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Efficiency of fresh fish and clam meat as a maturation diet in Sebae clownfish, *Amphiprion sebae* (Bleeker 1853)

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The influence of different combinations of fresh fish and clam meat as feed on gonadal development and maturation in *Amphiprion sebae* was evaluated in a 60-day study. The study used four different combinations of diet *viz.* control diet (C: commercial feed with 50 % protein (C)), Treatment 1 (T1: fresh fish alone (F)), Treatment 2 (T2: fresh fish in combination with squid meat (FSQ)) and Treatment 3 (T3: fresh fish in combination with clam meat (FCM)). The experiment was conducted in triplicate, with Clown fish (mean weight: 15 g) stocked in tanks of 200 L capacity. The stocked fish were fed on alternate days with the respective assigned diets. On completion of the 60 days study period, maturation of the fish was assessed based on gonado-somatic index (GSI), fecundity and histology. The present study found highest GSI in fish fed with a diet of fresh fish and clam meat on alternate days (0.493 %), followed by fish fed with a diet of fresh fish and squid meat in alternate days (0.349 %) while the lowest GSI was observed in the group fed with the control diet (0.100 %). Average fecundity observed for *A. sebae* during the present study was 2204±137.4 eggs. Among the different diets, fish fed fresh fish and clam meat (T3) on alternate days had better gonadal development. On 60th day, the ovary of T3 showed late vitellogenic oocytes with an appearance of yolk vesicles. Thus, it could be concluded that fresh fish in combination with clam meat can be used as maturation diet for marine ornamental fish *A. sebae* to attain early maturation in captivity.

[**Keywords**: *Amphiprion sebae*, Fecundity, Feed combination, Maturation, Size at first maturity]

Introduction

In the recent decades, marine aquarium keeping has gained momentum among the people all over the globe. Recent developments in aquarium technology have favored keeping of marine ornamental fish as an important aspect in households. A greater portion (> 95 %) of the global trade in marine aquaria industry depends on fishes caught from the delicate coral reef ecosystems especially those of the developing countries[1]. This is of notable importance in the south East Asian countries such as Philippines and Indonesia. Anemone fish or the more popularly known clownfish, is one of the highly preferred tropical marine ornamental fish[2], due to its small size, attractive color, peculiar behaviour patterns, and the symbiotic association with sea anemones. It is an omnivorous fish which attains a standard length of about 100 mm, depending on the species. They are highly adaptable species and display interesting behaviour in captivity[3]. Alava & Gomes[4] first noted the association between a giant sea anemone and a beautiful clown fish in the South China Sea. Clownfish belong to the family Pomacentridae, inhabiting tropical and subtropical seas. Members of this family are dispersed in four subfamilies comprising of 29 genera and 350 species[5]. Clown fish is protandrous *i.e.* it begins its life as a male and later on changes itself into a female and these are reported to have a total life span of 12 years. The time period for the conversion of anemone fish from a functional male into a female or from an immature male is size dependent and may take several months to a year. Brood stock nutrition and feeding plays a major role in enhancing the quality of egg and sperm and seed production in captivity. The availability of food and essential nutrients has significant effects on the gonadal development of continuous spawners particularly those fishes with short vitellogenic stages.

The availability of adequate quantities of good quality feed significantly influences the reproductive performance of fish[6-8]. Broodstock nutrition directly and indirectly influences gonadal development making it one of the essential areas in aquaculture research[9]. Brood stock nutrition studies are limited and relatively expensive. Presently, marine hatcheries operating on a commercial basis depend greatly on wet diets obtained from the sea or oceans. Fresh fish as well as other aquatic animals are used as broodstock feed and have proved to fulfill the nutritional requirements of the fishes ensuring good quality gametes especially eggs[9]. In case of fishes with a short vitellogenic period, provision of enriched diets shortly before or during spawning can manipulate the gonadal development and fecundity positively[10]. Kumar *et al.*[11] reported breeding of clown fish *Amphiprion sebae* in captivity and also rearing of clown fish larvae in brackish water conditions. In their study, clown fish were fed with different feeds such as live *Acetes* sp., trash fish, clam meat, mussels and squids and the fishes have spawned successfully. A larval diet comprising of artemia nauplii and rotifer, *Brachionus plicatilis* were fed to achieve maximum survival rate in the clownfish larvae[11]. According to Dhaneesh *et al.*[12], the maximum number of spawning in *Amphiprion sebae* was observed in summer and spring months and the brood fish showed higher reproductive efficiency when they were fed live *Acetes* sp. The present study deals with evaluation of the effects of different feed combinations on gonadal development and maturation of sebae clown fish. Anemone fishes have high energy requirements during spawning period owing to protracted spawning, somatic growth even after sexual maturity and the exhaustive parental care. Consistent spawn quality can be effectively obtained if all these energy demands are met via dietary manipulations[6].

The tropical clownfish, *A. sebae*, show a significant difference in reproductive performance when fed different feeds, such as live *Acetes* sp., polychaete worms, cuttlefish, clam, squid and trash fish[9,12,13]. Ablated *Penaeus indicus* when fed with live mysids and fresh clam, gained early maturation and spawning[14]. Akiyama[15] reported that clam powder-based diet for *P. indicus* resulted in better conversion. Clam meal possesses various essential nutrients and chemo-attractant which influence the maturation process of shrimp. Normally, clam meal consists of 56.5 % protein and 8.8 % lipid, which meets the protein and lipid requirement of broodstock[16]. Squid rich in protein (84.5 %) and low in lipid content (3.1 %) as against other fresh feed like polychaete, clams and oysters, has been observed to be rich source of highly unsaturated fatty acids (HUFA)[16,17]. Squid meal is rich in DHA (> 12 %) and EPA (> 9 %)[47]. Of the total lipid present in squid mantle, 18.0 % is cholesterol, which is higher than any other animal feed ingredient[1]. These qualities make squid a good source of maturation diet.

There is scarcity of information regarding nutrition of marine ornamental fish particularly in regard to nutritional requirements for maturation and gonadal development. Seed production in marine fish hatcheries is highly dependent on the proper (qualitative and quantitative) nutrition of broodstock and larvae. But, studies are concentrated on captive maturation and spawning of clown fish. The role played by different feed combinations on the growth, maturation, and spawning of clownfish is yet to be properly acknowledged. The present investigation was hence designed in order to give more insights on the influence of different feed combinations on the maturation and spawning of sebae clownfish.

Materials and Methods

Experimental design

The study followed a completely randomized design which consisted of three experimental diets and one control diet. The experiment was conducted for 60 days. The clownfish, *Amphiprion sebae*, were procured from Kilakarai, Ramanathapuram district, Tamil Nadu, India, and were transported to the centre, located in the same area, using the oxygenated polythene bags. At the center, the fish were treated with formalin at 50 ppm for 1-hour (Francis-Floyd, R. 1996)[18], and then they were conditioned in clean sea water for 10 days. After conditioning, active fish (average weight 15 g) were segregated and randomly stocked in the 12 Fiber Reinforced Plastic tanks (each 200 L capacity) provided with biological filters. In a tank two pairs of fish were stocked, and each pair was separated from other pair by a partition, to prevent fighting. The partition frame was made using 0.5-inch PVC pipe and polypropylene net (Fig. 1).

Feeding and rearing

Four different diets *viz.*, control diet (commercial feed with 50 % protein (C)), Treatment 1 (fresh fish daily (F)), Treatment 2 (fresh fish in combination with squid meat on alternate days (FSQ)), Treatment 3 (fresh fish in combination with clam meat on alternate days (FCM)) were prepared. The experimental fish were fed thrice daily (at 9:00, 13:00 and 17:00 h) throughout the experimental period of 60 days. Feeding was done until apparent satiation. After each feeding, uneaten feed particle and faeces were siphoned out. Proximate composition of each of the experimental diets was calculated following standard protocol and is tabulated in Table 1. Physico-chemical parameters of the culture water such as salinity, temperature, pH, phosphate, dissolved oxygen, ammonia, nitrate and nitrite were maintained at optimal level with 30 % water exchange daily. The parameters were recorded twice a week following standard procedures.

Sampling of fish

The growth performance of the experimental fish (n = 12/treatment) was measured, once in 20 days, to find out the increments in length and weight. Clownfish are territorial and in normal breeding procedure only one pair of fish is kept in a tank to avoid fighting between pairs. Therefore, in each treatment 6 pairs were kept in individual tanks. One pair of fish were sacrificed in each treatment (n = 2/treatment) for every twenty days to observe gonadal development and GSI of the fish (Fig. 1). Fishes were euthanized by injecting an overdose of tricaine methanesufonate (MS 222; Sigma-Aldrich Inc., St Louis, MO, USA).

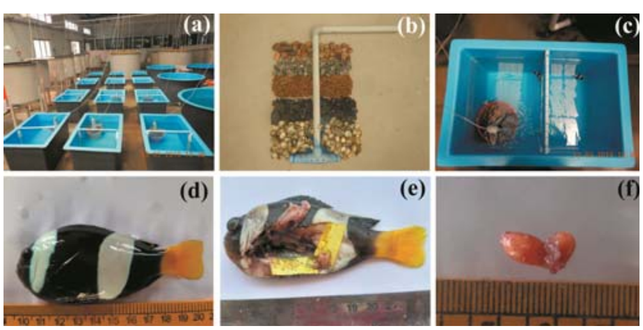

Fig. 1 — Methodology: a) Experimental set- up; b) Cross section view of biofilter; c) Fish in the experimental tank; d) Specimen of *A.sebae*; e) Dissection of fish to remove ovary; and f) Mature ovary of *A. sebae*

Gonadosomatic Index (GSI) was calculated by using the formula given by Kristan *et al.*[8].

$$\text{Gonadosomatic Index (GSI)} = \frac{\text{Weight of gonad (g)}}{\text{Body weight of fish (g)}} \times 100$$

Histological studies were performed on the gonads under standard protocol. The gonads were collected, placed in casettes, dehydrated for tissue processing and finally they were embedded in paraffin wax. 5 µm thick sections were cut from the wax embedded organs. These sections were dewaxed, dried and stained with hematoxylin and eosin using standard procedure as described by Humason[20]. Developmental stages of the gonads were categorized according to major morphological characteristics. These observations were based on the microscopic and macroscopic features of the ovaries[21-25].

Statistical analysis

The data obtained from the present study was analyzed in the statistical software SPSS 16.0. The data were tested for variance among treatments in one way ANOVA and the comparison of means of the treatments was done using Duncan's multiple range test (DMRT) setting statistical significance at $p < 0.05$.

Results

In the present study, water quality parameters were maintained without much variation during the course of the study. The water quality parameters were as follows: dissolved oxygen (6.4 – 8.32 mg/l), ammonia (0.001 – 0.010 ppm), temperature (27.7 – 32.2 °C), pH (7.92 – 8.30), salinity (33 – 38 ppt), nitrite (0.007 – 0.015 ppm), nitrate (0.198 – 0.428 ppm) and phosphate (0.064 – 0.072 ppm). They were maintained at optimal level to support normal growth of clown fish. Among the various feed combinations used in the experiment, squid displayed highest crude protein level of 81.93 % and the control feed showed the highest crude lipid (Ether Extract) level of 11.06 %. Clam meat had a crude protein content of 58.47 % and crude lipid content of 9.01 %.

Table 1 — Proximate composition of different experimental diets on dry weight basis

Feed	Crude protein (%)	Crude lipid (%)	Crude fiber (%)	Moisture (%)	Total ash (%)	Gross energy (Kcal/kg)
Commercial feed (C)	48.49	11.06	1.36	9.02	13.27	4511
Fresh fish (F)	77.59	8.30	1.00	7.64	6.95	5168
Squid (SQ)	81.93	5.73	1.00	8.18	5.39	5171
Clam (CM)	58.47	9.01	1.33	7.78	12.71	4636

Influence of different feed combinations on the body weight gain of *Amphiprion sebae*

The mean body weight was 0.83, 0.68, 0.65, 0.74 g for the control, T 1, T 2 and T 3, respectively (Fig. 2). Fish fed with commercial feed showed more increment in weight followed by fish fed with fresh fish and clam on alternate days.

The mean body length gain in the control diet fish was 0.31 cm. The mean body length gain of *Amphiprion sebae* was calculated to be 0.30, 0.15 and 0.15 cm for T 1, T 2 and T 3, respectively (Table 2).

Gonadosomatic Index

At the end of the experiment, the highest GSI was observed in fish fed with fresh fish in combination with clam meat (T3) (0.493 %), followed by fish fed with fresh fish in combination with squid (0.349 %). The control diet fish had the lowest GSI (0.10 %), while other experimental diets had resulted in higher GSI (Table 3).

Relationship of body length, body weight and GSI

Fish fed with control diet showed more increment in length as well as in weight in comparison with other combinations of diet. Among the treatments, fish fed with fresh fish in combination with squid on alternate days had least increment in weight; whereas fish fed with fresh fish in combination with clam and squid on alternate days had lesser increment in length than the control. In contrast, fish fed with fresh fish in combination with clam on alternate days recorded the maximum GSI followed by fish fed with fresh fish in combination with squid. Fish fed with commercial feed had least increment in GSI (Fig. 3).

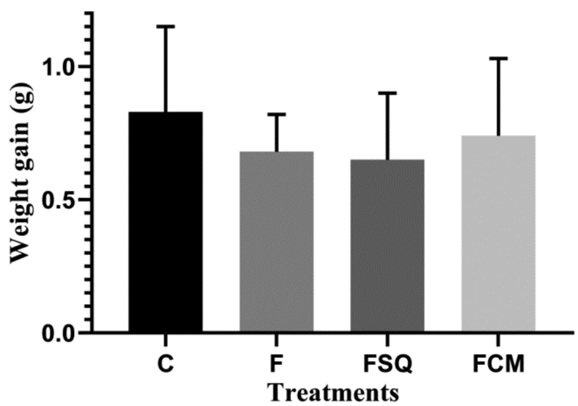

Fig. 2 — Influence of different feed combinations on the weight gain of female *A. sebae*

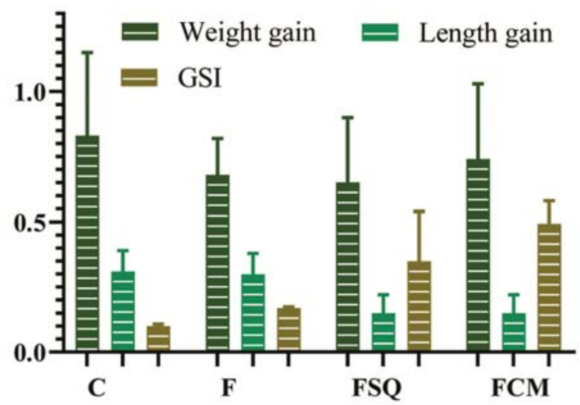

Fig. 3 — Relationship between weight and length gain and GSI

Table 2 — Biometrics of *A. sebae* (L: Length and W: Weight) when fed with different feed combinations

Treatment	1st day		20th day		40th day		60th day		Mean	
	L	W	L	W	L	W	L	W	L	W
Control	9.25 ± 0.79^{b}	19.08 ±1.95^{b}	9.31	19.26	9.56	12.84	9.56 ±0.38^{b}	12.92 ± 2.00^{b}	0.31±0.08	0.83±0.32
Treatment 1 (F)	9.83 ±1.42^{ab}	24.32±4.72^{ab}	10.06	24.64	10.13	25.01	10.13±0.54^{ab}	25.02± 4.75^{ab}	0.30±0.08	0.68±0.14
Treatment 2 (FS)	9.35 ±0.80^{ab}	19.33±1.74^{ab}	9.46	19.91	9.48	19.95	9.50±0.28^{ab}	19.98± 1.52^{ab}	0.15±0.07	0.65±0.25
Treatment 3 (FCM)	9.50 ±1.03^{a}	24.00±3.38^{a}	9.63	24	9.65	24.21	9.65±0.44^{a}	24.74 ± 4.03^{a}	0.15±0.07	0.74±0.29
P-value	0.039	0.132					0.065	0.130		

Values in the same column with different superscript differ significantly ($P < 0.05$). One-way ANOVA was used following Duncan multiple ranges testing of SPSS 16.0(ref. 45)

Table 3 — Observations on Gonadosomatic Indices (GSI) and fecundity of *A. sebae*

Treatments	20th day	40th day	60th day	Fecundity
Control (C)	0.095 ± 0.010^{c}	0.100 ± 0.005^{c}	0.100 ± 0.014^{c}	1700 ± 201.1
Treatment 1 (F)	0.063 ± 0.012^{b}	0.127 ± 0.007^{c}	0.169 ± 0.003^{bc}	1900 ± 121.5
Treatment 2 (FSQ)	0.085 ± 0.009^{c}	0.156 ± 0.005^{b}	0.349 ± 0.11^{ab}	2090 ± 167.8
Treatment 3 (FCM)	0.136 ± 0.011^{a}	0.313 ± 0.014^{a}	0.493± 0.050^{a}	2204 ± 137.4
P-value	0.001	0.000	0.009	

Values in the same column with different superscript differ significantly ($P < 0.05$). One-way ANOVA was used following Duncan multiple Ranges testing of SPSS 16.0(ref. 45)

Gonad features

There was significant variation observed in gonad development when fish fed with different feed combinations. Fish fed with control diet did not show much difference in gonad development. The gonad was small in size, white in color and in immature stage with no development of eggs. Similarly, fish fed with fresh fish also did not show much development on gonads. Ovary was small in size, white in color and was in immature condition. On the 60th day of study, the eggs started to develop. Gonads of fish fed with fresh fish and squid on alternate days did not show any development up to 40th day. Ovaries were small in size and in immature stage. However, on 60th day, the ovary colour changed from deep pale yellow to bright yellow indicating the beginning of maturation of ovary. Most of the eggs were in pre-vitellogenic stage. In fish fed with fresh fish and clam meat on alternate days showed a significant development in gonads. On the 20th day the ovary was pale yellow in color, small in size was in immature state. On the 40th day the ovary was dark orange in color indicating the maturation of ovary. On the 60th day ovary was fully matured and brownish in color with eggs in late vitellogenic stage.

Histology of the ovary

The dissected female fish showed complete absence of testicular tissues in the ovary, indicating complete sex change. The fish fed with control diet exhibited an immature ovary, which can be identified by the presence of undeveloped ovary, with numerous primary oocytes, mostly in the perinucleolar phase (Figs. 4a & b). In T1 and T2 the histological sections revealed the growth of oocytes with the presence of previtellogenic and early vitellogenic oocytes. In T3, ovary showed late vitellogenic oocytes with the appearance of yolk granules and globules.

Discussion

In the present study, the broodstock fish showed only slight growth improvement in terms of body weight gain. This increase in body weight was however, not statistically significant. Fish did not show much increase in length, however, there was a slight increase in weight, especially in fish fed with fresh fish and clam on alternate days. In the present study maturation of ovary was observed in 2 months, in fish fed with a combination of fresh fish and clam meat on alternate days. Ignatius *et al*.[26] have reported similar maturity level in *A. sebae* fed with a diet of clam meat, supplemented with polychaete worms for 3 months. Among the other treatments, fresh fish and a combination of fresh fish and squid on alternate days, showed lower weight gain, which was similar to the findings of Dhaneesh *et al.*[12], who reported that *A. sebae* fed with trash fish and commercial feed had lower maturation efficiency.

Previous studies observed that the relationship among body length, weight and GSI value differed significantly between males and females. Body size was positively correlated with the GSI value, as shown for other fish species such as Common dace (*Leuciscus leuciscus*) and Dark chub (*Zacco temmincki*)[27-28]. In the present study matured fish had positive correlation between body weight and Gonado Somatic Index (Fig. 3). Fish fed with fresh fish + clam meat and fresh fish + squid on alternate days showed increment in body weight but not much increment in length. This increase in body weight may be due to development of ovary. The present findings are similar to the earlier reports on Mountain mullet, *Agonostomus monticola* where GSI values corresponded closely with the developmental changes of the gonad[29]. Further, this higher GSI generally corresponds to the breeding season of the fish[30].

A. sebae are protandrous, which was confirmed by the presence of both ovarian and testicular tissues in male gonad and by the absence of testicular tissues in the ovary of females. Similar observations were also reported in gonads of *Amphiprion* species from Japan coast[31], *A. clarkii*[32-34]; Gobiid fish and Wrasses[34]; *A. akallopisos*[35] and in *A. ocellaris*[22]. The pattern of development of ovaries in *A. sebae* was confirmed to be similar to that of most teleosts[36-39]. The present study observed four development stages in ovary based on its colour, size and oocytes, which was similar to the observation in *A. ocellaris*[40]. In the first stage, the ovary is immature, small, white to pale yellow in color. In second stage, ovary turns pale yellow to bright yellow colour. Premature females showed this stage of ovary. In the third stage, the ovary was matured, bright orange in colour, vitellogenic oocytes present, found mostly in mature females. In the fourth stage, the ovary was fully matured, bigger in size, brownish in colour, and the eggs on late vitellogenic stage. The fecundity of *A. sebae* recorded in the present study was 2204±137.4 eggs per female, which was similar to the earlier reports in *A. sebae*[12], *A. ocellaris*[40] and *A. melanopus*[2]. The increase in fecundity with length and weight of body was consistent with the observations in other

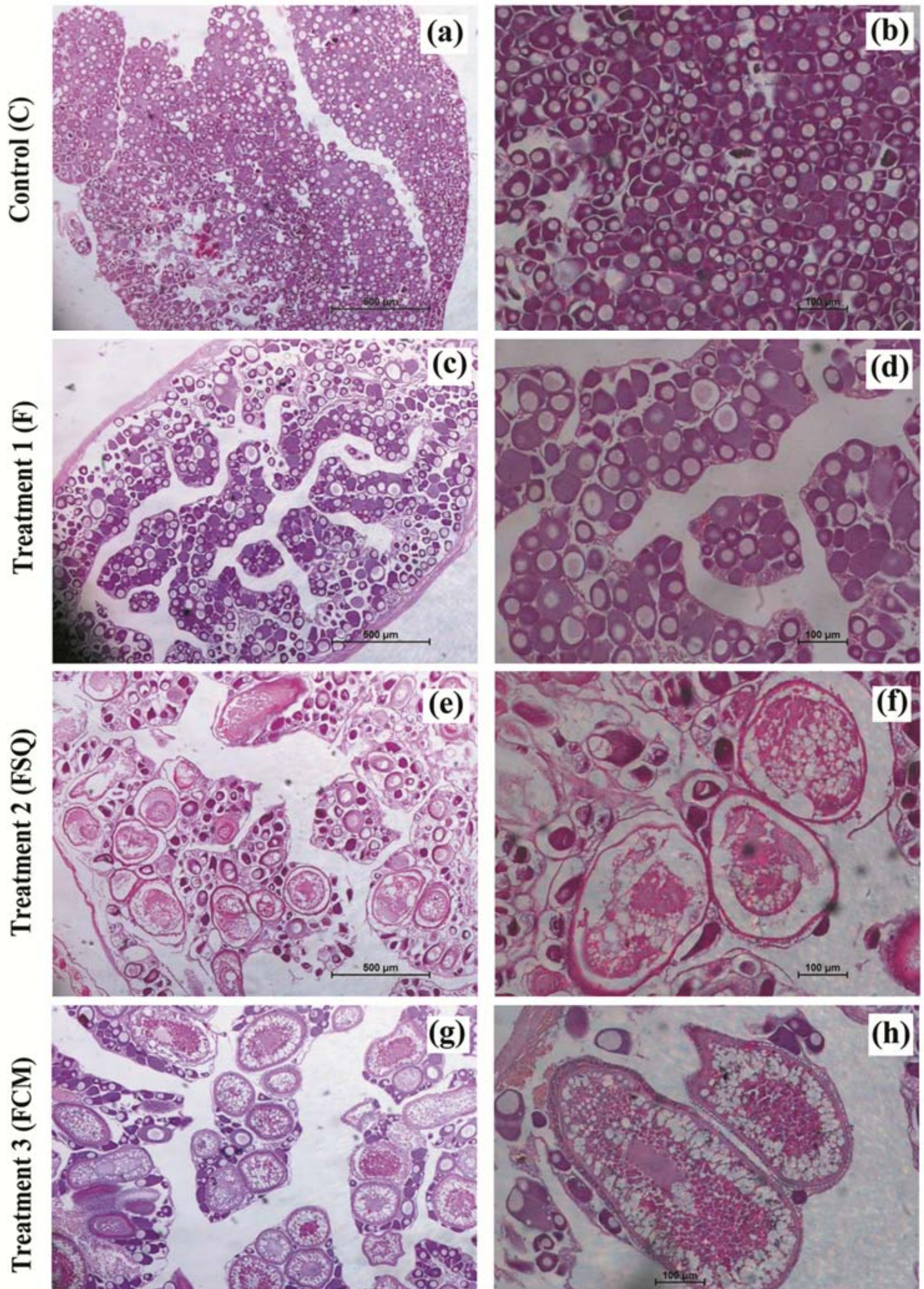

Fig. 4 — Histology of ovary on 60th day: a & b) Control (C); c & d) Treatment 1 (F); e & f) Treatment 2 (FSQ); and g & h) Treatment 3 (FCM). Magnification = 4X (left); and 10X (right)

Amphiprion species[41-42], which supported the reliability of length and weight of body as indicators of the capacity of egg production[43].

The histology of control fish revealed initial phase of ovarian development while the treatments revealed different stages of ovarian growth. Among the three treatments, on 60th day of sampling, fish fed with fresh fish and clam meat on alternate days exhibited late vitellogenic phase of ovary. This confirms that fresh fish in combination with clam meat on alternate days can be used effectively for inducing maturation in *A. sebae* under captivity.

During spawning, the energy requirement of *Amphiprion sebae* was higher; it may be attributed to their protracted spawning nature and extensive parental care behavior. These energy-demanding processes need to be catered through proper nutrition to produce good quality spawn. Traditionally, fresh natural diets were used to feed broodstock which is found to be the most effective way of fulfilling the nutritional needs of fish and ensuring good quality eggs[44].

Conclusion

The present study revealed the importance of proteins and crude lipids as major nutrients influencing the maturation. A diet with 55 to 60 % protein and 9 to 12 % lipid content positively affected the broodstock performance in Sebae anemone fish[4]. In the present study, clam meat (58.47 % and lipid of 9.01 %) gave the best performance in terms of maturation of *A. sebae* due to its nutritional composition. Squid meat had higher protein content (81.93 %), but less lipid content (5.73 %) and performed poorer with respect to maturation. Commercial feed had higher lipid content (11.06 %), and less protein content (48.49 %), which might have led to good physical growth but resulted in low GSI. Therefore, it could be concluded that clam meat and fresh fish, fed on alternate days can be used as a maturation diet for successful gonad maturation of *A. sebae* in captivity.

Ethical statement

This study was approved by ethical committee of Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Tamil Nadu, India. The authors conducted the experiments as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) issued by the Animal Welfare Division of the Ministry of Environment and Forests, Govt. of India.

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Conflict of Interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Author Contributions

All the authors have contributed immensely in the design and plan of experiments, data collection and compilation, and consolidation of results and discussion the outcomes.

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