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Effects of diet containing enriched *Artemia* with unsaturated fatty acids and vitamin C on growth, survival and stress resistance of swordtail *Xiphophorus hellerii* fry

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Abstract. The effect of essential fatty acids (EFA) and vitamin C enriched *Artemia* nauplii on red swordtail *Xiphophorus hellerii* Heckel, 1848 fry growth, survival rate and resistance to high temperature stress (34ºC) were determined. Fry were fed 6-times daily with practical diet (P), P + *Artemia*, *Artemia* + essential fatty acid (EFA), *Artemia* + EFA + 0.5 g vitamin C and *Artemia* + EFA + 1 g vitamin C for 17 weeks in glass aquaria. Results showed that P + *Artemia* was the most effective diet in growth indices including weight gain (2744.8 ± 32.6 mg), length increasing (47.6 ± 4.6 mm), the specific growth rate (SGR) with 3.7 ± 0.2%, and the average daily gain (ADG) with 22.9 ± 2.2%. *Artemia* + EFA + 1 g vitamin C resulted in the highest survival rate in 120 day-old fish (95.8 ± 2.5%) and highest resistance of 10 day-old fry to high temperature with 96.4 ± 1.3%. Feeding live food as supplement, reduced the mortality rate, increased growth performance, and increased the larval resistance to high temperature stress, which showed that using live food is beneficial for larviculture of the ornamental fish.
Key words: aquarium fish, Poeciliidae, stress, SGR, ADG.

Introduction. The nutritional condition of broodstocks and larvae can impact on several larviculture aspects of fish and other aquatic organisms; this includes growth performance and survival rate (Xu et al 1994; Marsden et al 1997; Perez-Velazquez et al 2002; Wouters et al 2002; Racotta et al 2003). Lipids and amino acids are major sources of metabolic energy during the embryonic and prefeeding larval stages in fishes. At hatch, yolk-sac larvae have high levels of these energy sources, but they are dramatically reduced during the endogenous feeding stage (Evans et al 2000). Thus, start-feeding larvae require a live feed that provides sufficient levels of these energy sources. Studies have shown that essential fatty acids (EFA), such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20: 5n - 3) are also important in larval fish nutrition (Takeuchi 1997; McEvoy et al 1998; Estevez et al 1999; Sargent et al 1999). However, the live feeds commonly used for the first-feeding larval stages, such as rotifers and *Artemia*, which are naturally poor in these fatty acids, so enrichment of live foods with lipids rich in EFA is necessary to achieve better growth and survival through metamorphosis (Rainuzzo et al 1997; Binh & Serrano 2012). Recently, absolute and relative levels of DHA and EPA in the diets of fish larvae have received considerable attention (Sargent et al 1999; Harel et al 2002; Bell & Sargent 2003). Other specific benefits of feeding DHA enriched diets to fish larvae include successful metamorphosis, reduced pigmentation problems, enhanced vision capabilities, improved neural development and stress resistance (Watanabe 1993). In the case of freshwater ornamental fish culture, there is little information on the nutritional requirements to cover larvae needs. Since common food sources for ornamental species are live

organisms such as *Artemia* and *Daphnia* (Godin & Dugatkin 1996), their larviculture performance could vary depending on the culture conditions as the type of food. It is demonstrated that the energy and nutritional contents of zooplanktons strongly depend on the biochemical composition of the diet they receive (Sorgeloos & Léger 1992). Therefore, live foods such as *Artemia urmiana* can be used as carrier of some nutritive materials such as long chain unsaturated fatty acids and vitamins, especially vitamin C (Coutteau et al 1997). Previous studies indicated that fatty acids play a critical role in the reproductive physiology of teleost fishes (Tuncer & Harrell 1992; Kim et al 1996; Furuita et al 1996b; Ershad et al 2009) such as maintaining immune function, osmoregulatory systems and endocrine system function (Ako et al 1994; Awaiss et al 1996; Furuita et al 1996a; Koueta & Noel 2002). Ershad et al (2009) conducted similar tests using *Pterophyllum scalare* fed-enriched *Artemia urmiana* that has been fed fatty acids and vitamin C. Vitamin C plays an important role in maintaining immune response and is required for numerous biological functions in fish and other vertebrates, for example, maintaining skeletal integrity, growth and survival and physiological responsibilities such as resistance against stressors, poisoning (Merchie 1997; Gapasin et al 1998; Lim 2002a; Dhert et al 2004). Also immune activities improved in different species of aquatic larvae with usage of vitamin C complements (Dhert & Sorgeloos 1995; Hanaee Kashani et al 2011).

In Poeciliidae, red swordtail *Xiphophorus helleri* is one of the most important species in ornamental fish culture (Mousavi-Sabet & Ghasemnejhad 2013; Mag-Muresan & Pop 2004; Petrescu-Mag et al 2013). Livebearers are an important group of relatively large and often colorful aquarium fishes. The family Poeciliidae comprises about 37 genera and about 304 species (Moyle 2002; Nelson 2006).

Understanding the nutritional requirements of early larval swordtail, especially of EFA such as DHA and EPA, is important for successful mass production. So, in this study the effects of feeding swordtail fry with *Artemia urmiana* nauplii which had been specifically fed an emulsion of unsaturated fatty acids and vitamin C was tested to determine if feeding these enriched *Artemia* would improve the efficiency of growth and survival of the fish. Which its results can be also useful for other species.

Materials and Methods. A stock of *X. hellerii*, comprising males and females was procured from a local ornamental fish dealer in Tehran. After acclimation to the laboratory conditions, animals were disinfected (Robertson et al 1993) and stocked in separate aquariums containing recirculating water (26 ± 1°C) and exposed to a 12 (light): 12 (dark) photoperiod, where oxygen concentration was kept above 5.5 mg/L by continues aeration (Mousavi-Sabet et al 2012). To remove chlorine, water for the aquaria was drawn from 1000 liter tank which had been intensely aerated for at least 24 hours. The experimental stocking density was adjusted to eight fish per aquaria at a sexual ratio of two males and six females (Garcia-Ulloa & Garcia-Olea 2004; Mousavi-Sabet et al 2012; Mousavi-Sabet & Ghasemnejhad 2013). Each experimental aquarium was filled up to a culture volume of 50 L with municipal freshwater. Daily, faeces and other particles were extracted out from the bottom of each aquarium by siphoning and 50% of water volume was changed every two days (Mousavi-Sabet et al 2012). All of the swordtail fry used in these treatment studies was obtained from these broodstocks. In order to prevent fry from being eaten by their parents, the ripe females were kept in plastic nets (Mousavi-Sabet et al 2012). The fry were immediately removed from the aquariums, counted and placed in 18 small glass aquariums, each containing 40 L of freshwater that was continuously aerated with a 5-cm air stone and filtered by a normal sponge filter.

Six experimental diets were considered through the 120 days of trial includes: 1) a control diet which was a practical diet for ornamental fish (distributed by Energy Company, Tehran, Iran; crude protein 40%, crude fat 6%, crude fiber 5%, moisture 12%, ash 10%), 2) The practical diet + *Artemia* nauplii (un-enriched), 3) *Artemia* nauplii (un-enriched), 4) Enriched *Artemia* nauplii with fatty acids, 5) Enriched *Artemia* nauplii with fatty acids and 0.5 g vitamin C, and 6) Enriched *Artemia* nauplii with fatty acids and 1.0 g vitamin C (Table 1). Live *Artemia* nauplii were enriched in 1.5 L conical cylinders with 33 g/L salt, 28 °C water temperature and mild aeration. To enrich the *Artemia* by

unsaturated fatty acid the following feeding regime was followed: *Artemia* was suspended the enriching dishes containing an emulsion (ICES 30/4/C, INVE Co., Belgium) which consist of 30% unsaturated fatty acid methyl esters at 30% dry weight of the emulsion. This emulsion was 4 to 1 of dicosahexenoic (DHA) and eicosapentaenoic (EPA). Preparation of standard emulsion is done on the basis of a standard method (Leger 1989). Five mL of the fatty acid emulsion was mixed with 50 mL dechlorinated fresh water and blended by an electric blender for 3 minutes at room temperature. To *Artemia* enriching with vitamin C, ascorbyl palmitate (Serva, USA) was added at a ratio of either 10% or 20% of the fatty acid emulsion as proposed by Merchie et al (1995, 1997) and Agh & Sorgeloos (2005). The ascorbyl palmitate at 0.5 g or 1 g per 5 mL of emulsion was blended with 50 mL of water. Five mL of this mixture was then added to the 1.5 L enriching dishes in order to enrichment the live *Artemia*. These prepared emulsions were stored in tightly sealed containers which excluded light under a nitrogen head space to limit oxidation and the suspension kept in the refrigerator for up to 10 days until it was used for enriching the *Artemia urmiana* (Ershad et al 2009).

Table 1

EPA and DHA fatty acid concentration in different diet treatments (percentage of total fatty acids)

Fatty Acid	P	Un-enriched *Artemia*	*Artemia* + EFA	*Artemia* + EFA + 0.5 g Vitamin C	*Artemia* + EFA + 1.0 g Vitamin C
EPA	4.25 ± 0.56	1.86 ± 0.10	2.13 ± 0.14	3.08 ± 0.21	3.47 ± 0.46
DHA	1.59 ± 0.34	0.00 ± 0.00	2.43 ± 0.46	6.75 ± 1.01	6.39 ± 0.97

To *Artemia* enrichment, 2 mL of the emulsion described above were added to 1 liter water containing live *Artemia* twice a day (every 12h) which meaning 4 mL/L in 24h (Leger 1989; Coutteau & Sorgeloos 1997; Ershad et al 2009). At this point, we wrapped the *Artemia* in a clean and wet zooplankton filter (mesh 400µ) and held them in a refrigerator (10°C) until use. Keeping the enriched *Artemia* in the mentioned condition preserve them from utilizing their fatty acids and vitamin C contents. The enriched *Artemia* were fed within 15 hours to the fry. Fish were fed 4 times a day at 7:00, 12:00, 17:00, and 22:00. The control group was fed with a commercial practical starter food using the same feeding schedule by 5% of wet body weight of the fry per day.

Ten day-old swordtail fry were subjected to temperature stress test following the method described by Ako et al (1994) and Kanazawa (1995). The test involved immersing 10-larvae/replicate in 34ºC for a period of one hour. The fish from each replicate were gently caught and placed to new container with 40 L, and water temperature raised from 26 to 34 ºC. The mortality was recorded every 5 min intervals. Other remained larvae (non-stressed) of each treatment were cultured with their diet to 120 days after birth, and the survival rate of the 120-day-old fish (which are ready to sale as marketable fish) was recorded. To determine uptake of long chain fatty acids in the enriched *Artemia urmiana*, fatty acid profiles were measured (n=3) through Metcalf et al (1996) and detected by gas chromatography system model Unicam 4600 (Unicom company, NY, USA). The important growth parameters such as BW and total length were recorded at 120 day-old swordtails. Specific growth rate (SGR) was calculated by SGR = 100 (lnW1 – lnW0) $(t)^{-1}$, where W0 and W1 were wet body weight of fish at the start and end of the experiment. Average daily gain (ADG) was calculated by ADG = weight gain/experimental period (d), and condition factor (CF) was calculated by CF = 100W/ L3, where W is the body weight and L is the total length (Mousavi-Sabet 2007; Mousavi-Sabet et al 2011).

The Kolmogorov-Smirnov test was applied first to check the normality of the data. Differences in mortality and differences in body weight and length between groups were tested by one-way analysis of variance by ranks (SPSS ver. 16.0 for Windows) followed by the Duncan's test in $p < 0.05$, which was applied to study the effect of diets on larviculture factors. All percentage data were transformed to arc-sin prior to statistical analysis and then ANOVA was applied. Values for each evaluated parameter represent treatment means (± SD).

Results and Discussion. The EPA and DHA contents are presented in Table 1. The concentration of EPA and DHA in the *Artemia* increased after enrichment by the emulsion to 3.47 and 6.39 percent of the total fatty acids, respectively. The levels of EPA and DHA in practical diet were 4.25 and 1.59 %, respectively. The comparison of weight, length, and growth indices are shown in Table 2.

Table 2

Effects of different diets on growth indices (mean ± SE) of *Xiphophorus hellerii*

Diet	Weight gain (mg)	Length increase (mm)	SGR (%)	ADG (%)	CF (%)
P	1871.5±18.5	39.2±3.7	3.4±0.1	15.6±1.6	1.81±0.03
P + *Artemia*	2744.8±32.6	47.6±4.6	3.7±0.2	22.9±2.2	1.61±0.03
Artemia	1931.3±19.8	39.9±4.3	3.4±0.3	16.1±1.7	1.79±0.02
Artemia + EFA	2254.4±25.8	43.1±4.1	3.6±0.2	18.8±2.1	1.69±0.02
Artemia + EFA + 0.5 g Vitamin C	2311.9±24.8	43.7±5.3	3.6±0.2	19.3±2.1	1.68±0.02
Artemia + EFA + 1.0 g Vitamin C	2285.1±29.7	43.3±4.6	3.6±0.1	19.0±2.1	1.70±0.03

As shown in Table 2, P + *Artemia* was the most effective diet in growth indices including weight gain (2744.8 ± 32.6 mg), length increasing (47.6 ± 4.6 mm), the specific growth rate (3.7 ± 0.2%), and the average daily gain (22.9 ± 2.2%). *Artemia* + EFA + 1 g vitamin C resulted in the highest survival rate in 120 day-old larvae (95.8 ± 2.5%) and highest resistance of 10 day-old larvae to high temperature with 96.4 ± 1.3%, which had the most output compared with other treatments ($p < 0.05$). Adding vitamin C increased larval survival, and larval resistance to high temperature ($p < 0.05$).

The fry which fed enriched *Artemia* (fatty acids + vitamin C) exhibited more resistance to high temperature stress (Fig. 1) ($p < 0.05$), also all other treatments (enriched and un-enriched *Artemia*) showed more resistance compare with control group. The highest mortality rate was observed in control group ($p < 0.05$).

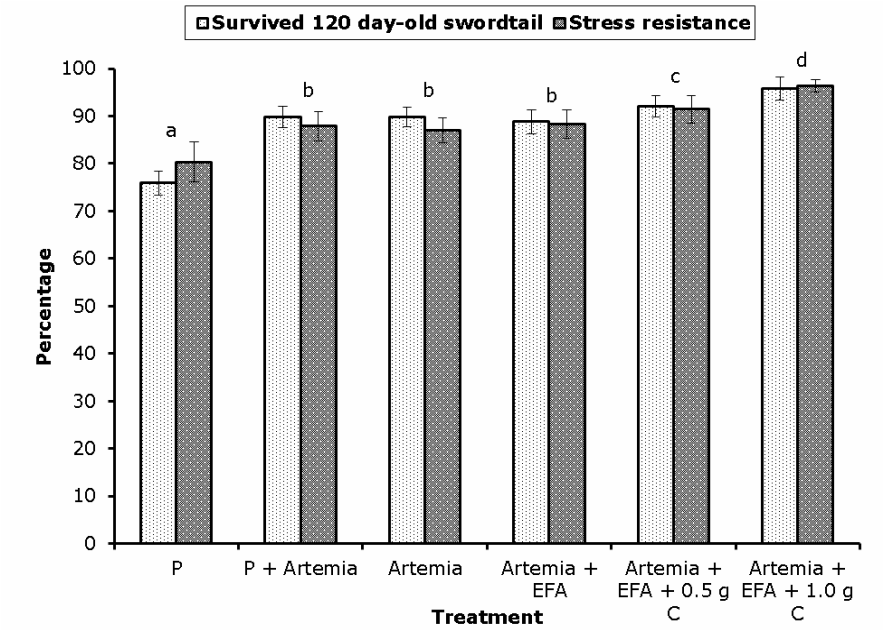

Figure 1. Percentages of survived 120-day-old swordtail (marketable fish), and survival rate of high temperature stress (34ºC) in 10-day-old swordtail in different treatments (means ± S.D., n = 3 replicates per treatment). Different letters indicate significant differences (P < 0.05).

In the present study the effect of essential fatty acids (EFA) and vitamin C enriched *Artemia urmiana* nauplii on *Xiphophorus hellerii* fry growth performance and larvicaulture factors including weight gain, length increasing, SGR, ADG, larvae survival rate, and

larval resistance to temperature (high) stress were determined. The use of *Artemia* biomass as sole live food provides energetic and nutritional profits for the predator organism (Sorgeloos et al 1986) and is a very common practice in ornamental aquaculture. The effect of enriching live *Artemia* with long chain unsaturated fatty acids and vitamin C has been evaluated in different aquatic species in marine and freshwaters, but only in a few ornamental fishes. Numerous studies have reported the importance of dietary HUFA for early survival of marine (Sorgeloos & Sweetman 1993; Watanabe 1993) and freshwater (Tuncer & Harrell 1992; Awaiss et al 1996) fishes, and the importance of EPA and DHA, in particular in marine larval rearing have been demonstrated (Watanabe 1993). The importance of DHA in fish development is also well documented (Kanazawa 1995; Furuita et al 1996a; Furuita et al 1996b). Similar research with *Acipenser* sp., Indian white shrimp post larvae (*Fenneropenaeus indicus*), rainbow trout larvae (*Oncorhynchus mykiss*), giant freshwater prawn (*Macrobranchium rosenbergii*) and milk fish larvae (*Chanos chanos*) reported that enriched *Artemia* with unsaturated fatty acids and vitamin C increased larval survival rate (Dhert et al 2004; Gapasin et al 1998; Girri et al 2002; Lim 2002a). In the present study, we observed an increase in survival between the control and the treatments using fatty acid and vitamin C enrichments.

A study on freshwater angel fish *Pterophyllum scalare* showed the effects of using live food include enriched foods on increasing reproductive performance (Ershad et al 2009). Lim (2002ab, 2003), Dhert et al (2004) and Ershad et al (2009) studied on *Poecilia reticulata*, *Xiphophorus helleri*, *Xiphophorus maculatus*, *Poecilia sphenops*, *Hyphessobrycon herbertaxelrodi*, *Pterophyllum scalare* and *Symphysodon aequifasciata* and reported that uses enriched *Artemia* with unsaturated fatty acid and vitamin C in broodstocks diet improved fecundity and larvae survival rate. We also found that growth increased by live diets compared with control. Significant differences were observed in survival rate between all treatments and the control group. Using live *Artemia* enhanced survival, supporting the findings of earlier researchers. This is likely due to the higher concentration of unsaturated fatty acids in treatment diets. Adding *Artemia* with unsaturated fatty acids and vitamin C to larvae diets improved larval survival rate (Merchie 1997; Ershad et al 2008). Since vitamin C decreases stress negative effects (Dhert & Sorgeloos 1995; Merchie 1997; Gapasin et al 1998; Lim 2002a; Dhert et al 2004), as result; decreasing in environmental stresses effects on larvae caused the increase its survival in treatment which had been enriched with ascorbic acid. Increasing unsaturated fatty acid and vitamin C contents in the larvae food could transmit beneficial effects on immune and health factors and increasing larvae survival.

Several studies have established that lipids are an optimal energy substrate for embryogenesis and larvae growth (Middleditch et al 1980; Xu et al 1994; Naessens et al 1997). Godin & Dugatkin (1996) pointed out that under optimal conditions, guppy fish (*Poecilia reticulata*) broods are produced every 27-30 days per female and the diet of broodstocks is one of the most affective factors on this period.

Milkfish larvae given *Artemia* enriched with HUFA + vitamin C showed higher survival after a stress test (Gapasin et al 1998). Ako et al (1994) and Gapasin et al (1998) observed no or few mortalities among fish fed *Artemia* enriched with menhaden oil (high DHA:EPA ratio) compared to high mortalities among fish fed un-enriched *Artemia*. Red sea bream (*Pagrus major*) and marble sole (*Euryglossa orientalis*) larvae given diets containing DHA and lecithin tolerated temperature and salinity changes, low oxygen and air exposure better than the larvae given DHA and lecithin-free diets (Kanazawa 1995). Furuita et al (1995ab) reported that yellowtail larvae and red sea bream juvenile fed *Artemia* enriched with DHA exhibited higher survival in the stress test than those fed *Artemia* enriched with EPA. In the present study, the fry which fed enriched *Artemia* with fatty acid + vitamin C showed better resistance to high temperature than those given un-enriched diet with vitamin C and the commercial diet. This result is similar to *Chanos chanos* (Gapasin et al 1998) and *Sepia officicinalis* (Koueta et al 2002) which fed by enriched live foods with fatty acid and vitamin C. When subjected to salinity stress test, 20 days old *Clarias gariepinus* larvae fed ascorbate-supplemented diet exhibited significantly low mortality than those larvae fed an ascorbate free diet (Merchie et al 1995). Survival of 120-day-old swordtail fed various

diets were significantly different supporting the results of Ako et al (1994) and Gapasin et al (1998).

Conclusion. As conclusion in present study and about *X. hellerii* fry growth, using live food such as *Artemia*, as a supplement to commercial diet increased growth performance. Enriching the *Artemia* with EPA, DHA and incorporate additional vitamin C, increased larval survival and larval stress resistance. Improving efficiency of hatchery operations would make them more economically viable and also increase the number of produced offspring.

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