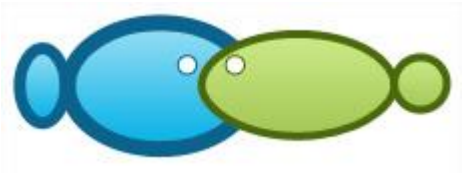

Improvement of reproductive performance of clown anemonefish, *Amphirion ocellaris* (Cuvier, 1830), with vitamin C

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Abstract. An enhancement in broodstock nutrition can significantly influence the reproductive success of fish species. This study investigated the impact of vitamin C supplementation in broodfish diets on the reproductive performance, egg quality, and larvae parameters of clown anemonefish (*Amphiprion ocellaris*). Five diet treatments with varying levels of vitamin C in form of L-Ascorbyl-2-Polyphosphate (0.0, 200, 400, 600, and 800 mg kg^{-1} diet) were implemented over a 12-month period. Results indicated positive effects of vitamin C supplementation on fecundity, egg quality, hatching rate, larvae survival, and malformation rate ($p<0.05$). The treatment with 600 mg kg^{-1} diet exhibited the highest hatching rate (94.61%), lowest egg loss rate (12.86%), and lowest malformation rate (0.519%). Egg diameter and larval size did not significantly differ among treatments ($p>0.05$). Although vitamin C supplementation reduced re-maturation time and increased spawning frequency, the differences were not statistically significant ($p>0.05$). Overall, a dietary supplementation of 600 mg kg^{-1} vitamin C is recommended to improve reproductive performance in clownfish broodstock.
Key Words: larvae of clownfish, malformed rate, reproductive quality, survival rate, vitamin C.

Introduction. Marine ornamental fish have witnessed a surge in global popularity, captivating enthusiasts with their vibrant colors and unique behaviors. Among these captivating species, the clown anemonefish (*Amphiprion ocellaris* Cuvier, 1830) stands out as one of the most beloved and sought-after marine inhabitants. With its striking appearance and endearing charm, *A. ocellaris* has garnered widespread admiration among hobbyists and aquarists worldwide. Efforts to cultivate *A. ocellaris* through seed production and growth culture have been done (Anil et al 2010; Kumar & Balasubramanian 2009; Loc et al 2011; Raheem et al 2021). However, despite these advancements, challenges persist in achieving optimal reproductive efficiency (Loc et al 2011) with low reproductive efficiency and unsatisfied quantity and quality. Recognizing the pivotal role of broodstock nutrition in enhancing reproductive outcomes, efforts are underway to address this critical aspect. Broodstock nutrition holds significant importance in both freshwater and marine aquaculture settings (Varghese et al 2009). The dietary composition provided to broodstock exerts direct influence on crucial parameters such as fertilization rates, egg quality, embryo development, and larvae quality. Insights from studies (Bromage 1998; Izquierdo et al 2001) further highlight the profound impact of broodstock diet on the overall success of fish hatcheries.

In fish species characterized by short re-maturation and spawning periods, the optimal development of gonads and reproductive efficiency hinges upon the provision of a nutrient-rich diet immediately before and post-spawning (Izquierdo et al 2001). Consequently, there is a pressing need to enhance both seed quality and the efficacy of seed production hatcheries through the formulation of short-term or long-term nutritional

strategies tailored to broodstock. During the early larval stage of fish, the availability of endogenous nutrients within the yolk sac is crucial for sustenance. These nutrients, in turn, can be influenced by the dietary intake of broodstock. Vitamin C (VC) emerges as a vital nutritional component essential for normal growth and physiological functions in fish. However, it is noteworthy that many fish species may lack the ability to synthesize VC internally due to the absence of the enzyme L-gulonolactone oxidase or ascorbate (Chatterjee 1973; Ching et al 2015). Therefore, the supplementation of VC through dietary sources becomes imperative to facilitate the optimal growth and development of fish.

The VC, in its water-soluble form such as ascorbic acid (AA), serves as a crucial antioxidant in fish physiology, mitigating stress factors that fish may encounter (Ortuño et al 2003). Its involvement spans various metabolic processes, encompassing collagen synthesis for tissue repair, protection of cell membranes, facilitation of metal absorption, and detoxification of xenobiotics (Adeyemi-Doro & Iyiola 2018; Dabrowski & Ciereszko 2001). Moreover, VC emerges as an indispensable micronutrient within the reproductive cycle of fish (Sandnes & Braekkan 1981). Elevated levels of VC within steroid-producing tissues, notably the ovaries and adrenal glands, suggest its potential involvement in steroid hormone formation, potentially acting as a cofactor or regulator in estrogen synthesis within follicular cells (Levine & Morita 1985; Tolbert et al 1975). The influence of VC extends to hormone synthesis, spermatogenesis, and the quality of oocytes and gametophytes (Waagbø et al 2000). Reports indicate that AA supplementation positively impacts reproductive performance in fish species, as evidenced by improvements in reproductive organ indices, antioxidant capacity of gametes, prevention of DNA damage, and hormonal regulation during crucial periods such as re-maturation and spawning (Dabrowski & Ciereszko 2001). During ovarian development, an increase in AA concentration has been observed within oocytes and ovaries across various fish species (Halver et al 1975; Hilton et al 1979; Sandnes & Braekkan 1981). Thus, dietary supplementation of AA emerges as a pivotal factor in enhancing fish reproductive performance during adulthood and throughout the spawning cycle across diverse fish species (Valdebenito et al 2015).

The requirement for AA varies depending on the species of fish and their developmental stage. Studies have indicated specific AA requirements for broodstock of various fish species, such as rainbow trout *Salmo gairdneri* (Sandnes et al 1984), Atlantic cod *Gadus morhua* (Eskelinen 1989; Mangor-Jensen et al 1994), rainbow trout *Oncorhynchus mykiss* (Blom & Dabrowski 1995), channel catfish *Ictalurus punctatus* (Chatakondi et al 2010). These AA levels range approximately around 357 mg AA kg^{-1} feed. It's noted that the AA requirement for adult fish tends to be significantly higher than for juveniles (Blom & Dabrowski 1995). For instance, a concentration of 1250 mg kg^{-1} feed of AA has been shown to enhance the reproductive performance of tilapia (*Oreochromis mossambicus*) (Soliman et al 1986). VC dietary supplementation has been demonstrated to impact the spawning and reproductive performance of fish in various studies (Gao et al 2014; Nguyen et al 2012; Sinjal 2010). AA supplementation in the diet of tilapia (*Clarias gariepinus*) has been found to increase gonadal maturity and accelerate oocyte production (Sinjal 2010). Similarly, supplementation of 150 mg AA kg^{-1} feed in the diet of tilapia (*Oreochromis niloticus*) has been associated with an improved gonadosomatic index (GSI) (Martins et al 2016). Transfer of AA from broodstock to fish eggs, embryos, and larvae has shown to enhance larval quality and reduce mortality and deformities in fingerlings. Blom & Dabrowski (1995) reported that salmon (*Oncorhynchus mykiss*) which was fed a high AA concentration could significantly improve the survival rate of larval. The absence of AA in diets can lead to a decrease in available VC in the ovary, resulting in reduced egg numbers, hatchability rates, increased rates of larvae malformations, and mortality (Fracalossi et al 1998; Navarro et al 2009). Despite this wealth of knowledge, there remains a dearth of evidence concerning the effects of VC dietary supplementation on *A. ocellaris* broodstock's reproductive performance, egg quality, and larvae.

Therefore, this paper aims to fill this gap by investigating the effects of VC on *A. ocellaris* broodstock's reproductive performance using indicators such as egg loss rate, hatching rate, survival rate, and malformed rate of larvae. The findings from this

research may provide valuable insights into the potential supplementation of VC in *A. ocellaris* broodstock feed, thereby enhancing the efficacy of reproductive protocols.

Material and Method

Experimental diets and feeding practices. Five experimental diets were formulated with varying levels of VC supplementation, ranging from 0 mg kg^{-1} to 800 mg kg^{-1}. The VC utilized was L-Ascorbyl-2-Polyphosphate (L-2APP or AA) in powdered form. The composition of these diets is detailed in Table 1. The AA concentrations in the diets containing 200 mg kg^{-1}, 400 mg kg^{-1}, 600 mg kg^{-1}, and 800 mg kg^{-1} were analyzed using reverse-phase high-performance liquid chromatography (HPLC, HP 1100, USA). The results revealed AA concentrations of 195.67 mg kg^{-1}, 391.33 mg kg^{-1}, 587 mg kg^{-1}, and 782.67 mg kg^{-1}, respectively, in the corresponding feeds.

Table 1

Ingredient of experimental diets for *Amphiprion ocellaris*

Ingredient	*Treatment diets (mg VC kg^{-1} feed)*				
	0	*200*	*400*	*600*	*800*
Shrimp meat (%)	70	70	70	70	70
Molluscs (%)	30	30	30	30	30
L-Ascorbyl−2-polyphosphate (mg kg^{-1})	0	200	400	600	800
α-tocopheryl acetate (mg kg^{-1})	375	375	375	375	375
Astaxanthin (mg kg^{-1})	150	150	150	150	150

All ingredients, excluding VC, were thoroughly mixed using a blender. The VC was dissolved in water and subsequently added to the prepared diets, followed by another round of mixing to ensure uniform distribution. The experimental diets were then stored at -30°C and utilized within one month to maintain freshness and nutritional integrity. During feeding sessions, the frozen food was thawed at room temperature and then broken into small pieces. These pieces were further cut into smaller portions using a spoon, facilitating easy consumption by the fish. This feeding method ensured that the fish could readily ingest the diet and derive maximum nutritional benefit from the supplemented the VC.

Experimental design

Experimental tank. The experiment was conducted over a duration of 12 months in experimental tanks with a volume of 120 L each (Figure 1).

Figure 1. The experiment system for *Amphiprion ocellaris* treatment (a), with a detailed tank #25 (b) and couple *Amphiprion ocellaris* in the tank (c).

These tanks were equipped with a biological filter system comprising a volume of 70 L, which included a clay pot serving as substrate for the aquatic environment. This setup ensured adequate filtration and maintenance of water quality throughout the experimental period, providing a suitable habitat for the *A. ocellaris* broodstock.

Broodstock prepare. Twenty pairs of pre-mature *A. ocellaris*, each measuring approximately 5 cm in total length (TL), were sourced from artificial breeding efforts conducted at the Institute of Oceanography – Vietnam. Prior to the initiation of the feeding trial, these fish were acclimated and grown in separate experimental tanks for a duration of six months. During this period, the fish were fed a diet consisting of fresh shrimp meat twice daily, with feeding levels set at 5–10% of the total biomass weight. To maintain water quality, any food waste remaining in the tank after an hour of feeding was promptly siphoned out. Additionally, the breeding tanks underwent daily cleaning procedures to ensure optimal environmental conditions for the fish. Following the six-month culture period, the fish reached maturity, and 15 pairs were selected for participation in the feeding trial. The selected male fish exhibited a mean length of 5.29±0.38 cm, while the female counterparts measured approximately 7.87±0.65 cm in length. These matured pairs were used to assess the effects of dietary supplementation on the reproductive performance of nemo fish broodstock.

Feeding trial. Each experimental diet was administered to 3 pairs of nemo broodstock, ensuring that each treatment was replicated in triplicate. The feeding regime consisted of twice-daily feedings at 8 a.m. and 4 p.m. Following each feeding session, the tanks were siphoned to remove any excess food or waste, maintaining optimal water quality. Environmental parameters were meticulously monitored, with measurements taken daily at 2 p.m. Temperature readings were obtained using a mercury thermometer with an accuracy of 1°C, while pH levels were assessed using a test kit (JBL). Salinity levels were determined using a refractometer with an accuracy of 1‰. Additionally, the concentrations of inorganic nutrients (NH_3/NH_4^+ and NO_3^-) were analyzed every two weeks following the method of Rice et al (2017).

Data collection and analyses

Reproductive performance parameters. Absolute fecundity (the number of eggs per spawning per female): The total number of eggs after fish lay within 4h was counted directly through a magnified image of the entire egg nest with the Canon PowerShot A2200HD 14.1 megapixels camera. Spawning frequency (SF) and egg loss rate (ELR - %) were calculated using the following formulae:

$$SF = \frac{N_{FS}}{t}$$

Where:
N_{FS} - the number of fish spawning during the experimental period;
t - the experimental period in month.

$$ELR\ (\%) = \frac{NE_t}{NE_{D1}} \times 100$$

Where:
NE_t - the number of eggs left after incubation;
NE_{D1} - the number of eggs laid on the first day.

Egg quality *parameters*

Diameter of eggs.

After 40-60 minutes of spawning, every five eggs per 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 determined using a microscopic ruler. The eggs hatching rate (HR - %) was calcultaed using the following formula:

$$HR\ (\%) = \frac{NF_H}{NE_H} \times 100$$

Where:
NF_H - the number of fish hatched;
NE_H - number of eggs transferred to the hatchery.

Before hatching, the eggs were transferred to a separate tank. The total number of eggs remaining before the transfer was determined from the percentage of eggs loss rate. The eggs which did not hatch and sinked to the bottom were collected by siphoning the bottom of the tank. Any other unhatched eggs left on the nest were counted visually on the substrate.

Larvae quality parameters
Survival rate of 3-day post-hatch larvae fish (SR_3) was calculated using the following formula:

$$SR_3\ (\%) = \frac{NF_H - NF_D}{NF_H} \times 100$$

Where:
NF_H - number of fish hatched;
NF_D - number of dead fish within 3 day from the time the eggs hatched.

The rate of malformation of newly hatched fish larvae (NMR - %) was measured by the following method: After 12 h, all newly hatched fish larvae which were dead, weak, lying on the bottom, swimming close to the bottom or lethargic were siphoned. These samples were fixed in 4% formol and observed on a microscope. Malformation larvae were larvae with abnormal shapes (curved body, crooked body, short body and short mouth).

$$NMR\ (\%) = \frac{NF_M}{NF_{NH}} \times 100$$

Where:
NF_M - the number of malformed fish;
NF_{NH} - the total number of newly hatched fish.

Statistical analysis. The values of the means were statistically compared by one-way ANOVA. Data were processed in SPSS software for Windows (version 26). The data were presented mainly as mean±Standard Error (SE).

Results

Survival rate of broodstock during the experiment. Throughout the experiment period, no mortality among the nemo broodstock was observed. This indicates that the supplementation of VC in the diet did not exert any adverse effects on the survival rate of the broodstock. Moreover, it underscores the suitability of the VC supplementation for supporting the growth and development of nemo fish. These findings suggest that VC can be incorporated into the diet regimen for nemo fish broodstock without compromising their overall health and well-being.

Reproductive performance of nemo broodstocks fed different VC supplemented diets. The re-maturation time and spawning frequency of the nemo broodfish remained unaffected by the diet supplemented with VC ($p>0.05$). However, noteworthy positive effects were observed on fecundity and egg loss rate as a result of the VC supplementation (Table 2). These findings suggest that while VC supplementation may not influence the timing and frequency of re-maturation and spawning events, it plays a beneficial role in enhancing fecundity and reducing egg loss, contributing to improved reproductive outcomes in nemo broodfish.

Table 2
Reproductive efficiency of *Amphiprion ocellaris* broodstock fed with different experimental diets during 12 months experimental period.

Reproductive efficiency	*Vitamin C supplementation (mg VC kg^{-1} feed)*				
	0	*200*	*400*	*600*	*800*
Re-maturation and spawning (day spawning^{-1})	13.57±0.08^{a}	13.41±0.07^{a}	13.32±0.07^{a}	13.25±0.09^{a}	13.52±0.05^{a}
Spawning frequency (nests month^{-1})	2.21±0.01^{a}	2.24±0.01^{a}	2.26±0.01^{a}	2.26±0.02^{a}	2.22±0.01^{a}
Fecundity (eggs nest^{-1})	421.25±0.85^{a}	451.08±3.83^{b}	477.89±6.64^{c}	499.69±9.29^{c}	466.64±5.66^{bc}
Egg loss rate (%)	15.12±0.10^{a}	14.24±0.20^{b}	13.52±0.20^{b}	12.86±0.41^{b}	13.69±0.28^{b}

Value: mean value±SEM. Letters on the same line showed significant difference ($p<0.05$).

After 12 months of experimentation, the re-maturation time, spawning time, and spawning frequency of nemo broodstock did not exhibit significant differences across the experimental treatments ($p>0.05$). The re-maturation time ranged from 13.25 to 13.57 days per spawning event, while the spawning frequency ranged between 2.21 and 2.26 times per month. However, notable differences were observed in fecundity and egg loss rate among the experimental groups. The absolute fecundity of nemo fish in the VC-supplemented treatments ranged from 451.08±3.83 to 499.69±9.29 eggs nest^{-1}, significantly higher than the control treatment's average of 421.25±0.85 eggs nest^{-1}. Furthermore, the egg loss rate in the VC-supplemented treatments ranged from 12.86±0.41 to 14.24±0.20%, significantly lower than the control treatment's average of 15.12±0.10% ($p<0.05$). The treatment supplemented with 600 mg VC kg^{-1} feed demonstrated the most favorable reproductive efficiency, although no significant differences were observed between this treatment and the others ($p>0.05$).

In the VC-supplemented treatment, the egg count of nemo broodstock was significantly higher compared to the control treatment during the fourth month of the experiment ($p<0.05$). Across all treatments, the number of eggs exhibited an upward trend with increasing levels of VC supplementation. However, it's noteworthy that supplementation with 800 mg kg^{-1} feed of VC led to a reduction in absolute fecundity from the sixth month onwards. Among the different VC supplementation levels, the optimal absolute fecundity was observed with the supplementation of 600 mg VC kg^{-1} feed in the diet. This level resulted in absolute fecundity ranging from 439.3 to 533.7 eggs per clutch. Interestingly, there was no significant difference in absolute fecundity between diets supplemented with 400 and 600 mg VC kg^{-1} feed ($p>0.05$), indicating that both levels of supplementation were equally effective in promoting fecundity (Figure 2).

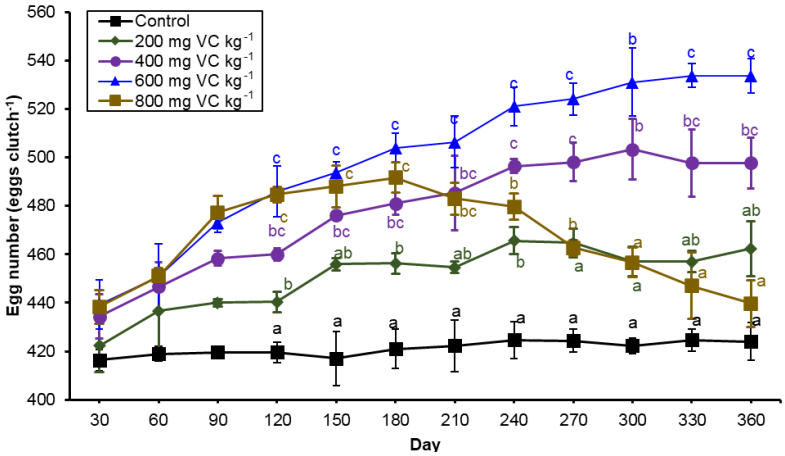

Figure 2. Fecundity (mean±SE) of *Amphiprion ocellaris* broodstock fed diets containing different levels of vitamin C for 12 months. Letter a, b, c showed significant difference in the same day ($p<0.05$).

The egg loss rate showed an improvement in diets supplemented with VC; however, over the course of the experiment, no significant differences were observed between the treatments, including those with or without VC supplementation ($p>0.05$). Nevertheless, it's noteworthy that the diet supplemented with 600 mg kg^{-1} feed of VC exhibited the lowest egg loss rate, while the control treatment showed the highest (Figure 3). Despite these trends, statistical analysis did not reveal significant disparities among the treatments regarding egg loss rate over time.

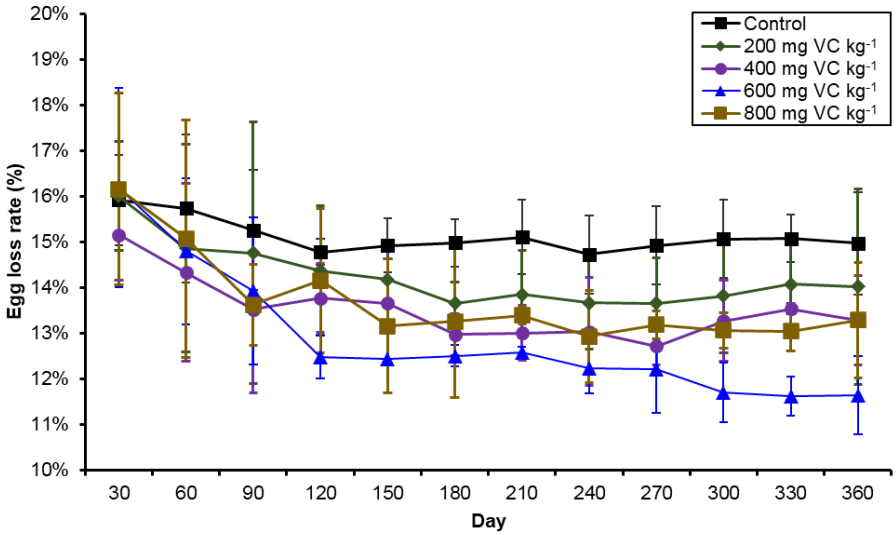

Figure 3. Egg loss rate (mean±SE) of *Amphiprion ocellaris* for 12 months. There was no difference within treatments ($p>0.05$).

Egg and larvae quality of A. ocellaris broodstocks fed different VC supplemented diets. Table 3 illustrates that supplementation of feed with VC at various levels did not exert significant effects on egg diameter or the size of 3-day post-hatch larvae fish. However, it notably improved the hatching rate, survival rate of 3-day post-hatch larvae fish, and reduced malformation rate ($p<0.05$).

Table 3

Seed quality of *Amphiprion ocellaris* fed diets containing different levels of vitamin C for 12 months

Seed quality parameters		*The VC supplementation (mg C kg^{-1} feed)*				
		0	*200*	*400*	*600*	*800*
Egg size (mm)	Width	1.20±0.50^{a}	1.28±0.03^{a}	1.36±0.13^{a}	1.31±0.04^{a}	1.24±0.01^{a}
	Length	2.44±0.03 ^{a}	2.45±0.08^{a}	2.52±0.09^{a}	2.61±0.08^{a}	2.44±0.05^{a}
Larvae size (mm)		3.34±0.02^{a}	3.35±0.02^{a}	3.36±0.03^{a}	3.37±0.03^{a}	3.30±0.03^{a}
Hatching rate (%)		92.27±0.20^{a}	93.03±0.18^{a}	94.28±0.24^{b}	94.61±0.32^{b}	93.08±0.18^{a}
Survival rate of 3 days old larvae (%)		91.47±0.128^{a}	91.83±0.184^{a}	92.62±0.186^{bc}	93.89±0.183^{b}	93.01±0.165^{c}
Larvae malformation rates (%)		0.74±0.033^{a}	0.68±0.029^{a}	0.65±0.029^{a}	0.52±0.031^{b}	0.88±0.033^{c}

Value: mean value±SEM. Letters on the same line showed significant difference ($p<0.05$)

Specifically, hatching and larval survival rates were significantly higher in treatments supplemented with 400-600 mg VC kg^{-1} feed ($p<0.05$). The highest hatching rate, larval survival rate, and lowest malformation rate were observed in treatments with VC at 600

mg kg^{-1} feed. Nevertheless, these rates did not exhibit significant differences compared to other treatments over time ($p>0.05$) (Figures 4 and 5).

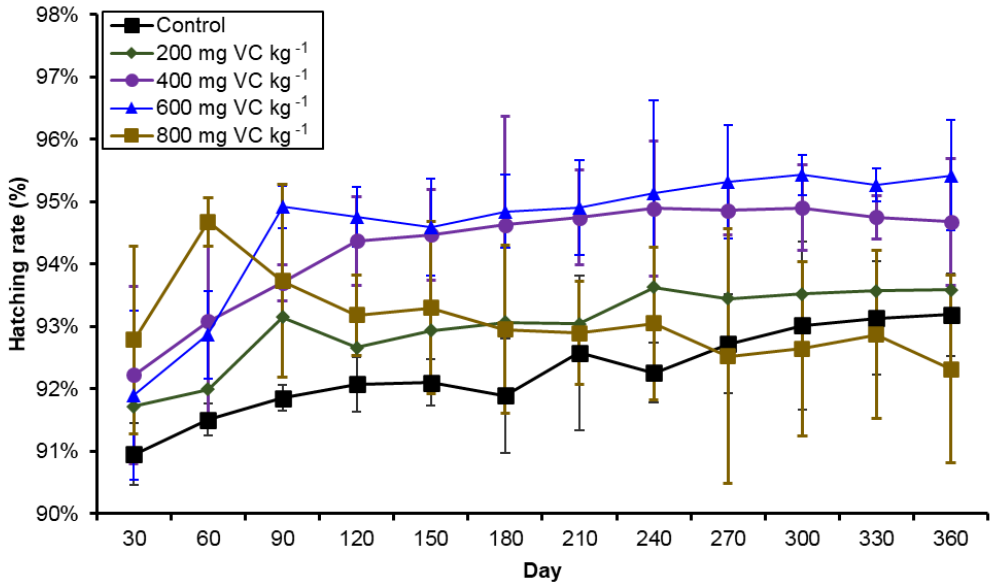

Figure 4. Hatching rate (mean±SE) of *Amphiprion ocellaris*. There was no difference within treatments ($p>0.05$).

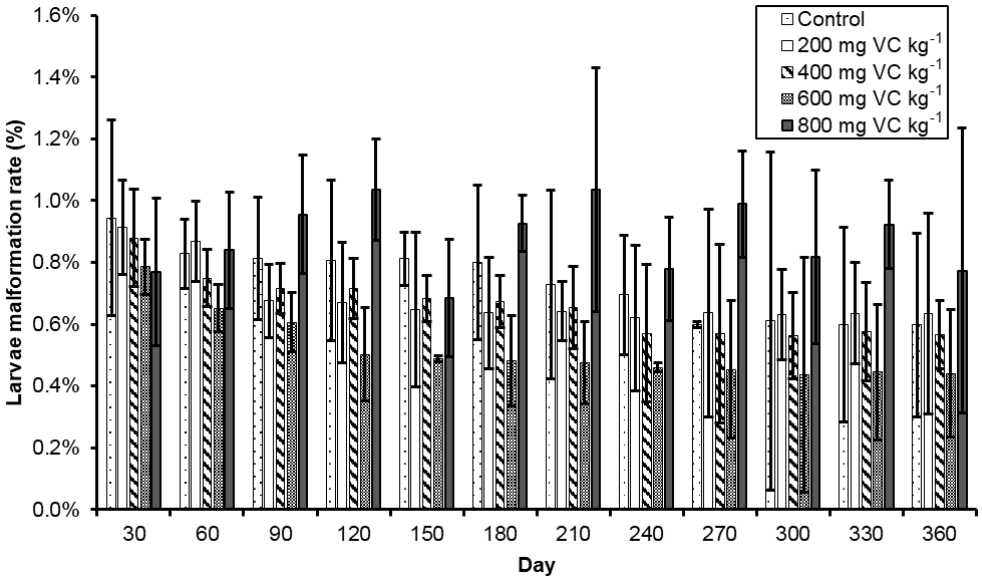

Figure 5. Larvae malformation rate (mean±SE) of *Amphiprion ocellaris*. There was no difference within treatments ($p>0.05$).

Furthermore, the survival rate of 3-day post-hatch larvae fish showed significant differences between groups with and without VC supplementation in the feed ($p<0.05$) during the periods of 1-60 days and 300-360 days. Notably, the treatment with 600 mg kg^{-1} feed demonstrated the highest survival rate among larvae within the period of 120-360 days (Figure 6).

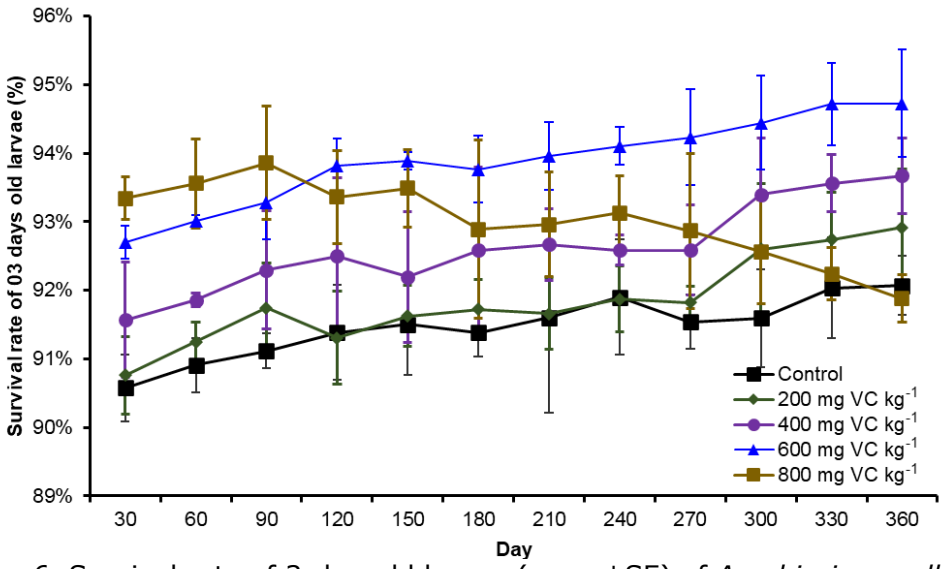

Figure 6. Survival rate of 3-day-old larvae (mean±SE) of *Amphiprion ocellaris*.

Discussion. The experiment was conducted in a controlled environment within a room equipped with air conditioning, maintaining a consistent temperature range of 27–28°C throughout the culture period. Consequently, the fluctuations in temperature remained negligible across all treatments. Over the experimental duration, water quality parameters were carefully monitored, ensuring optimal conditions for the incubation of eggs (Hoff et al 1996). Specifically, the water environment maintained a salinity range of 33-35‰, pH levels within the range of 7.8–8.3, water temperature consistently at 27–28°C, dissolved oxygen concentration ranging from 4.4 to 5.6 mg O_2 L^{-1}, and NH_3/NH_4^+ concentration consistently below 0.01 mg N L^{-1}. These stringent measures ensured that the water quality remained conducive for the development and hatching of eggs throughout the experimental period. *A. ocellaris* have the potential to spawn throughout the year in captivity, with their fecundity typically increasing during the spring and summer months (Hoff et al 1996). This highlights the importance of dietary enrichment for broodstock, as it can enhance both egg and sperm quality, thereby improving the overall reproductive performance of the fish (Izquierdo et al 2001). Given these considerations, our experiments conducted in a controlled environment with stable room temperature conditions enabled the broodstocks to breed at the expected times. This consistency in breeding patterns underscores the efficacy of dietary supplementation in facilitating optimal reproductive outcomes for nemo fish.

The experimental findings from the current study reveal that dietary supplementation with VC at various levels in the broodstock diet over a 12-month period (Table 2) had no significant effect on the time to re-maturation and spawning frequency ($p>0.05$). However, VC supplementation exhibited a significantly positive impact on absolute fecundity and egg loss rate. Additionally, supplementation with VC did not influence egg size or the size of 3-day post-hatch larvae fish, but it led to improvements in hatching rate, survival rate of 3-day post-hatch larvae fish, and decreased malformation rate. These findings are consistent with various studies investigating the effects of VC on the broodstock of numerous marine fish species (Sandnes et al 1984; Soliman et al 1986; Eskelinen 1989; Terova et al 1998; Blom & Dabrowski 1995; Dabrowski & Ciereszko 2001; Furuita et al 2009b; Mehrad et al 2011). Furthermore, previous research has reported positive impacts of VC supplementation on the reproductive performance of fish (Blom & Dabrowski 1995; Dabrowski & Ciereszko 2001; Valdebenito et al 2015). However, our study, conducted over a 12-month period, did not find significant effects of VC supplementation on fish reproduction, as indicated by insignificant differences in re-maturation and spawning periods, spawning frequency, egg diameter, and larvae size. This finding aligns with similar observations reported in the literature (Blom & Dabrowski 1995; Lavens et al 1999; Mehrad et al 2011; Terova et al 1998). Specifically, Blom & Dabrowski (1995) found that egg size was insignificantly

affected by VC supplementation in the diet for rainbow trout fish (*Oncorhynchus mykiss*). Additionally, VC supplementation in the broodstock diet for sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) did not positively impact egg size, fertilization rate, reproduction rate, and hatching rate (Terova et al 1998). This evidence was also reported for milkfish brood (*Chanos chanos*) supplemented with 0.1% VC or 0.05% Vitamin E (VE) over a 3-year observation period (Emata et al 2000). However, previous studies have confirmed that VC supplementation in the diet improved fish reproduction (Omoniyi & Ovie 2018; Sandnes 1991; Setyaningrum et al 2017).

In our study, observations revealed that diets supplemented with VC over a 12-month culture of broodstock nemo fish significantly and positively affected absolute fecundity and egg loss rate ($p<0.05$). These findings align with previous observations in rainbow trout fish, where supplementation with ascorbic acid (AA) for broodstock fish cultured over 10 months resulted in increases in both the number and total mass of eggs produced (Blom & Dabrowski 1995). For instance, the highest number of eggs (3729±613 eggs fish^{-1}) was recorded in the treatment with a diet supplemented with 870 mg AA kg^{-1} feed, whereas the lowest (2288±426 eggs fish^{-1}) was observed in the control diet without VC supplementation (Blom & Dabrowski 1995). Similarly, a diet supplemented with 1000 mg AA kg^{-1} feed, fed to zebrafish (*Danio rerio*) for 20 weeks, led to a significant increase in reproduction (Mehrad et al 2011). Additionally, Dabrowski & Ciereszko (2001) reported increased fecundity and survival rates with the supplementation of AA in the diet for cultured rainbow trout. Moreover, the use of VC-supplemented diet during the rearing period of brood platy fish (*Xiphophorus maculatus*) for 20 weeks resulted in significantly higher Gonadosomatic Index (GSI), fertility, and fry number in groups supplemented with 1600 mg VC kg^{-1} feed compared to the control group and other experimental groups (Mahsa & Allah 2011). However, it is noteworthy that Mangor-Jensen (1994) indicated that dietary VC had an insignificant effect on total egg production in codfish. Moreover, the VC stored in the female gonads of brood fish can be transferred to and stored in eggs, providing essential support for larval growth and development until the initiation of independent feeding (Falahatkar et al 2011; Furuita et al 2009a; Soliman et al 1986). VC deposition and contribution occur during vitellogenesis and embryogenesis, where it plays a crucial role in collagen synthesis, positively impacting reproduction and larval survival rates. In our study, supplementation of L-ascorbic acid for nemo broodstock fish resulted in significantly improved hatching rates, 3-day post-hatch larvae survival, and reduced malformation rates in newly hatched larvae ($P<0.05$). These findings are consistent with previous studies (Blom & Dabrowski 1995; Dabrowski & Ciereszko 2001; Eskelinen 1989; Furuita et al 2009b; Mahsa & Allah 2011; Mehrad et al 2011; Sandnes et al 1984; Soliman et al 1986). Supplementation with 115 mg AA kg^{-1} feed significantly increased the number of hatched eggs in rainbow trout (Sandnes et al 1984). Feeding tilapia brood fish with 1250 mg AA kg^{-1} resulted in significantly improved egg hatching rates and reduced fry malformation rates. However, some studies have reported conflicting results regarding the contribution of VC supplementation to fertilization rates and larval survival rates (Mangor-Jensen et al 1994; Terova et al 1998). Mangor-Jensen et al (1994) found that VC supplementation in the diet of Atlantic cod insignificantly affected fertilization and survival rates. Similarly, supplementation of AA in the reproductive process of female rainbow trout did not significantly improve the hatching rate (Blom & Dabrowski 1995). Similar observations were made in sea bass and sea bream (Terova et al 1998).

In this study, the reproductive performance of nemo broodstock was monitored monthly, revealing significant increases in absolute fecundity, indicating the number of eggs, starting from the fourth month of experimentation ($p<0.05$). Broodstock fish fed with a diet supplemented with 600 mg VC kg^{-1} feed exhibited the highest egg production, which remained stable throughout the experiment. The survival rate of larvae was influenced by VC supplementation from the first month of the experiment ($P<0.05$), with no significant differences observed during the 3rd to 9th months. However, discrepancies emerged from the 10th month onwards. The highest survival rate (>94%) was observed with dietary supplementation of 600 mg VC kg^{-1} feed. In the natural fish reproductive cycle, VC supplementation must accumulate over time to exert its full effects. Thus, the

appropriate VC concentration and timing of supplementation in the broodstock diet can significantly influence fish breeding outcomes. Our findings highlight that the treatment supplying 600 mg VC kg^{-1} feed yielded the most favorable reproductive indicators for nemo fish larvae, including fecundity rate, hatching rate, and survival rate of 3-day post-hatch larvae, as well as reducing egg loss and malformation rates. Therefore, the optimal VC supplementation in the broodstock diet for nemo fish reproduction is 600 mg kg^{-1} feed, ideally continued for at least 10 months before and during the spawning period to enhance reproductive performance.The optimal VC supplementation for nemo fish reproduction observed in this study was higher than that reported for other species, such as in rainbow trout fish at 115-375 mg VC kg^{-1} feed (Blom & Dabrowski 1995; Sandnes et al 1984), in turbot at 375 mg kg^{-1} feed (Lavens et al 1999); in sea bass, sea bream at 2000 mg kg^{-1} of feed (Terova et al 1998), zebrafish at 1000 mg kg^{-1} of feed (Mehrad et al 2011), in platy *Xiphophorus maculatus* at 1600 mg kg^{-1} of feed (Mahsa & Allah 2011), and tilapia at 1250 mg AA kg^{-1} feed (Soliman et al 1986). These variations may be attributed to differences in fish species, size, VC formulation, and experimental conditions across studies (Lovell 1998).

Furthermore, VC serves a crucial role as an antioxidant, effectively scavenging free radicals and reactive oxygen species. This function helps prevent cellular damage caused by radicals, safeguard cell membranes and cytosolic components, and aid in the restoration of VE. VC assists in the recycling or sparing of VE from membrane α-tocopherol radicals, vital for the antioxidant function in fish (Jiménez-Fernández et al 2015). The coexistence of VE and VC at appropriate ratios can generate synergistic antioxidant effects, regulating oxidative stress indices, and averting stem cell damage while preventing lipid peroxidation (Arrigoni & De Tullio 2002). However, excessive doses of VC or VE may elevate phospholipid peroxidation and dehydroascorbic reductase activity, leading to adverse effects on physiological function (Moreau et al 1999).

In nemo broodstock, studies examining the supplementation of VE and astaxanthin (Ax), for broodstock rearing over 13 months, indicated no significant effects on re-maturation and spawning, spawning frequency, fecundity, egg diameter, or larvae size (Dao et al 2018; Nguyen et al 2020). Generally, the addition of VE, Ax, and VC to the diet of nemo broodstock showed insignificant differences in certain reproductive parameters. However, when specifically supplementing VC, an increase in fecundity and a reduction in the rate of egg loss were observed compared to supplementation with VE and Ax alone. While the formulation of a diet containing VE and Ax suitable for nemo fish reproduction was established in the VC experiment, the interaction impact of Ax, VE, and VC on the reproductive performance of nemo broodstock has not been assessed. Nevertheless, previous studies have reported on the combinations of VC-VE and their interactions with fish reproduction (Emata et al 2000; Furuita et al 2009a; Gao et al 2014; Nguyen et al 2012). Therefore, further research is necessary to elucidate the roles and interaction relationships of these vitamins in the reproduction of nemo fish.

Conclusions. The analysis of experimental data reveals a significant positive impact of VC supplementation on the reproduction of broodstock nemo fish. VC supplementation in the diet exhibited beneficial effects on actual fertility, egg loss rate, hatching rate, 3-day survival rate of larvae, and malformation rate of juveniles. Our findings suggest that the optimal vitamin C requirement for nemo broodstock participating in spawning is 600 mg kg^{-1} feed. This level of supplementation has the potential to enhance reproductive efficiency and improve the quality of nemo larvae, thereby contributing to the overall success of nemo fish aquaculture.

Conflict of interest. The authors declare no conflict of interest.

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