AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

The effect of ascorbic acid on hatching performance and tolerance against environmental stressor (high temperature) by immersion of Prussian carp (*Carassius gibelio*) fertilized eggs

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Abstract. The aim of this study was to evaluate the influence of L-ascorbic acid (AA) in three levels (0, 100, 1000 and 2000 mg L^{-1}) on eyed egg and hatching rate, growth and viability of larva, and larval tolerance against high temperature stress of Prussian carp *Carassius gibelio* (Bloch, 1782). The fertilized eggs were placed in water containing different levels of AA for 3 h. The percentage of eyed egg and hatching were measured after 2 and 3 days respectively. After larva absorbed their yolk sac, half of them were challenged by high temperature (30°C) and the others were reared for 45 days and growth factors and survival were calculated. The result shown that the highest eyed egg and hatching rate were in 2000 mg L^{-1} and had significantly differece with other treatments ($P<0.05$). The significant differences in larval tolerance against high temperature stress were observed in 1000 and 2000 mg L^{-1} compared to 0 and 100 mg L^{-1} treatments. No significant differences were observed between growth parameters of treatment batches ($P>0.05$). Viability was different between experimental groups, but it was not significant between 0 and 100 mg L^{-1}. According to our results when broodstocks of Prussian carp do not have enough vitamin C in their ovaries, immersion of fertilized eggs in 2000 mg L^{-1} of AA may be beneficial.

Key Words: Prussian carp, acid ascorbic, fertilized eggs, high temperature stress**.**

چکیده: هدف از این مطالعه بررسی اثر ال- اسید اسکوربیک (AA) در 3 سطح (0، 100، 1000 و 2000 میلی گرم در لیتر) بر روی میزان چشم زدگی و تخمه گشایی، رشد و زنده مانی لارو و دامنه تحمل لاروهای ماهی قرمز *Carassius gibelio* (Bloch, 1782) در برابر استرس دمایی بالا (30 درجه سانتی گراد) بود. تخم های بارور شده به مدت 3 ساعت در آب حاوی سطوح مختلف AA قرار داده شده و درصد چشم زدگی و تخمه گشایی آنها پس از 2 و 3 روز محاسبه شد. لاروهای حاصل از هر تکرار پس از جذب کیسه زرده به دو قسمت تقسیم شدند. نیمی از آنها تحت تاثیر چلنج دمایی بالا قرار گرفتند و مابقی به مدت 45 روز پرورش داده شده و پارامترهای رشد و بقا محاسبه گشت. نتایج نشان داد که بالاترین میزان چشم زدگی و تخمه گشایی در تیمار 2000 میلی گرم در لیتر مشاهده شد که تفاوت معنی داری با تیمارهای دیگر داشت ($P<0/05$). تفاوت معنی داری در میزان مقاومت لاروها در برابر استرس دمایی بالا در تیمارهای 1000 و 2000 میلی گرم در لیتر نسبت به تیمارهای 0 و 100 میلی گرم در لیتر مشاهده شد. هیچ اختلاف معنی داری بین شاخص های رشد گروه های آزمایشی مشاهده نشد ($P>0/05$). نرخ زنده مانی بین تیمارها اختلاف داشته اما بین تیمارهای 0 و 100 میلی گرم در لیتر معنی دار نبود. با توجه به نتایج حاصل از این مطالعه می‌توان بیان کرد، هنگامی که مولدین ماهی قرمز ویتامین C کافی در تخمدان خود ندارند، غوطه وری تخم های بارور شده آنها در 2000 میلی گرم در لیتر از AA می‌تواند سودمند باشد.

کلمات کلیدی: ماهی قرمز، اسید آسکوربیک، تخم های بارور شده، استرس با درجه حرارت بالا

Introduction. Vitamin C is an essential vitamin for normal physiological functions in animals including fish (Wilson & Poe 1973; Lim & Lovell 1978). The ascorbic acid requirement for different teleost fish has been well documented (Dabrowski 2001). Tissues vary considerably in their concentration of ascorbic acid, but gonads represent organs having one of the highest levels, several-fold higher than blood plasma (Blom & Dabrowski 1995; Ciereszko & Dabrowski 1995). High concentrations of ascorbic acid in fish gonads indicate its particular relevance to reproduction. The lack of dietary AA also resulted in lower egg hatching rates (Sandnes et al 1984), egg strength (Mangor-Jensen & Holm 1994), and poor fry survival (Soliman et al 1986).

On the other hand when eggs absorb water, it is possible to introduce compounds and micronutrients, such as vitamins and mineral elements, into the eggs with the water solution before hardening. In rainbow trout, immersion the fertilized eggs in enrichment water by vitamin C had significantly effect on TAA (total acid ascorbic) concentration at the eyed stage, and in hatched alevins (Falahatkar et al 2006). Useful effects of complementary ascorbic acid in broodstock diets on fish fertility have been shown in

rainbow trout, *Oncorhynchus mykiss* (Sandnes et al 1984; Blom & Dabrowski 1995b), tilapia, *Oreochromis niloticus* (Soliman et al 1986), cod, *Gadus morhua* (Mangor-Jensen & Holm 1994), yellow perch, *Perca flavescens* (Lee & Dabrowski 2004), and guppy, *Poecilia reticulata* (Mehrad & Sudagar 2010).

Also AA is known to take part in several biochemical reactions within the cells, all related to its ability to undergo reversible oxidation and reduction (Conklin 1997). AA has been shown to improve immune response (Li & Lovell 1985), and tolerance to environmental stressors (Ishibashi et al 1992; Merchie et al 1995).

It has been established that vitamin C is required by all animals for body maintenance, growth and other biological performances and the vitamin C level needed for these functions varies with the species and culture environment (Delong et al 1958; Lovell 1972). Lee et al (1998) reported that by increasing of vitamin C level in the diet to 1500 mg kg^{-1} diet, the best growth performance and feed utilization for Korean rockfish (*Segastes schlegeli*) were obtained. Similarly, the influence of dietary levels of vitamin C on growth rates of *Heterobranchus longifilis* fingerlings was studied by Ibiyo et al (2007) who mentioned that the dietary level of vitamin C required for maximum growth of this species is 100 mg kg^{-1} diet.

The aims of this investigation were to evaluate the effect of immersion fertilized Prussian carp eggs in enrichment water by different levels of L-ascorbic acid on hatching performance (eyed egg and hatching percent), tolerance against high temperature stress, some of growth factors and viability of larva.

Material and Method

Collection of gametes and vitamin treatments. The experiments were conducted from March to June 2010 in Aquaculture Research Center in agricultural science and natural resources university of Gorgan, Iran. 20 Prussian carp female (mean weight, 63±4.3 g) and 30 Prussian carp male (mean weight, 42±5.6 g) transferred to the place of experiment and acclimated for 2 weeks in 1000 L tanks. Broodstocks were injected with 0.5 mg kg^{-1} Ovaprim (sGnRH+Dompridon) and 12 hours after injection treatment females were stripped. Fresh milt also was collected from males 12 h after injection and stored in syringe.

Four different concentrations of ascorbic acid including 0 (control), 100, 1000 and 2000 mg L^{-1} of L-ascorbic acid (AA) (Sigma, St Louis, MO, USA) were added to each experimental aquarium (with 80 liter aerated water). Each treatment was performed in three replicate.

Fertilization and incubation. Approximately 1 g (~1000 oocytes) were used for each replicate and placed in petri dish (10 cm diameter). Sperm motility was checked before experimentation (Ciereszko & Dabrowski 1993), and semen samples with > 90% initial motility were pooled and used for fertilization. Ova from the all of 20 females were mixed together. 200 Microliters of semen was used for each replicate. Obtained eggs were fertilized by milt and were placed in aquarium containing different levels AA for 3 h, after hardening their water emptied and used fresh water (without AA) and aeration was performed. Eggs were incubated in these aquariums at 20°C. The percentage of eyed egg and hatching rate was measured after 2 and 3 days respectively.

Larva cultivation and high temperature challenge. After yolk sac absorption, larva were divided in 2 groups. For evaluation of the newly hatched larval quality, half of the larva were challenged by high temperature. In this propose, larva were transferred to other aquariums and temperature was increased to 30°C (10°C higher than incubation temperature) and survival duration was calculated.

The other half of larva were reared for 45 days. Larva were fed with artemia naupli diet during this period. Fish from each aquarium were counted and weighed at 2-week intervals to monitor growth and mortalities were recorded.

Calculations and statistical analysis. 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)

Daily growth rate (DGR) = $[(W_t - W_0)/ t] \times 100$ (De Silva & Anderson 1995)

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

W_t and W_0 were final and initial larva weights (g), respectively; N_t and N_0 were final and initial numbers of larva in each replicate, respectively; and t is the experimental period in days.

Results are presented as means±SD. Significant differences among treatments were determined by analysis of variance (ANOVA), and the differences between means were tested with Duncan's multiple-range test using SPSS 16.0 programme. Differences were considered significant at $P<0.05$.

Results

Effect of vitamin C on eyed egg and hatching rate. The result shown that eyed egg and hatching rate were increased with increasing the level of vitamin C and were significant between 2000 mg L^{-1} with other treatments ($P<0.05$). The highest percentage of eyed egg and hatching (84.3±2.1 and 89.53±5.23) and lowest percentage of eyed egg and hatching (67.2±2.28 and 74.1±3.2) was observed in 2000 and 0 mg L^{-1} respectively. Differences were not significant between 0, 100 and 1000 mg L^{-1} treatments (see Figure 1).

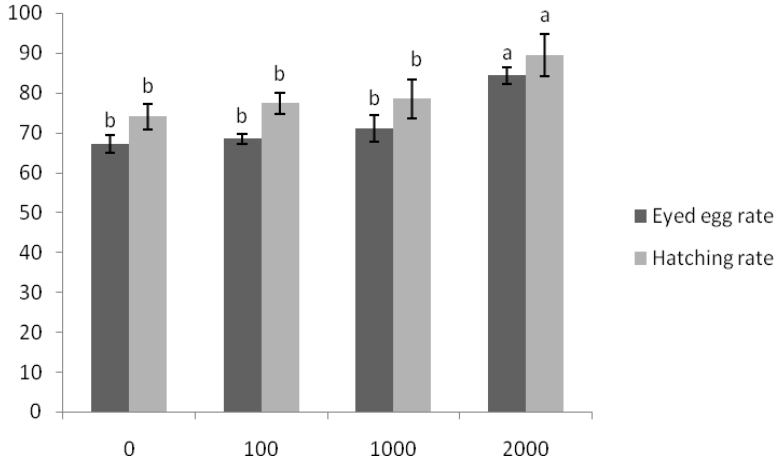

Figure 1. The percentage of eyed egg and hatching rate in groups treated by vitamin C.

Effect of vitamin C on larval tolerance. As see in the Figure 2, differences in larval tolerance against high temperature stress (30°C) were observed between experimental groups, and they were significant between 0 and 100 with 1000 and 2000 mg L^{-1} treatments. Highest and lowest times of survival in 30°C were observed in 2000 and 0 mg L^{-1} respectively.

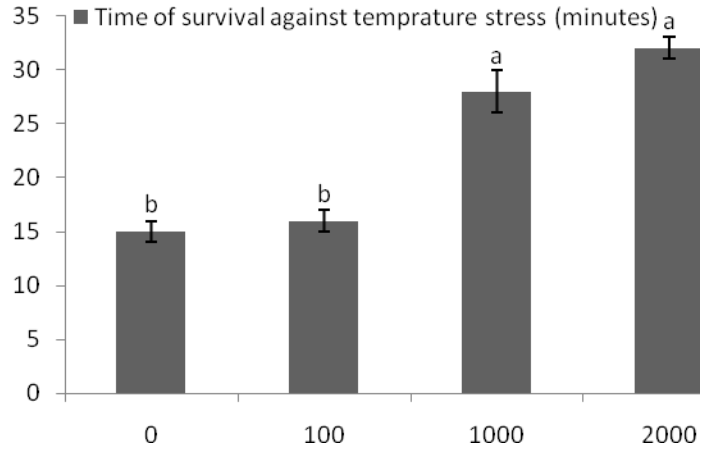

Figure 2. Effect of vitamin C on larval survival duration against high temperature (30°C).

Effect of vitamin C on growth and viability. Growth parameters (BWI, SGR and DGR) were not significantly different between treatments. Highest and lowest BWI, SGR and DGR were observed in 1000 and 100 mg L^{-1} respectively. Growth parameters in 2000 mg L^{-1} were higher than 0 mg L^{-1}, and lower than 100 and 1000 mg L^{-1} treatments but these differences were not significant (see Table 1).

Table 1

Growth factors of Prussian carp fry after 45 days (Mean ± SE)

Vitamin	0 (mg L^{-1})	100 (mg L^{-1})	1000 (mg L^{-1})	2000 (mg L^{-1})
BWI	221±4.34^a	217±5.12^a	239±3.64^a	226±4.74^a
SGR	11.23±2.56^a	10.54±1.76^a	12.17±2.43^a	11.42±3.37^a
DGR	521.48±3.2^a	518.23±4.16^a	531.11±5.26^a	522.56±5.24^a

Values of different superscripts in a row are significantly different at ($P<0.05$)

As see in the Figure 3, survival rate were increased with increasing the level of vitamin C. But significant difference was not observed between 0 and 100 mg L^{-1}. Highest survival (73%±2.1) and lowest survival (47.2%±4.56) were observed in 2000 and 0 mg L^{-1} treatments.

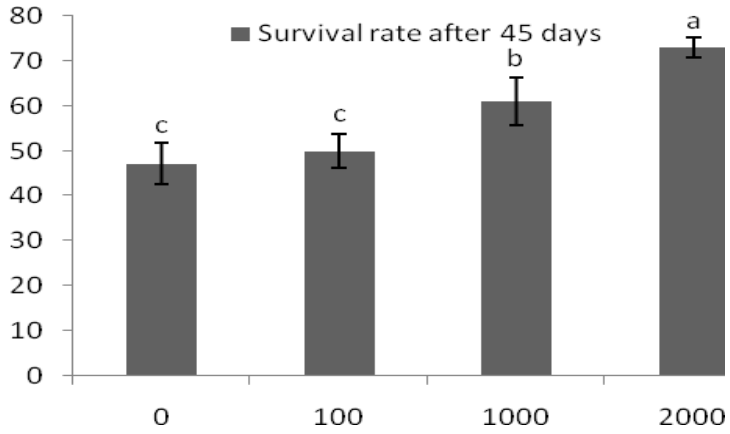

Figure 3. Survival rate of fry after 45 days.

Discussion. The present study confirmed that additional AA is useful for the propagation of Prussian carp broodstock and affected positively on percentage of eyed egg, hatching rate and larval performance. The importance of high ascorbic acid concentrations in female fish gonads for embryo vitality has been reported (Dabrowski 1991; Blom & Dabrowski 1995a; Dabrowski & Ciereszko 2001).

Ascorbate transfer actively from yolk reserves into larval fish body. For instance, Terova et al (1998) argued that in some scenarios, ascorbate concentration increases significantly between unfertilized egg and yolk sac larvae. In other species, a decrease of approximately 20-50% was observed during embryonic development and endogenous feeding (Knox et al 1988; Blom & Dabrowski 1998). The most likely need for the ascorbic acid storage in egg yolk reserves is for the synthesis of collagens during the development of the embryo and for proline and lysine hydroxylation.

In the present study, we found increased eyed egg and hatching rate in the eggs after immersion with ascorbic acid solutions and they were maximum in 2000 mg L^{-1}

treatment. The application of this procedure may be helpful in balancing out individual variations among different females and may decrease susceptibility to vitamin C deficiency in broodstocks fish. Bylund & Lerche (1995), Fitzsimons (1995), Fisher et al (1996) and Amcoff et al (1998) used different concentrations of thiamin to prevent M74 disease (Baltic Sea salmon), EMS (salmonids in Great Lake) or CS (Cayuga syndrome; *Salmo salar* in Finger Lakes region). Their results indicated that the concentration of thiamin after immersion of eggs in thiamin solutions was increased and the mortality of eggs and embryos decreased. Falahatkar et al (2006) suggested that when broodstock rainbow trout do not have enough vitamin C in their ovaries, immersion of eggs in 1000mg L^{-1} of neutralized AA (with NaOH) may be useful.

Treatment of Prussian carp fertilized eggs with ascorbic acid increased larval tolerance against high temperature stress at 30°C. Cavalli et al (2003) evaluated the effect of dietary supplementation of vitamins C and E on maternal performance and larval quality of the prawn *Macrobrachium rosenbergii*. They tested the tolerance of newly hatched and 8-day-old larvae of *M. rosenbergii* to ammonia exposure. Their results shown newly hatched and 8-day-old larvae tolerance tended to increase with increasing levels of AA and higher dietary levels of α-tocopherol acetate did not affect the tolerance to ammonia of newly hatched larvae, but it positively augmented the ammonia tolerance of 8-day-old larvae.

Also eggs treated during water hardening indicates that survival increased with increasing AA but had not affect on growth parameters. Ibiyo et al (2007) evaluated the requirements of vitamin C (ascorbic acid) in *Heterobranchus longifilis* fingerlings and indicated the survival and growth of *H. longifilis* fingerlings improved significantly with increasing supplementation of dietary ascorbic acid, and its growth reached a plateau at between 100 to 200 mg AA kg^{-1} diet.

Conclusion. According to our results, we suggested a dose of 2000 mg AA L^{-1} to enrich water of Prussian carp eggs incubation.

Acknowledgements. We are grateful to the staff of the central lab in Gorgan University. The authors would like to thanks the staff of the fish Aquaculture station, Gorgan, Iran.

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Received: 15 August 2010. Accepted: 06 September 2010. Published online: 06 September 2010.
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How to cite this article:
Taati M. M., Mehrad B., Shabani A., 2010 The effect of ascorbic acid on hatching performance and tolerance against environmental stressor (high temperature) by immersion of Prussian carp (*Carassius gibelio*) fertilized eggs. AACL Bioflux **3**(3):219-225.