Sri Lanka J. Aquat. Sci. **12** (2007): 39 - 60

Evaluation of the effects of dietary vitamin C, E and Zinc supplementation on reproductive performance of Nile tilapia (*Oreochromis niloticus*)

M. GAMMANPILA[1]*, A. YAKUPITIYAGE[2] AND A. N. BART[2]

[1]National Aquatic Resources, Research and Development Agency, Crow Island, Mattakkuliya, Colombo 15, Sri Lanka.
[2] Aquaculture and Aquatic Resources Management, Agricultural and Aquatic Systems, and Engineering Program, School of Environment, Resources and Development, Asian Institute of Technology, P.O. Box 4, Klong Luang Pathumthani 12120, Thailand.

*Corresponding author (E-mail: gMeneke@yahoo.com)

Abstract

A 3×2 factorial experiment was conducted to evaluate the effects of dietary vitamin C, E and Zinc supplementation on growth, survival and reproduction of *Oreochromis niloticus* in hapa based system installed in earthern ponds for 5 months. The triplicate groups consisting 30 fish were fed three levels of vitamin C (0, 50 and 1250 mg kg^{-1} diet), two levels of vitamin E (0, 600 mg kg^{-1} diet). An additional treatment that supplemented Zinc at a rate of 120 mg kg^{-1} with higher vitamin C and E levels was also included in the experiment. The results revealed that vitamin C deficient female fish exhibited significantly lower weight gain compared to the fish fed with diet supplemented with higher vitamin C. The spawning efficiency, total seed production and number of eggs per spawner were not affected by dietary vitamin C or E supplementation. However, the total number of spawns in hapa were significantly higher in fish fed with the Zinc supplemented diet compared to those fed with other experimental diets. The brood fish fed diets supplemented with 1250 mg kg^{-1} vitamin C and 600 mg kg^{-1} vitamin E had a significantly higher mean clutch size compared to the fish fed with other diets except the fish fed with 50 mg kg^{-1} vitamin C and 600 mg kg^{-1} vitamin E in the diet. The hatching rate and larval survival rate of the fish were significantly affected by feeding the fish 1250 mg kg^{-1} of vitamin C. Fish fed with a diet without vitamin C had reduced sperm viability and sperm motility compared with the fish fed with supplementary vitamin C (1250 mg kg^{-1}) in the diet. The present study suggests that supplementation of vitamin C, E and Zn would improve the total number of spawns, total seed production, mean

fecundity, hatching rate, sperm motility and sperm viability of *O. niloticus*. However consistent interactive patterns among tested levels of vitamin C, vitamin E and Zn were not evident in *O. niloticus* in relation to the reproductive performance parameters tested.

Introduction

Tilapia is the second most important farmed fish in the world, after carps (El-Sayed 2002). Despite the great potential of tilapia, shortage of fry production to meet increasing global demands remains one of the major obstacles that limit the expansion of intensive culture of tilapia (Jalabert and Zohar 1982). An improvement in broodstock nutrition and feeding should be reflected by high quality eggs, sperms and also a quality and quantity of seed produced.

Some of trace elements and vitamins have been linked with brood fish growth and egg quality (Sandnes et al. 1984). Vitamin C is considered to be an essential component in diets for teleost fish (Haliver 1985; Dabrowski and Ciereszko 2001,). Numerous studies have shown that the dietary supplemented ascorbic acid has a positive effect on the reproductive performance of Rainbow trout (*Salmo gairdneri*) (Sandnes et al. 1984; Waagbo et al. 1989), Milkfish (*Chanos chanos*) (Emata et al. 2000), Tilapia (*Oreochromis mossambicus*) (Soliman et al. 1986b) and Atlantic salmon (*Salmo salor* L.) (Eskelinen 1989). Ascorbic acid requirements may be correlated with fish ontogeny, for instance larval metamorphosis or gonadal maturation and also may have particular relevance to completion of reproduction and quality of gamates and fertility (Dabrowaki and Clereszko, 2001). Sandnes et al. (1984) and Soliman et al. (1986b) pointed out that brood fish dietary ascorbic acid is transferred to the eggs, where it is stored for supporting growth and development of the larvae until the first feed intake. Increased ascorbic acid content in gonads during maturation has been shown for carp, *Carassius carassius* (Seymour 1981) indicating that the ascorbic acid of brood fish is important for successful breeding. The role of vitamin C was found to be related to vitellogenesis as well as maintaining semen quality (sperm concentration and sperm motility) in rainbow trout, *Oncorhynchus mykiss* (Ciereszko and Dabrowski 1995; Ciereszko and Dabrowski 2000; Ciereszko et al. 1999).

Vitamin E (∝-Tocopherol) is another important micronutrient that affects the reproductive performance of fishes. Increasing vitamin E in the diet increases spawning success, egg survival, hatchability and larval survival of Ayu (*Plecoglossus altevilis*), red sea bream (*Pagrus australis*), increase the gonado-somatic index and vitellogenesis of common carp (*Cyprinus carpio*) (Watanabe and Takashima 1977; Kanazawa 1985,) and big head carp (*Aristichthys nobilis*) (Santiago and Gonzal 2000). Watanabe (1985) showed that the vitamin E content is high in eggs and low in tissues of brood fish

after spawning, suggesting some physiological function of this vitamin in spawning, fertilization and hatching.

Zinc is an important trace element in fish nutrition as it is involved in several metabolic pathways (Watanabe et al 1997). It has been suggested to be important for fish reproduction (Satoh et al. 1983). Based on the past literature, Gatlin and Wilson (1984) suggested to evaluate the Zinc essentiality for fish growth.

Present study investigates the effects of vitamin C, vitamin E and their interactive effect on brood fish and seed quality of Nile tilapia (*Oreochromis niloticus* L.). Additionally, supplementation of higher level of Zinc on gamete quality was also evaluated.

Materials and Methods

Experimental location and experimental units

The study was conducted for 5 months in the earthen ponds at the Asian Institute of Technology, Bangkok, Thailand. The experiments were conducted using twenty-one 3× 2 × 1.2 m fine mesh nylon hapas (cages) installed three ponds (200 m^2). Female and male *Oreochromis niloticus*, chitralada strain (size range 20-30 g) was stocked at male and female ratio of 1:1 and density of 5 fish m^{-2} in each hapa. The batch weights of male and female in each hapa were recorded to the nearest gram before stocking in the net cages. The ponds were fertilized weekly at a rate of 3 kg N ha^{-1} day^{-1} and 1.5 kg P $ha^{-1}day^{-1}$ using urea and TSP.

Experimental diet

The experimental diets were formulated with three levels of vitamin C (0.0, 50 & 1250 mg kg^{-1} in the diet) and two levels of vitamin E (0.0 & 300 mg kg^{-1} in the diet) and their combinations (Table 1). After the first month dietary vitamin E level increased up to 600 mg kg^{-1}. To evaluate the effect of Zinc supplementation, additional diet was added with higher vitamin C and E with 120 mg kg^{-1} Zinc sulphate. Fish were fed two times a day with experimental feeds for 5 months at a rate of 6% of the body weight per day during first month experimental period, and thereafter at a rate of 4% of the body weight per day. The feeding rate was adjusted using batch weight every month.

Nutrient analysis of diets and fish

Moisture content was determined by oven drying at 100^{0}C for 24 h. Crude protein level was tested by using a Kjeltec machine (model Tecator Kjeltec System 1026 Manual, 1987). Crude lipid was determined using the Soxhlet system (model Tecator Soxtec System HT 1043-001 Manual. 1983) for extracted lipid samples by petroleum ether. Tecator fibertec system 1010 heat extractor was used in acid and alkali digestion of samples to determine

crude fiber content. Ash content was determined by ignition of samples in a muffle furnace at 550 ^{0}C for 16 hours. Nitrogen free extract (NFE) was calculated by the difference.

The L-Ascorbic-2-polyphosphate in gonadal tissues was determined using reserved phase high performance liquid chromatography (HPLC) with UV detection at 254 nm as described by Keck and Schuep (1988). The alkaline saponifications of samples were used to determineα-tocopherol concentration as described by Manz and Philipp (1988).

Spawning efficiency and seed production

The spawned females in each treatment unit was tagged by using PIT tags. Eggs were removed weekly from brooder's buccal cavity and number of eggs in each spawn and the brooder's body weight were recorded. Egg clutches were classified based on observation according to Juntana (1990). The total number of eggs per individual fish was estimated by taking total weight of eggs and the mean weight of sub samples containing 100 eggs into account. The mean dry weight of egg was determined to the nearest 0.0001 g by taking random samples containing 50 eggs and then oven dried at 50^{0}C for 24-48 hrs. (Rana 1985). Ten eggs were examined under calibrated binocular microscope to measure egg diameter. Since eggs were ellipsoid-shape, both axes (long and short axes) were measured in order to calculate mean egg diameter (Coward and Bromage 1999).

Egg hatching

One hundred stage 1 eggs (undeveloped eggs) from individual spawning were loaded in to the down-welling incubation units. After 20 hrs, samples were removed from each incubator and fertilization success was determined as the percentage of eggs undergoing embryonic development. After eggs were hatched, yolk sac larvae were transferred to 40×25 cm. aluminum trays with a flow through water system. The fertilization rate refers to the ration of fertilized eggs to the total number of eggs as a percentage. The percentage of egg hatchability is the ratio of number of eggs hatched to the total number of fertilized eggs as a percentage Larval survival rate is the ratio of number of larvae survived until completely yolk sac absorption to the total number of eggs hatch as percentage.

Female and male in each experimental unit were checked once a month to determine number and weight of batch to calculate growth and survival rate. A three females and three males were randomly sampled for the determination of final gonadosomatic index (GSI).

Table 1: The ingredients and proximate composition of experimental diets (g 100^{-1} g of feed)

Ingredient	Treatment diets						
Composition (%)	C0/E0	C50/E0	C1250/E0	C0/E600	C50/E600	C1250/E600	C1250/E600/Zn
Fish meal	25	25	25	25	25	25	25
Soy bean meal	35	35	35	35	35	35	35
Rice bran	27	27	27	27	27	27	27
Fish oil	1	1	1	1	1	1	1
Cassava starch	10	10	10	10	10	10	10
Mineral premix	1	1	1	1	1	1	1*
Vitamin-C	0	0.015	0.36	0	0.015	0.36	0.36
Vitamin-E	0	0	0	0.06	0.06	0.06	0.06
Vitamin premix	0.566	0.566	0.566	0.566	0.566	0.566	0.566
Ricehull (carry)	0.434	0.419	0.074	0.374	0.359	0.014	0.014
Proximate composition							
Moisture	7.78	7.74	7.31	7.54	6.53	8.16	8.36
Crude protein	36.90	37.3	37.39	36.81	36.7	36.64	36.48
Crude lipids	9.00	8.88	8.92	9.17	9.23	8.88	8.49
Ash	10.89	10.88	10.93	10.67	10.65	10.98	10.87
Crude fiber	4.58	4.7	5.18	4.15	4.48	4.49	5.12
NFE	38.62	38.24	37.59	39.2	38.94	39.02	39.05

Mineral premix (mg/100 g. of diet): Manganese-5.4, Iron – 14.2, Copper - 1.0, Zinc - 2.9, Na-3.3, Iodine - 0.019, K- 0.0009, Cobalt - 0.0011, others- 1.0. Vitamin premix- Rovi mix 2118 (mg/100 g. of diet): Vitamin A-13.4, Vitamin D - 0.02, Vitamin K_3 (Menadione sodium bisulfite) - 6.8, Vitamin B_1 (Thiamine mononitrate) - 7.283, Vitamin B_2 (Riboflavin) - 12.5, Vitamin B_6 (Pyridoxine hydrochloride) - 9.756, Vitamin B_{12} - 1.35, Niacin - 53.0, Calpan (Calcium D. Pantothenate) - 29.444, Folic acid - 4.125, Biotin - 16.75, Inositol - 135, Endox - 0.2, Sipernat – 20.
* supplemented 0.012 g Zn/100 g. feed

Sperm quality

Sperms were collected once a month from randomly selected three male tilapia, in each experimental units. Number of males having milt were counted. Total volume (ml) of milt from three randomly selected males were measured by using a measuring clinger. The sperm samples were stored in ice for further analysis. One µl of thoroughly mixed sperm sample was taken using micropipette and the sub sample was diluted by adding 199 µl of distilled water to a microscope vial. Total number of sperm heads in a sub sample was counted using haemocytometer-counting chamber under binocular a light microscope with high magnification (× 40).

Motility of sperm in each individual sample was evaluated within two hours after collection. One µl. of sperm sample was thawed and placed on a microscope slide. Additional 20µl of distilled water was added and mixed thoroughly and quickly (1-5 sec.). Sperm motility was estimated by counting the sperms that are actively swimming in a forward motion using 40 × magnification of a compound microscope. A scale from 0 to 100% was used, where 0, 25, 50, 75 and 100% respectively corresponded to all sperm immotile, most sperm immotile or with few slight vibrating, few sperm progressively motile, most sperm progressively motile and all sperm progressively motile. Eosin-nigrosin dye was used to differentiate live or dead sperm, which become white or pink/violet in color respectively (Hambananda and Mongkonpunya 1996).

Statistical analysis

Experimental data were analyzed using 3×2 factorial analysis of variance (ANOVA) with CRBD to detect the effects of vitamin C, E and their interaction on various performance parameters. Duncan's multiple range test was used to identify the significant differences of mean values of seven treatments at 5% ($p<0.05$) probability level to detect the effect of vitamins and Zinc. Percentage data were subjected to arcsine transformation before statistical analysis. Microsoft Excel and SPSS software were used for data analysis.

Results

Growth and survival of brood fish

Vitamin C deficient female fish exhibited significantly lower daily weight gain (0.47 ± 0.05 g day^{-1}) compared to the supplementation of higher vitamin C in the diet (0.56 ± 0.03 g day^{-1}) ($p< 0.05$). However supplementation of vitamin E had no effect on growth parameters. There was no effect of supplementation of either vitamin C or vitamin E on the daily weight gain of male brood fish ($p > 0.05$). The survival of male or female brood fish was not affected by supplementation of dietary vitamin C or E or Zinc in the diet (Data not given).

Nutritional parameters

Brood fish fed with diet without vitamin C and E showed elevated moisture content compared to the other diets (Table 2). There were no significant ($p>0.05$) effect of vitamin C and E on protein, fibre and NFE contents in fish body. Even though there were significant differences among diets in relation to the lipid content, no consistent pattern was seen among the fish groups, probably due to the interative effects of dietary components. Supplementation of vitamins C, E and Zinc significantly increased carcass ash content compared to the diet without vitamin C or E .

Spawning efficiency

The percentage of spawning and frequency of spawning in fish did not significantly increase by dietary vitamin C, E or Zinc supplementation (Table 3). Diet supplemented with 120 mg kg^{-1} Zinc ($C_{1250}/E_{600}/Zn$) significantly increased total number of spawns when compared to all other dietary treatments ($p<0.05$).

Seed production

The highest total seed production (10,991/ hapa) during three months egg collection period was observed in fish fed with 120 mg kg^{-1} Zinc (with higher vitamin C and E) supplemented dietary treatment (Table 4). However, differences were not statistically significant when compared with the fish fed with 50 mg kg^{-1} vitamin C alone (C_{50}/E_0) or the diet supplemented with 50 mg kg^{-1} vitamin C and 600 mg kg^{-1} vitamin E (C_{50}/E_{600}). Data on 3×2 factorial analysis of variance (ANOVA) showed that mean clutch size (mean fecundity) of *O. niloticus* fed with diet without vitamin C suplementation significantly reduced the clutch size compared to the fish fed with vitamin C 1250 mg kg^{-1} and vitamin E 600 mg kg^{-1} diet (Table 4). The mean number of egg produced per spawner (absolute fecundity) did not significantly increase ($p>0.05$) by feeding fish with higher levels of vitamin C and E. No significant differences were observed between treatments in relation to the number of seed /spawner and number of seed/female/day ($p>0.05$) (Table 4).

Seed quality

High concentration of vitamin C, E or Zinc in *O. niloticus* brood fish diet did not significantly affect the egg diameter, dry weight of eggs and lipid content of eggs.

The mean fertilization rate was not affected by dietary vitamin C, E or Zinc supplementation in the diet ($p>0.05$). The maximum and minimum hatching rate based on percentage of total fertilized number of eggs were 46.51% (for C_{50}/E_{600}) and 19.13% (for C_0/E_0), respectively. The supplementation of only 1250 mg kg^{-1} vitamin C 39.93% (for C_{1250}/E_0) or supplementary vitamin C and E in the diets (46.51%, 45.92% and 38.59%) significantly increased the hatching rate compared with control (C_0/E_0) (19.13%) diet. Supplementation of vitamins C, E and Zinc in the diets appears to increase the larval survival rate compared to the fish fed with no supplementation of vitamin C, E and Zinc. However the differences were not significant (Table 4).

Table 2: Initial and terminal proximate composition (mean± SD) of *Oreochromis niloticus* fed with three different levels of vitamin C (0, 50, 1250 mg kg^{-1}) with two levels of vitamin E (0, 600 mg kg^{-1}) and supplementation of 120 mg kg^{-1} Zn in the experimental diet during five months experimental period (dry matter basis).

Nutrients	Treatment diets							
%	Initial	C0/E0	C50/E0	C1250/E0	C0/E600	C50/E600	C1250/E600	C1250 /E600/Zn
Moisture	77.03	77.42±1.58^{a}	71.27±1.68^{bc}	70.89±1.76^{c}	72.66±1.05^{bc}	72.45±2.44^{bc}	72.16±1.80^{bc}	74.87±0.48^{ab}
Crude protein	67.33	63.92±1.00^{a}	66.53±2.22^{a}	64.10±3.04^{a}	64.37±3.72^{a}	65.40±2.11^{a}	66.54±3.09^{a}	66.26±1.36^{a}
Crude lipids	4.89	13.07±3.64^{a}	8.31±1.59^{abc}	10.78±3.76^{ab}	6.90±2.57^{bc}	8.35±2.67^{abc}	11.75±3.24^{ab}	4.09±0.28^{c}
Ash	17.36	18.13±1.68^{bc}	17.65±1.56^{bc}	16.12±2.24^{c}	21.08±1.17^{ab}	17.65±2.38^{bc}	19.42±2.19^{abc}	22.44±2.09^{a}
Crude fiber	0.69	0.74±0.57^{a}	0.45±0.32^{a}	0.14±0.06^{a}	0.40±0.12^{a}	0.47±0.27^{a}	0.21±0.15^{a}	0.39±0.05^{a}
NFE	9.73	4.13±0.51^{a}	7.06±2.54^{a}	8.87±6.27^{a}	7.26±2.78^{a}	8.12±2.02^{a}	2.07±1.92^{a}	6.83±0.40^{a}

Figures in the same rows with different superscripts are significantly different ($p<0.05$).

Table 3: Spawning efficiency of *Oreochromis niloticus* fed with different experimental diets during 5 months experimental period.

Spawning efficiency	Treatment diets						
	C0/E0	C50/E0	C1250/E0	C0/E600	C50/E600	C1250/E600	C1250/E600/Zn
Spawning percentage	48.89±7.7[a]	64.44±7.7[a]	57.78±21.43[a]	60.00±29.06[a]	55.56±36.72[a]	42.22±20.37[a]	80.00±28.28[a]
Total spawns/hapa	11±5[b]	15±3[b]	12±5[b]	12±6[b]	15±11[b]	11±7[b]	25±6[a]
Spawning frequency	1.51±0.53[a]	1.59±0.43[a]	1.36±0.21[a]	1.34±0.09[a]	1.55±0.48[a]	1.63±0.55[a]	2.17±0.24[a]

Figures in the same rows with different superscripts are significantly different ($p<0.05$).
Spawning percentage = Number of spawn females/Number of total females
Spawning frequency = Total number of spawns/Number of female spawned

Table 4: Seed production and seed quality of *Oreochromis niloticus* fed with different experimental diets during 5 months experimental period. Figures in the same rows with different superscripts are significantly different (P<0.05), Number of seed/spawner = Total seed produced/Number of female spawned, Clutch size = Total seed produced/Total number of spawns

Parameter	Treatment diets						
	C0/E0	**C50/E0**	**C1250/E0**	**C0/E600**	**C50/E600**	**C1250/ E600**	**C1250/ E600/Zn**
Seed production							
Clutch size	480±84^{bc}	510±95^{bc}	476±28^{bc}	412±26^{c}	556±96^{ab}	626±80^{a}	438±57^{bc}
Seed/ spawner	719±243^{a}	795±222^{a}	650±143^{a}	554±56^{a}	831±143^{a}	995±269^{a}	956±227^{a}
Seed/kg body weight	6303±626^{ab}	6237±514^{ab}	6081±923^{b}	4773±651^{c}	6546±844^{ab}	7462±588^{a}	6425±180^{ab}
Total seed production/hapa	5304±2217^{b}	7576±1617^{ab}	5768±2738^{b}	5093±2742^{b}	7441±5299^{ab}	6711±3984^{b}	10991±1334^{a}
Seed/m^2/day	10±4^{b}	14±3^{ab}	11±5^{b}	9±5^{b}	14±10^{ab}	12±7^{b}	20±2^{a}
Seed /female/day	8±3^{a}	9±2^{a}	7±2^{a}	6±1^{a}	9±2^{a}	11±3^{a}	11±2^{a}
Seed quality							
Egg diameter (mm)	2.13±0.02^{abc}	2.07±0.07^{bc}	2.14±0.05^{ab}	2.20±0.07^{a}	2.04±0.03^{c}	2.12±0.08^{abc}	2.11±0.01^{bc}
Egg dry weight (mg)	1.90±0.11^{a}	1.65±0.23^{a}	1.86±0.10^{a}	2.05±0.26^{a}	1.67±0.07^{a}	1.88±0.29^{a}	1.76±0.01^{a}
Egg lipid percentage (%)	21.03±2.71^{a}	22.10±1.21^{a}	20.90±1.22^{a}	23.00±2.00^{a}	19.98±1.05^{a}	21.06±1.06^{a}	23.13±1.43^{a}
Fertilization rate (%)	76.19±5.58^{a}	84.01±3.17^{a}	79.50±10.40^{a}	83.67±4.37^{a}	80.88±9.78^{a}	88.89±4.68^{a}	88.57±6.36^{a}
Hatching rate (%)	19.13±8.56^{c}	24.63±6.49^{bc}	39.93±1.57^{ab}	24.65±7.53^{bc}	46.51±13.82^{a}	45.92±6.33^{a}	38.59±13.26^{ab}
Larval survival rate (%)	57.74±15.02^{a}	73.74±12.66^{a}	80.18±6.75^{a}	65.03±16.24^{a}	78.69±4.45^{a}	81.09±2.93^{a}	73.03±10.05^{a}

Final GSI of males and females (the means) ranged from 0.5% to 0.66% in males and from 1.86% to 3.47% in females. However final GSIs were not affected by supplementation of the vitamin C or vitamin E or zinc ($p>0.05$). (Result not shown)

Sperm quality

The number of males having milt, mean individual milt volume and sperm concentration in males did not reveal any significant differences between the treatments ($p>0.05$) (Table 5). The mean sperm concentration among males varied from 2.01×10^9 to 4.28×10^9 number ml^{-1} between treatments. Results of 3×2 factorial analysis of variance (ANOV A) showed that the sperm motility was increased in the fish fed with higher levels of vitamin C (1250 mg kg^{-1}) compared with the control and the fish fed diet containing 50 mg kg^{-1} of vitamin C.

The sperm viability was significantly higher in fish fed with vitamin C at a rate of 1250 mg kg^{-1} in the diet compared with control diet ($P<0.05$). However, there were no significant differences in sperm viability between fish fed diets containing 50 and 1250 mg kg^{-1} vitamin C.

Ascorbic acid and ∝-tocopherol level in the gonads

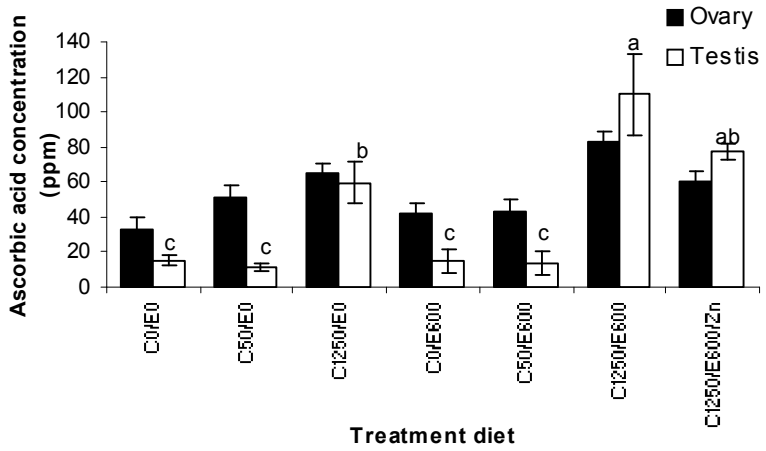
The ovarian ascorbic acid concentration increased with increasing dietary ascorbic acid levels, but there were no significant differences (Fig. 1a). The ascorbic acid level in the testis significantly increased with higher dietary vitamin C in the diet ($p<0.05$). The tissue levels of α-tocopherol in ovary and testis were significantly higher in the fish fed with a diet supplemented with vitamin E(α-tocopherol) (Figure 1b).

Discussion

Growth and survival and body composition of fish

Vitamin C is shown to be necessary for growth, reproduction and immune response. The results presented in this study also indicated the beneficial effect of dietary ascorbic acid level on growth response of female Nile tilapia. Soliman et al. (1986a) showed significantly lower ($p<0.05$) specific growth rate results in juvenile *Oreochromis niloticus* fed with ascorbic acid free diet compared to a diet supplemented with 125 mg ascorbic acid per 100g feed. The weight gain of juvenile hybrid tilapia (initial body weight =1.53g) was highest when the dietary supplementation of L-ascorbic acid at 90 mg kg^{-1} in the diet (Shiau and Hsu 1995).

(a)



(b)

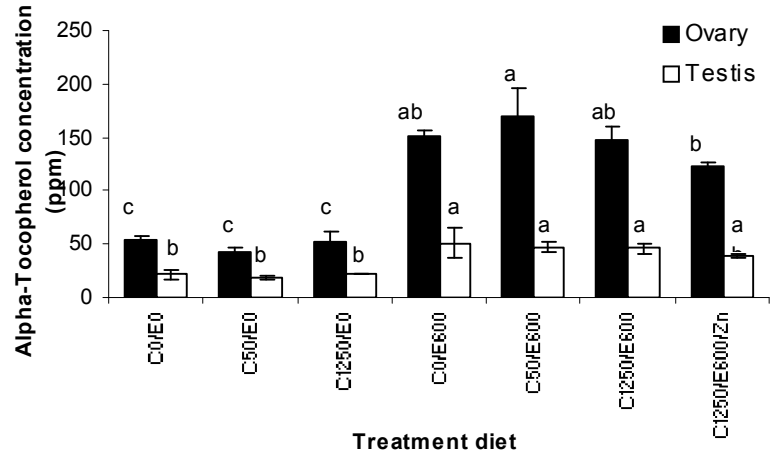


Figure 1. (a) Gonadal concentration of ascorbic acid (ppm) and (b) Gonadal concentration of α-tocopherol (ppm) in *Oreochromis niloticus* fed with seven experimental diets. For ovary and testis, each bar with different letters represents significantly different mean values among dietary treatments ($p<0.05$). Error bars are ± standard deviation.

Dietary ascorbic acid requirement for maximum growth and survival of common carp larvae is about 45 mg kg^{-1} (Gouillou – Coustans et al. 1998). The major reason for the positive effect of ascorbic acid on growth is due to important role in collagen formation, which is necessary for normal growth (Masumoto et al. 1991).

The vitamin C requirement of larvae and juvenile Indian carp,*Cirrhinus mrigala* was estimated to be 700 mg kg^{-1} in diet (Mahajan and Agrawal 1980), which was much higher than other cyprinid fish species. Nitzan et al. (1996) who conducted an experiment on hybrid tilapia in pond system, suggested that inclusion of vitamin C does not cause an increased growth rate enrichment at a level of 458 mg kg^{-1} feed with ascorbic acid polyphosphate. However, Anadu et al (1990) recommended to add ascorbic acid at a level of 3000 mg kg^{-1} for better growth and development of*Tilapia zillii* in intensive culture.

Vitamin C requirements may depend on various factors such as fish species, size, age, growth rate, stage of sexual maturity, smoltification, type of diet, processing and storage time of the diet as well as environmental stressors (disease, water temperature, water quality) and levels of environmental toxicants (Gouillou-Coustans and Kaushik 2000). Moreover, exact vitamin C requirement also depends on interactions between ascorbic acid and other nutrients.

Juvenile hybrid tilapia fed with vitamin E deficient diets supplemented with dietary vitamin C (80 mgkg^{-1} diet), showed lower weight gain than the fish fed with both vitamins C and E containing diet (Shiau and Hsu 2002). However, interactive effect of vitamin C and E on growth of Nile tilapia brood fish was not observed in the present study. Gatlin et al. (1986) reported that the vitamin C does not have a sparing effect on supplementation vitamin E in channel catfish diet. Recent work on *O. niloticus* fingerlings fed with excessive ascorbic acid (2000 mg kg^{-1}) and excessive ∝-tocopherol (240 mg kg^{-1}) in the diet significantly increased their weight gain, but there was no synergistic effect between vitamin C and E on growth performance (Kim et al. 2003). Similar to the results of present study, previous studies on several other fish species, such as Indian major carp, *Labeo rohita* (Sahoo and Mukherjee 2002), Rainbow trout, *Salmo gairdneri* (Blazer and Wolke 1984) and Atlantic salmon, *Salmo salar* (Hardie et al. 1990), channel catfish, *Ictalurus punctatus* (Jarboe et al. 1989; Silva et al. 1994) have shown no effect of dietary vitamin E on growth rate. In the present experiment relatively high level of vitamin E (12 times higher than normal requirement of tilapia as recommended by NRC, 1993) was supplemented in the diet. The feed ingredients in present experimental diets should have supplied adequate amount of vitamin E for growth of fish. Moreover, natural food sources in environment could also may have contribution to fulfill the vitamin E requirement of tilapia and ascorbic acid has the ability to reduce α-tocopheroxyl radicals and thereby regenerate toα- tocopherol.

There were no significant diet related differences in percentage of crude protein and fibre content in whole fish at the end of the study period. But there were differences in percentage of moisture, lipid and ash. In earlier studies fish fed with diet containing no ascorbic acid exhibited elevated moisture content in Nile tilapia (Soliman et al. 1994) and grass carp (Takeuchi et al 1992).

Spawning efficiency, seed production and seed quality

Results of present study show that there was no significant effect of supplementation of vitamin C and E on the spawning percentage, spawning frequency and total spawns hapa^{-1}. Emata et al. (2000) pointed out that the total egg production, mean number eggs per spawning and number of spawns were not affected by supplementation of 1000 mg kg^{-1} vitamin C and 500 mg kg^{-1} vitamin E in the diet of milk fish. Recent studies on Nile tilapia by Areechon et al. (2003) reported that the higher vitamin E (600 mg kg^{-1}) in the diet increased number of spawners.

The present study found that the supplementation of high levels in the diet vitamin C enhanced hatching rate rather than total egg production. Emata et al. (2000) pointed out that the essentiability of supplementation vitamin C in milkfish, *Chanos chanos* feed. In the Milk fish vitamin E did not affect the total seed production or egg quality. This is agreeable with the results of the present study obtained for Nile tilapia. The elevated levels of antioxidant vitamins in the diet of fish, above the required level for growth, affect phagocytosis, antibody production and may stimulate the immune response. However, little information is available on negative effects of mega doses of vitamin C and E on fish growth or reproduction. Excess tocopherol appears to be reasonably harmless, perhaps because it can be easily stored in lipids (Guillaume et al. 2001). Nevertheless some other studies, Tokuda and Takeuchi (1995) showed that excess doses of α-tocopherol might induce lipid peroxidation in tissues of rainbow trout. When the amount of vitamin C is not enough to scavenge all α-tocopheroxyl radical in the tissues, it produce a lipid alkoxyl radicals that react with lipid substract and further reacts with oxygen to generate other lipid peroxyl radicals (Kaewsrithong et al 2001). These free radicals are very harmful to cell organelles and even to macromolecules. The terminal compounds are stable but are toxic (Guillaume et al. 2001). The lipid reserves are important for reproduction and insufficiency of reserves leads to decrease in fecundity and gonadal development. This is a plausible reason behind observed reduction in fecundity and seed production in fish fed with diet without supplementation of vitamin C and higher vitamin E. Thus, the balance between vitamin C and E in biological tissues might be one of the most important factor affecting the physiological condition of fish. Further studies are needed to clarify the interactive physiological role between vitamin C and E in fish.

Supplementation of vitamin C significantly improved the egg hatchability. Larval survival rates appear to be improved by vitamin C. However earlier studies showed that there was no significant improvement of the hatching rate of tilapia supplemented with either 1000 mg kg^{-1} vitamin C or 600 mg kg^{-1} vitamin E and the combination (Areechon et al. 2003). Mangor-Jesen et al (1994) described that there were no significant differences in total egg production, mean fertilization, survival of embryos and concentration of ascorbic acid in developing ovaries of cod (*Gadus morhua*) fed 0, 50, 500 mg kg^{-1} vitamin C in the diet.

Table 5: Sperm quality of *Oreochromis niloticus* fed different experimental diets.

Reproductive parameters	Treatment diets						
	C0/E0	C50/E0	C1250/E0	C0/E600	C50/E600	C1250/ E600	C1250/ E600/Zn
Number of males running milt	3.44±1.26[a]	4.22±0.96[a]	4.44±1.07[a]	4.11±1.50[a]	4.00±1.53[a]	4.78±2.50[a]	3.06±0.92[a]
Total milt volume (ml)	0.12±0.02[a]	0.09±0.04[a]	0.15±0.06[a]	0.08±0.04[a]	0.11±0.02[a]	0.10±0.02[a]	0.08±0.06[a]
Sperm concentration (Number/ml)× 10^9	2.01±0.65[a]	2.81±1.26[a]	3.30±1.61[a]	3.18±1.48[a]	3.28± 0.80[a]	3.48±0.58[a]	4.28±0.42[a]
Sperm motility (%)	61.11±19.69[a]	51.39±12.03[a]	78.51±19.28[a]	58.33±14.43[a]	61.11±14.63[a]	75.93±6.69[a]	61.67±12.58[a]
Sperm viability (%)	42.18±5.19[a]	60.35±9.80[a]	69.64±8.90[a]	62.16±13.39[a]	51.64±9.58[a]	66.42±9.61[a]	68.64±10.49[a]

Figures in the same rows with same superscripts are not significantly different (P>0.05).

Soliman et al. (1986b) and Sandnes et al. (1984) showed that high ascorbic acid levels in the brood stock ovaries get transferred to the eggs, where it is stored for growth and development of the larvae until first feed intake. The main reason for the positive effect of ascorbic acid in fish egg hatchability and fry survival might have resulted from deposition of ascorbic acid during the period of vitellogenesis and embryogenesis. In the present study highest ovarian ascorbic acid concentrations (64.45 and 82.63 ppm) were recorded for fish fed with higher vitamin C in the diet compared with fish fed with diet without supplementation ascorbic acid. This would explain why higher hatching and larval survival recorded for the fish fed with high vitamin C diets.

The survival in eying stage of the fertilized eggs get reduced when ayu (*Plecoglossus altivelis*) fed diets with low ∝- tocopherol levels (Takeuchi et al 1981). Cahu et al. (1995) studying on *Penaeus indicus* showed that egg hatching rate and egg ∝- tocopherol concentration were positively correlated. Gupta et al (1991) treated common carp, *Cyprinus carpio* fed with vitamin E diets showed higher gonado somatic index, bigger ova and more eggs. The histological studies demonstrated that when ∝- tocopherol deficient ovaries did not accumulated yolk-granules or yolk – vesicles in oocytes and retarded oocyte development (Watanabe and Takashima 1977). In the present study, dietary vitamin E supplementation was provided to the broodstook for 5 months, yet Gonado-Somatic Index and egg diameter were not enhanced. King et al. (1983) showed no differences in rainbow trout growth, pre spawning mortality rate, egg development or egg hatchability between the two diets, one containing ∝-tocopherol and one lacking it. High concentrations of ∝- tocopherol in the Atlantic salmon brood stock diet did not increase survival of eggs and fry (Eskelinen 1989).

Sperm quality

Fish fed with a vitamin C free diet significantly reduced the sperm concentration, total sperm production and sperm motility of rainbow trout (Ciereszko and Dabrowski 1995). However, in the present study, fish fed diet without ascorbic acid supplementation did not show significant effect on sperm concentration. Ciereszko et al (1999) and Dabrowaki and Ciereszko (1996) mentioned that low antioxidant levels have been implicated in oxidative damage to sperm DNA. Vitamin C-free diet caused severe histological damage to reproductive tissues and resulted in increase in sperm pathology. Moreover, vitamin C and E are strong scavengers and have been shown to have a protective role against the action of free radicals, which were responsible for deteriorating egg membranes and membrane integrity (Izquierdo et al. 2001).Waagbo et al. (2000) pointed out that high concentration of vitamin E in the diets act as a pro-oxidant, especially when ascorbic acid concentration is low. This could be a reason for not having any negative effect on sperm quality of fish fed diet supplemented with higher vitamin E and low vitamin C in the present study. Ciereszko and Dabrowski (2000) evaluated supplementation of ascorbic acid on motility and fertilization ability of rainbow trout, *Oncorhynchus mykiss*, sperm after storage. They suggested that the positive effect of ascorbic acid on sperm quality is related to its long-term effects during

spermatogenesis. Ciereszko and Dabrowski (1995) also showed that two year old rainbow trout were maintained on diet supplemented with 0, 30, 110, 220, 440 and 810 ppm ascorbyl monophosphate influenced sperm quality. The improvement of sperm quality (sperm viability, motility and sperm concentration) with supplementation of vitamin C in brood stock diets is less critical for external fertilization because several other factors such as, osmotic pressure, ionic composition, pH and water temperature are the most important factors determine the activation of sperm.

In conclusion, the present study support the use of vitamin C, E and Zn supplementation in the diet for the improvement of certain reproductive performance parameters in *O. niloticus* such as total number of spawns, total seed production, fecundity, hatching rate, sperm motility and sperm viability. However consistent interactive patterns among tested levels of vitamin C, vitamin E and Zn were not evident *in O. niloticus* in relation to the reproductive performance parameters tested.

Acknowledgements

This research was conducted for a Master thesis of M. Gammanpila for Aquaculture and Aquatic Resources Management program at School of Environment, Resources and Development, Asian Institute of Technology, Thailand. The financial support is from a Government of the Netherlands and Roche Aquaculture Centre, Asia Pacific.

References

Anadu, D. I., O. C. Anozie & A. D. Anthony 1990.
Growth responses of *Tilapia zillii* fed diets containing various levels of ascorbic acid and cobalt chloride. Aquaculture 88: 329-336.

Areechon, N., C. Thaitoungchin, R. Yoongpundh & S. Bunnom 2003.
Functions of antioxidant vitamins in tilapia broodstock nutrition. The 9th Aquaculture conference Asia Pacific. Bangkok, Thailand pp. 60-74.

Blazer, V. S. & R. E. Wolke 1984.
The effects of ∝- tocopherol on the immune response and non-specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). Aquaculture 37: 1-9.

Cahu, C. L., G. Cuzon & P. Quazuguel 1995.
Effect of highly unsaturated fatty acids, alpha-tocopherol and ascorbic acid in brood stock diet on egg composition and development of *Penaeus indicus*. Comparative Biochemistry and Physiology 112(3-4): 417-424.

Ciereszko, A. & K. Dabrowski 1995.
Sperm quality and ascorbic acid concentration in rainbow trout semen are affected by dietary vitamin C; an across-season study. Biology of Reproduction, 52(5): 982-988.

Ciereszko, A. & K. Dabrowski 2000.

Effect of ascorbic acid supplement in vitro on rainbow trout sperm vialibility. Aquaculture international, 8(1): 1-8.

Ciereszko, A., K. Dabrowski, F. Lin & L. Liu 1999.
Protective role of ascorbic acid against damage to male germ cells in rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences, 56(2): 178-183.

Coward, K. & N. R. Bromage 1999.
Spawning periodicity, fecundity and egg size in laboratory held stocks of a substrate-spawning tilapiine, *Tilapia zillii* (Gervais). Aquaculture 171: 251-267.

Dabrowski, K. & A. Ciereszko 1996.
Ascorbic acid protects against male infertility in a teleost fish. Experientia 52(2): 97-100.

Dabrowski, K. & A. Ciereszko 2001.
Ascorbic acid and reproduction in fish: endocrine regulation and gamate quality. Aquaculture research, 32: 623-638.

Ei – Sayed, A. M. 2002.
Effect of stocking density and feeding levels on growth and feed efficiency of Nile tilapia (*Oreochromis niloticus* L.) fry. Aquaculture Research. 33: 621-626.

Emata, A. C., Bohongan, I. G. & J. P Damaso 2000.
Dietary vitamin C and E supplementation and reproduction of milk fish, *Chanos chanos* Forsskai. Aquaculture Research 31: 557-564.

Eskelinen, P. 1989.
Effects of different diets on egg production and egg quality of Atlantic salmon (*Salmo salar* L.) Aquaculture, 79: 275-281.

Gatlin, D. M. III., W. E. Poe, R. P. Wilson, A. J. Alnsworth & P. R. Bowser 1986.
Effects of stocking density and vitamin C status on vitamin E- adequate and vitamin E-deficient fingerling channel cat fish. Aquaculture 56: 187-195.

Gatlin, D. M. III & R. P Wilson 1984.
Zinc supplementation of practical channel catfish diets. Aquaculture 41: 31-36.

Gouillou-Coustans, M. F., P. Bergot & S. J. Kaushik 1998.
Dietary ascorbic acid needs of common carp (*Cyprinus carpio*) larvae. Aquaculture 161(1-4): 453-461.

Gouillou-Coustans, M. F. & S. J. Kaushik 2000.
Ascorbic acid requirement in fresh water and marine fish: Is there a differences. In: Ascorbic acid in aquatic organisms (K. Dabrowski ed), CRC press. pp. 49-68.

Guillaume, J., S. Kaushik, P. Bergot & R. Metailler 2001.
Nutrition and feeding of fish and crustaceans. Praxis publishing, UK. pp.111-130.

Gupta, S. D., Khan, H. A. & R. M. Bhowmick 1991.
Observations on the effect of vitamin E and growth hormone on the gonadal maturity of carps. Journal of the Inland Fisheries Society of India 19(2): 26-31.

Halver, J. E. 1985.
Recent advances in vitamin nutrition and metabolism in fish. In: Nutrition and feeding in fish (Cowey, C. B., Mackie, A. M., & J. G. Bell eds), pp. 415-429,Academic press, London..

Hambananda, A. & K. Mongkonpunya 1996.
Cryopreservation of milt of striped cat fish,*Pangasius sutchi*. Proceedings of the 34th Kasetsart university annual conference, pp. 320-328, .Kasetsert university press, Bangkok, Thailand.

Hardie, L. J., T. C. Fletcher & C. J. Secombes 1990.
The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.).Aquaculture 87: 1-13.

Izquierdo, M. S., H. Fernandez – palacios & A. G. J. Tacon 2001.
Effect of brood stock nutrition on reproductive performance of fish. Aquaculture 197: 25-42.

Jalabert, B. & Y. Zohar 1982.
Reproductive physiology in cichlid fishes, with particular reference to Tilapia and Sarotherodon. In: The biology and culture of Tilapias (R. S. V. Pullin & R. H. Lowe-McConnell eds), pp. 129-140, ICLARM Conference Proceedings 7,432.p. International center for living aquatic resources management, Manila, Philippines.

Jarboe, H. H., H. R. Robinette & P. R. Bowser 1989.
Effect of selected vitamin E supplementations of channel catfish production feeds. Progressive fish-culturist Washington DC 51(2): 91-94.

Juntana, J. 1990.
Mass incubation of Nile tilapia, *Oreochromis niloticus* in relation to it's proposed introduction in to Udornthani province. MSc. Thesis No As–90–27, Asian Institute of Technology, Bangkok, Thailand.

Kaewsrithong, J., T. Ohshima, H. Ushio, R. Nagasaka, M. Maita & M. Sawada 2001.
Effects of an excess dose of dietary α-tocopherol on hydroperoxide accumulation and erythrocyte osmotic fragility of sweet smelt,*Plecoglossus altivelis* (Temminck et Schlegel). Aquaculture Research 32 (1): 191-198.

Kanazawa, A. 1985.
Nutritional factors in fish reproduction. In: Reproduction and culture of milk fish (Lee, Cheng- Sheng & Liao, I-Chiu eds), pp.115-125, Proceedings of a workshop held at the Tungkang marine laboratory, Taiwan.

Keck, E & W. Schuep 1988.
Determination of L-Ascorbic-2-polyphosphate in complete feeds and premixes. In: Analytical methods for Vitamins and Carotenoids (Keller H. E. ed), F. Hoffmann-La Roche & Co. AG, Basel, Switzerland. pp. 98.

Kim, K. W., X. Wang, S. M. Choi, G. J. Park, J.W. Koo & S. Bai 2003.
No synergistic effects by the dietary supplementation of ascorbic acid,∝ tocopheryl acetate and selenium on the growth performance and challenge test of *Edwardsiella tarda* in fingerling Nile tilapia,*Oreochromis niloticus* L. Aquaculture Research 34, 1053-1058.

King, I., R. W. Hardy & J. E. Halver 1983.
The effect of dietary vitamin E on the distribution of α- tocopherol in Rainbow trout (*Salmo guirdneri*) during ovarian maturation. In: International symposium on salmonid reproduction (R. N. Iwamoto & S. Sower eds), pp.111-112. Bellevue, W. A., Washington sea grant program. Univ. Washington, Seattle, W.A.

Mahajan, C. L. & N. K. Agrawal 1980.
Nutritional requirement of ascorbic acid by Indian major carp, *Cirrhina mrigala*, during early growth. Aquaculture 19: 37-48.

Manz, U & K. Philipp 1988.
Determination of alpha-Tocopherol in Complete Feeds, Premixes and Vitamin C concentrates with the aid of HPLC. In: Analytical methods for Vitamins and Carotenoids, (Keller, H. E., ed), pp.12-14, F. Hoffmann-La Roche & Co. AG, Basel, Switzerland.

Masumoto, T., H. Hosokawa & S. Shimeno 1991.
Ascorbic acids role in Aquaculture nutrition. In: Proceedings of the Aquaculture feed processing and nutrition workshop (Akiyama, D. & R. K. H. Tan eds), pp. 42-48.

National Research Council. 1993.
Nutrient requirements of fish. Washington, D.C. National academy of science.

Nitzan, S., H. Angeonl & N. Gur 1996.
Effects of ascorbic acid polyphosphate (AAPP) enrichment on growth, survival and disease resistance of hybrid tilapia. The Israeli Journal of Aquaculture – Bamidgeh 48(3): 133-141.

Rana, K. J. 1985.
Influence of egg size on the growth, onset of feeding, point of-no-return, and survival of unfed *Oreochromis mossambicus* fry. Aquaculture 46: 119-132.

Sahoo, P. K. & S. C. Mukherjee 2002.
Influence of high dietary∝- tocopherol intakes on specific immune response, non specific resistance factors and disease resistance of healthy and aflatoxin B_1-induced immunocom promised Indian major carp, *Labeo rohita* (Hamilton). Aquaculture Nutrition 8: 159-167.

Sandnes, K., Y. Ulgenes, O. R. Braekkan, & F. Utne 1984.
The effect of ascorbic acid supplementation in brood fish seed on reproduction of rainbow trout (*Salmo guirdneri*). Aquaculture 43:167-177.

Santiogo, C. B & A. C. Gonzal 2000.
Effect of prepared diet and vitamins A, E and C supplementation on the reproductive performance of cage- reared bighead carp *Aristichthys nobilis* (Richardson). Journal of Applied Ichthyology 16 (1): 8-13.

Satoh, S., T. Takeuchi, Y. Narabe & T. Watanabe 1983.
Effects of deletion of several trace elements from fishmeal diets on growth and mineral composition of rainbow trout fingerlings. Nippon Suisan Gakkaishi. 53: 1861-1866.

Seymour, E. A. 1981.
The effects of powdered carp pituitary on ovarian development, ovarian ascorbic acid and ovulation in *Carassius carassius* L. exposed to various photoperiod and temperature regimes. Journal of Fish Biology 19: 675-682.

Shiau, S. Y. & T. S. Hsu 1995.
L-Ascorbyl-2-sulfate has equal antiscorbutic activity as L-ascorbyl-2-monophosphate for tilapia, *Oreochromis niloticus× O.aureus.* Aquaculture 133: 147-157.

Shiau, S. Y. & C. Y. Hsu 2002.
Vitamin E sparing effect by dietary vitamin C in juvenile hybrid tilapia, *Oreochromis niloticus × Oreochromis aureus*. Aquaculture 210: 335-342.

Silva, P. L., E. Robinson, J. O. Hearnsberger & J. L. Silva 1994.
Effect of dietary vitamin E enrichment on frozen channel catfish (*Ictalurus punctatus*) fillets. Journal of Applied Aquaculture 4(3): 45-55.

Soliman, A. K., K. Jauncey & R. J. Roberts 1986a.
The effect of varying forms of dietary ascorbic acid on the nutrition of juvenile tilapias (*Oreochromis niloticus*). Aquaculture 52:1-10.

Soliman, A. K., K. Jauncey & R. J. Roberts 1986b.
The effect of dietary ascorbic acid supplementation on hatchability, survival rate and fry performance in *Oreochromis mossambicus* (Peters). Aquaculture 59; 197-208.

Soliman, A. K., K. Jauncey & R. J. Roberts 1994.
Water-soluble vitamin requirements of Tilapia; ascorbic acid (vitamin C) requirement of Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture and Fisheries Management 25: 269-278.

Takeuchi, M., S. Ishii & T. Ogiso 1981.
Effect of dietary vitamin E on growth, vitamin E distribution and mortalities of the fertilized eggs and fry in ayu, *Plecoglossus altivelis*. Bulletin of the Tokai Regional Fisheries Research Laboratory 104:111-122.

Takeuchi, T., K. Watanabe, S. Satoh & T. Watanabe 1992.
Requirement of grass carp fingerlings for ∝-tocopherol. Nippon Suisan Gakkaishi 58(9): 1743-1749.

Tokuda, M & M. Takeuchi 1995.
Effects of excess doses of α-tocopherol on the lipids and function of rainbow trout liver. Journal of Nutritional Science and Vitaminolog, 41 (1): 25-32.

Waagbo, R., T. Thorsen & K. Sandnes 1989.
Role of dietary ascorbic acid in vitellogenesis in rainbow trout (*Salmo gairdneri*). Aquaculture 80:301-314.

Waagbo, R., K. Hamre & A. Maage 2000.
The impact of micronutrients on the requirement of ascorbic acid in crustaceans and fish. In: Ascorbic acid in aquatic organisms (K. Dabrowski ed), . pp. 105-131,CRC press. USA.

Watanabe, T. 1985.
Important of the study of brood stock nutrition for further development of aquaculture. In: Nutrition and feeding in fish (C. B. Cowey, A. M. Mackie & J. G. Bell eds), pp 395-414, Academic press, London.

Watanabe, T. & F. Takashima 1977.
Effect of α tocopherol deficiency on carp. Deficiency symptoms and changes in fatty acid and triglyceride distribution in adult carp. Bull. Jap. Soc. Sci. Fish 43: 819-830.

Watanabe, T., V. Kiron & S. Satoh 1997.
Trace minerals in fish nutrition. Aquaculture 151: 185-207.