EFFECT OF DIETARY SELCO ON REPRODUCTIVE PERFORMANCE, EGG AND LARVAE QUALITY OF CLOWFISH *AMPHIRION OCELLARIS* (CUVIER, 1830)

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Abstract. Broodstock nutrition is one of the most important research field in aquaculture. The study was conducted to evaluate the influence of HUFA (A1-DHA Selco-Selco, Inve) supplemented in the broodfish diets on reproductive performance, egg and larvae quality of clownfish *Amphirion ocellaris* (Cuvier,1830). There were 5 treatments with 5 diets supplemented Selco with levels: 0.0 (as the basal diet), 50 g, 100 g, 150 g and 200 g Selco/kg feed, respectively. Each diet treatment was repeated in triplicate and the experimental period was 12 months. The results showed that spawning frequency, re-maturation and spawning, egg size and larvae were not affected by the addition of Selco to broodstock diets. Diet supplemented with Selco 50 and 100 g/kg feed had positive effects on absolute fecundity, egg loss rate ($p<0.05$). The hatching rate and survival rate of 3 days posthatch larvae fish in the treatment supplemented Selco 100 g/kg feed were the highest and had significantly difference (94.73±0.19 % and 94.56±0.93%, respectively) ($p<0.05$). The malformation rate of fish larvae in the treatment supplemented with Selco 150 g/kg feed was the lowest (0.55 ±0.022 %) but there were no differences from the diet treatments supplemented 50 and 100 g Selco/kg feed. The study showed that supplementation of Selco with 100 g Selco/kg feed in broodfish diet had significantly increased absolute fecundity, hatching and survival rate of larvae.

Key words: *Amphiprion ocellaris*, Clownfish nemo, DHA Selco, HUFA.

1. Introduction

The success of the breeding marine ornamental fish depends on many factors such as: culture system, culture technique, nutrition, stocking density, care regimes, environmental factors, disease. In which, broodstock nutrition is one of the important factors contributing to improving fecundity, hatching and survival rate in rearing, growth of fish species (Fernández-Palacios et al., 2011). An improvement in broodstock nutrition has been shown to greatly improve not only egg and sperm quality but also seed production (Izquierdo et al., 2001). The fatty acid composition of fish eggs is directly affected by the fatty acid content of the broodstock diets (Fernández-Palacios et al., 1995). There have been numerous studies on effect of n-3 HUFAs (highly unsaturated fatty acids) supplied in broodstock diets to fecundity fertilization and hatching rate of egg, larvae quality. Requirement n-3 HUFAs in marine fish species such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) due to they cannot themselves synthesize these fatty acids (Rainuzzo et al., 1997; Tocher, 2010). In addition the n-3 HUFAs, n-6 fatty acids such as arachidonic acid (ARA) are also essential for growth, reproduction, and larval quality (Bell et al., 2003; Furuita et al., 2003). Many studies have demonstrated that dietary lipids and fatty acids enhance the reproductive performance of broodstock. Lipids and their fatty acids in dietary composition affect reproductive development (Nzohabonayo et al., 2017),

reproductive performance (Zakeri et al., 2011) as well as egg and larval quality (Noori et al., 2019). Despite being given considerable attention over the past two decades, understanding the requirements for EFA in marine fish remains complicated by the fact that requirements may vary with species, environmental conditions and life stage (Tocher, 2010). It has also been suggested that future research focuses not only on the quantities of DHA, EPA and ARA but also on the ratios between them as being critical in further understanding their effects on fish growth (Bell et al., 2003; Tocher, 2010). In particular, the lipid and fatty acid composition of broodstock diets have been shown to affect reproduction and larval survival in marine species such as Gilthead sea bream (*Sparus aurata*) (Fernandez-Palacios et al., 1995), European sea bass (*Dicentrarchus labrax*) (Bruce et al., 1999), Japanese flounder (*Paralichthys olvaceus*) (Furuita et al., 2002), crescent sweetlips (*Plectorynchus cinctus*) (Li et al., 2005), Chilean flounder (*Paralichthys adspersus*) (Wilson, 2009) and yellowfin sea bream (*Acanthopagrus latus*) (Zakeri et al, 2011). However, some studies have shown that excess n-3 HUFA can also have a negative impact on egg quality (Furita et al., 2002; Li et al., 2005). Most of the nutritional studies are on commercial fish, there are very few studies of ornamental fish, especially nemo fish.

The aquarium trade is a rapidly growing industry worldwide with marine ornamental fish sourced mainly from coral reefs. Marine ornamental fish is one of the potential fisheries products in the domestic and overseas markets because of its abundant and beautiful colors that attract the interest of aquarists and as a result, a large number of aquarium businesses developed. However, the ornamental fish trade is still dependent on catches from the wild. This causes considerable pressure on the ecosystem such as over-exploitation of natural populations, damage to coral reefs and environmental degradation. Therefore, it is necessary to develop marine ornamental fish cultivation to reduce pressure on the ecosystem. *Amphiprion* sp. is known as the most popular tropical marine ornamental fish with unique colouration and sequential hermaphroditic, social hierarchy and monogamous characteristics and the clownfish *Amphiprion ocellaris* has become one of most popular in the world. Nemo clownfish is a fish that reproduces continuously, with a short vitellogenic period, so a complete diet will shorten the time to re-maturing, increase the reproductive efficiency of the fish, improve the hatching rate of eggs and the rate of growth, the survival of larvae, thereby increasing seed production (Ha Le Thi Loc, 2010). However, there is few research on the nutritional requirements and effects of supplements on the reproductive quality of nemo fish. Therefore, this study was carried out to evaluate the effect of HUFA (A1DHA Selco) on egg and larval quality as well as hatching rate, survival rate and malformation rate of larvae.

2. Materials and methods

2.1. Experimental diets and feeding preparative

The HUFA used in this experiment was A1 DHA Selco with five diets: 0 g/kg feed (control), 50, 100, 150 and 200 g/kg feed (with fatty acid as 1.11; 1.72; 2.22; 2.72 and 3.26% were mixed with basic diet). Basic diet ingredients include 70% shrimp meat, 30%

mollusca, astaxanthin 150 mg/kg feed, vitamin E (α-tocopheryl acetate) 375 mg/kg feed. Food was stored at -40^{o}C and used within 1 month. The food was defrosted at room temperature and cut into small pieces for the fish to eat.

2.2. Experimental design

Broodstock prepare. Twenty pairs of pre-mature nemo fishes from artificial breeding at the Institute of Oceanography, Vietnam were selected and growth in a separated experimental tank for six months prior to feeding trials. The fish were fed by fresh shrimp and molluscs meat twice a day (at 8 h and 16 h), at a level of 5–10% of total biomass weight. Food waste in the tank were siphoned after an hour of feeding. Breeding tanks were cleaned daily. After six months, the fishes were matured and 15 pairs of the fishes (male length of 5.24 ± 0.44 cm; female length of 0.67 ± 0.73 cm) were selected for the feeding trial.

Experimental tank. There were 15 glass tanks and the pair of fish have been paired but have never participated in spawning, without anemone were stocked in 120 liters/tank with a clay pot as a substrate. The experiment was carried out in door with circulating filter system for 12 months.

Management. The waste in the tank will be siphoned after about an hour of feeding. The culture tank was cleaned daily and supplemented with fresh water to maintain salinity as well as evaporation throughout the experiment period.

2.3. Data collection and analyses

2.3.1. Reproductive performance parameters

Absolute fecundity (The number of eggs per spawning per female). The total number of eggs after fish lay within 4h were counted directly through a magnified image of the entire egg nest using the Canon PowerShot A2200HD 14.1 megapixels camera.

Spawning frequency = Number of times the fish spawn during the entire experimental period/month (number of spawning times/month).

Egg loss rate (%) = The total number of eggs before hatching × 100/number of eggs laid on the first day. The total number of eggs at the time before hatching will be counted directly by eye through a magnified image of the entire egg nest with using the Canon PowerShot A2200HD 14.1 megapixels camera

2.3.2. Evaluation of the quality of eggs

*Diameter of eggs***.** After 40-60 minutes of spawning, 5 eggs/nest were randomly taken using a pinwheel and were put into 1.5 mL Eppendorf tubes containing 4% formol fixation solution. The diameter of the eggs was measured by light microscopy using a calibrated ocular micrometer.

Hatching rate of eggs (%) = Number of newly hatched larvae/ The total number of eggs before hatching × 100%. The number of newly hatched larvae were determined indirectly

through the number of eggs at the time before hatching minus the number of remaining unhatched eggs. The number of remaining unhatched eggs were sinked eggs at the bottom tank and left unhatched eggs on the substrate.

2.3.3. Evaluation of the quality of larvae

Survival rate of 3 day posthatch larvae fish (%) = (number of fish hatched - number of dead fish) x 100/number of hatched fish. Every day, dead fish were siphoned on the entire bottom of the tank. The dead fish were counted for 3 days from the time the eggs hatch.

The rate of malformation of newly hatched fish larvae (%): After 12 h, all newly hatched fish larvae were dead, weak, lying on the bottom, or swimming close to the bottom, lethargic were siphoned. These samples were fixed in 4% formol and observed using light microscope. Malformation larvae were larvae with abnormal shapes (curved body, crooked body, short body and short mouth). Malformation rate of newly hatched larvae (%) = Number of malformed fish x100/total number of newly hatched fish.

2.3.4. Statistical analysis

The performances of reproduction, egg quality was statistically analysed by using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS 18 for Windows. Significant differences were based on the $P < 0.05$ level. The data are presented as mean ± SE.

3. Results

3.1. Spawning of broodstock in the experimental

The results showed that the maturation process in female fish started with the appearance of enlarged abdomen and expanded oviduct from the genital hole of female, then the parent pair of fish together cleaned the egg laying area, and spawned at 11 a.m-15.30 p.m and the eggs were cared by the male broodstock during incubation time. The colour of egg will changed from orange on the first day to silver eye on the seventh day, and the eggs hatched at 6.00-8.00 p.m on the eighth day.

3.2. Survival of broodstock during the experimental period

During the experiment, there were no fish died in the treatments. This result showed that the A1 DHA Selco supplemented in the diet did not affect the survival rate of nemo broodstock, this result again confirmed the feed used in the experiment (inherited from previous studies) were suitable for the growth and development of memo fish.

3.3. Reproductive efficiency of broodstock

The re-maturation time and spawning frequency of nemo broodfish were not affect by the Selco supplemented diet ($p>0.05$) (Table 1), but it had significantly positive effects on the fecundity and the egg loss rate.

Table 1. Effect of Selco on the productive efficiency (mean value ± SE).

Reproductive efficiency	Selco supplementation (g/kg feed)				
	0	50	100	150	200
Re-maturation and spawning (day/spawning)	13.41±0.198[a]	13.24±0.103[a]	12.99±0.214[a]	12.93±0.203[a]	13.20±0.201[a]
Spawning frequency (nests/month)	2.24±0.032[a]	2.27±0.018[a]	2.31±0.036[a]	2.32±0.038[a]	2.28±0.034[a]
Fecundity (eggs/nest)	430.00±2.605[a]	503.31±4.024[b]	512.08±4.901[b]	472.94±3.487[c]	441.81±5.359[a]
Egg loss rate (%)	14.82±0.158[a]	12.74±0.164[bc]	12.62±0.151[b]	13.50±0.248[c]	14.45±0,228[a]

Note: Letters after value on the same line showed significant difference (p<0.05).

The results showed that the number of eggs per spawning of fish in the treatment added with Selco at 50-150 g/kg feed was higher (472.94-503.31eggs) than not supplemented (430 eggs) or supplemented at 200 g/kg of feed (441.18 eggs). Although the number of eggs depends on the size of the female fish and the number of eggs may improve in the next spawning, the difference of the eggs number may be due to diet. This result is contrary to the study on seabream (*Acanthopagrus latus*), supplementing with HUFA with concentrations of 6.67; 4.26 and 2.92%, respectively, the reproductive performance of fish was significantly improved (p<0.05). Accordingly, the relative fecundity was highest in broodstock fed the 6.67% HUFA diet (1.642.477±1534 eggs), followed by the fish fed the supplemented diet 4.26%. HUFA (1.473,557±1846 eggs) and the lowest was 1.20.020±1073 eggs while the diet supplemented with 2.92% HUFA (Zakeri et al., 2011). However, the results in *Paralichthys olvaceus* showed that egg production was highest in fish fed the highest n-3 HUFA level (6.2%) but egg quality parameters, such as percentage of buoyant eggs, hatching rate and percentage of normal larvae were significantly higher in the group fed the lowest n-3 HUFA diet (2.1%) (Furuita et al., 2002), whereas in *Sparus aurata*, adding 1.6% had a significantly higher number of eggs than adding 1.3; 2.18; 3.15%, the results of this research also showed that fish fecundity was reduced and yolk sac hypertrophy appeared in the newly larvae hatched from the broodstock fed the highest HUFA (3.15%) (Fernandez-Palacios et al., 1995). Thus, the effectiveness of HUFA depends mainly on the fish species studied.

After 12 months of the experiment, the time of re-maturation and spawning and spawning frequency of nemo broodstock were not statistically significant different between treatments (p> 0.05). The fecundity of nemo fish broodstock fed diets containing different levels of Selco for 12 months (Fig. 1). The results showed that the number of eggs were increased in the diets with Selco supplemented at 50-150 g/kg of feed and there were differences between groups without Selco and 200 g/kg of feed and the highest number of eggs was 100 g/kg of feed added (p<0.05).

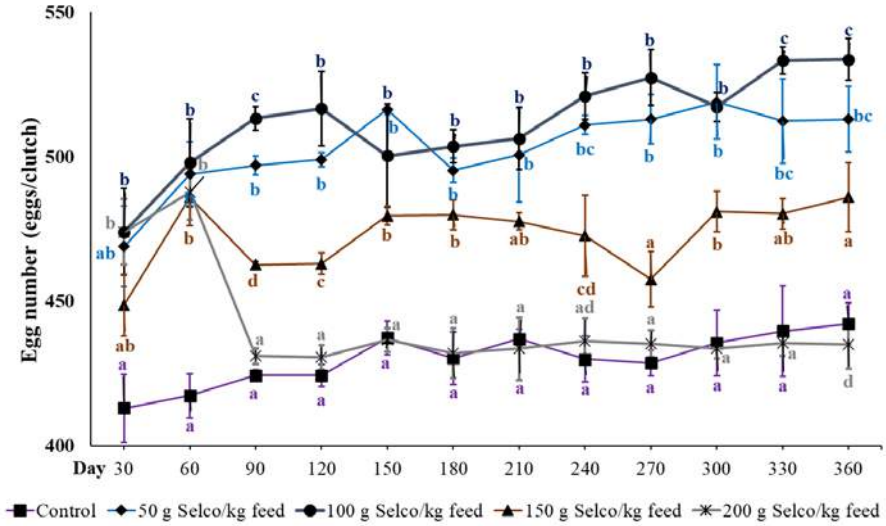

Figure 1. Effect of A1DHA Selco on fecundity.

Table 1 also shown that the addition of Selco to broodstock also improved the loss rate of nemo eggs ($p < 0.05$). The egg loss rate was significantly reduced in groups were fed Selco at 50-150 g/kg of feed (12.74-13.50%) compared with 14.82% in the control group and 14.45% in the diet supplemented Selco at 200 g/kg feed. However, statistical analysis of broodstock spawning data by month also showed that the supplementation of Selco reduced the percentage of eggs lost (Fig. 2). The egg loss rate of the Selco-supplemented treatments were lower than in the control (12.00-15.91 and 14.27-16.02%, respectively), in which the loss rate was lowest in the treatment supplemented with 100 g/kg of feed (12.00-13.89%), but there were no difference between treatments. Thus, this supplement has made an important contribution to improving the reproductive efficiency of nemo broodstock.

Hatching rate can be considered as an indicator to evaluate the egg quality of aquatic products. The hatching rate of nemo broodstock in the groups of fish supplemented with Selco were significantly different from that of the group without A1DHA Selco ($p<0.05$), in which the highest hatching rate was the supplemented diet of 100 g/kg of feed (Fig. 3). The effect of HUFA on hatching rate of eggs was also mentioned in research on flame angelfish (*Centropyge loriculus*), the broodstock supplemented with 3.6% n-3 HUFA had a higher hatching rate than other groups supplemented with 1.8%, 2.9% and markedly improved compared with the control (Callan et al., 2012). According to Zakeri et al. (2011), the study on seabream (*Acanthopagrus latus*) also showed that the diet supplemented with high HUFA concentration (6.67%) had a higher hatching rate (59.81±2.4%) than fish groups supplemented 4.26% and 2.92% (43.55±2.7% and 39.1±1.9%, respectively) ($p<0.05$).

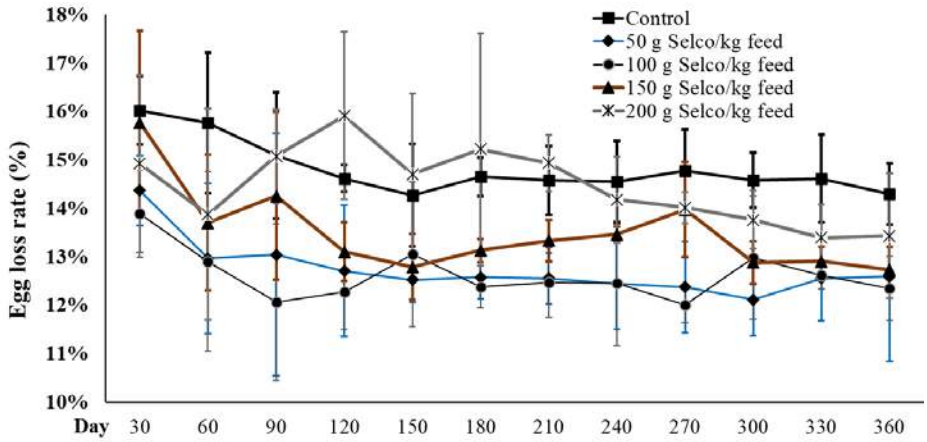

Figure 2. Effect of A1DHA Selco on egg loss rate.

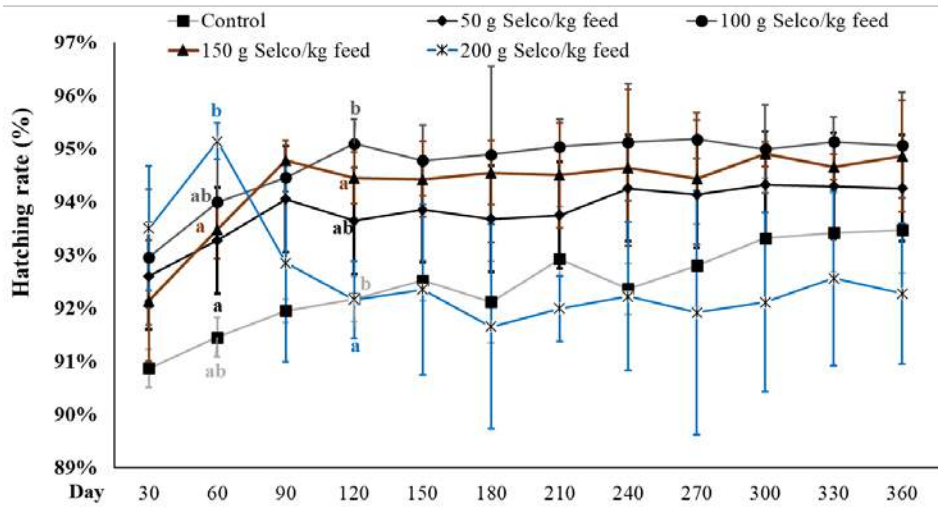

Figure 3. Hatching rate (mean ± SE) of nemo fish.

3.4. Effect of A1DHA Selco on egg and larval quality

Diets supplemented with Selco at different levels did not affect the eggs diameter and the size of the 3 days posthatch larvaes fish, but it did improve the hatching rate, the survival rate of 3 days posthatch larvae fish, and decreased malformation rate ($p<0.05$) (table 2). The diameter of nemo eggs was larger in the Selco 150 g/kg feed diet (2.49±0.081 mm) than in the other groups (1.97-2.41 mm), but no correlation was found between the egg diameter and dietary Selco levels ($p>0.05$). Similarly, HUFA supplementation in the broodstock diets had no positive effect on larvae size.

Table 2. Seed quality of nemo fish

Seed quality parameters	Selco supplementation (g/kg feed)				
	0	**50**	**100**	**150**	**200**
Egg length (mm)	1.97 ±0.486^{a}	2.38± 0.084^{a}	2.39 ±0.089^{a}	2.49± 0.081^{a}	2.41 ±0.054^{a}
Egg width (mm)	1.6 ±0.408^{a}	1.25 ±0.027^{a}	1.33 ±0.118^{a}	1.28 ±0.037^{a}	1.22 ±0.006^{a}
Larvae size (mm)	2.55 ±0.775^{a}	3.44 ±0.009^{a}	3.50 ±0.024^{a}	3.51 ±0.009^{a}	3.56 ±0.032^{a}
Hatching rate (%)	92.45 ±0.231^{a}	93.85 ±0.147^{b}	94.73 ±0.189^{c}	94.33 ±0.224^{bc}	92.57 ±0.272^{a}
Survival rate of larvae (%)	92.273±0.146^{ab}	92.728±0,208^{bd}	94.556±0.93^{c}	93.072±0.158^{d}	91.906±0.124^{a}
Larvae malformation rates (%)	0.72 ±0.036^{a}	0.60 ±0.025^{ab}	0.61 ±0.022^{ab}	0.55 ±0.022^{b}	0.96 ±0.048^{c}

Note: *Letters after value on the same line showed significant difference (p<0.05).*

The malformation rate of nemo fish in the experiment was significantly affected by the concentration of Selco supplement (Fig. 4). The results showed that the malformation rate of fish was lowest in the treatment supplemented with 150 g Selco (0.55%), followed by the treatment 50 and 100 g Selco (about 0.6%) and this rate was quite high in the control (0.72%) and the highest at Selco 200 g/kg feed (0.96%) ($p < 0.05$). However, monthly data analysis showed that the malformation rate of nemo fish did not differ much (Fig. 4). The study on seabream (*Acanthopagrus latus*) showed that the fertilization rate and the malformation rate of fish larvae were not significantly different between the concentrations of HUFA n-3 supplemented ($p > 0.05$) (Zakeri et al., 2011).

The survival rate of 3 days posthatch larvae was different between the experimental treatments ($p < 0.05$) (Fig. 5). The survival rate of 3 days posthatch larvae fish was highest in the treatment supplemented Selco with 100 g/kg feed (94.55%), followed by the fish groups supplemented with 50 and 150 g Selco/kg feed (92.72 and 93.07%, respectively) and the lowest in the group supplemented with 200 g Selco/kg (91.90%).

According to the results of this research, it was shown that the optimal HUFA concentration requirement for nemo broodstock to improve reproductive performance was 100 g/kg of feed (equivalent to fatty acids of 2.22%). The concentration of HUFA supplemented for broodstock in this study may be different from other studies, this difference may be influenced by species, experimental conditions.

Nutrition study (n-3 HUFA) for European seabream broodstock showed that egg quality, hatching rate and survival rate of 3 days posthatch larvae of fish were improved with increasing n-3 HUFA concentration 1.6%. Two nutritional studies of nemo broodstock also showed that diets supplemented with vitamin E and astaxanthin also improved hatching, survival and malformed rate of lavae (Dao et al., 2018; Nguyen et al., 2020). Specifically, the optimal vitamin E concentration supplemented to broodstock nemo showed the highest hatching rate, survival rate and lowest malformation rate (89.24%; 94.48%; 0.67%, respectively) (Dao et al., 2018). Similarly, 150 mg/kg feed of Astaxanthin

supplementation also improved survival, hatching and malformed rate of lavae (92.14%; 93.57% and 0.55%, respectively) (Nguyen et al., 2020).

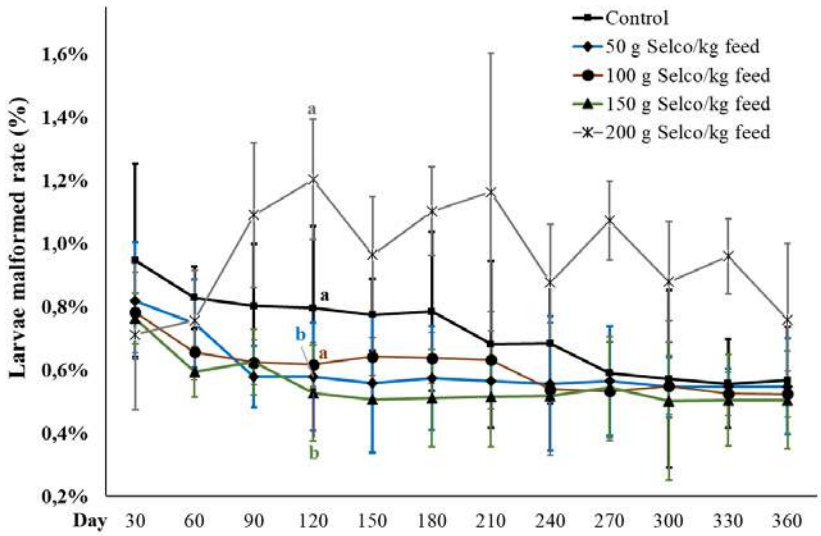

Figure 4. Effect of A1DHA Selco on larvae malformed rate.

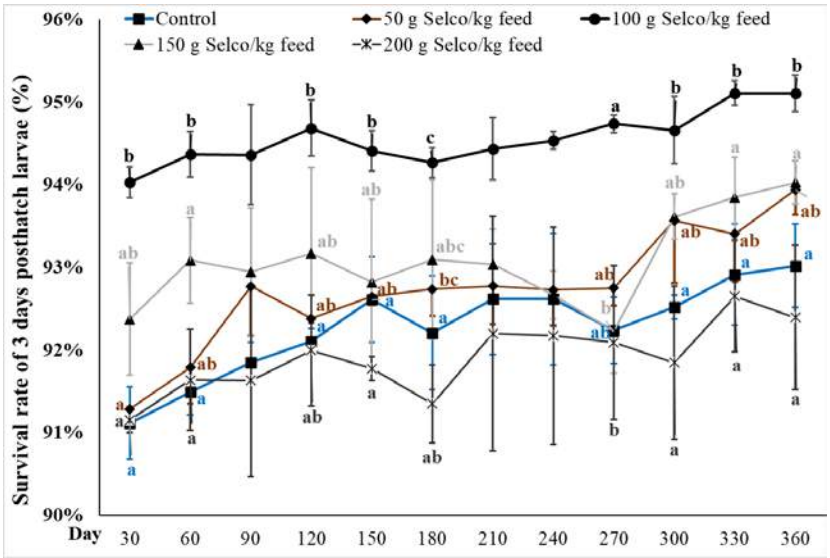

Figure 5. Effect of Selco on survival rate of 3 days posthatch larvae.

Conclusions

The addition of different levels of Selco to the nemo broodstock feed before and during spawning period did not affect the re-maturation time, egg diameter and size of the broodstock nemo but improved significantly absolute fecundity, hatching rate, malformation rate and survival rate of 3 days posthatch larvae fish.

The present study showed that the suitable fatty acid concentration of A1DHA Selco supplemented with 100 g/kg feed for nemo broodstock significantly affected reproductive performance and egg quality.

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ẢNH HƯỞNG CỦA CHẾ ĐỘ ĂN BỔ SUNG SELCO ĐẾN HIỆU QUẢ SINH SẢN, CHẤT LƯỢNG TRỨNG VÀ ẤU TRÙNG CỦA CÁ KHOANG CỔ NEMO *AMPHIRION OCELLARIS* (CUVIER, 1830)

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Tóm tắt. Dinh dưỡng cho cá bố mẹ là một trong những lĩnh vực quan trọng trong nuôi trồng thủy sản. Nghiên cứu được thực hiện để tìm ra ảnh hưởng của việc bổ sung HUFA (sản phẩm thương mại A1 DHA Selco gọi tắt Selco, Inve) đến hiệu quả sinh sản, chất lượng trứng và ấu trùng của cá khoang cổ nemo *Amphiprion ocellaris* (Cuvier, 1830). Thí nghiệm được bố trí 5 nghiệm thức với 5 chế độ ăn có bổ sung Selco vào thức ăn cá bố mẹ lần lượt là 0 g, 50 g, 100 g, 150 g và 200 g Selco/kg thức ăn. Mỗi nghiệm thức có ba lần lặp, thời gian thí nghiệm là 12 tháng. Kết quả thí nghiệm cho thấy tần suất sinh sản, thời gian tái thành thục sinh dục, kích thước trứng và ấu trùng không bị ảnh hưởng của việc bổ sung Selco vào các chế độ ăn cá bố mẹ ($p>0,05$). Bổ sung Selco 50 và 100 g/kg thức ăn có tác động tích cực đến sức sinh sản tuyệt đối, tỷ lệ hao hụt trứng ($p<0,05$). Tỷ lệ nở và tỷ lệ sống của ấu trùng 3 ngày tuổi ở nghiệm thức bổ sung Selco 100g/kg thức ăn là cao nhất và sai khác có ý nghĩa (lần lượt là 94,73±0,19% và 94,56±0,93%) ($p<0,05$). Tỷ lệ dị hình của ấu trùng cá ở nghiệm thức bổ sung Selco 150g/kg thức ăn là thấp nhất (0,55 ±0,022%) nhưng không có sai khác với nghiệm thức bổ sung 50 và 100 g Selco/kg thức ăn. Nghiên cứu đã chỉ ra việc bổ sung Selco ở mức 100 g/kg thức ăn vào chế độ ăn cho cá bố mẹ có thể nâng cao hiệu quả sinh sản và chất lượng ấu trùng của cá khoang cổ nemo.

Từ khóa: *Amphiprion ocellaris*, cá khoang cổ nemo, DHA Selco, HUFA