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Reproductive Output of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), Fed Increasing Levels of Ascorbic Acid

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Abstract: Possible effects of increasing dietary levels of ascorbic acid (AsA) on rainbow trout *Oncorhynchus mykiss* (Walbaum) reproductive parameters such as fecundity, egg diameter, hatchability, embryo and sac-fry mortality, as well as the percentage of the total fry produced, were analysed. A total of four hundred rainbow trout broodstock were equally divided into four groups; four diets (AsA-0, AsA-600, AsA-1200, AsA-2400), one for each group, were fed to fish for a period of 144 days. No additional AsA was incorporated in diet AsA-0, whereas diets AsA-600, AsA-1200 and AsA-2400 were supplemented with AsA to contain 600, 1200 and 2400 mg kg^{-1}, respectively. From the statistical analysis and evaluation of the research results it was established that the above-mentioned quantities of AsA in the broodstock diet did not cause a significant difference to fecundity or the mean diameter of the eggs ($P>0.05$). On the contrary, AsA supplementation significantly affects hatchability, sac-fry mortality and the percentage of the total fry produced ($P\leq0.01$). It could be suggested that the addition of 1200 mg AsA kg^{-1} into rainbow trout broodstock diet increases not only hatchability, but the viability of sac-fry and the percentage of the total fry produced, as well.

Key words: Ascorbic acid, vitamin C, rainbow trout, *Oncorhynchus mykiss*, fish reproductive output

INTRODUCTION

L-ascorbic acid (AsA), a co-factor in the biosynthesis of steroid hormones (Ito *et al*., 1992), plays an important role in the natural functioning of gonads in superior animals (Claesson *et al*., 1949; Lutwak-Mann, 1958; Schmidt-Elmendorff and Loraine, 1962; Hershberger *et al*., 1965; Koed and Hamburger, 1967). It seems that the same occurs with fish, where high levels of AsA have been detected in the ovaries and the ovarian follicles of several species, such as those of crucian carp, *Carassius carassius* (L.), cod, *Gadus morrhua* L., silver bream, *Vimba vimba* L., rainbow trout, *Oncorhynchus mykiss* (Walbaum) and arctic charr, *Salvelinus alpinus* L. (Halver *et al*., 1975; Dabrowski, 1976; Hilton *et al*., 1978, 1979; Agrawal and Mahajan, 1980; Sandnes and Braekkan, 1981; Seymour, 1981; Dabrowski, 1991).

AsA could affect the newborn fish as well. It is long known that maternal demand for specific nutrients increases during reproduction period, in general (Sandnes, 1987). More specifically, the maternal reserves of nutrients are of vital importance for fish because, in the period of vitellogenesis, substances such as AsA ends up in the yolk to be used during the embryo development (Dabrowski and Blom, 1994; Blom and Dabrowski, 1995a). In tilapias, *Oreochromis mossambicus* (Peters), lack of AsA supply in broodstock fish leads to fry deformity. On the contrary, AsA supplementation on broodstock diet improves hatchability and fry condition (Soliman *et al*., 1986).

Rainbow trout lacks the ability to synthesize the required amount of AsA in order to maintain its needs under rearing conditions (Gavriilidou, 1998). Therefore, AsA is considered as an essential nutrient in rainbow trout diet. It is well known, that AsA is involved in rainbow trout growth performance (Gavriilidou, 1998), but the very role of AsA in reproductive output is still under consideration.

Sandnes *et al*. (1984) indicated that rainbow trout broodstock should be fed with adequate amounts of AsA to provide eggs with more than 20 mg AsA g^{-1}, which approximates the lower limit for normal fry development. Waagbo *et al*. (1989) also found that AsA was related to endocrine functions in the maturing rainbow trout, concerning the hormones oestradiol-17β and vitellogenin, in particular. More recently, Dabrowski and Blom (1994) reported that AsA deficiency in the broodstock rainbow trout diet, provided for several months prior to spawning, considerably depletes AsA reserves in eggs, but has no significant effect on the embryo's survival. In another study, they also reported that the rainbow trout's reproduction requirements of AsA might be higher than

those demanded for the growth of immature fish. Their findings indicate that a level of 400 mg kg^{-1} is necessary to optimize tissue AsA levels and achieve maximum reproductive success (Blom and Dabrowski, 1995ab). They also suggested that high levels of AsA in the eggs, provided from the broïdstock, has beneficial effects on the young fry (Blom and Dabrowski, 1996).

The aim of the present study was to obtain more information about the effect of dietary AsA on the rainbow trout reproductive output, involving the parameters of fecundity, diameter of eggs, hatchability, embryo and sac-fry mortality and, the percentage of the total rainbow trout fry produced.

MATERIALS AND METHODS

Rearing conditions: The experiment was conducted at the Edessa's Hatchery, Greece. A random sample of 400 rainbow trout, of 18 months of age, with a mean body weight of 438.07±4.42 g (mean±SEM) was used, obtained from Hatchery's broodstock (Batzios, 1999). Fish were equally divided into 4 groups, ensuring a relative degree of homogeneity between groups (Batzios, 1995). Each group was separately placed in a rectangular concrete tank (approximate volume $1m^3$), with an average water temperature of 13±0.1°C and a pH level of 7.12±0.02 (mean±SD), during the experimental period. The average dissolved oxygen was 9.83±0.19 mg l^{-1} and 9.38±0.6 mg l^{-1}(mean±SD), at the supply and the drainage point, respectively. Water flow was set at a rate of 3.3 l sec^{-1}.

Dietary treatment: Four diets, AsA-0, AsA-600, AsA-1200 and AsA-2400, were randomly assigned, one for each group. All diets were identical in composition, except for the supplementation of AsA (pure crystalline L-ascorbic acid provided by La Roche). More specifically, diet AsA-0 was not supplemented with AsA, while the diets AsA-600, AsA-1200 and AsA-2400 were supplemented with 600, 1200 and 2400 mg AsA kg^{-1}, respectively. The diets were prepared in the laboratory and their composition was calculated to meet with the nutritional requirements of the fish (Halver, 1976, 1982, 1989; Lovell, 1991). An electrical blender was employed for mixing the ingredients; then diets were cold pelleted. L-ascorbic acid was incorporated in the diets as a prepared mixture in wheat middlings. The wet pellets, 8mm in diameter, were freshly prepared every 15 days, immediately placed in sealed plastic bags and stored at –23°C in order to minimize possible loss of L-ascorbic acid (Sandnes and Utne 1982; Li and Lovell 1985; Albrektsen *et al.*, 1988). Diets were tested for AsA concentration by the official method employed in the European Union for the analysis of ascorbic acid in feeding stuffs (HMSO., 1982). No significant differences were observed among the concentrations of AsA added in the diets and those after storage. Table 1 presents the basal composition of the diets used.

Table 1: Basal composition of the diets fed to broodstock rainbow trout for a period of 144 days before reproduction

Ingredient	% in diet
Fish meal	45.0
Meat meal	10.0
Soya meal	14.4
Fish oil	7.0
Wheat middlings[a]	22.5
Choline chloride	0.6
Methionine	0.3
Vitamin- mineral premix (free AsA)[b]	0.166

[a] Amount to be adjusted for AsA added

[b] The premix contained the following ingredients per kg of feed: Vitamin A 24000 IU, Vitamin D 2500 IU, Vitamin E 160 mg, Vitamin K 25 mg, Vitamin B_1 23 mg, Vitamin B_2 30 mg, Vitamin B_6 20 mg, Vitamin B_{12} 0.1 mg, Niacin 225 mg, Pantothenic acid 90 mg, Folic acid 8 mg, Biotin 1.5 mg, Inositol 300 mg, BHT 100 mg, Fe 45 mg, Zn 45 mg, Cu 5 mg, Co 1 mg, I 1.5 mg, Se 0.1 mg, Mn 50 mg, Mg 45 mg

Fish were given the experimental diets for 6 days a week, manually, at quantities equivalent to 1% (dry matter) of fish body weight per day, for a period of 144 days before reproduction. A sample of 10 fish from each group was randomly selected and weighed every two weeks, to adjust feeding rates.

Broodstock weight evaluation: At the end of the feeding trial, broodstock fish of each group were individually weighed, under anaesthesia (solution of aqueous of 4-methyl-2-pentanol, 1ml l^{-1}).

Evaluation of reproductive output: In order to obtain the eggs, experimental fish were macroscopically examined and females that were considered to be mature were stripped. Eventually, 15 females were used from group AsA-0, 17 from AsA-600, 20 from AsA-1200 and 15 from group AsA-2400.

The ova were dry fertilized by the addition of the seminal fluid of a male to the eggs collected from two females, all from the same experimental group (Pneumatikatos, 1993). At this point, dead eggs were removed, considered as unfertilized. The remaining eggs of each group, considered as the fertilized eggs, were placed on incubator trays, set in separate units. Water was distributed to the units at a rate of 5 l min^{-1} at a temperature of 12.5±0.2°C, pH 7.0±0.1 and dissolved O_2 12.0±0.5 mg l^{-1}, during the whole incubation period. Fecundity (number of eggs per kg of female) was calculated using a random sample of $n_1 \cong 50$ eggs, taken from each female's weighed ova. Furthermore, a mean value of the egg diameter was estimated using a random sub-sample of $n_2 = 20$ eggs, which were individually measured.

Table 2: Reproductive output parameters of rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction. Groups AsA-0, AsA-600, AsA-1200 and AsA-2400 represent fish fed with 0, 600, 1200 and 2400 mg kg^{-1} AsA, respectively

	Groups			
Dependent variable	AsA-0	AsA-600	AsA-1200	AsA-2400
Number of females	15	17	20	15
Fecundity (per kg of female)	1445±118[a]	1581±84[a]	1588±102[a]	1518±88[a]
Egg mean diameter (mm)	4.32±0.08[a]	4.44±0.11[a]	4.56±0.11[a]	4.46±0.09[a]
Total number of eggs (estimations)	18350	20434	23847	17707

Values are expressed as mean±SEM
Mean values in the same row with a superscript in common do not significantly differ (P>0.05)

Table 3: Incubation period from eggs fertilization to hatching (days and D°C), obtained from rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction

	Groups			
Incubation period	AsA-0	A-600	AsA-1200	AsA-2400
Days	29	24	27	27
D°C	362.5	300	337.5	337.5

Table 4: Mean daily changing rates (%) of embryo and sac-fry mortality, obtained from rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction

	Groups			
Dependent variable	AsA-0	AsA-600	AsA-1200	AsA-2400
% Mortality of embryos	6.85[a]	9.62[a]	7.29[a]	6.32[a]
% Mortality of sac-fry	6.6[a]	1.97[b]	8.76[a]	2.4[b]

Mean daily changing rates in the same row with a superscript in common indicate no significant difference between the respective initial regression coefficients (P>0.05)

Dead eggs were removed from each incubator tray and were counted in order to estimate the mortality of the embryos at a regular basis. Two days after hatching, the trays were removed and sac-fry were set free in the incubator units. In each unit, the dead sac-fry were counted for a period of 38 days and at a regular basis. This period was considered as the proper time for the yolk sac absorption.

Statistical analysis: Several analytical techniques were applied for the statistical analysis of the experimental data. To determine possible effects of diet on broodstock body weight, fecundity and egg diameter, one-way analysis of variance were applied (the completely randomized design) in case of homogeneity of variances, with or no transformed data. Variances were tested for homogeneity using Levene's test. Differences between groups were tested for significance by Duncan's new multiple range test. Significance of $P \leq 0.05$ was used, unless otherwise noted (Kanji, 1994).

Chi-square procedure (χ^2 test) was applied to test frequencies homogeneity of hatchability, final sac-fry mortality and the percentage of the total fry produced, among groups (Katos, 1986).

Regression analysis was used and especially the Ordinary Least Squares method (OLS), to estimate the mean daily changing rates, of embryos mortality for the incubation period, as well as of sac-fry mortality for the 38-days period of the yolk absorption. More specifically, the mean daily changing rates were estimated using the model $Y=A*(1 + j)^t$, where Y = dependent variable, A = parameter for estimation, j = daily changing rate (%) and t= time (Batzios, 1991, 1999). Student's test was used to evaluate differences concerning the estimated regression coefficients between experimental groups (Allen and Cady, 1982).

RESULTS

No signs of AsA deficiency were recorded in broodstock fish fed with the unsupplemented diet. No significance difference (P>0.05) was found between groups, concerning the broodstock body weight at the end of the 144-days feeding trial, either. However, fish fed with the unsupplemented diet (group AsA-0), finally showed a relatively higher body weight (707.92±22.66 g, mean value±SEM), in relation to the fish fed with the AsA supplemented diets (664.39±18.85, 661.49±17.45 and 679.67±17.55 g for groups AsA-600, AsA-1200 and AsA-2400, respectively).

The mean values of parameters such as the fecundity (number of the eggs per kg of female's body weight) and the egg diameter showed different levels among the experimental groups (Table 2). More specifically, the estimated mean value of fecundity was 1445±118 for group AsA-0 and 1581±84, 1588±102 and 1518±88 for groups AsA-600, AsA-1200 and AsA-2400, respectively. Furthermore, the mean value of the egg diameter was

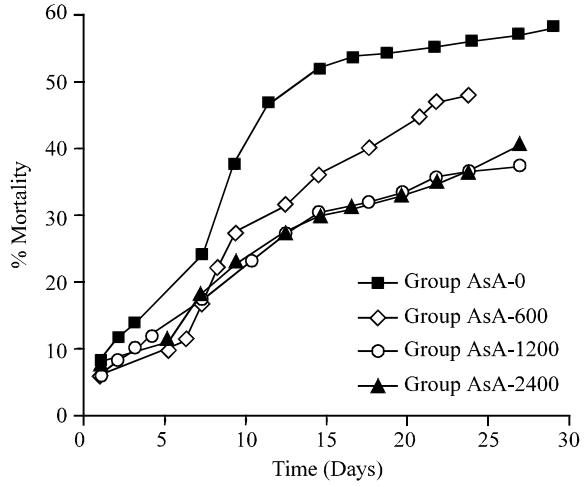

Fig. 1: Mortality (%) of the embryos, obtained from rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction, during incubation period

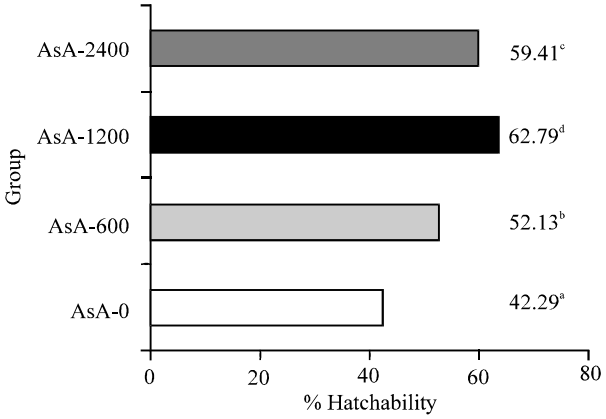

Fig. 2: Hatchability (%) of the eggs, obtained from rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction {values with a superscript in common are not significantly different}

estimated to be 4.32±0.08 mm for group AsA-0 and 4.44±0.11, 4.56±0.11 and 4.46±0.09 mm for groups AsA-600, AsA-1200 and AsA-2400, respectively. Statistical evaluation of the results above (one-way analysis of variance) revealed that the observed differences between the experimental groups are not significant (P>0.05).

The incubation period from the egg fertilization to hatching ranged from 24 to 29 days, for the four groups (Table 3).

Mortality of embryos (%) calculated at specific points of time during the incubation period, for the four

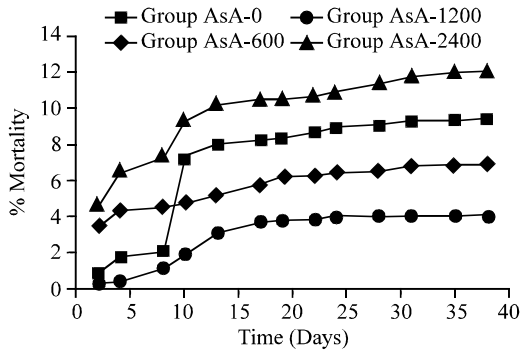

Fig. 3: Mortality of the sac-fry, obtained from rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction

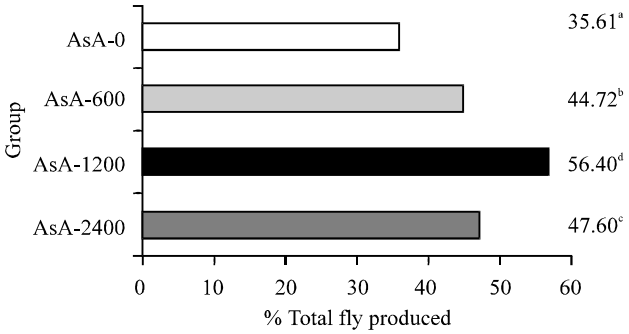

Fig. 4: Percentages of the total fry produced, obtained from rainbow trout broodstock fed increasing levels of AsA for a 144-day period before reproduction {values with a superscript in common are not significantly different}

experimental groups, is shown in Fig. 1. At the beginning of the incubation period (2nd day), the embryo mortality was calculated at 8.21, 6.08, 5.52 and 7.64% for groups AsA-0, AsA-600, AsA-1200 and AsA-2400, respectively. Up to the 8th day, the embryo mortality followed an increasing pace of change reaching the 23.64, 16.82, 17.22 and 17.90% for groups AsA-0, AsA-600, AsA-1200 and AsA-2400, respectively. Three days later embryo mortality more than doubled in all groups, while after the 12th day of the incubation period a diminishing rate of increase followed. At the end of the incubation period the mortality of the embryos reached 57.71% for the unsupplemented fish and 37.20% for fish fed with 1200 mg kg^{-1} dietary AsA. Fish on diets supplied with 600 and 2400 mg kg^{-1} dietary AsA reached 47.87 and 40.59% of embryo mortality, respectively.

Calculations involving (%) egg hatchability (expressed as the number of eggs hatched divided by the number of fertilized eggs) are presented in Fig. 2. From

this figure it is shown that fish on the 1200 mg kg^{-1} AsA supplemented diet exhibited a relatively higher hatchability level (62.79%). In contrast, the lowest level was noticed for fish fed with the unsupplemented diet (42.29%). Testing the observed frequencies for homogeneity (χ^2- test) has shown that, at a level of significance α=1%, the addition of increasing levels of AsA to broodstock diet causes significantly different results, concerning hatchability.

Concerning sac-fry mortality level, for the time elapsed from egg hatching to the time of yolk absorption (38th day), a different behaviour was noticed among the four groups (Fig. 3). More specifically, one day after hatching the mortality level was calculated at 0.76, 3.33, 0.17 and 4.48% for groups AsA-0, AsA-600, AsA-1200 and AsA-2400, respectively. Up to the 13th day, the sac-fry mortality followed increasing rates almost in all groups. For the rest of the period under study, sac-fry mortality followed diminishing rates of increase, resulting to the levels of 9.37, 6.77, 3.94 and 12.01%, for groups AsA-0, AsA-600, AsA-1200 and AsA-2400, respectively. Having tested the observed frequencies for homogeneity (χ^2- test) it has been concluded that, at a level of significance α=1%, the addition of increasing levels of AsA to broodstock diet causes significantly different results, concerning sac-fry mortality 38 days after hatching.

Table 4 presents the mean daily changing rates (%) of the embryo mortality for the incubation period, as well as of the sac-fry mortality for the 38-days period of the yolk absorption, obtained from the respective estimated regression equations. Having compared the respective regression coefficients among experimental groups (t-test) it has been concluded that there are no significant differences in the mean daily embryo mortality changing rate ($P>0.05$). On the other hand, the mean daily sac-fry mortality rate among experimental groups differs significantly ($P\leq0.05$).

Fig. 4 presents the percentage of the total fry produced (%), calculated as the total number of fry finally produced to the total number of eggs initially received. Having tested frequencies for homogeneity among groups (χ^2-test) it is concluded that increasing levels of AsA to broodstock diet causes significant differences as to the percentage of the total fry produced. Fish on the diet supplemented with 1200 mg kg^{-1} dietary AsA showed the significantly highest level (56.40%), though fish on the unsupplemented diet showed the lowest one (35.61%).

DISCUSSION

The findings of the present study show that there were no clinical signs, associated with AsA dietary absence, in rainbow trout broodstock. Fish fed with the unsupplemented diet reached the highest, though not significantly different, mean body weight, compared with fish fed diets supplemented with 600, 1200 and 2400 mg AsA kg^{-1}, after the 144-day experimental period.

Literature reports absence of dietary AsA in diet doesn't seem to affect, when rainbow trout weigh 11g (Sato *et al*., 1978), 20 g, (Skelbaek *et al*., 1990), 219 g (Volker and Fenster, 1994) and 1581 g (Dabrowski *et al*., 1994) neither when trout are of 11 months (Primbs and Sinnhuber, 1971), 3-4 years (Waagbo *et al*., 1989) and 1 year of age (Dabrowski *et al*., 1990). Similar results were reported for common carp, *Cyprinus carpio* L., (Ikeda and Sato, 1964), channel catfish, *Ictalurus punctatus* (Rafinesque) (Li and Lovell, 1985) and Atlantic salmon, *Salmo salar* L. (Hardie *et al*., 1991).

Rainbow trout can synthesize a limited amount of AsA (Yamamoto *et al*., 1978); which is insufficient for the needs required at an early age. As fish grow older (i.e. our experimental broodstock fish), it seems that the dietary AsA needs decrease (Sato *et al*., 1978) due to lower AsA requirements for certain biochemical functions or to an increased AsA storage capacity of fish, combined with efficiently endogenous reuse (Waagbo *et al*., 1989). Moreover, it has been suggested that AsA dietary supplementation was not considered important when fish were reared under normal condition (Primbs and Sinnhuber, 1971). On the contrary, AsA should be added to the diet when fish were reared under an abnormal condition, independently of the fish age. This suggestion supports the negative effect of dietary AsA absence on the growth of 2-year-old rainbow trout, kept under low temperatures and having their spawning delayed (Dabrowski *et al*., 1994).

Statistical evaluation of our results revealed that feeding rainbow trout broodstock with a diet devoid of AsA or supplemented with 600, 1200 and 2400 mg AsA kg^{-1}, for a 144-day period before reproduction, did not significantly affect the mean fecundity of fish, or the mean egg diameter ($P>0.05$). It should be noted that fish on the unsupplemented diet produced a relatively lower number of eggs per kg of female, in relation to fish on the supplemented diets. Among the four experimental groups the highest value was noticed at AsA-1200. Similar results were also observed for the mean egg diameter.

It must be noted that fish fed with the unsupplemented diet showed the lowest fecundity numbers, but at the same time revealed the highest body weight. On the contrary, fish provided the 1200-supplemented diet showed the highest fecundity and the lowest body weight. It is known that during vitellogenesis the ovary grows rapidly at the expense of muscle growth (Sandnes *et al*., 1984). In the case of the supplemented fish in our experiment, AsA may act in a way that the dietary energy was used for reproduction, at the expense of growth.

Sandnes *et al*. (1984) experimenting with two groups of rainbow trout broodstock fed without AsA, or with the addition of 115 mg kg^{-1} AsA, did not report significant differences, concerning the number or the diameter of the eggs, although values were higher in group fed with the supplemented diet. Concerning the mortality of the eggs, a significant difference was noted until eye stage. It could be referred that the above mentioned study was conducted under water with 30% of salinity. Moreover, the addition of 300 mg AsA kg^{-1} to rainbow trout broodstock diet didn't cause significant changes on fecundity, compared to broodstock fed with AsA-free diet (Dabrowski and Blom, 1994). It must be noted, though, that the fish of this study were kept at prolonged light cycles. On the contrary, the addition of a greater amount of ascorbic acid (1250 mg kg^{-1}) to tilapia (*Oreochromis mossambicus*) progenitors significantly increases the hatching of the eggs (Soliman *et al*., 1986).

In our study, the group of fish on the 1200-supplemented diet showed significantly higher hatchability (62.79%). The same group showed the lowest sac-fry mortality, after the 38 days period needed for yolk absorption (3.94%). In addition, fish of group AsA-1200 exhibited the highest percentage of the total fry produced (56.40%).

The group of fish fed with the 600 mg kg^{-1} supplemented diet showed 52.13% hatchability and 6.77% of final sac-fry mortality. The percentage of the total fry produced was calculated at 44.72%.

The group provided the highest level of AsA (2400 mg kg^{-1}) exhibited a slightly lower hatchability (59.41%) than that of the group AsA-1200 and a slightly higher one than that of the group AsA-600. It also showed the highest level of final sac-fry mortality of all groups (12.01%). Furthermore, the percentage of the total fry produced was 47.60%, slightly lower than that of the group AsA-600.

Fish on the unsupplemented diet provided 42.29% of hatched eggs, which was the lowest value among the experimental groups. Furthermore, this group exhibited a relatively high final sac-fry mortality (9.37%) and the lowest percentage of the total fry produced, among all groups (35.61%).

Moreover, the mean daily changing rate (%) of the sac-fry mortality for the 38-day period of the yolk absorption was considered to differ significantly between experimental groups. This should be co-evaluated along with the mortality rates estimated at the starting-point (one day after hatching). In contrast, the mean daily changing rate (%) of the embryo mortality for the incubation period showed a similar behaviour among the four experimental groups.

Many researchers have argued that a certain quantity of AsA is probably transferred from the progenitors to the eggs and is consequently used for the development of the larva until they receive food for the first time (Sandnes *et al*., 1984; Soliman *et al*., 1986; Sato *et al*., 1987; Waagbo *et al*., 1989). It is also well known that AsA plays an important role in collagen formation of sea urchin embryo archenteron (Mizoguchi and Yasumasu, 1982). Soliman *et al*. (1986) indicate metabolism of maternal AsA during embryonic development, in *Oreochromis mossambicus*. They also suggest that AsA may be transferred from female's ovary to the egg and then to the fry, providing the fry with a store of AsA after hatching. In our experiment, the lack of AsA in the unsupplemented group might lead to AsA deficiency in the developing embryo, resulting in the lowest performance.

Guinea pig pups obtained from females on a high AsA supplemented diet showed accelerated mortality after fed with an AsA-free diet (Norkus and Rosso, 1975). In addition, pups from mothers on high AsA levels, exhibited higher AsA requirements (Basu, 1985). In our experiment, a similar mechanism, related to an after birth AsA dependency, might lead to a high sac-fry mortality, when fish were obtained from female on the 2400-supplemented diet. This assumption is still under investigation.

In conclusion, according to the results of the present research, the addition of ascorbic acid, in certain amounts, to the broodstock diet leads to the reduction of the consequences arising from the lack of ascorbic acid during the first stages of life of the rainbow trout fry. It could be suggested that the addition of 1200 mg AsA kg^{-1} in broodstock diet increases hatchability and sac-fry viability.

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