Agr. Nat. Resour. 57 (2023) 951–962

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Effects of vitamins C and E on semen quality and reproductive hormones in yellow mystus (*Hemibagrus spilopterus*)

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Article Info

Article history:
Received 1 September 2023
Revised 3 November 2023
Accepted 6 November 2023
Available online 31 December 2023

Keywords:
Reproductive hormones,
Semen quality,
Vitamin C,
Vitamin E,
Yellow mystus

Abstract

Importance of the work: Breeding success depends partly on sperm quality which could be enhanced by vitamin supplementation in feed.

Objectives: To investigate the effects of vitamins C and E and their combination on semen quality and sex hormones in yellow mystus.

Materials & Methods: Male yellow mystus aged 1 yr were stocked in three cages (each 4 m^2) and fed with 32% protein commercial feed supplemented with: 1) no vitamins as the control; 2) vitamin C (Vit C) at 1,000 mg/kg; 3) vitamin E (Vit E) at 500 mg/kg; 4) Vit C at 1,000 mg/kg + Vit E at 500 mg/kg. In each experimental group, the growth, semen quality and sex hormones were investigated.

Results: The results showed that vitamin supplementation did not affect the growth of yellow mystus. On day 30, the Vit C, Vit E and Vit C + Vit E groups had higher gonadosomatic index values than the control ($p < 0.05$). On days 30 and 60, the Vit C + Vit E group had the highest mean sperm motility (87.33 ± 3.05%; $p < 0.05$). On day 30, the Vit C and the Vit C + Vit E groups had higher sperm viability than the control ($p < 0.05$), while on day 60, the Vit C, Vit E, and Vit C + Vit E groups had higher sperm viability ($p < 0.05$). In addition, the Vit C + Vit E group had significantly higher testosterone levels compared to the control and the Vit C group measured on day 60.

Main finding: Adding Vit C + Vit E in the diet enhanced the semen quality and sex hormones of yellow mystus. This approach could serve as an effective method to increase the fingerling production of yellow mystus.

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https://doi.org/10.34044/j.anres.2023.57.6.05

Introduction

The yellow mystus, *Hemibagrus spilopterus* (Ng & Rainboth, 1999), also known as the green catfish, inhabits freshwaters throughout Southeast Asia. It is extensively farmed in countries, including Thailand and Malaysia (Mesomya et al., 2002; Adebiyi et al., 2011). It is valued for its delicious taste and high nutritional content, making it popular in both domestic and international markets, especially in Indonesia (Aryani and Suharman, 2014). Yellow mystus culture depends on hatchery-bred fry of which the production is still limited due to the poor performance of broodstock, especially males (Aryani et al., 2018).

Vitamin supplementation has been widely used to enhance reproductive performance and growth in fish, with vitamin C, also known as ascorbic acid (Gasco et al., 2018) protecting cells from oxidative stress and supporting various physiological functions (Liang et al., 2017; Reddy et al., 2018; Ibrahim et al., 2020). It also modulates the production and activity of reproductive hormones, stimulates gonadal development and regulates sex steroid hormone synthesis in Nile tilapia (*Oreochromis niloticus*; Sarmento et al., 2017) and increases the levels of plasma estradiol and testosterone involved in reproductive processes in rainbow trout (*Oncorhynchus mykiss*; Dabrowski et al., 1995). Additionally, it mitigates the negative effects of stress, reducing cortisol levels and protects gonadal tissues from oxidative damage (Rurangwa et al., 2004), thus maintaining sperm cell functionality (Sönmez et al., 2005; Betancor et al., 2012). Furthermore, it affects reproduction and enhances fish growth. For example, supplementing vitamin C at a level of 1,000 mg/kg in the diet of the Thai climbing perch (*Anabas testudineus*) resulted in higher final weight, weight gain and specific growth rate and a lower feed conversion ratio (Pimpimon and Ungsetthaphan, 2013).

Vitamin E (tocopherols and tocotrienols) can improve the quality of fish eggs, leading to enhanced fertilization rates and hatching success. For example, vitamin E improves egg viability and reduces deformities (Fu et al., 2022), as well as influencing the synthesis and release of gonadotropins, which play a crucial role in gonadal development and reproductive behavior (Vasudhevan et al., 2017). Furthermore, vitamin E mitigates the toxic effects of pollutants by reducing oxidative stress and protecting gametes and reproductive tissues, thereby enhancing egg quality and improving fertilization rates, viability and embryo development (Khara et al., 2016; Fu et al., 2022). Like vitamin C, vitamin E also enhances fish growth, including goldfish (Kashani et al., 2012), guppy (*Poecilia reticulata*; Mehrad and Sudagar, 2010), zebrafish (*Danio rerio*; Mehrad et al., 2012), channel catfish (*Ictalurus punctatus*; Yildirim-Aksoy et al., 2008) and juvenile hybrid striped bass (*Morone chrysops* ♀ × *M. saxatilis* ♂; Sealey and Gatin, 2002).

Both vitamin C and vitamin E are known for their antioxidant properties and their effects on reproductive systems have been studied (Takhshid et al., 2012). In addition, they impacted sperm concentration and motility *in vitro*, as well as sperm motility, egg fertilization and the penetration of egg cells (Keogh et al., 2017). Furthermore, both vitamins are effective antioxidants that can improve male reproductive performance (Martin-Hidalgo et al., 2019). In the absence of these vitamins, epithelial degeneration occurs in the seminal ducts, including the Leydig cells within the seminiferous tubules (Muhammad, 2017). Notably, vitamins C and E have shown synergistic effects in the enhancement of reproductive performance (Gammanpila et al., 2007; Hidayatik et al., 2021), albeit with some exceptions (Nguyen et al., 2012). Although such enhancement effects of these vitamins have been reported in diverse species of fish, study is lacking regarding yellow mystus. Specifically, there have been no prior reports on the interaction between these two vitamins concerning semen quality and sex hormones.

Therefore, the current study was conducted to investigate the effects of vitamin C and vitamin E supplementation on the growth, semen quality and sex hormones in yellow mystus. While other reproductive aspects have been reported already, this study was the first examination of semen quality and sex hormones in this particular fish species. The results obtained should benefit the hatchery production of yellow mystus.

Materials and Methods

Animal preparation

The experiment was conducted using 360 male yellow mystus aged 1 yr, with an initial average weight ± SD of 218.95 ± 7.52 g. Before the experiment, the fish were acclimated for 2 wk in cages fixed in an earthen pond. The water was changed every week. They were fed floating commercial pellet feed (FishFirst brand no. 4643) containing 32% protein twice a day, morning and evening, at a rate of 3% of their body weight for 2 wk. After 2 wk of acclimatization, the fish were allocated to 12 netted cages, each measuring 2 m × 2 m × 1.5 m, with each unit containing 30 fish.

Feed preparation and feeding trial

This experiment used commercial floating pellets containing 32% protein (FishFirst brand no. 4643). In the treatment with the vitamin C (L-ascorbic acid) supplement, vitamin C was mixed with water at a 5% ratio and subsequently was evenly sprayed onto the feed to make a concentration of 1,000 mg/kg feed. To prepare the feed with vitamin E supplement, alpha-tocopherol vitamin E (α-tocopherol acetate; Sigma; Saint Louis, MO, USA) was mixed with vegetable oil at a 5% ratio and evenly sprayed onto the feed to reach a concentration of 500 mg/kg feed. Then, the sprayed feed was dried in the shade and stored in a refrigerator at 4°C. The nutritional value of the experimental diets was analyzed using the method described by Association of Official Analytical Chemists (2016).

Experimental design

The experiment was conducted following a completely randomized design with four treatments each with three replications, with the experimental fish being fed one of the following diets: 1) feed without any supplementation of vitamins (control); 2) feed supplemented with vitamin C (Vit C) at 1,000 mg/kg; 3) feed supplemented with vitamin E (Vit E) at 500 mg/kg; and 4) feed supplemented with both Vit C at 1,000 mg/kg and Vit E at 500 mg/kg.

Feeding occurred twice daily, at 0800 hours and 1600 hours at 3% body weight. The experiment spanned 60 d, involving random sampling and data collection on both day 30 and day 60, while water quality assessments were conducted on a weekly basis.

Data collection

Growth rate

In each experimental group, 5 fish/replication were randomly taken and measured for weight and length every 30 d. Daily weight gain (DWG) and specific growth rate (SGR) were calculated using Equations 1–2 (Roa et al., 2019):

$$DWG = \frac{\text{Final weight - Initial weight}}{\text{Number of rearing days}} \quad (1)$$

$$SGR = \frac{[\text{Ln (Final weight) - Ln (Initial weight)}] \times 100}{\text{Number of rearing days}} \quad (2)$$

After that, fish samples were taken for analysis of semen quality and sex hormones on day 30 and day 60 of rearing.

Analysis of semen quality

Due to the location of the testes under the gastrointestinal tract, it was necessary to dissect the stomach to extract the testicles. Any blood present was wiped away prior to recording any data. The assessment of semen quality was performed according to the methods of Shamspour and Khara et al. (2016) and Uddin et al. (2017), which included the measurement of visual appearance and detailed examination using microscopy.

Sperm motility percentage: Sperm motility was assessed based on six levels of sperm motility: 0%, 20%, 40%, 60%, 80% and 100% (Vuthiphandchai and Zohar, 1999; Ponchunchoovong and Plime, 2010). For evaluation, 1 µL of fresh semen was placed on a glass slide and added with 50 µL of 0.4% NaCl. A needle was used to gently transfer fresh semen onto a droplet of distilled water, followed by gently mixing the two. Subsequently, sperm motility was assessed under a microscope within 30 s, using magnifications in the range 40–100 times.

Sperm viability percentage: Sperm viability was assessed based on differential staining of live and dead sperm using a nigrosin-eosin solution. Eosin cannot penetrate living sperm cells, so dead sperm are stained pink with eosin (Björndahl et al., 2003).

Sperm concentration (measured in cells per milliliter): The semen was diluted 100 times with 0.9% normal saline. Then, the diluted semen was placed on a hemocytometer covered with a glass slide designed for counting blood cells. The slide cover glass was gently pressed back and forth to allow excess semen solution to flow out of the counting chamber. The sperm concentration was calculated using Equation 3, according to the method of Hajirezaee et al. (2010):

$$\text{Sperm concentration} = C \times 2 \times 10^6 \text{ spermatozoa/mL} \quad (3)$$

where C = the number of sperm counted in 25 channels, with all counting undertaken by one person, with three replicates per sample.

Analysis of sex hormones

Sex hormone analyses were conducted on day 30 and day 60 of the experiment. Briefly, blood samples were collected, placed in an iced flask and transported to the laboratory within 6 hr. Subsequently, they were centrifuged at 3,000×*g* for 15 min, with the serum fraction being carefully collected from sterile test tubes into 1 mL plastic tubes and stored at -20°C. To detect cortisol and testosterone levels, a competitive enzyme-linked immunosorbent assay (ELISA) was performed using a microplate reader at an absorbance of 405 nm. The ELISA kit used had the lowest reading of 0.0023 ng/mL.

Water quality

Water samples were collected weekly at 0600 hours and 1500 hours and were determined for dissolved oxygen content (DO; model 52; YSI; OH, USA), pH (model pH100; YSI; OH, USA), temperature (model HI93150; Hanna Instruments, RI, USA), and total ammonia based on the phenate method, according to Boyd (1990).

Statistical analysis

The data on body weight, body length, semen quality and sex hormones were expressed as mean ± SD values and analyzed using a one-way analysis of variance. The means were compared using Duncan's new multiple-range test at the 95 percent confidence level.

Ethics statements

The research procedures and methods were carried out under the supervision of the Sub-committee on Animal Husbandry and Use at Rajamangala University of Technology Isan, Thailand. Committee oversight occurred regarding the moral and ethical aspects of using laboratory animals for scientific work; the study obtained permission under No. U1-04362-2559.

Results

Growth rate

Yellow mystus fed with supplements of Vit C, Vit E and a combination of Vit C + Vit E were monitored for growth rate on day 30 and day 60. The results showed that vitamin supplementation did not significantly affect the growth of yellow mystus (Table 1).

Reproductive performance

Gonadosomatic index and sperm motility

The gonadosomatic index (GSI) of the yellow mystus fed Vit C or Vit E supplemented diets for 30 d and 60 d were significantly different on day 30. The results clearly showed that supplementation of either individual vitamins (Vit C or Vit E) or a combination of the two significantly enhanced the GSI compared to the control without vitamins (Table 2). However, on day 60, there were no significant differences in the GSI values among all experimental groups. On day 30, the highest percentage of sperm motility was recorded for the group that had been fed a combination of Vit C + Vit E (sperm motility = 87.33 ± 3.05%), which was not significantly different from the group fed the diet solely supplemented with Vit E (85.33 ± 1.52%). The group fed with the Vit C supplement tended to have improved sperm motility (76.67%) over that of the control (73.33%) although there was no significant difference. When the treatment was continued until day 60, the percentages of sperm motility in the groups fed the Vit C supplement (86.33 ± 3.21 %) or the Vit C + Vit E supplement (86.00 ± 3.60 %) were significantly higher than the control (73.33 ± 5.77%). Notably, the sperm motility of the group fed the Vit E supplement was not significantly different from the two former groups, nor from the control (Table 2).

Sperm viability and sperm concentration

Sperm viability and sperm concentration are crucial factors in fertility, as they can serve as indicators of sperm quality.

Table 1 Mean ± SD of growth traits of yellow mystus fish fed with vitamin C, vitamin E, and vitamin C + vitamin E supplements

Parameter	Vitamin supplement				*p*-value
	No Vit	Vit C	Vit E	Vit C + Vit E	
Day 30					
Initial length (cm)	23.66±1.53	23.03±1.03	23.85±1.15	23.00±0.88	0.82
Final length (cm)	26.66±1.52	27.00±1.00	27.33±0.57	28.33±0.58	0.43
Initial weight (g)	209.00±5.56	206.00±4.58	211.00±4.58	215.00±3.00	0.18
Final weight (g)	223.33±5.03	221.00±7.81	224.00±4.35	230.66±3.05	0.23
Daily weight gain (g/day)	0.48±0.02	0.500±0.384	0.43±0.07	0.522±0.084	0.95
Specific growth rate (%/day)	0.22±0.01	0.23±0.18	0.20±0.03	0.23±0.04	0.96
Day 60					
Final length (cm)	28.33±3.21	29.66±2.88	27.66±2.51	29.00±1.00	0.91
Final weight (g)	227.35±7.19	232.96±10.56	229.66±18.98	237.29±10.17	0.79
Daily weight gain (g/day)	0.31±0.04	0.45±0.10	0.31±0.30	0.37±0.134	0.72
Specific growth rate (%/day)	0.14±0.01	0.20±0.04	0.14±0.13	0.16±0.05	0.67

Vit = vitamin; Vit C = vitamin C; Vit E = vitamin E

It was observed that on day 30, the percentages of viable sperm in the Vit C and Vit C + Vit E groups were significantly different compared to the group without vitamin supplementation. After another 30 d (on day 60), the Vit C, Vit E and Vit C + Vit E groups had significantly higher sperm viability than the control group (Table 2). There were no significant differences among the results for sperm concentration among the groups on day 30. However, after an additional 30 d (on day 60), there were increases in sperm concentration in the groups using vitamin supplements. The highest sperm concentration was in the Vit C + Vit E group (208.00 ± 27.05×10^6 cells/mL), although this was not significantly different from the Vit E group (194.66 ± 10.50 × 10^6 cells/mL). Furthermore, there were no significant differences in the sperm concentrations between the Vit C and Vit E groups. The group without vitamin supplements had the lowest sperm concentration (115.00 ± 13.74×10^6 cells/mL), being approximately 1.5 times lower than that of the Vit C + Vit E group, demonstrating the profound effects of either Vit C + Vit E supplementation or Vit E supplementation alone on the enhancement of sperm concentration at day 60 (Table 2).

Testosterone and cortisol levels

Serum testosterone levels were measured in yellow mystus after feeding them with Vit C and Vit E for 30 d and 60 d. On day 30, there were no significant differences in the serum testosterone levels among all treatments. However, at day 60, the group receiving Vit C + Vit E had a significantly higher serum testosterone level compared to the control group and the group receiving only Vit C. Similar to the effect on sperm concentration, the addition of Vit C to Vit E tended to enhance the effect of Vit E on increasing testosterone in yellow mystus. Additionally, serum cortisol levels in yellow mystus in this experiment varied considerably within each treatment, as can be seen from the relatively large SD values. Overall, there were no significant differences among the serum cortisol levels in the fish fed diets without added vitamins or with either Vit C or Vit E or with Vit C + Vit E for 30 d and 60 d (Table 3).

Water quality

Water quality in the experimental ponds was within the range set in the standards (American Public Health Association,

Table 2 Gonadosomatic index, sperm motility, sperm viability and sperm concentration of yellow mystus fish fed with a range of vitamin C and vitamin E supplements

Reproductive performance	Vitamin supplement				*p*-value
	No Vit	Vit C	Vit E	Vit C + Vit E	
Day 30					
Gonadosomatic index (GSI)	0.29±0.04^{a}	0.44±0.09^{b}	0.41±0.04^{b}	0.46±0.05^{b}	0.043
Sperm motility (%)	71.66±7.63^{a}	76.67±5.78^{ab}	85.33±1.52^{bc}	87.33±3.05^{c}	0.017
Sperm viability (%)	67.66±6.80^{a}	80.00±2.00^{b}	74.48±5.01^{ab}	80.66±4.04^{b}	0.035
Sperm concentration (10^6 cells/mL)	156.00±39.34	193.00±35.59	218.66±96.54	201.00±30.04	0.610
Day 60					
Gonadosomatic Index (GSI)	0.42±0.07	0.57±0.17	0.50±0.12	0.65±0.06	0.190
Sperm motility (%)	73.33±5.77^{a}	86.33±3.21^{b}	82.00±7.21^{ab}	86.00±3.60^{b}	0.046
Sperm viability (%)	70.66±6.11^{a}	86.00±4.58^{b}	87.67±2.08^{b}	94.00±2.64^{b}	0.001
Sperm concentration (10^6 cells/mL)	115.00±13.74^{a}	155.33±27.59^{b}	194.66±10.50^{bc}	208.00±27.05^{c}	0.003

Vit = vitamin; Vit C = vitamin C; Vit E = vitamin E

Mean ± SD values in a row with different lowercase superscripts are significantly ($p < 0.05$) different.

Table 3 Testosterone hormone and cortisol of yellow mystus fish fed with feed without vitamin supplement and supplemented with vitamin C, vitamin E or vitamin C + vitamin E

Vitamin supplement	Testosterone hormone level (ng/mL; 1:1)		Cortisol hormone level (ng/mL; 1:10)	
	Day 30	Day 60	Day 30	Day 60
No Vit	179.40±66.27	244.68±47.42^{a}	127.42±23.16	088.57±89.05
Vit C	333.83±120.26	284.93±79.47^{a}	151.86±62.61	209.93±30.54
Vit E	189.23±39.18	373.27±271.96^{ab}	192.88±82.97	106.90±75.73
Vit C + Vit E	275.68±122.48	661.11±203.77^{b}	147.02±30.80	086.24±120.98
p-value	0.219	0.047	0.558	0.308

Vit = vitamin; Vit C = vitamin C; Vit E = vitamin E

Mean ± SD values in a column with different lowercase superscripts are significantly ($p < 0.05$) different.

2017). The values of DO were in the range 4.10–4.90 mg/L, temperature were in the range 28.30–32.00°C, pH were in the range 6.40–7.20 and NH_3 was at 0.001 mg/L, indicating that the water was suitable throughout for fish raising.

Discussion

Effects of vitamin supplementation on growth

While other studies have documented growth promotion in fish when they received Vit C-supplemented feed (Ibiyo et al., 2006; Zhou et al., 2012; Pimpimon and Ungsetthaphan, 2013), the current study indicated that supplementation with Vit C did not have a significant impact on growth, which was consistent with a study conducted by Andrade et al. (2007), who reported no significant impact on weight and survival rates with vitamin supplementation levels of 500 mg/kg, 800 mg/kg and 1,200 mg/kg in Amazon snakehead (*Arapaima gigas*). In contrast, other research has shown that Vit C supplementation improved the growth rate and weight gain in common carp (*Cyprinus carpio*) (Aboseif et al., 2022) and juvenile largemouth bass (*Micropterus salmoides*) (Chen et al., 2015). Furthermore, there have been reports of Vit E supplementation enhancing growth parameters, including body weight and length, in yellow drum (*Nibea albiflora*) (Wang et al., 2019) and Pacific white shrimp (*Litopenaeus vannamei*) (Yowaphui et al., 2016), while such growth enhancement was not observed in the current study. This disparity in results may be attributed to the fact that as fish mature, their growth responses naturally slow down; thus, the enhancement effects might have been masked. Nevertheless, it is important to note that the primary focus of the current study was on reproductive traits rather than growth.

Effects of vitamin supplementation on reproductive performances

Overall, the current results demonstrated the positive effects of Vit C and Vit E on the reproductive performance of male yellow mystus. The synergistic effects of these two vitamins were supported by the statistical analysis of semen quality and reproductive hormones in the study. In general, Vit C is a potent water-soluble antioxidant, protecting germ cells from oxidative stress, maintaining DNA integrity and promoting collagen synthesis (Liang et al., 2017; Ibrahim et al., 2020), which is essential for the structural integrity of reproductive tissues (Waagbo et al., 2000). Vit C can help protect gametes (sperm and eggs) from oxidative damage, which is important for successful reproduction (Kashani et al., 2012). Vit E, a lipid-soluble antioxidant, shields germ cell membranes from oxidative damage, preserving the cell structure and genetic material (Böhm, 2018). Additionally, the impact of Vit E on hormone regulation may influence germ cell maturation (Asadi et al., 2017; Muhammad, 2017; Naderi et al., 2017). In addition, research has shown that Vit E can increase sperm motility. The research by Dzyuba et al. (2016) and Legendre et al. (2016) indicated that ATP-dependent sperm motility was associated with creatine phosphate in mitochondria. Positive effects of Vit E on sperm motility have also been documented (Cosson, 2013; Fedorov et al., 2015). The combined supplementation of vitamins C and E may create synergistic effects, enhancing overall antioxidant defenses and fostering a favorable cellular environment for germ cell development (Andrade et al., 2007; Khara et al., 2016; Keogh et al., 2017; Betsy et al., 2021). While specific references are needed to substantiate these claims, research indicates the potential of these vitamins to positively impact germ cell quality and reproductive health in various species (Lee and Dabrowski, 2003; Ortuño et al., 2003; Chen et al., 2004; Gao et al., 2014). There are two main mechanisms involved in the interaction between vitamins C and E: one is the simultaneous protection of the water and lipid phases against oxidation, while the other is the regeneration of Vit E from the Vit E radicals by Vit C (Ortuño et al., 2001).

Notably, in the current study there were significant differences in the GSI values between the fortified groups and the non-fortified group only at day 30 and not at day 60. This indicated the effects of both vitamins in triggering the early development of testes. Despite the GSI value of male yellow mystus not being provided in other publications, the GSI value in the current study falls in the maximum range reported for the congener, *H. nemurus* (male GSI = 0.15–0.44%, Handayani et al., 2020). The GSI is a parameter used to assess the reproductive status of organisms, particularly in relation to gonad development and maturation (Flores et al., 2019). It was found to be highly correlated with gonad growth and vitellogenin gene expression in *Notopterus notopterus* and *Anematichthys armatus* (Panprommin et al., 2015). The results of the current study were consistent with the results reported by Liang et al. (2017), working with juvenile yellow catfish (*Pelteobagrus fulvidraco* Richardson), which showed that Vit C supplementation significantly improved the GSI. Similar findings have been reported in Nile tilapia males, using Vit C at concentrations in the range 599–942 mg/kg to improve the performance and quality of semen (Sarmento et al., 2017).

Sperm motility significantly increased with the administration of Vit E, which plays a crucial role in cell protection and maintaining the integrity of sperm membranes as has been observed in various fish species, including Senegalese sole (*Solea senegalensis*; Beirão et al., 2015), Curimba (*Prochilodus lineatus*; Navarro et al., 2014), rainbow trout (Naderi et al., 2017; Canyurt and Akhan, 2008) and Atlantic salmon (*Salmo salar*; Figueroa et al., 2018). Notably, 60 d of Vit E treatment resulted in no significant improvement in sperm motility compared to the control group, possibly because Vit E may stimulate semen motility for a limited period of time, as there was a significant impact after 30 d of Vit E supplementation, but not at 60 d. Notably, the effects of Vit E on sperm motility can vary between different fish species and other factors may also influence the outcomes, such as dosage, duration of supplementation and the specific reproductive stage of the fish. Other studies that did not find a significant effect of Vit E supplementation on sperm quality (sperm concentration, motility and viability) included the report on Nile tilapia (Gammanpila et al., 2007). The current study demonstrated that supplementation with Vit C, Vit E or a combination of both led to improvements in sperm motility and viability. Both Vit C and Vit E play roles in protecting cell membrane integrity (Higgins et al., 2020), and their synergistic interaction provides better protection against oxidative damage. The study results suggested that the supplementation with Vit C, Vit E or their combination positively affected sperm quality (Table 2). Similarly, Xu et al. (2015) observed increased sperm motility and viability, indicating improved reproductive performance in turbot (*Scophthalmus maximus*), rainbow trout (Canyurt and Akhan, 2008), fertilization in Atlantic salmon (Figueroa et al., 2018), and in *Rhamdia quelen* (Xavier et al., 2021). According to the current results, when given in combination, Vit C and Vit E were likely to synergistically influence hormone synthesis and hormone production, including prostaglandin synthesis. In Thai walking catfish, the administration of 600 mg/kg of Vit E resulted in the highest average sperm concentration of 50.38 ± 3.14×10^6 cells/mL, with no significant ($p > 0.05$) difference from the control group (Somnuek et al., 2019). Similar effects were observed in Atlantic salmon (Figueroa et al., 2018), and kuruma shrimp (*Marsupenaeus japonicas*; Nguyen et al., 2012). When Vit E was administered alone for 60 d, the sperm concentration increased more than from administering Vit C, depending on factors such as type, age and size. Vitamins C and E have been studied for their potential effects on various aspects of fish reproduction, including increased sperm density compared to the control group (Cabrita et al., 2011).

Effects of vitamin supplementation on testosterone

At day 60, the testosterone levels in the yellow mystus supplemented with Vit C + Vit E were higher than for those not fortified and for those fortified with Vit C only. Notably, on day 30, the Vit C tended to result in higher testosterone levels than for the other groups; however, due to a high SD value, the difference was not significant. The effect of the vitamins was not clear on day 30; however, a positive result was apparent on day 60. This disparity may have arisen from the possibility that the current doses of Vit C and Vit E supplementation might have been insufficient to significantly stimulate testosterone production as early as at 30 d. However, owing to the fact that the testosterone level is related with sperm quality, a slight improved sperm quality at day 30 may suggest the increased testosterone level at that time point. The combined vitamin supplementation (Vit C + Vit E) which resulted in the highest testosterone level, demonstrated either their synergistic effect or the additive effect of the dosage of the combined vitamins.

Vit E functions as an antioxidant, helping to protect cell membranes and prevent lipid peroxidation that can negatively impact reproductive functions (Mutalip et al., 2018). It has been suggested that Vit E supplementation enhanced the synthesis and release of testosterone in fish. For example, a significant increase in blood testosterone was observed in Nile tilapia (Zhang et al., 2021), and turbot (Huang et al., 2019) fed diets supplemented with Vit E. Overall, adequate Vit E intake is associated with improved testicular health and function, which can impact testosterone production (El-Sayed and Izquierdo, 2022). Likewise, some studies have suggested that Vit C supplementation may support healthy testosterone levels by maintaining the health of Leydig cells and reducing oxidative stress (Fernandes et al., 2011; Rahayu et al., 2019). The combined supplementation of vitamins C and E may have synergistic effects on oxidative stress reduction and overall antioxidant defense. This, in turn, might contribute to maintaining testosterone levels within a healthy range (Hidayatik et al., 2021). Similarly, in hybrid male abalone, Wang et al. (2023) reported that dietary supplementation with Vit C and Vit E significantly increased the testosterone levels in the treated group compared to the control group.

Effects of vitamin supplementation on cortisol

There were no significant differences in the serum cortisol levels among all experimental groups, perhaps because the fish in the current study had similar adaptation mechanisms

to stress due to their shared environment. The current result was inconsistent with a study in gilthead seabream (*Sparus aurata*) in which dietary supplementation of Vit C or vit E influenced cortisol levels in fish subjected to crowding stress (Montero et al., 1999). Cortisol is commonly used as an indicator of stress and supplementation with Vit C and Vit E can assist fish in maintaining normal stress indicators when subjected to stressful conditions (Ortuño et al., 2003). Furthermore, cortisol itself is closely linked to many of the adverse consequences of chronic stress, including effects on growth, reproduction and the immune system (Lemos et al., 2023). Consequently, the extent of cortisol elevation following exposure to a standardized stressor has been adopted as a trait of physiological significance on which a selection procedure may be based. High-responding (HRC) and low-responding (LRC) individuals continued to display divergent cortisol responses to confinement up to 21 mth after the start of a study (342 ± 34 ng/mL and 208 ± 21 ng/mL, respectively; Pottinger and Carrick, 1999). However, cortisol levels may also be influenced by the ability of fish to rapidly adapt to stressful conditions or by other acclimatization mechanisms external to the fish. Iwama et al. (2005) reported that cortisol levels peaked after 1 hr of stress and returned to the baseline level after 6 hr. Similarly, a study by Morales et al. (2006) demonstrated an immediate increase in cortisol levels following manipulation, followed by a return to the baseline level after 8 hr. Notably, in certain species, the magnitude of the stress response can vary depending on prior acclimatization to stress factors (Lankford et al., 2003).

Appropriateness of current dosage

The dosage levels of vitamins used in the current study (500 mg/kg for Vit E and 1,000 mg/kg for Vit C), fall within ranges reported for several fish species. For example, in stinging catfish (*Heteropneustes fossilis*, Bloch, 1792), the optimum level of Vit C to enhance growth was 1,200 mg/kg (Alam et al., 2009). In Nile tilapia, the reported optimum level of Vit C was 400 mg/kg (Ibrahim et al., 2020), while 1,250 mg/kg of Vit C increased sperm motility (Gammanpila et al., 2007). Furthermore, the optimum dose of Vit C for Japanese seabass (*Lateolabrax japonicas*) was approximately 489.0 mg/kg in the diet (Ai et al., 2004). The optimum level of Vit E in Thai walking catfish (*Clarias macrocephalus*) was 600 mg/kg in the diet (Somnuek et al., 2019). For turbot, Vit E at 721.60 mg/kg in the diet improved the sperm concentration and motility duration and maintained normal sperm morphology (Xu et al., 2015). The combination of vitamins C and E at the fortified levels of 800 mg/kg Vit C and 500 mg/kg Vit E increased the weight and the survival rate in Amazon snakehead (Andrade et al., 2007). In common carp, Vit C at 400 mg/kg + Vit E at 400 mg/kg were reported to have positive effects (Rahimi et al., 2019). Additionally, for guppy, the combination of 1,000 mg/kg Vit C and 300 mg/kg Vit E had a beneficial effect on growth parameters and survival rate (Şahin and Aral, 2021). Notably, the optimum levels of vitamins C and E for fish reproduction may vary depending on the fish species, life stage, environmental conditions and the specific research objectives. The effects of combining both vitamins (C+E) may also depend on their interaction and the presence of other dietary components.

Conclusion

The impact of Vit C, Vit E or Vit C + Vit E supplementation on the semen quality and sex hormones of the yellow mystus was explored for the first time in this study. The findings indicated that after 30 d of supplementation, these vitamins had notable positive effects on both the GSI and semen quality. Furthermore, on day 60, Vit C and Vit E had a positive impact on semen quality, while Vit C + Vit E positively affected both semen quality and sex hormones. In conclusion, dietary supplementation using Vit C and Vit E positively influenced the semen quality and sex hormones in yellow mystus fish. Specifically, supplementation using 1,000 mg/kg of Vit C combined with 500 mg/kg of Vit E for 60 d enhanced the semen quality. However, it is important to note that this supplementation may not lead to an increase in semen volume, as indicated by the absence of a significant difference in the GSI. This approach could serve as an effective method to increase the production of yellow mystus fingerlings.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin campus provides funding. The Fisheries Laboratory, Rajamangala University of Technology Isan, Surin campus and the Animal Science

Laboratory, Faculty of Agriculture and Agricultural-Industry, Surindra Rajabhat University provided assistance and support.

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