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Research Article

Effect of selenium-enriched microalgae and rotifers for the efficacious larval survival of Pink Skunk Clownfish, *Amphiprion perideraion*

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Selenium is an essential microelement for the normal functioning of fish health. It was reported that selenium-enriched zooplankton has given reasonable survival rates in food fish larvae. However, limited literature is available on clownfish. Hence, the present experiment was designed to improve the assessment of the larval survival of pink skunk clownfish, *Amphiprion perideraion*, with selenium-enriched microalgae *Nannochloropsis* sp. Different concentrations of Se [0 (Control), 2, 4, 10 and 50 mg/L] were added to the stock culture of microalgae. For this experiment Walne medium was used at the prescribed level to culture *Nannochloropsis* sp. In each tank, 100 larvae of *A. perideraion* (3.5 – 3.8 mm) were stocked immediately, after hatching. The rotifer, *Brachionus plicatilis* were fed to different groups of selenium-enriched *Nannochloropsis* sp. and same was introduced in tanks (6 – 8 no./ml). There was no colour change in Se-enriched microalgae at concentrations of 0, 2, and 4 mg/L, whereas the microalgae at 10 and 50 mg/L turned pale after the 3rd day. Maximum larval survival was observed in 2 and 4 mg/L groups, followed by control and 10 mg/L. However, meagre survival was noticed in 50 mg/L. Early metamorphosis (11th day) was recorded in the 2 and 4 mg/L groups; in the control group, it occurred after the 15th day. The present study concluded that selenium-enriched microalgae at specific concentrations are recommended for the larval rearing of pink skunk clown; however, this may also be adopted for other species of clowns.

[**Keywords**: Algae enrichment, *Amphiprion perideraion*, Growth, Larvae survival, Selenium]

Introduction

Clownfish (Family: Pomacentridae), also known as anemone fish, are among the most attractive marine ornamental fish. Clownfish make up 43 % of the global marine aquarium trade, and only 25 % of the global trade comes from the hatchery, resulting in decreased densities of the species in exploited areas[1]. Due to the short time taken for maturity and parental care for eggs, clownfish are considered one of the best candidates among marine ornamental fishes for captive breeding programs. Successful larval and juvenile rearing is a major hurdle in captive breeding and seed production of marine ornamental fishes. Copepods, the primary food source of the larvae in wild[2], is still facing snags in raising them in mass, in captivity. Comparative analysis between cultured rotifers and wild copepods indicated that rotifers have lower concentrations of many essential minerals, especially Selenium (Se), between 0.08 – 0.09 mg Se/kg dry weight, than recommended (0.25 – 0.3 mg Se/kg DW) for fish growth[3].

Selenium (Se) is considered an essential microelement for the normal functioning of a fish and the essential components of an enzyme such as Glutathione Peroxidase, which protect cells against oxidative damage[4]. The concentration of Se supplemented through feed is known to influence the activity level of Glutathione peroxidase[5]. The central role of Se is to protect the biological compounds, especially DNA, proteins and lipids. It further provides protection against free radicals, resulting from normal metabolism by reducing hydroxides and peroxides, after converting them into more stable alcohol and water[6]. Selenium is also involved in regulating the expression of bone morphogenetic protein (*bmp*) and osteocalcin (*oc*) genes, which encode for two important proteins involved in bone cell differentiation and mineralization, which is strongly dependent on dietary Se inclusion[7-8]. Thyroid hormone is vital in early development and plays a regulatory role in growth, reproduction and metamorphosis[9]. Thyroid hormone levels in fish bodies are considered to be regulated by the concentration of Se supplied through feed. Se intake influenced the thyroid hormone levels in Senegalese sole (*Solea senegalensis*) larvae and juveniles[5].

Selenium is an essential nutrient for fish, but can be toxic, if administered at higher concentrations[10]. Selenium toxicity occurs through two main mechanisms. The first is that Se can disrupt proteins via substituting as Sulphur in Sulphur bonds, resulting in incorrect protein shape and dysfunctional enzymes. The second mechanism is oxidative stress caused by excess unbound Se[(ref. 11)].

The mineral recommended for the growth of fish larvae or juveniles can be supplemented through its feed. Short- or long-term enrichments can enrich the rotifers with such a diet. The process of long-term enrichment is advised as it facilitates greater uptake, assimilation and stabilization of nutrients in the rotifer body, than short-term enrichment[12-13]. The Se concentration in the rotifers can be improved by feeding Se-enriched microalgae, which can absorb soluble inorganic minerals and convert them into organic molecules[14]. Hence, enrichment of rotifers and other zooplankton by mineral-enriched microalgae is considered a better alternative than directly applying the insoluble forms of minerals. Sodium selenite (NaSe) is the most commonly used source of inorganic Se for addition to fish feeds and is found to be less available to fish in comparison to organic forms of Se, such as hydroxy-selenomethionine (OH-SeMET).

Se supplementation in artificial diets is known to enhance the growth and development of rainbow trout, *Oncorhynchus mykiss*[15], grouper *Epinephelus malabaricus*[16], red sea bream *Pagrus major*[2] and gilthead seabream, *Sparus aurata*[17]. However, limited research is available on marine ornamental fishes. Among clownfishes, few works have been done on *Amphiprion percula*, *A. ocellaris*, *A. frenatus* and *P. biaculeatus*; however, from Skunk clade, which includes *A. perideraion*, *A. akallopisos* and *A. sandaracinos*, no work has been attempted. Hence, the main objective of the present study is to document the effects of different concentrations of Se-enriched algae and rotifers on the survival of *A. perideraion* larvae and the duration required to complete the metamorphosis stage.

Materials and Methods

The experiment was conducted at the ICAR-NBFGR and Mangrove Foundation clownfish breeding facility, Airoli, Maharashtra. The microalgae *Nannochloropsis* sp. and rotifer *Brachionus plicatilis* were obtained from the live feed stock culture unit of the said facility. In this experiment, a commercial product, Sel-Plex® (Alltech, USA) was used to enrich microalgae and rotifers. Sel-Plex consists of 63 – 66 % methioseleonin, 33 – 36 % of low molecular weight Se and < 0.5 % inorganic selenium, which were used to enrich the microalgae. Marine microalgae, *Nannochloropsis* sp. was selected for bioaccumulation of Se and further for the enrichment of rotifers, due to its higher levels of mineral content and optimum level of Poly Unsaturated Fatty Acids (PUFA)[18].

Nannochloropsis sp. was cultured with Walne medium and incubated for four days, under optimised conditions (Salinity 28±1 PSU, pH 8.2, temperature 24±1 °C[(ref. 18)] & 24 h of photoperiod[1]). Differe nt concentrations of Se (0 (Control), 2, 4, 10 and 50 mg/L) were enriched with the microalgae culture. Ahmadifard *et al.*[12] were followed for the long-term enrichment of *Nannochloropsis* sp. and rotifers with selenium. Rotifer strains obtained from the NBFGR hatchery were cultured in 20 l plastic buckets with continuous aeration and were regularly fed with *Nannochloropsis* sp. The rotifer culture was maintained, adopting the same parameters followed for algae. For enrichment, rotifers were continuously fed with different groups of selenium-enriched algae [0 (Control), 2, 4, 10 and 50 mg/L] for four days, with no water exchange.

The enriched rotifers were added to the larval rearing tank at a density of 6 – 8 no/ml as the initial feed[19]. The larval rearing experiment was carried out for 20 days in 50 l glass tanks. For each treatment, three replicates were maintained. In each experimental tank, 100 newly hatched larvae of *A. perideraion* (3.5±0.3 mm) were stocked. The water quality parameters were maintained as salinity 30±1 PSU, pH 8.2±0.1, temperature 28±1 °C, and dissolved oxygen 6.5±0.3 ppm throughout the larval rearing period. About 5 % water exchange was provided daily to avoid ammonia accumulation. After 5 days (1, 5, 10, 15 and 20 DPH), larvae were randomly sampled from each tank and anaesthetized with clove oil, followed by 5 % formalin fixation. The total and standard lengths were measured for all sampled larvae, using a 102 microscopic measurement system, including a stereomicroscope (Discovery V8, Zeiss, 103 Germany) equipped with a digital camera (AxioCam, HSm). The survival rate of larvae was calculated from the average number of larvae survived in the tanks. The effect of selenium enrichment on larval growth was analysed by One-way ANOVA followed by Tukey-Kramer *post hoc* test. All the statistical analyses were carried out using SPSS version 16.0 (SPSS Inc. SPSS for Windows).

Results

Effect of Se enrichment on the survival

The results revealed that rearing larvae with Se-enriched microalgae and rotifers had positive effects on survival. *Artemia* feeding started 8 – 9th day onwards, but up to 11 days, a mixture of *Artemia* and rotifer was given. This was continued for a period of 11 to 20 days. After 20th day of culture, a better survival (62 %) was reported in the treatment with 2 mg/L Se and 45 % in 4 mg/L group, followed by the control (10 %) (Fig. 1). However, low survival (8 – 10 %) was recorded in higher concentration of 10 mg/L and meager survival (< 5 %) was noticed in the tank with 50 mg/L Se concentration. In the first two days of culture, higher mortality than the control group was observed in 10 mg/L & 50 mg/L treatment groups. It's noted that with the increase in Se concentration, there was a decrease in the survival rate of the larvae.

Effect of Se enrichment on the growth

After 20 days of rearing, juveniles showed significant differences in growth rate. Also, a higher growth rate of 22.5 % was observed in the tanks with 2 mg/L and 4 mg/L Se concentration (21.5 %), followed by 20 % in the 10 and 50 mg/L treatment groups, whereas 0 mg/L Se (control) had a lower growth rate of 17.5 % (Table 1).

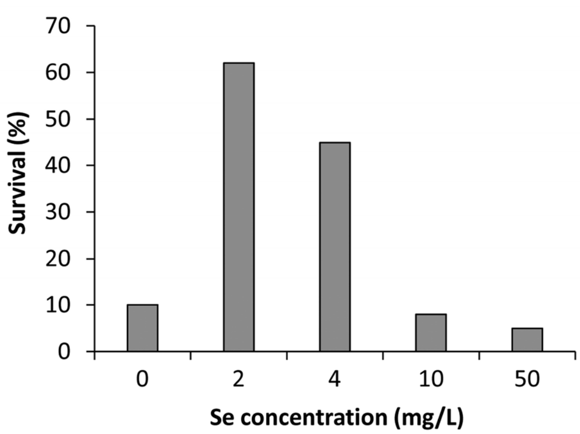

Fig. 1 — Effect of Se concentration on *A. perideraion* larval survival

Morphometric parameters, including total and standard lengths, significantly differed between Se-enriched treatment groups and the control (0 mg Se/L). The highest length was recorded in 2 mg Se/L (8.0±0.2 mm) and 4 mg Se/L (7.8±0.2 mm). The lowest length (7±0.3 mm) and lowest specific growth rate (17.5 %) were reported in the control group.

Effect of Se enrichment on the duration required to complete metamorphosis of A. perideraion larvae

Early metamorphosis was recorded in the treatment group of 2 mg/L on 11th DOC (Days of Culture), followed, 4 mg/L, 10 mg/L and 50 mg/L (13th DOC). Delayed metamorphosis (starting after 15th DOC) was reported in the control tank (Fig. 2). Higher selenium concentration in experimental tanks might have led to delayed metamorphosis, likely due to stress conditions. In stressed environment, fish showed behaviors like congregating in corners and had black body colouration instead of pink. They also fed less actively on live feed, resulting in slower growth.

Discussion

It's important to understand the nutritional needs for the successful rearing of early larval stages, particularly marine fishes. Rotifers are commonly used as the initial food for many marine fishes, like clownfish, because they are small, slow-moving, reproduce quickly, and can be fed on an enriched diet[19]. However, cultured rotifers may lack certain essential fatty & amino acids, vitamins, and minerals, such as selenium, compared to wild copepods[20-21]. To address this, specific nutrients like selenium can be increased in live feeds by feeding them with enriched microalgae or yeasts for short or long-term durations[21-22]. Additionally, rotifers can absorb minerals more efficiently when incorporated through microalgae rather than directly fed[14]. Selenium is crucial for various biological functions throughout the fish's life cycle, including in early larval stages[5].

In this context, the present experiment analysed the effects of different concentrations of selenium (Se) on

Table 1 — Effect of Se enrichment on the growth of *A. perideraion* larvae

Treatment	Total length (mm)	Initial length (mm)	Specific growth rate (%)	Standard length (mm)
0 mg/L	7.0±0.3^{a}	3.5±0.04^{a}	17.5±1.15^{a}	5.8±0.1^{a}
2 mg/L	8.0±0.2^{b}	3.5±0.2^{a}	22.5±0.4^{b}	6.5±0.09^{b}
4 mg/L	7.8±0.2^{ab}	3.5±0.4^{a}	21.5±0.6^{b}	6.4±0.1^{b}
10 mg/L	7.5±0.25^{ab}	3.5±0.1^{a}	20±1.15^{ab}	6.5±0.08^{b}
50 mg/L	7.5±0.25^{ab}	3.5±0.2^{a}	20±0.7^{ab}	6.5±0.1^{b}

Superscripts ^{a,b} denote significant variations ($P < 0.05$) according to Tukey-Kramer *Post hoc* test

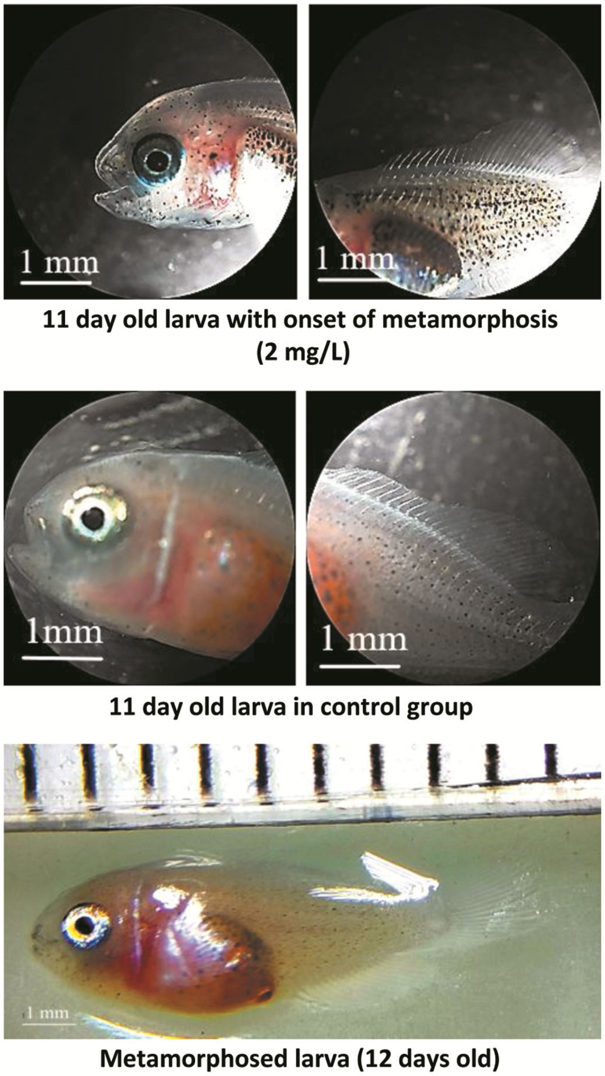

Fig. 2 — Metamorphosis process of *A. perideraion* larvae in Se treated and control groups

the survival, growth, and metamorphosis duration of pink skunk larvae. The results indicated that rearing the larvae with Se-enriched microalgae and rotifers positively impacted their survival. After 12 days, the survival rate was 62 % with the 2 mg/L Se treatment and 45 % in the 4 mg/L group. However, the concentration of Se was not quantified in microalgae and rotifers after enrichment. Moreover, the larvae in the 2 mg/L and 4 mg/L Se concentration groups exhibited better growth, with a size of 8±0.2 mm, compared to the control group (0 mg/L Se) with a growth rate of 7±0.3 mm. It was observed that when Se was present in the feed below the recommended level, adverse effects on fish growth were reported. The deficiency of Se was found to have adverse effects on the survival and growth of marine fish larvae, leading to decreased activity of Glutathione peroxidase[2,21]. Furthermore, Se deficiency was known to induce various negative effects such as loss of appetite, reduced growth, increased mortality, decreased muscular tone, and oxidative damage to cells and membranes.

Like most marine vertebrates, clownfish also undergo a biphasic life cycle, where pelagic larvae transform into miniature adults by losing their elongated body shape and colouration with an ovoid body and dark pigmentation with one to three white bars. A post-embryonic developmental process triggered by thyroid hormones and characterised by ecological, behavioural, morphological, and physiological changes is called metamorphosis[23]. For the pink skunk clownfish, *A. perideraion*, metamorphosis involves the acquisition of one vertical white band on the opercular region and another horizontal white band at the base of the dorsal fin. Metamorphosis in *A. perideraion* larvae start on 15 DPH (Day Post Hatching) and the process completes on 22nd DPH[ref. 24]. In the present experiment, an early start of metamorphosis was recorded on 11th day in the treatment group of 2 mg/L, followed by 4 mg/L (14th day), 10 mg/L (14th day) and 50 mg/L (14th day). However, the delayed metamorphosis was noticed (on 15th day) in the control group. Ribeiro *et al.*[5] found high thyroid hormone levels in sole (*Solea senegalensis*) larvae fed with Se-enriched live feed than in the control group.

Early metamorphosis by day 4 and improved survival were reported in yellowtail clownfish (*Amphiprion clarkii*) treated with 0.01 ppm T3 (Triiodothyronine) compared with the control group. At the same time, body deformations were observed in the treatment with 0.1 ppm T3 concentration. Additionally, maximum survival of 60.85±0.14 % was observed in the 0.01 ppm T3 group, followed by 28.95±0.04 % (0.1 ppm T3) and 24.53±0.13 % (control)[25]. In the present study, different concentrations of Se were used to enrich microalgae & rotifer and assess its effects on the growth and survival of the pink skunk larvae. Lower survival for larvae was reported in the treatment groups with higher concentrations of Se, such as 10 mg/L and 50 mg/L. The margin line between the required level and a higher level of Se causing stress depends upon the species, size/age of the fish, and type of Se incorporated *i.e.* either in organic (Selenomethionine) or inorganic form (Sodium selenite)[2]. The algae and rotifers efficiently absorb the organic form of Se compared to its inorganic form. The signs of severe stress include reduced growth and feed intake, increased oxidative stress, and disturbance of fatty acid metabolism[26]. Further studies need to be conducted to analyse the effects of higher Se on the clownfish larvae.

Conclusion

The study concluded that selenium-enriched microalgae at specific concentrations (2 to 4 mg/L) were more beneficial in the survival, growth, and physiological development of pink skunk larvae than at higher concentrations (10 and 50 mg/L). Therefore, to improve the larval survival and growth of pink skunk clownfish, providing live feeds enriched with the optimum concentration (2 to 4 mg/L) of selenium is essential. Since the clownfish larval rearing protocols are almost similar, this finding can be adopted for the other 29 species of clown, which provide promising results in juvenile production and play a role in the sustainable marine aquarium trade.

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Conflict of Interest

The authors declare that they do not have any conflict of interest in this manuscript.

Author Contributions

MN: Experimentation and manuscript preparation; NBD: Experimentation; PRD: Manuscript editing and review; TTAK: Concept, guidance and manuscript reviewing; and UKS: Overall guidance and facilitating the work.

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