46 $\pm$ 0.49 ^{b,c}	15.95	3,8	0.001*
	50	0.15 $\pm$ 0.02 ^a			
	100	1.53 $\pm$ 0.20 ^b			
	150	2.84 $\pm$ 0.27 ^c			
DHA	Wild	4.23 $\pm$ 0.14 ^b	27.71	3,8	0.000*
	50	1.16 $\pm$ 0.21 ^a			
	100	3.53 $\pm$ 0.37 ^b			
	150	6.13 $\pm$ 0.63 ^c			

Note: Means were expressed as Mean $\pm$ SD (standard deviation).

Abbreviations: ALA, Alpha-linoleic acid; ARA, Arachidonic acid; DHA, Docosahexanoic acid; EPA, Eicosapentanoic acid; LA, Linoleic acid; MA, Margaric acid; MOA, Moroctic acid; OA, Oleic acid; PA, Palmitic acid; SA, Stearic acid.

* $p < 0.001$; alphabet superscripts indicate significant levels based on Duncan's Multiple Range Test (DMRT).

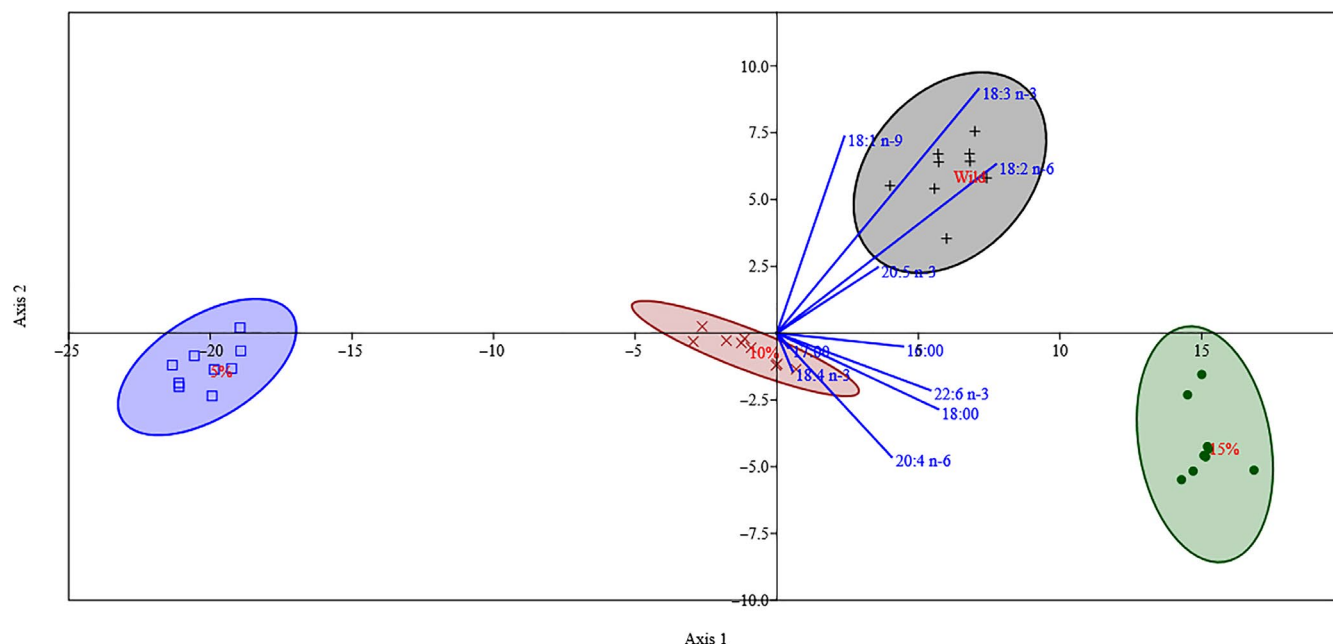


FIGURE 4 A linear discriminant analysis (LDA) displayed with 4 treatment centroids by comparing the fatty acid profile of different treatment groups (5%, 10%, 15% and Wild). Variations in the fatty acid profile were observed with 95% confidence levels

lipid-enriched diet than the wild-caught shrimp) compared to the PUFA values of embryos from the wild-caught shrimps. Moreover, it is well known that the marine shrimps have limited capability to elongate and desaturate PUFA, in order to produce HUFA (Kanazawa et al., 1979).

Lastly, the highly unsaturated fatty acids (HUFA) which mainly include the arachidonic acid (ARA), eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) displayed higher values in the extruded embryos from the experimental groups, especially 100 and 150 g/kg lipid-enriched diets compared to the values from the embryos of wild-caught shrimps. Many studies have suggested that ARA supplementation in the diet has greatly enhanced the female reproduction in both fresh water and marine shrimps (Calado et al., 2010; Coman et al., 2011; Kangpanich et al., 2016). Since, ARA acts as a fatty acid precursor molecule for prostaglandins E (PGE2) and F (PGF2) of the series II, it is important for crustacean reproduction and has been extensively studied in penaeid shrimps (Tahara & Yano, 2004; Wuttisak et al., 2013) and *Macrobrachium* sp. (Spaziani et al., 1995; Sumpownon et al., 2015). Therefore, increasing ARA content through enriched diets would continue to be reserved in the ovaries, eggs and larvae to produce prostaglandins (E and F). Next, the EPA and DHA content of the extruded embryos from the lipid-enriched experimental groups observed in this study would be a reflective of the fatty acid requirements of this specific marine ornamental shrimp *R. durbanensis*, since the EPA and DHA values in our study strongly agreed with that of the extruded embryo of the wild-caught shrimps. Surprisingly, the EPA and DHA pattern in the embryos vary significantly and always exhibit contrasting values as previously reported for the marine shrimps (Kattner et al., 1994; Morais et al., 2002). However, Cavalli et al. (1999) suggested that

the dietary inclusion of both EPA and DHA have produced positive effects on the fecundity and offspring quality, in the freshwater shrimp *M. rosenbergii* brood stock.

Our study focused on enhancing the reproductive performance of marine ornamental 'hinge-beak' shrimp *R. durbanensis*, which is a species with considerable market value in the aquarium trade (Prakash et al., 2017). The significance of providing lipid-enriched diets is to produce a greater number of high-quality embryos and larvae that can survive and grow better based on the paternal diets are clearly observed in this study. Further, it must be noted that essential fatty acid requirements for embryos and larvae should not be analysed as a proportion of the diet, but as a function of the total essential fatty acids in that diet (Glencross & Smith, 2001). Nevertheless, several other questions remain to be addressed in future studies: first, whether fatty acid content of the newly hatched larvae would produce the same values as the lipid-enriched maturation diet provided to the adults? Second, whether tissue fatty acid content of adult shrimps in the experiment and wild collected shrimps are same to produce high-quality embryos and larvae? Third, what will be the changes in the energy reserves during starvation in the newly hatched larvae when fed with lipid-enriched diets that can be comparable to the newly hatched larvae of the wild collected shrimps? Fourth, whether early feeding of larvae within 24 h would benefit the survival and metamorphosis from zoea I to zoea II in the experimental groups? Finally, whether the feed ingestion rate of the females is same or different when fed with increasing concentration of lipid-enriched diets and its interaction during gonadal maturation?

Lastly, the questions provided above need to be supported with further experimental trials using marine ornamental shrimps to address the intra- or inter-specific comparisons on the utilization of

total fatty acids for maturation. Researchers must be conscious whether using a commercially formulated diet to feed the shrimps could be capable to produce high-quality embryos and larvae throughout the year. We believe that the present study would act as a baseline and first-of-its-kind information in the formulation of lipid-enriched diets which can trigger the gonadal maturation in the brood stocks of marine ornamental shrimps. Hence, future studies could be more focused on investigating the function of individual essential fatty acids in the diets to ascertain the link between the specific elements and their role in improving the reproductive performance of *R. durbanensis* and other marine ornamental decapods.

ACKNOWLEDGEMENTS

SP is indebted to the management of Sathyabama Institute of Science and Technology, Chennai for the facilities and the Vice Chancellor Dr. T. Sasipraba for her constant support and encouragement. TTAk extending his gratitude to the Director, ICAR-NBFR. SP also acknowledges Science and Engineering Research Board (SERB), Department of Science and Technology (DST) for the award of Early Career Research Award (ECR) (grant number: ECR/2015/000213). SP is thankful to Ms. P. M. Vatsala for improving the English language of the manuscript. Lastly, the authors are grateful to two anonymous reviewers and the associate editor whose constructive comments have greatly improved the final version of the manuscript.

CONFLICT OF INTEREST

The authors did not have any conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

SP, TM and TTAk designed the experiment. SP and TM formulated the feed and conducted the experimental trials, analysis and interpretation. JB helped with the statistical analysis and interpretation. SP, TM, JB and TTAk wrote the manuscript and finalized the draft.

DATA AVAILABILITY STATEMENT

All the data generated in this study were included in the manuscript.

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How to cite this article: Prakash S, Marudhupandi T, Balamurugan J, Ajith Kumar TT. Influence of lipid-enriched diets on the reproductive performance of marine ornamental 'hinge-beak' shrimp *Rhynchocinetes durbanensis* (Caridea: Rhynchocinetidae). *Aquaculture Research*. 2020;00:1-13. <https://doi.org/10.1111/are.14991>