Biochemical Profile of Spiny Lobsters *P. homarus* and *P. ornatus*

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ABSTRACT

**Background:** Lobsters are highly expensive and demanded sea food due to their taste and nutritional value. Especially people around the world prefer more and pay huge for live lobsters. They are being exported in different forms like frozen, whole cooked, whole chilled frozen tails and as lobster meat. As there is an extreme targeted catch these lobster resources are being exploited. The spiny lobsters, *P. homarus* and *P. ornatus* are one among the major distributed lobster species along the Gulf of Mannar, South east coast of India. The two lobster species are flavored seafood in many countries because of their more nutrient content are fine flavor.

**Methods:** The experiment was conducted for a period of one year (2018-2019). The spiny lobsters *P. homarus* and *P. ornatus* were cultured under captivity in FRP tanks for a period of one year. After the study period the animal muscle was taken lyophilized and stored at 20°C and used for further analysis.

**Result:** This experiment was conducted to evaluate the crude protein, carbohydrates, crude lipids, moisture, ash, amino acids and fatty acids from harvested spiny lobster, *P. homarus* and *P. ornatus*. The maximum protein content carbohydrate, lipid, moisture, ash and amino acids, fatty acids was noted in *P. ornatus*. The biochemical compositions amino acids and fatty acids were observed moderate and lowest in *P. homarus*.

**Key words:** Biochemical composition, Gulf of Mannar, *P. homarus*, *P. ornatus*, Sea food.

INTRODUCTION

Spiny lobsters are one among the worlds most highly priced and valuable seafood species that are captured and marketed in more than 90 countries (Fitzgibbon *et al.* (2014); Jeffs (2010)). This crustacean has become one of favourite seafood’s and has a high price in the restaurant (Smith *et al.* (2011)). These organisms are believed to have the content of nutrition that is good for human health. On the other hand, information on the nutritional content of lobster is not available, especially those living in the Gulf of Mannar Southeast coast of India, Tamil Nadu. Continuing stability over 30 years of these global landings, there have been recent dramatic declines in production from the three largest spiny lobster fisheries, apparently due to a decrease in recruitment (Fitzgibbon *et al.* (2014)). Due to the lobster decline several researchers are experimenting for more than 100 years at captive rearing the larvae of lobsters around many parts of the world including the United States of America, Japan, Australia, India, Vietnam, Brazil, UK, Mexico and New Zealand (Cox, *et al.*, 2004 and Hattori, *et al.*, 1899).

However, these efforts have always encountered major difficulties around supplying appropriate feeds due to the lack of understanding of the dietary requirements of these larvae Phillips and Sastry (1980). Biologically, lobsters need protein to grow (Floreto, *et al.*, 2000) even since being at the larval stage and through the life cycle including metamorphosis and becomes an adult (Francis *et al.* 2013; Jensen, *et al.*, 2013; Simhachalam *et al.*, 2015; Supriyantini *et al.*, 2007). Survivorship of larval stage of marine organisms can also be improved by good quality of feed (Rao *et al.*, 2010). It has been reported that lobster had better growth if the protein content in food greater than 50% of the diet and lower fat levels AOAC (2005). It has been shown that the quality of the feed given greatly affect the nutrient content of an organism Lowry *et al.* (1951). Excess energy obtained from food will be stored in the form of proteins, lipids and carbohydrates. Despite the enormous ecological and economic importance of spiny lobsters (*Palinuridae*) worldwide, relatively little is known about their unusual larval biology, especially the feeding and nutritional requirements of their phyllosoma larvae. This is because the oceanic pelagic habitat of the phyllosoma and their cryptic behaviour has made in situ field observations of larval feeding impossible Rudd *et al.* (2001).

*P. homarus* and *P. ornatus* is an important tropical lobster of food value and efforts to standardize the culture...
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**MATERIALS AND METHODS**

The harvested spiny lobster *P. homarus* and *P. ornatus* was collected from Gulf of Mannar and transported live to the experimental laboratory and cultured in FRP tanks under captivity for a period of one year. After the study period the animal muscle was taken and washed with distilled water extracted with 0.1 M NaOH and the insoluble substances was lyophilized and stored at 20°C.

**Biochemical analysis**

Biochemical analysis was done for crude protein, carbohydrate, crude lipids, moisture, ash and amino acid and fatty acids analysed after harvesting spiny lobsters.

**Estimation of moisture and ash**

On drying of the 50 grams of lobster muscle samples in an oven at 80°C for 24 hrs and the moisture was expressed in percentage. The total ash was found by heating the dried sample of 2g in silica dish at 600°C for 6 hrs Bligh et al. (1959).

**Estimation of protein**

Protein estimation was done by Floreto et al. (1996) 5 g mussel dry powder was weighed and grinded well with 10 ml of distilled water in mortar and pestle. After vortex for 2 minutes, at 5000 rpm for 10 mins the tubes were centrifuged. The supernatant was taken and made up the volume to 1ml with distilled water and 3 ml of reagent (50 ml of reagent A (2% sodium carbonate in 0.1 N sodium hydroxide) and 1 ml of reagent B ( 0.5 % copper sulfate in 1% potassium sodium tarterate) were added. After adding 0.2 ml of Folinicciolateu reagent, tube was incubated for 30 mins at room temperature. Bovine Serum Albumin (BSA) was used as standard at the range of 20-100 mg/ml. The samples and standards all were prepared in triplicates and in the wavelength of 750 nm absorbance was measured.

**Estimation of carbohydrate**

The carbohydrate content was estimated by the method of Simpson et al.(1976). An aliquot (100 µl) of the supernatant was diluted to 1ml with extraction buffer and 1 ml of 5% phenol (aqueous w/v). 5 ml of concentrated sulphuric acid mixed well was added rapidly and the tubes were incubated at 37°C for 10 mins. At the wavelength of 490 nm using UV-Vis Spectrophotometer (Shimadzu-1800, Japan) the colour development was estimated. Blank is the reagent without sample. Using a standard graph using D glucose was used at the concentration of 10-100 µl/ml for the amount of sugar to be estimated.

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\text{Carbohydrate} = \frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of the sample taken}} \times 100 \]

**Estimation of lipids**

The lipid content of the dried powder was done by solvent extraction method as described by Stocchi et al. (1999). Typically, the cells were harvested by centrifugation at 850 rpm for 5 mins and washed once with distilled water. After samples were dried using freeze drier, the samples were pulverized in mortar and pestle and extract by using mixture of Chloroform: Methanol (2:1 v/v) ratio. For every gram of dried sample 50 ml of solvents were used in the extraction step. The sample being stirred using magnetic stirrer bar for 5 hrs and ultrasonicated for 30 min at 3000 rpm for 10 mins the samples were centrifuged. With the help of filter paper (Whatman No. 1 filter paper) the solid phase was separated carefully. Inrotary evaporator at 40 to 45°C two pieces of filter papers was applied twice to complete separation.

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\text{Lipid} \% = \frac{\text{Amount of lipid in the sample}}{\text{Weight of the sample taken}} \times 100 \]

**Fatty acid analysis**

Fatty acid methyl ester (FAMEs) preparation from lipids was done as above mentioned Battilana et al.(2018). Tricosanoic acid (23:0) was used as the internal standard. The FAMES were taken up in hexane and assayed by GC-FID (Hewlett Packard 5890A) using capillary column (Omega axe 320; 30 m=0.32 mm; film thickness, 0.25 mm) Operating parameters were: column temperature, 210°C; FID temperature, 250°C and carrier gas He, 30 ml.min⁻¹. By comparing relative retention times and equivalent chain lengths of the peaks with those of authentic standards (Sigma) and cod liver oil FAMEs, The FAMEs were identified. Induplicate each sample was analyzed.

**Amino acid analysis**

With norleucine as an internal standard and hydrolyzed in vacuo with 4 N methanesulfonic acid the tissue samples (2.0±0.5 mg) were spiked for 22 h at 110°C Glencross et al. (2001). Dabsyl amino acids were prepared from the hydrolysates Gnaiger (1983) and assayed by HPLC (Hewlett Packard Series 1050 with automatic sampler) using a Supelcosil LC-DABSTM column (15 cm × 4.6 mm, 3µm) To prepare the standard calibration mixtures, a commercial amino acid mixture (Standard H, Pierce Chemical, Rockford, IL) and individual amino acids (Sigma, St. Louis, MO) were used. In duplicates each sample was analyzed.

**RESULTS AND DISCUSSION**

Biochemical composition of harvesting lobster

Biochemical such as moisture, crude protein, carbohydrate, crude lipid and ash were absorbed in (Table 1) and shown that the significantly increased values when compared to the *P. ornatus*. The minimum moisture (83.52±1.52%), crude protein (56.38±1.52% g/100g), carbohydrate (7.26±0.52% g/100g), crude lipids (7.18±0.22% g/100g) and ash (36.19±4.28% g/100g) were present in *P.homarus*. The minimum
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moisture (84.35±1.26%), crude protein (57.82±4.28%/100g), carbohydrate (7.95±0.73%/100g), crude lipids (7.63±0.45%/100g) and ash (37.90±4.13%/100g) were recorded in *P. ornatus* followed by *P. homarus* respectively.

**3.2. Amino acid profiles of *P. homarus* and *P. ornatus***

The total essential amino acids of *P. homarus* were found to be as 50.31±0.07% and nonessential amino acids were 49.93±0.15% similarly the total essential amino acids of *P. ornatus* were found to be as 51.25±1.28% and nonessential amino acids were 49.99±0.55%. Among the essential amino acids, the leucine was found to be high as 8.83±0.16% on dry matter basis and the other essential amino acids percentage were found fluctuating between 8.29±0.25% > arginine 5.52±0.12% > lysine 5.92±0.25% > Threonine 5.52±0.12 > Histidine 4.42±0.22% > Methionine 4.28±0.16% > Phenylalanine 3.98±0.20% > isoleucine 3.36±0.13% > valine 3.15±0.23 > Tyrosine 2.41±0.08 > and Tryptophan 0.28±0.09 in *P. homarus*. In *P. ornatus* the essential amino acids of leucine were found to be high as 8.83±0.16% on dry matter basis and the other essential amino acids percentages were found fluctuating between Leucine 8.83±0.16% > Arginine 7.41±0.85% > Lysine 6.15±0.30% > Threonine 5.80±0.15 > Methionine 4.33±0.20 > Histidine 4.30±0.55% > cystine 0.84±0.55% > Phenylalanine 4.13±0.28% > Isoleucine 3.40±0.22 > Valine 3.25±0.40 > Tyrosine 2.45±0.05 > Cystine 0.84±0.11 > and Tryptophan 0.36±0.11.

Among the nonessential amino acids of *P. homarus* and *P. ornatus* reported, the Glutamic acid, proline, Alanine, serine and Aspartic acid were found to be predominant compounds at the following levels of 18.52±0.55 and 18.62±0.15; 11.08±0.61 and 11.02±0.82; 5.62±0.35 and 5.52±0.58; 5.30±0.25 and 5.12±0.12; 4.35±0.18 and 4.42±0.18 respectively (Table 2).

**Fatty profile in *P. homarus* and *P. ornatus***

The *P. homarus* and *P. ornatus* fatty acid composition is shown here in the table (Table 3). Total fatty acids (mg/g dry weight), as well as total n-3 fatty acids content (mg/g dry weight), did not significantly vary between *P. homarus* and *P. ornatus*. However as a ratio of total fatty acids, *P. homarus* contained significantly lower proportions of 16:1 n-7 and total mono unsaturated, but significant higher proportion of 18:2 n-6; 18; 4n-3; 20:4n-6; 20:5n-3; 22:6n-3 total poly unsaturated fatty acids (PUFA) total n-3 and n-6 PUFA respectively.

There is only a slight difference between the two lobsters (P>0.05). On the separate chemical compounds of *P. homarus* and *P. ornatus* are summarized in table.1 four main biochemical compounds corrected to changes in the three variables (Moisture, crude protein and Ash). The proximate composition of *P. homarus* and *P. ornatus* were having slight changes during the carbohydrate and crude lipids of the spiny lobster. The estimates of biochemical for moisture protein, lipids, carbohydrate and ash ranged from the *P. homarus* and *P. ornatus* were (83.5±1.521 and 84.35±1.26%; 56.83±2.85 and 57.82±3.11; 7.26±0.52 and 7.95±0.73; 7.18±0.22 and 7.63±0.45 and 36.19±4.28 and 37.90±4.13 respectively.

Similarly Bayer and Lagarias (1989) reported that the protein and fat content in lobster meat was (Floreto et al. 2000; Hattori and Oishi 1989), respectively. Chemical composition of lobster indicated that protein, fat, moisture and ash contents of male and female lobster were not significantly different (P>0.05). The five biochemical content of the above spiny lobster is closely related to *P. homarus* and *P. ornatus* in the present study. The American lobster homarus can us studied by Prince, (1997) proved that the proximate (% of dry weights) (Milue Edwards) affected with shell- disease, were compared with those of healthy, unaffected animals. Muscle tissues of affected lobster had significantly lower levels of carbohydrate and the protein profile had significantly. Higher levels of protein 35% less lipid and 26.8% higher levels of ash than healthy lobster. Very lower amount of calcium and phosphorus were found in the dead lobster.

**Table 1:** A comparison of the proximate composition (% of dry) of *P. homarus* and *P. ornatus*. Mean±SE. Treatments with different letters are significantly different from each other (P<0.05).

| Proximate compositions | *P. homarus* | *P. ornatus* |
|------------------------|--------------|--------------|
| Moisture               | 83.5±1.52a   | 84.35±1.26a  |
| Crude protein          | 56.58±2.85a  | 57.82±3.11a  |
| Carbohydrate           | 7.26±0.52b   | 7.95±0.73a   |
| Crude lipids           | 7.18±0.22b   | 7.63±0.45a   |
| Ash                    | 36.19±4.28a  | 37.90±4.13a  |

**Table 2:** A comparison of the amino acid (%) profile of *P. homarus* and *P. ornatus*. Mean±SE. Treatments with different letters are significantly different from each other (P<0.05).

| Essential amino acid %  | *P. homarus* | *P. ornatus* |
|--------------------------|--------------|--------------|
| Arginine                 | 7.35±0.72    | 7.41±0.85    |
| Histidine                | 4.42±0.22a   | 4.30±0.55a   |
| Isoleucine               | 3.36±0.13    | 3.40±0.85    |
| Leucine                  | 8.29±0.35a   | 8.83±0.16a   |
| Lysine                   | 5.92±0.25a   | 6.15±0.30a   |
| Methionine               | 4.28±0.16    | 4.33±0.20    |
| Cystine                  | 0.72±0.08    | 0.84±0.11    |
| Phenylalanine            | 3.98±0.20a   | 4.13±0.28a   |
| Tyrosine                 | 2.41±0.08    | 2.45±0.05    |
| Threonine                | 5.52±0.12    | 5.80±0.15    |
| Tryptophan               | 0.28±0.09    | 0.36±0.11    |
| Valine                   | 3.15±0.23    | 3.25±0.40    |
| Total                    | 50.31±0.07   | 51.25±1.28a  |

**Non-essential amino acid %**

| Aspartic acid            | 4.35±0.18    | 4.42±0.18    |
| Glutamic acid            | 18.62±0.15   | 18.52±0.55   |
| Serine                   | 5.30±0.25    | 5.12±0.08    |
| Glycine                  | 11.02±0.82   | 11.08±0.61   |
| Alanine                  | 5.02±0.20a   | 5.3±0.80a    |
| Proline                  | 5.62±0.35    | 5.5±0.58     |
| Total                    | 49.93±0.15a  | 49.99±0.55a  |
in the exoskeleton of the lobster being poorly mineralized. Crisp et al. (1976); Gnaiger, (1983) increased proportions of moisture, protein and lipid in the affected lesions were expected. Hence compared to the present study the bio chemical composition were similar in all those species. There was a very lower sum protein of haemocytes circulating of the affected lobster and blood cells that they were post cultured were destroyed through phagocytosis and lysis towards the infection Zagalsky et al. (1991). The present investigation revealed that the maximum level of protein content in Panulirus homarus and Panulirus ornatus followed by 56.38± 2.85and 57.82 ± 3.11 g/100g.

The protein value Phomarus was comparatively less than Pnoratus where as the lipids content was slightly lower in Phomarus when compared to Pnoratus. It requires higher oxygen for the combustion of lipids compare to protein and carbohydrates. 2.0×10³, 1.2×10³ and 0.8×10³ ml O₂/g for lipid protein and carbohydrate, respectively Race et al. (1984) twice the amount of energy per unit weight is produce from the combustion of lipids Syafri et al. (1995) and hence lipids are more efficient for energy storage Race et al. (1984). Arnanda et al. (2005) studied that the lobster carapace Carteno protein; alpha-crustacycin a possible role for tryptophan in the bath chronic spectral shift of protein bound astaxanthin Purwaningsih et al. (2012) studied that the relationships between the weight and chemical composition of exuvia and whole body of the black tiger prawn Penaeus monodon. The relationships between the weight and chemical compositions of Exuvia and whole body of the tiger prawn Penaeus monodon was studied by Purwaningsih et al. (2012).

Human health is strongly influenced by the quality of the food intake Picincu, (2018), Daniello, (1980) the nutritional content of food in often associated with high protein content in particular is also associated with growth. This protein is generally supplied from animal products, Jenkins and Watts (1968) Spiny lobster as one of the important fishery products, it can also function as a supplier of animal protein, especially aquatic animals this study illustrates that the spiny lobsters have good nutritional value, Phomarus and Pnoratus is the best type for consumption. According to JACQUOT (1961) classification, the tail muscle of P. interruptus could be classified as high protein, low fat.

The building blocks of cells and tissues are proteins. The human body needs these proteins to build these muscles to fight the germs and to be recovered from the illness. There are certain proteins that help to transport oxygen and transmit messages between the cells and also some cells use their energy to facilitate certain chemical reactions. Nutrients are made up of amino acids which are of two types essential and non essential amino acids they are the reason to initiate various bodily functions, such as hormone synthesis and cellular repair. Most of these nutrients are derived from food and beverages. These are being attached by peptide bonds to each other. The protein being breakdown into amino acids upon ingestion of them. There are 20 different types of amino acids each having a different function and characteristic needed by the body in various ways Chandrasekhar and Deosthale (1993).More amount of amino acid leucine is found in P. homarus and P. ornatus are as leucine phosphate is being served as a transformer of energy Çagiltay et al. (2011). Muscle contraction of invertebrates are also used a source of energy Yokoyama et al. (2007)

Certain essential amino acids like acids leucine, Arginine lysine, Histidine, Methionine and nonessential amino acids glutamic acid, glucose, Alanine, proline, serine, Aspartic acid were found more in two species of the spiny lobsters comparing other amino acids (Table 2) variation in amino acid composition of fish is reported by Carroll and Woodward, (1989) which occur only on certain species especially evident with Arginine and Histidine contents.

### Table 3: A comparison of the study fatty acid profiles of P. homarus and P. ornatus

| Fatty acids (Mg/g dry weight) | Phomarus | Pnoratus |
|-----------------------------|----------|----------|
| 16:0 b                    | 1.08±0.35 | 1.11±0.17 |
| 15:0 a                    | 1.03±0.11 | 1.08±0.09 |
| 16:0 b                    | 15.28±0.72 | 15.41±0.52 |
| 17:0 b                    | 0.63±0.05 | 0.72±0.09 |
| 18:0 a                    | 3.98±0.28 | 4.19±0.68 |
| 22:0 a                    | -         | -        |
| Total                     | 22.56± 0.55 | 22.53±0.40 |

### Monounsaturated

| 16:1 n-7      | 6.08±0.28 | 6.15±0.20 |
| 16:1 n-5      | 0.35±0.10 | 0.52±0.19 |
| 1.23± 0.14    | 1.30±0.16 | -        |
| 18:1 n-9      | 11.68±0.25 | 12.08±0.29 |
| 18:1 n-7      | 7.05±0.50 | 6.93±0.32 |
| 19:1 a        | -         | -        |
| 20:1 n-11     | 2.93±1.08 | 3.11±0.55 |
| 20:1 n-9      | 1.05±0.70 | 0.95±0.19 |
| 22:1 n-9      | -         | -        |
| Total         | 30.37±0.53 | 31.04±0.40 |

### Polynsaturated

| 16:4 n-3      | -         | -        |
| 18:2 n-6      | 0.95±0.13 | 1.12±0.16 |
| 18:3 n-3      | 0.37±0.08 | 0.47±0.15 |
| 18:4 n-3      | 0.55±0.11 | 0.72±0.28 |
| 20:4 n-6      | 3.11±0.09 | 3.18±0.13 |
| 20:4 n-3      | -         | -        |
| 20:5 n-3      | 26.36±1.65 | 26.55±1.90 |
| 22:5 n-3      | -         | -        |
| 22:6 n-3      | 13.19±0.92 | 13.21±1.08 |
| Total         | 44.53±0.52 | 45.25±0.68 |
| Σ n-3         | 39.92±0.52 | 41.67±0.68 |
| Σ n-6         | 3.98±0.47  | 3.28±0.19  |
| Σ n-3 (mg/g dry weight) | 10.03±1.03 | 9.32±1.05 |
| 98.46%        | 99.52%    |
Challem, (1998) studied the proximate composition, amino acid mineral and trace element content of the edible muscle of 20 Indian fish’s amino acid profiles of 21 seafood species was done by Floreto et al. (2000). The muscles of spiny lobsters contained fewer amounts of amino acids cystines and tryptophan. Crustacyanin astaxanthin binding and vitamin A n proteins is done by tryptophan and the melanization is due to tyroinase Arnanda et al. (2005)as there is a lack of idea of amino acids in crustacean metabolism it is difficult to explain why the amino acid was highly reduced in the protein of various affected lobsters P. homarus and P. ornatus has elevated leucine and arginine and tryptophan and aspartic acid level was lowered at lysine elevated levels of P. ornatus. The glycine level is elevated in the muscle and the levels of phenylalanine and threonine of P. ornatus and P. homarus (Table 2).

The key nutrients that affect the early growth, development and nutrition are the fatty acids which results in fatal chronic diseases. Other type of fat called as mono unsaturated fatty acid (MUFA) is referred to be a good fat. As studies show how they help for bringing the cholesterol level and protect from the risk of heart disease. There are decosahexaenoicacid (DHA) (PUFA) intakes here have physiological benefits over blood pressure, heart rate triglicerides and likely inflammation, endothelial function and cardiac death at consumption of 250 mg per day DHA plus EPA by Yokoyama et al. (2000).

There are 24 different fatty acids and totally 97.46% and 99.26% was found in P.ornatus and P.homarus. They are also called as size saturated fatty acids (MUFA) and nigen polyunsaturated fatty acids. The major saturated fatty acids were 16:10 and 18: 0 in (15.28 ± 0.72) (3.98 ± 0.28) was higher in amount, the dry weight basis total n-3 content was not different from spiny lobster essential fatty acids are associated with intelligence decrease incidence of cardiovascular disease Carroll and Woodward (1989) and used against lupus and multiple sclerosis anti immune diseases an also used as a therapy for arthritis Challem, (1998). Floreto et al. (2000) noted the amino acids phenylalanine and threonine was slightly different 18:2n-6 and 20:4 n-6was significant in the healthy lobster. Oleic acid was one of the most unsaturated fatty acids found to be 17.11 and 19.73% on different male and female lobsters. Moreover high concentration of plastic was also found. The palamtic, oleic acid and decosahexaenoic were the predominant fatty acids present in P. homarus. MUFA and PUFA (18:1n-9 -16. 1n-7 and 20.5 n-3 22:6 n-3) the major acids found as 11.68±0.25 - 6.08±0.28 and 26.36±1.65 -13.19±0.92. The percentages availability of these two species P. homarus and P. ornatus SFA, MUFA and PUFA content was (22.56±0.55 -22.97± 0.40), (30.37±0.53 - 31.04±0.40) and (44.53±0.52 - 45.25±0.68) in spiny lobster. The fatty acid profiles is mentioned in the (Table 3). The HUFA's 20:5 n-3 and 22:6 n-3 constitutes the marine crustacean’s dietary essentials Abramo et al. (1981) and is associated into the phospholipids which constitute the cellular membranes. Important role are played by the phospholipids which actthemselves as structural compounds of cellular membranes and in nutrient transport.

CONCLUSION

Lobsters are one of highly priced marine crustaceans known for their flavour and taste. This study was done to examine the nutritional and biochemical content of the losters P. ornatus and P. homarus. Our study gives a insight on the biochemical composition of lobsters P.homarus and P. ornatus. The essential aminoacids, monounsaturated fatty acids and polyunsaturated amino acids are found to be higher in Panulirus ornatus after the analysis. This study shows the nutritional content of the losters which is a major help to know the nutritional factors of lobster. As lobster resource are been declining in a drastic rate new hatchery technologies of loster must be adapted.

Conflict of interest statement

We declare that we have no conflict of interest.

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