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Impact of different dietary vitamin C contents on growth, survival, fecundity and egg diameter in the zebrafish, *Danio rerio* (Pisces, Cyprinidae)

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Abstract. This study was conducted to examine the Impact of different dietary vitamin C contents on growth, survival, fecundity and egg diameter in the zebrafish, *Danio rerio*. Zebrafish were divided into 5 treatments each replicated three times, and fed with one of five diets for 20 weeks. The experimental vitamin C diets were formulated to contain 250, 500, 1000 and 2000 mg ascorbic acid kg^{-1} with 1 control group (0 mg kg^{-1}). In the vitamin C treatments, the body weight increase (BWI), specific growth rate (SGR) and fecundity of zebrafish increased significantly with increasing the levels of vitamin C ($P<0.05$). There were no significant differences in egg diameter observed between the treatments. The survival rate of zebrafish fed the diets containing 1000 and 2000 mg kg^{-1} AA was higher than other groups. The significance of the results herein obtained underlined the importance of diet in the reproductive process, supporting the hypothesis that feed additives can improve fecundity. Considering that the zebrafish has been clearly established as a vertebrate model for biomedical research, these results support the potentiality of feed additives such as vitamins can improve reproduction in all vertebrates, including humans.

Key Words: zebrafish, Vitamin C, Growth, egg diameter, Survival, fecundity

چکیده. این آزمایش به منظور تعیین اثرات ویتامین C جیره بر پارامترهای رشد (وزن بدن، نرخ رشد ویژه . . .)، بقاء، هماوری و قطر تخمک در ماهی‌گورخری انجام گرفت. مقادیر مختلف ویتامین C (ال-آسکوربیک اسید) شامل دوزهای 250، 500، 1000 و 2000 (میلی‌گرم در کیلوگرم جیره) در سه تکرار به ماهیان داده شد، همچنین یک تیمار بدون ویتامین به‌عنوان تیمار شاهد برای آزمایش با سه تکرار در نظر گرفته شد. زیست‌سنجي ماهیان هر 4 هفته یكبار انجام مي‌گرفت. پس از اینکه ماهیان علائم بلوغ را نشان دادند از هر آکواریوم 3 عدد ماهی انتخاب شده و پس از شکافتن شکم، میزان هماوری آنها محاسبه شد. همچنین از هر هماوری نیز 15 عدد تخمک بصورت تصادفی انتخاب شده و قطر آنها اندازه‌گیری شد. در تیمارهای ویتامین C، با افزایش سطح ویتامین، نرخ رشد ویژه، میزان افزایش وزن بدن و هماوری به‌صورت معنی‌داری افزایش یافتند (P<0/05). در میزان تلفات گروه‌های تغذیه شده با جیره حاوی 1000 و 2000 میلی‌گرم بر کیلوگرم ویتامین C اختلاف معنی‌داری را با سایر تیمارها نشان دادند (P<0/05). قطر تخمک اختلاف معنی‌داری را بین تیمارها نشان نداد. نتایج نشان می‌دهد که ویتامین C به عنوان یک ماده افزودنی به جیره می‌تواند توان تولیدمثلی را در مهره‌داران افزایش دهد.

کلمات کلیدی: ماهی گورخری، ویتامین C، رشد، قطر تخمک، بقاء، هماوری

Introduction. Nutrients like fatty acids, amino acids, minerals and vitamins have clear affects on reproduction as well as growth in fish. Vitamin C, L-Ascorbic acid (LAA) is a one of the micro nutrient elements, which plays an important role in all fish species (Benitez & Halver 1982). Although nutritional requirements of fish were previously reported for breeding purposes, LAA requirements for fish showed variation in respect to their physiology and developmental stage particularly if it the larval stage or gonad development (Dabrowski & Ciereszko 2001). Also LAA plays a critical role on gamete quality of fish like other vertebrates (Ciereszko & Dabrowski 2000). Antioxidants such as LAA protect germ cells against DNA damage and oxidation of seminal plasma proteins with reactive oxygen radicals (Liu et al 1995; Ciereszko et al 1999). Teleost fish cannot synthesis LAA like other vertebrates due to a lack of active gulconolactone oxidase, which is the terminal enzyme in the LAA synthesis pathway (Ciereszko et al 1999). Therefore, a fish's LAA requirements need to be provided within the diet.

The nutritional status of the broodstock can affect offspring quality. The accumulation of essential nutrients such as essential fatty acids and LAA are dependent on the nutrient reserves in the mother animal, and consequently on the dietary input of broodstock in the period preceding gonadogenesis (Blom & Dabrowski 1996; Bell et al 1997). Food or vitamin shortages may have caused suspension of vitellogenesis, resorption of oocytes, and decreased fecundity in the goldfish, *Carassius auratus* (L.) (Bekker 1958).

Zebrafish (*Danio rerio, Cyprinidae*) serves as an important vertebrate model for studying development, genetics, production and diseases (Kimmel 1989; Briggs 2002; Ingham 1997; Ward & Lieschke 2002). Zebrafish is a group-spawning, egg-scattering member of the Cyprinid family found throughout southern Asia (Laale 1977). Its ease of maintenance and ability to produce large numbers of transparent eggs has made it an ideal species for studies (Grunwald & Eisen 2002).

The main purpose of the present experiments was to investigate the influence of different vitamin C levels on reproductive success. This was considered an urgent task because nutritional studies on fecundity and fertility in fish have been mainly concerned with only quantity of food.

Material and Methods

Aquaria: Fifteen aquaria (40X30X50 cm) were used in this experiment. The water temperature was kept at 26±2 °C. The aquarium water was permanently aerated. Before starting the experiments water was dechlorinated. Once a week pH was checked and total hardness in water (dH) was examined titrimetrically. Oxygen content was recorded weekly.

Experimental diets: The basal experimental diets were formulated with the commonly available ingredients (see Table 1). Five graded levels of vitamin C (L-ascorbic acid, AA) at 0, 250, 500, 1000 and 2000 mg Kg^{-1} diets were included in the basal diet (AA was supplemented separately to the basal diet at the expense of wheat flour). The ingredients were grinded, milled, weighed, mixed and pelleted with a meat mincer through a 0.8 mm die. After cold pelleting, the feeds were air dried and put in an air-tight container. All diets were stored at -20 °C until fed.

Experimental fish and feeding regime: Zebrafish (*D. rerio*) were obtained from an Institute of Ornamental Fish Hatchery (Golestan), and were transferred to the place of experiment and acclimated for 2 weeks. Zebrafish were fed a vitamin C-free diet (a basal diet which finally served as the control diet) for 2 weeks while acclimating to experimental conditions. Twenty uniform fish were randomly selected and stocked into each of the 15 aquariums, which in turn were randomly assigned in triplicate to each treatment. Fish were fed approximately 5% of their body weight daily, and it was divided into four equal feedings (08:00, 12:00, 16:00 and 20:00 h) for 5 months. Feed preparation was carried out bi-weekly to prevent long storage. The all of fish from each aquarium were counted and weighed at 2-week intervals to monitor growth and adjust feed rations. Mortalities and general health were recorded. Any dead fish were removed and not replaced during the experiment.

Calculations and statistical analysis: Sampling was carried out fortnightly. The following variables were calculated:

Body weight increase (BWI) = $W_t - W_0$ (Tacon 1990)

Specific growth rate (SGR) = $(\ln W_t - \ln W_0) \times 100\ t^{-1}$ (Hevroy et al 2005)

Body weight gain (BWG) = $(W_t - W_0) \times N_t$ (De Silva & Anderson 1995)

Survival = $N_t \times 100\ N_0^{-1}$ (Ai et al 2006)

Gonadosomatic Index = $GW/BW_t \times 100$

W_t and W_0 were final and initial fish weights (g), respectively; N_t and N_0 were final and initial numbers of fish in each replicate, respectively, t is the experimental period in days, GW was gonad weight and BW was final body weight (g).

Pregnant females were recognized by the convex abdomen and other signs. At the estimated time for parturition, 3 females from each experiment were killed by tricaine methanesulphonate. Fixation was achieved in 4% formalin. Total body length and total body weight (somatic weight plus gonad weight) (Lagler et al 1962) were recorded. The number of fish oocyte (fecundity) of each female was counted and finally the egg diameters were measured (15 oocytes per fecundity).

The data obtained from the trial were subjected to one-way analysis of variance (ANOVA) and T-test (using SPSS 16.0 programme) to test for effects of dietary treatments. When ANOVA identified significant difference among groups, multiple comparison tests among means were performed using Duncan's new multiple range test. For each comparison, statistically significant differences were determined by setting the aggregate type I error at 5% ($P<0.05$).

Table 1

Formulation and proximate composition of the basal diets (dry weight)

Ingredients	*(%)*
Fish meal	60
Barley meal	7.5
Wheat flour	7.5
Corn meal	7.5
Soybean meal	7.5
Mineral mixture [a]	5
Olive oil	2
Fish oil	3
Proximate composition (%)	
Moisture	13.4
Ash	11.5
Crude protein	38.7
Crude lipid	13
Crude fibre	3.3
Carbohyrate	19

[a] Mineral mixture contain (mg/g mixture): Ca, 180000; P, 90000; Cu, 600; Zn, 300; Co, 300; I, 100; Co_3^{-2}, 100; Mg, 190000; Se, 1; Na, 60000; Mn; 200; Fe, 3000. Vitamin A, 500000 IU; Vitamin D_3, 100000.

Results. A significantly greater ($P<0.05$) increase in specific growth rate (SGR), and body weight increase (BWI) was recorded in diets supplemented with vitamin C levels of ≥500 mg kg^{-1} AA diet when compared to the control diet and 250 mg kg^{-1} AA diet (Figs 1 and 2). SGR and BWI were highest for zebrafish fed the supplemented diet using 2000 mg kg-1 AA diet (3.05 and 0.776 respectively) followed by 1000 mg kg^{-1} AA diet (2.97 and 0.745 respectively) and 500 mg kg^{-1} AA diet (2.69 and 0.682 respectively).

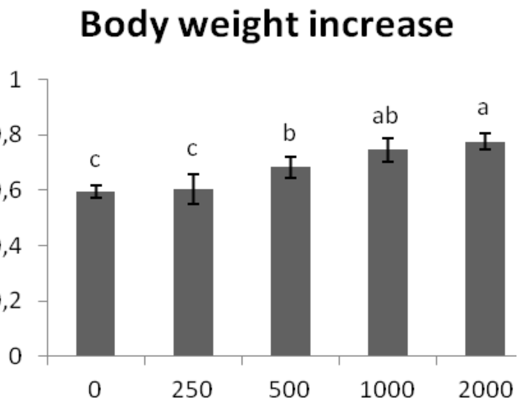

Figure 1. Effects of vitamin C on body weight increase of zebrafish.

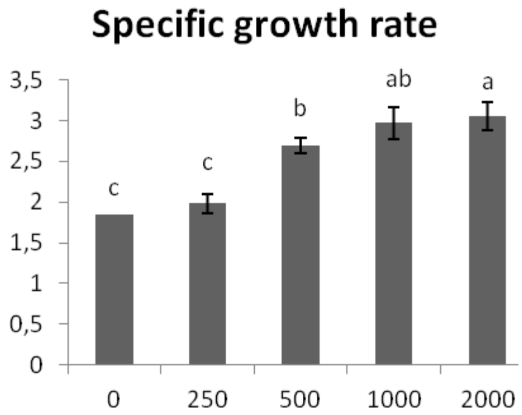

Figure 2. Effects of vitamin C on specific growth rate of zebrafish.

For body weight gain (BWG), a significant difference was observed between zebrafish fed the supplemented diet using 1000 and 2000 mg kg^{-1} AA diet compared to the other groups ($P<0.05$). The highest BWG was observed in fish fed the 1000 mg kg^{-1} AA diet (Fig 3).

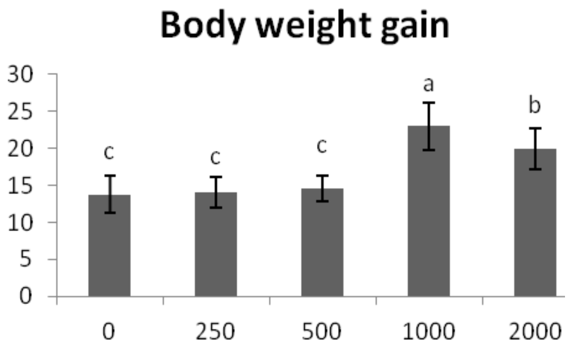

Figure 3. Effects of vitamin C on body weight gain of zebrafish.

The survival rate of zebrafish fed with diets containing graded levels of vitamin C are shown in Figure 4. The survival rate of zebrafish fed the diets containing 1000 and 2000 mg kg^{-1} AA was higher than other groups and the highest survival rate was observed in 1000 mg kg^{-1} AA and lowest survival rate was observed in 250 mg kg^{-1} AA (100% and 80% respectively).

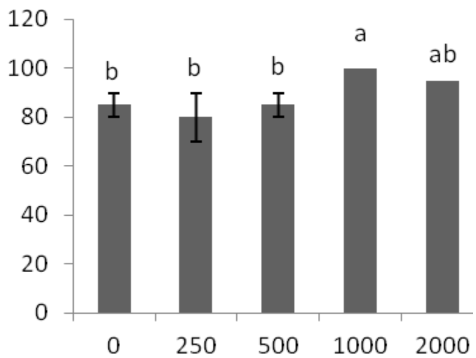

Figure 4. Effects of vitamin C on survival rate of zebrafish.

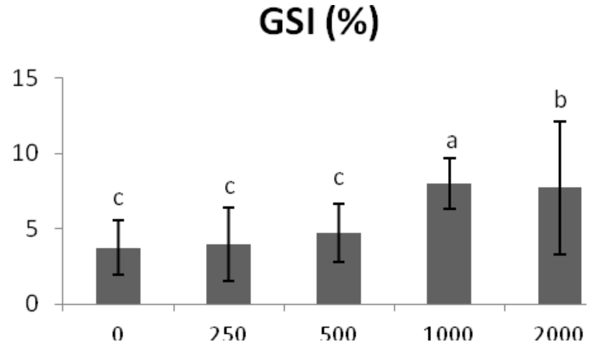

Figure 5. Effects of vitamin C on Gonadosomatic Index of zebrafish.

Mean values of GSI increased with increasing the level of vitamin C, and the results were significant ($P<0.05$). The results are shown in figure 5. In egg diameter there were no significant differences between groups, (Fig. 7). A significantly higher fecundity existed in the group of fish fed 1000 mg kg^{-1} AA diet compared with groups fed 0, 250 and 500 mg kg^{-1} AA diets. There was no significant difference between fish fecundity in fish fed 1000 and 2000 mg kg^{-1} AA diet and between fish fed 0 and 250 mg kg^{-1} AA diet (Fig. 8).

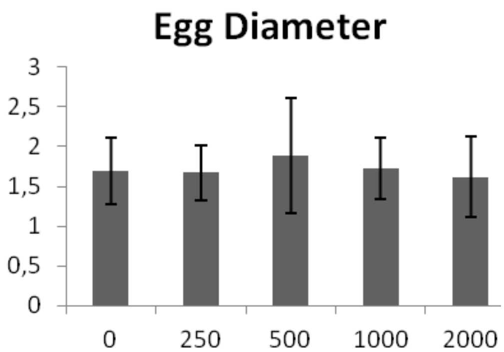

Figure 7. Effects of vitamin C on egg diameter of zebrafish.

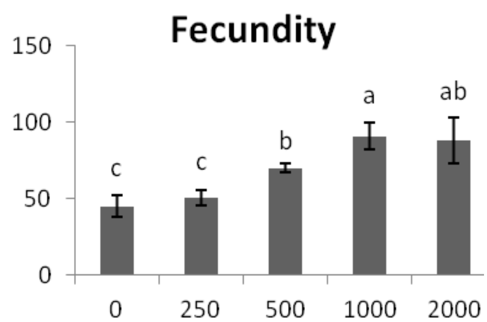

Figure 8. Effects of vitamin C on fecundity of zebrafish.

Discussion. Most fish species need a dietary supply of vitamin C to maintain normal growth because they lack the ability to convert L-gulonolactone to 2-keto-L-gulonolactone (NRC 1993). In our study, the growth of zebrafish improved significantly with increasing supplementation of dietary ascorbic acid. These results further confirm that zebrafish need adequate exogenous vitamin C to maintain normal growth and physiological functions. These results agree with previous studies on some other fish (Eya & Mgbenka 1990; Al-Amoudi et al 1992; Gouillou-Coustans et al 1998; Shiau & Hsu 1999; Sealey & Gatlin 1999; Wang et al 2003; Ai et al 2004; Ibiyo et al 2007; Mehrad & Sudagar 2010). Vitamin C is an essential coenzyme in certain oxidative processes, including the oxidation of tyrosine and phenylalanine (Brander & Pugh 1977). This probably explains the differences that occur in the body weight increase (BWI) with respect to the vitamin C free and enriched groups (Ibiyo et al 2007). Growth is a function of both the nutritional quality and the rate of consumption, among other things (Stickney 2000). In this research trial, a diet containing 1000 mg of ascorbic acid kg^{-1} diet was found to be the more optimal dietary requirement for zebrafish than other groups.

The ascorbic acid requirement value by zebrafish attained in this experiment was higher than the 10 and 25 mg kg^{-1} ascorbic acid as found for *Oreochromis aureus* and juvenile hybrid tilapia, *Oreochromis niloticus x O. aureus* (Shiau & Hsu 1999). Also, the requirement based on growth performance in this study was higher than that for *Oreochromis spilurus* (100-200 mg ascorbic acid kg^{-1} diet) (Al-Amoudi et al 1992). The difference is probably related to fish species, size, the form of vitamin C and experimental conditions in different studies (Lovell 1989).

The diet without ascorbic acid supplementation decreased the specific growth rate of zebrafish in compared to other groups that contain vitamin C, and this is in accordance with studies conducted by Ai et al (2004) who also observed declining specific growth rate with ascorbic acid deficient diet for seabass (*Scophthalmus maximus*).

A significant difference in survival rate was observed between the treatments containing vitamin C. This result conforms with a study conducted by Lee and Dabrowski (2004) on yellow perch, where they suggested survival of yellow perch fed with vitamin C diets were significantly higher than the fish fed with diet without vitamin C.

Blom & Dabrowski (1995) established for the first time the criteria to evaluate the fecundity and embryo survival with use of a range of dietary ascorbic acid levels. In this study a significantly higher fecundity existed in group of fish fed 1000 mg kg^{-1} AA diet compared with other groups, this result is in agreement with the study performed by Dabrowski & Ciereszko (2001) where in rainbow trout, both fecundity and survival of embryos increased with dietary ascorbate levels. However, the increase in GSI with increasing the level of vitamin C observed here is contrary to the results of Ronaldo et al (2003). They reported that GSI of females prawn *Macrobrachium rosenbergii,* were not affected by the different dietary vitamin C treatments. This difference may be due to the difference between fish and prawn.

Conclusions. The significance of the results herein obtained underlined the importance of diet in the reproductive process, supporting the hypothesis that feed additives can improve fecundity. Considering that the zebrafish has been clearly established as a vertebrate model for biomedical research, these results support the potentiality of feed additives such as vitamins, frequently used in the human diet, as a new technology to improve reproduction in all vertebrates, including humans.

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