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The Effects of Dietary Supplementation of Astaxanthin and B-caroten on the Reproductive Performance and Egg Quality of Female Goldfish (*Carassius auratus*)

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ABSTRACT

The present research was aimed to study the effects of different sources of carotenoids and their varying concentrations on the reproductive functions of goldfish. The study was carried out in seven treatments with three replicates at the Bony Fish Hatchery Complex (Rasht, Iran) from December 2011 to May 2012. Experimental diets containing 50, 100, and 150 mg kg-1 astaxanthin and 50, 100, and 150 mg kg-1 β-caroten along with a carotenoid free basic carp feed as control were utilized. The goldfish broodstock were fed with the formulated diets for a period of four months. In May, eggs obtained from the female goldfish were fertilized with the semen of identical male goldfish fed with control diet and the absolute, working and relative fecundities and egg fertilization along with egg survival rate were estimated for different treatments during incubation period. The results showed that there was no significant difference in the fecundity rates among different diet treatments. Nevertheless, the diameter and the number of egg per gram of the fertilized eggs in fish in the A_{150} (astaxanthin 150 mg kg-1) treatment were greater than those in the other treatments ($P \leq 0.05$) and this treatment showed higher egg survival rates in the incubation period ($P \leq 0.05$). Correlation of egg astaxanthin with fertilization rate and survival rate was significant. Moreover, there was significant correlation between β-caroten and survival rate ($P \leq 0.05$).

Keywords: A*staxanthin , β-caroten, carotenoid, fertilization rate, goldfish*

INTRODUCTION

Sexual maturity in fishes and most other aquatic animals occurs when gonads are fully developed. Many studies have been carried out on various fish species which were focused on a number of influential factors on egg quality, including the nutrition of broodstock, the environmental condition, and the methods of breeding (Bromage 1995; Bruce et al., 1999; Brown et al 2003).The role of nutrients and supplementary feed on boosting the reproductive physiology of broodstock have already been examined (Bromage 1995; Hardy, 1985 and Pavlov et al (2004).

The effect of carotenoid on improvement of sexual ripeness has been confirmed in trout (Swingle, 1961) shrimp (Quinitio et al., 1990; Harrison 1990, 1997; Dall, 1995) and female lobster (Linan-Cabello et al., 2004). Liver development normally occurs during ontogeny stages. The development of liver is associated with an increase in the liver indices among fish and most other crustaceans, which is due to the accumulation and concentration of nutrients suitable for the growth and development of the ovary during sexual maturity of spawners.

Carotenoid present in the diet, when consumed by aquatics, is accumulated in the liver and hypopancreas of crustaceans which is then transferred from the liver to the ovaries in the late stages of maturity.

Many researchers believed carotenoids have an elimination role of for free radicals of oxygen during embryonic development. This function protects eggs against free radical damages. Furthermore, carotenoids

can protect eggs against severe light during embryonic development. Recent research have shown that egg carotenoids have various functions during embryonic development and can protect egg health by `elimination of waste products (Paibulkichakakul et al., 2008).

A similar process was found in various Salmonid species (Bjerkeng et al., 2003),shrimp (Linan-Cabello et al., 2004, Paibulkichakakul et al., 2008) and goldfish (Benjamin and Del Tito,1988).

Fish fecundity is an interesting subject in recent research (Khara et al., 2012, Mousavi-Sabet et al., 2012). Also, a lot of research has been conducted on relationship between carotenoids and aquatic animals and plants (Mustafa and Turner, 2011; Pingret, 2012; Black et al., 2013; Mandal et al., 2012; Briers et al., 2013). In goldfish it has been proven that the β-caroten consumed by female spawners is reserved in the liver wherein it is transformed into vitamin A1. Afterward vitamin A1 turns into vitamin A2, moving later on to the eggs during the process of yolk formation (Benjamin and Del Tito,1988). Retinol or vitamin A is one of the basic compounds, necessary for vision improvement in fresh water fish larvae (West et al., 1966). This has been shown to be the case in salmonids. In these species (salmonidae), carotenoid such as β-caroten, lutenin and astaxanthin taken up by fish are mostly stored in the skin, muscles and liver tissues which are conveyed to the ovaries and yolk during their formation. These carotenoids are accumulated as esterifies in the fully developed eggs (Steven, 1948; Page, 2005).

It has also been shown that in rainbow trout (*Oncorhynchus mykiss*), different concentrations of astaxanthin during maturity stages tend to increase glycogen level in the liver. This can in turn, result in the improvement of liver structure and better performance of certain nutrients in the production of buffer substances (Swingle, 1961, Bromage 1995). Nevertheless, other research suggest that diets containing carotenoids may trigger higher chances of fertilization in eggs (Hubbs and Stavenbagen, 1958, Hubbs and Strawn, 1957, Deufel, 1965, Georgiev, 1971, Mikulin, 2003, Huang et al., 2008). However, some researchers such as Quantz (1980), Tveranger (1986), Totrrissen (1984), Christiansen et al (1995), and Choubert et al (1985) reported no correlation between diets containing varying levels of carotenoid and mortality rate, egg development and survival in alevin salmonids.

The goldfish (*Carassius auratus*) is one of the most precious ornamental fish in the world. For this reason, a large number of fish are annually propagated and farmed. Goldfish can be a good representative of the whole farmed Cyprinidae family (Sokolowska et al., 1984).Therefore, the present research can determine the role of carotenoids in increasing efficiency of various propagation procedures for goldfish and reveal the efficacy in the reproductive processes and embryonic developmental stages. In addition, the effects of diets with varying carotenoid contents, including astaxanthin and β-carotenoids on sexual maturity, egg quality and egg survival in the course of embryonic and larval developmental stages have been the focus of attention in this study.

MATERIALS AND METHODS

The experiment was carried out at the Bony Fish Hatchery Complex (Rasht, Iran) from December 2011 to May 2012. In order to compare diets containing various amounts of astaxanthin and β-caroten with each other and with the control diet, seven treatments with triplicate were designated for the study. To culture goldfish broodstock with the experimental diets, 21 fiberglass 4 m^3 tanks, with a diameter of 2m and depth of 1.2m, were used for the experiments.

One year old broodstock (n=1050) with an average weight of 47.21±2.19g and length of 14.71±0.31cm were selected (50 fish in each tank).Prior to the start of the experiment, fish were kept in 2 m^2 fiberglass tanks containing 5% brine for disaffection. During adaptation period, fish were fed with the control diet given twice daily (10.00 am and 3.00 pm).

Tanks were randomly designated for the study. Water temperature was examined daily (12.64±0.29 ^oC) .The average pH, dissolved oxygen, nitrate and ammonia concentrations were 7.43±0.28, 7.08±0.44 mg l^{-1}, 0.51±0.1 mg l^{-1} and 0.1±0.1 mg l^{-1}, respectively. Meanwhile, 10% of the total

tank water volume was replaced from the bottom each day.

Experimental diets

Astaxanthin was as Carophyll ® pink10% DSM and β-caroten was as β-caroten10% CWS. Experimental diets containing 50, 100 and 150mg kg-1 of astaxanthin along with three other rations consisting of 50, 100 and 150 mg kg-1 of β-caroten as well as one control diet free from any additive carotenoid were prepared for the study (Table1 and 2). The designated amount of astaxanthin and β-caroten were firstly added to 35 ^{o}C water and then mixed with the diets according to the procedure described by Page and Davies (2003). The feed was turned into paste using fish oil and water. They were then transferred to the California pelleting machine and finally formed into 2×3 mm pellet. The pellets were then placed in a drying machine for 24 hours at the temperature of 50 ^{o}C. The prepared feeds were put into dark plastic packs and kept in a freezer at -18 ^{o}C freezer. The feed preparation process was done once in a month. Fish kept in each tank, were fed up to 2% of their biomass for four months. During cold days when water temperature dropped below 6 ^{o}C feeding was not fulfilled.

In mid April, when water temperature was 21 ^{o}C, female fish received Ovaprime hormone injections ata dose of 0.2mg l^{-1} per kilogram body weight. About one month prior to the start of fish propagation, 300 male broodstock (average weight: 51±2.21g) were collected and fed with the control diet in order to homogenize the propagation procedures and eliminate male goldfish effects. Similar to females, the male fish were also injected at the same time with a dose of 0.1mg l^{-1} of Ovaprime hormone per kilogram body weight. Both male and female fish received the injections at the basal of their pectoral fins.

The male:female ratio was 1:2 and 4 broodstock were selected for each replicate in each step of propagation. As a result a total number of 12 fish were artificially propagated from each treatment. The average water temperature was 19^{o}C at the time of artificial fertilization. During propagation, egg diameter, absolute fecundity, working fecundity and relative fecundity were indentified for broodstock in each treatment.

Two hours after propagation and at the start of 4-cell divisions, sampling of eggs was carried out to determine the egg number per each gram and the percentage of egg fertilization. Shortly after this, the diameter of the fertilized eggs was measured.

The egg number per gram was determined soon after fertilization and at the start of gastrolation. The elapsed time between the fertilization and gastrolation stage was also recorded. At each propagation stage, a twenty g sample of eggs was extracted from each fish and transferred to a vase incubator 250 cc.

The water temperature was 21±1.1 ^{o}C in various incubation stages. During the course of incubation, egg samples were obtained at the start of the gastrolation stage and hatching. The samples were then stored in liquid nitrogen in terms of carotenoid and later kept in a freezer at -80 ^{o}C.

At the end of the incubation stage, the egg survival rate during gastrolation stage and the hatching rate of eggs were specified for each breeder along with the proportion of fertilized eggs and the obtained larvae to each breeder weight.

Finally, correlations between total carotenoid, astaxanthin and β-caroten with fertilization and survival rates were estimated.

Table 1. Composition of various diets (g/kg) of broodstock diets

Ingredients	A_{50}	A_{100}	A_{150}	control	B_{50}	B_{100}	B_{150}
Fish meal	290	290	290	290	290	290	290
Soybean meal	290	290	290	290	290	290	290
Corn Meal	200	200	200	200	200	200	200
Clupen fish oil	20	20	20	20	20	20	20
Wheat flour	164	164	164	164	164	164	164
Lime	4.5	4	3.5	5	4.5	4	3.5
Methionine	2	2	2	2	2	2	2
Lysine	2	2	2	2	2	2	2
Kavillamycinea	2	2	2	2	2	2	2
Dicalcium phosphate	5	5	5	5	5	5	5
Salt	5	5	5	5	5	5	5
Vitamin permixb	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Mineral permixc	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Astaxanthind	0.5	1	1.5	-	-	-	-
β-carotene	-	-	-	-	0.5	1	1.5
Total	1000	1000	1000	1000	1000	1000	1000

a. Kavillamycine: Contains 10,000 mg kg Avilomycin
b. Vitamin Permix.10g: ρ-aminobenzoic acid 10.0 mg; biotin 0.40 mg, inositol 400.0 mg; nicotinic acid, 40.0 mg; Ca-pantothenate, 60.0 mg; pyridoxine-HCl,12.0 mg; riboflavin, 8.0 mg; thiamin-HCl, 4.0 mg; menadione, 4.0 mg; cyanocobalamine, 0.08 mg; calciferol, 1.20 mg; folic acid, 0.80 mg; choline chloride, 120.0 mg.
c. Mineral Permix.100g:$K_2HPO_4$2.0g,$Ca_3(PO_3)_2$2.720g.$MgSO_47H_2O$3.04g
d. Astaxanthin =Carophyll ® pink10% DSM
e. β-caroten: β-caroten10% CWS
The amount of crude protein, total fat, carbohydrate, ash and moisture by the method described by AOAC, 1995 were measured (Table 2).

Table 2. Proximate analysis of experimental broodstock diets (means± standard error)

Treatment Proximate	A_{50}	A_{100}	A_{150}	control	B_{50}	B_{100}	B_{150}
Crude Protein(%)	33.16±0.26	33.16±0.05	34.11±0.34	33.81±0.70	34.50±0.48	33.97±0.50	33.84±0.16
Crude Fat (%)	7.98±0.70	7.67±0.68	8.75±0.43	7.03±0.52	8.04±0.43	8.62±0.31	8.57±0.34
Ash (%)	9.05±0.32	9.29±0.08	9.31±0.04	9.03±0.21	9.24±0.06	9.26±0.09	8.98±0.12
Fiber (%)	2.32±0.24	2.94±0.14	2.84±0.28	3.05±0.03	2.72±0.16	2.78±0.21	2.99±0.06
Humidity(%)	5.70±0.03	5.71±0.03	5.73±0.07	5.77±0.02	5.60±0.18	5.71±0.04	5.69±0.04
Total carotenoids mg/kg)	52.26±1.58	99.53±3.06	149.56±1.26	32.44±1.92	49.61±1.45	101.30±114	151.52±1.76

Carotenoid extraction

The fish eggs were collected during various sampling stages and kept at -80 ºC in liquid nitrogen. The 1.5 g samples were dried at -55 °C using Christ Freeze Dryer-Alpha-1-2 Ld plus model. The samples were then placed inside amber flasks in N_2 gas environment at -18 °C. Carotenoid extraction from the samples was carried out using pure acetone specific to HPLC as well as n-hexane (Hex, analytical grade) based on a hexane:acetone ratio of 1:3. Afterwards, 50 mg of the dried sample was stored in a glass bottle, wrapped with aluminum foil and 5 ml of hexane:acetone solution was added to the mixture. The procedure was followed by a 10 minute agitation of the mixture. The ambient temperature was 22 ºC under dim light condition during the extraction of carotenoid from the sample (Suhnel et al., 2009).

Total carotenoid measurement

To determine the amount of total carotenoids extracted, UV-visible spectrophotometry (Hitachi, U-1800) was used for a spectral window between 380 and 750 nm in triplicate. Carotenoid concentration was obtained using the Lambert-Beer law and for calculation purposes the following equation was applied to the absorbance values:

Total carotenoid (µg/mg) = [absorbance/Є × molecular mass × 1000 × sample volume (mL)]/ sample dry weight (mg)

The specific optical extinction coefficient Є 1×1cm of 124000(astaxanthin) at 460 nm was used (Mínguez-Mosquera et al., 2000) in conjunction with a molecular mass of 596.84

(astaxanthin). Absorbance measurements were performed in triplicate and the values previously averaged were applied in the above equation (Wallat et al., 2005). Correlation was estimated by means of Pearson method.

Measurement of carotenoid

To measure astaxanthin and β-caroten concentrations, their authentic standards were utilized for the experiment. The standard solutions were prepared with a 2.5-540 μg-l^{-1} concentrations (Sigma aldrich and Fluka). All the production and extraction procedures for the samples were carried out under the dim light and the ambient temperature of 22 ^{o}C. In order to extract carotenoid from the fish eggs, a 50 mg wet sample was homogenized in 3 ml acetone and vortexed for 30 seconds, followed by 5 minutes centrifuge (1500 RPM). A mixture of solvents, including 2 ml of hexane and 0.5 ml of water were added to 2.5 ml volume of the upper layer. Then it was vortexed for 30 seconds, followed by 5 min centrifuge (1500 RPM) and hexane layer dried in clean tube under a steam of nitrogen. 250 μl of methanol was added to the residue. 70 μl of this solution was injected into the HPLC [Younglin HPLC system equipped with a pump (SP930D), UV detector (730D), reodyne injector, and Autochro 2000 integrator software]. The HPLC separation condition includes: 1.4 ml min^{-1}; wave length: 470 nm; temperature: 25 ^{o}C; Column: C18, Inertsil ODS- 3V250×4.6mm.

The Mobile phase A included methanol-water (97/3) and mobile phase B consisted of methanol, THF and water (37:60:3) (Suhnel et al., 2009).

Statistical analysis

The comparison of the results obtained was reported based on mean number of replications and with the inclusion of standard error of measurement. To ensure the normality of data, one-sample Kolmogoram Sminow test was applied (Zar, 1999). Meanwhile, in order to examine the consistency of variance, the Levene test was utilized (Lavene, 1960).

The one-way analysis of variance was used to compare the data related to propagation normative. In order to find out whether or not there was any significant difference (if any) between the means, Duncan test was used, at $P < 0.05$ level of significance.

RESULTS

The mean of physical-chemical parameters measured during culture of broodstock and egg incubation did not show any significant difference ($P > 0.05$).

Morphological parameters and reproductive indices

The average length and weight of broodstock showed no significant differences in different treatments ($P>0.05$). The total mean weight of broodstock seemed to have increased in the third phase of sampling (February) due to an increase in the weight of gonads. However such an increase failed to show any significant difference in terms of absolute fecundity of broodstock among different treatments ($P>0.05$). The high absolute fecundity rate across treatments suggested the absence of any significant difference in the absolute fecundity of broodstock among different broodstock. The greatest absolute fecundity was observed in A_{150} treatment whereas the lowest one was found to be in the control group (Table 3). Following propagation and calculation of total amount of eggs extracted from broodstock in each treatment, the highest and lowest working fecundity were recorded in A_{150} and control treatments, respectively ($P>0.05$) Table 3. The highest relative fecundity was observed in A_{150} treatment and the lowest one in the control group. In spite of the higher relative fecundity value in A_{150} treatment, there appeared to be no significant difference among various treatments in terms of relative fecundity ($P>0.05$) (Table 3). The greatest egg number per gram was observed in treatment B_{100}. In other words, the eggs extracted out of control group fish were comparatively smaller than those in the treatment B_{50}, B_{100} and A_{150}, which causes a significant difference across different treatments in terms of weight ($P\leq0.05$) (Table 3).

Table 3. Morphological parameters and reproductive indices means (±standard error) during breeder artificial propagation (n=72)

B_{150}	B_{100}	B_{50}	control	A_{150}	A_{100}	A_{50}	Treatment Index
67.32±3.48a	63.91±3.89a	63.36±1.62a	63.33±1.15a	68.79±2.67a	62.71±3.51a	63.50±3.66a	Weight (g)
11.55±0.22a	11.30±0.25a	11.43±0.18a	11.30±0.07a	11.59±0.18a	11.22±0.24a	11.32±0.25a	Length (cm)
9278.55±605.9a	9018.32±1180.92a	8718.09±372.83a	8448.45±793.25a	11716.22±870.90a	9325.39±1051.75a	9668.66±1035.6a	Absolute fecundity
8729.92±595.9a	7968.84±1008.04a	7601.43±345.17a	7329.14±706.56a	10296.92±743.35a	8191.13±896.57a	8440.70±872.05a	Working fecundity
141.24±7.79a	139.02±13.63a	138.01±5.59a	132.16±10.64a	171.42±11.83a	148.68±15.52a	150.78±12.01a	Relative fecundity
877.31±41.76b	920.96±44.73b	916.222±41.80b	1111.87±54.96a	937.48±30.66b	966.53±62.69b	987.68±55.48ab	Oocytes number/g

*Means in each row followed by the same letters are not significantly different from each other (α=0.05).

Table 4. Artificial propagation parameters and incubation means (±standard error) at different treatments (n=12)

B_{150}	B_{100}	B_{50}	control	A_{150}	A_{100}	A_{50}	Treatment Index
444.95±11.05b	469.52±9.36ab	469.52±9.36b	457.65±6.31a	487.94±4.69b	453.40±10.67b	448.76±6.39a	Egg number/g (fertilization time)
2.26±0.05a	2.14±0.04ab	2.19±0.03ab	2.05±0.02c	2.17±0.04ab	2.21±0.01ab	2.05±0.03c	Egg weight (mg)
97.21±0.59a	97.75±0.32a	96.98±0.37a	87.70±0.13c	96.52±0.31a	96.94±0.49a	94.63±0.27b	Fertilization rate (%)
1.28±0.01a	1.27±0.01a	1.28±0.03a	1.21±0.02b	1.28±0.02a	1.31±0.01a	1.28±0.02a	Egg diameter (mm)
47.04±3.01b	46.89±6.20b	47.86±2.25b	45.56±4.53b	77.99±6.05a	69.51±8.06a	78.61±7.44a	Fertilized egg/ gram broodstock weight (number/g)
394.90±8.84cd	419.56±4.02b	413.79±4.43bc	455.32±7.26a	390.47±7.99c	410.57±5.42bc	440.13±5.27a	Egg number/g (gastrolation time)
2.55±0.06ab	2.39±0.02c	2.42±0.03c	2.20±0.03d	2.57±0.05a	2.44±0.03bc	2.28±0.03d	Egg weight (mg) (gastrolation time)
86.45±1.19a	80.13±1.86b	64.85±2.63d	57.1±1.60e	87.70±0.37a	70.42±1.85c	69.30±0.98cd	Survival rate (%) (gastrolation time)
11.18±0.44b	10.42±1.07b	9.51±0.77bc	6.73±0.84c	15.56±1.44a	9.43±1.23bc	9.87±1.02b	Egg weight increscent percentage (%)
38.03±1.31b	30.32±1.37c	25.61±1.36d	25.52±1.23d	45.56±1.17a	31.94±1.48c	30.85±1.46c	Hatchability rate (%)
20.10±1.47bc	15.78±1.99cd	13.55±0.95cd	12.51±1.40d	43.00±4.21a	23.95±2.48b	27.10±3.09b	Larvae number/ gram broodstock weight (number/g)

*Means in each row followed by the same letters are not significantly different from each other (α=0.05).

Artificial fertilization

The results obtained from the artificial fertilization of goldfish broodstock showed significant differences in various treatments in terms of most of their fertilization indices the summary of which is presented as follows:

The preliminary results of artificial fertilization show that the mean number of egg per each gram of fish across different treatments (two hours after fertilization) proved to be significantly different from one to another. The highest egg number per gram was in A_{50} diet and also the lowest one was in B_{150} (Table 4). However, the mean individual egg weight in A_{50} and control treatment was comparatively smaller than the rest of treatments (Table 4). The results related to fertilization rate across treatment also turned out to be statistically significant among various treatments (Table 4). The results suggest that treatment B_{100} accounted for the highest fertilization rate among treatments while the lowest one belonged to the control group (Table 4). The average fertilization rate in carotenoid treated groups did not show any significant difference. In addition, the results indicated that the egg diameter in the control group was significantly different from other treatments (Table 4).

The ratio of the average number of fertilized eggs to breeder's weight (in grams) across different treatments proved significantly different ($P \leq 0.05$). The highest ratio of fertilized egg number to breeder's weight was found in $A_{50,}$ while the lowest

was in the control group (Table 4). The results demonstrated that the treatments involving the use of astaxanthin privileged from the greater egg:fish weight ratio compared to the treatments of β-caroten diets.

Incubation phase

The mean egg diameter of broodstock at the state of incubation (post fertilization) showed significant differences among treatments ($P \leq 0.05$). The mean number of eggs per gram of broodstock in the gastrolation phase was also different ($P \leq 0.05$). The highest number of eggs per gram was observed in the control group and the lowest was in A_{150} during the gastrolation phase. The average weight of individual egg weight of broodstock across different treatments increased significantly at the start of gastrolation stage.

The results also showed the difference in egg weight gain of broodstock in A_{150} which was comparatively greater than the other treatments. In this treatment, the breeder's egg weight showed an increase by 15.56±1.44% from the beginning to the end of gastrolation. However, it should be noted that the egg weight increase was not significant in various treatments which used β-caroten. Nevertheless, all treatments ranked lower than A_{150} in terms of average weight gain (Table 4). Study of egg survival rate during incubation phase revealed that at the start of gastrolation, the survival of breeder's egg differed significantly (Table 4) indicating that the highest egg survival rate in the beginning of gastrolation belonged to A_{150} whereas the control treatment accounted for the least egg survival rate. In this phase, the average egg survival rate was not different between A_{150} and B_{150}. However, other treatments possessed much lower egg survival than A_{150} and B_{150} treatments (Table 4). At the end of the incubation period, the measurement of larvae obtained from each incubator detected the highest hatching rate in A_{150} and the lowest (25.51±1.23%) in the control group. However, B_{50} failed to show any significant differences with the control group ($P \leq 0.05$) (Table 4, Fig 1). At the termination of incubation phase and upon computation of larvae obtained from each incubator, a significant difference was noticed between ratio of larval number to body weight (in grams) across different treatments. The average ratio of larval number to broodstock' body weight in A_{150} was higher than that in the other treatments ($P \leq 0.05$). Comparison between astaxanthin and β-caroten treatments showed that excluding A_{150}, the A_{50}, and A_{100} treatments had equal larval number:fish weight ratio with B_{150} treatment (Table 4 and Fig 2).

Correlations between carotenoids with fertilization and survival rates

The results showed that there was no significant correlation between the total carotenoids with fertilization rate ($P > 0.05$). However, there was positive significant correlation between the egg astaxanthin with fertilization rate ($P \leq 0.05$). Moreover, results showed that there was significant positive correlation between the egg astaxanthin and survival rate during incubation period ($P \leq 0.05$) (Table 5). Additionally, there was significant positive correlation between the β-caroten and survival rate during incubation period ($P \leq 0.05$). However, there was no correlation between β-caroten and fertilization rate ($P > 0.05$) (Table 5).

Table 5. Correlations between carotenoids with fertilization and survival rate

	Total carotenoids	Astaxanthin	β-caroten	Fertilization rate	Survival rate
Total carotenoids	1	r = 0.78** n = 84	r = 0.31** n = 84	r = -0.07ns n = 56	r = 0.60** n = 84
Astaxanthin	r = 0.78** n = 84	1	r = 0.10ns n = 84	r = 0.38** n = 56	r = 0.54** n = 84
β-caroten	r = 0.31** n = 84	r = 0.12ns n = 84	1	r = 0.13ns n = 56	r = 0.49** n = 84
Fertilization rate	r = -0.07ns n = 56	r = 0.38** n = 56	r = 0.13ns n = 56	1	r = 0.04ns n = 56
Survival rate	r = 0.60** n = 84	r = 0.54** n = 84	r = 0.49** n = 84	r = 0.04ns n = 56	1

ns: non-significant **: significant at 0.01 level n= number

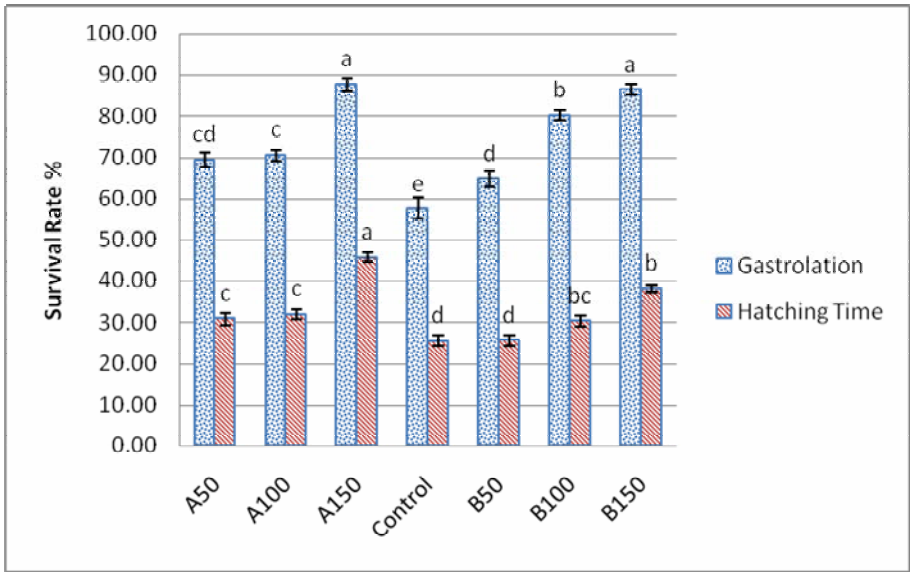

Fig. 1. Egg survival rate means during gastrolation and hatching time at various treatments**.**

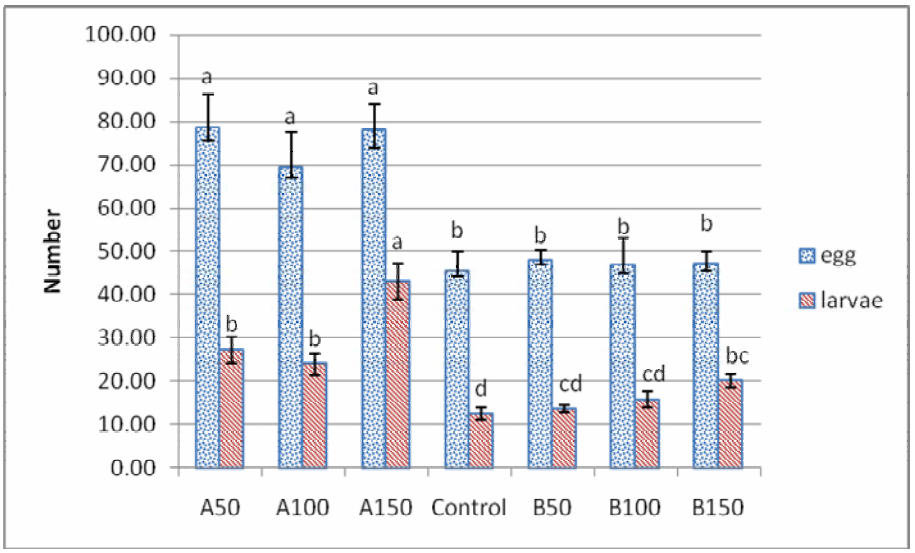

Fig. 2. Fertilized egg and produced larvae number for each gram broodstock weight at various treatments.

DISCUSSION

Several researchers have mentioned that the higher percentage of carotenoid in the broodstock' diet may give rise to the formation of synthetic lipoproteins in ovaries which play a significant role in the energy reserve of yolk (Wallace et al., 1967; Cheeseman et al., 1967; Harrison *et al*., 1990, Quinitio *et al* 1990). Meanwhile, a number of investigations reveal that astaxanthin present in fish eggs may act as an spermatozoa absorbent and thus increase fertilization chances (Hartmann *et al*., 1947; Quantz, 1980). Similar results have been achieved concerning other aquatics including crustaceans (Penaeus monodon) parrot fish (lytechinus variegate) and Barn swallows (hirund rustica) (George *et al* 2001; Salze et al., 2005, Huang et al., 2008). The results obtained from the present study shows that the mean number of broodstock' egg per gram in control and A150 groups were higher than other treatments. In other words, the average individual egg weight in these treatments was smaller than the

rest of treatments. The smaller breeder's egg weight after fertilization may be an indication of less reserved food in eggs (Heinimaa and Heinimaa., 2004). This might prove that carotenoid upgrades the fertilization percentage and survival rate of breeder's eggs in later stages. Beacham and Murray (1990) reported significant correlation between egg size and the yolk content accumulated in the yolk of Atlantic salmon. The egg size variations may directly affect the embryonic stage and larval survival.

Results on egg diameter (after fertilization) indicated that the egg diameter of control group was smaller than that of other treatments. There are a lot of evidence suggesting that carotenoid loaded diets affect fertilization of broodstock' eggs and other aquatics (Craik, 1985; Harris, 1984).

In the present research however, the average fertilization rate of eggs containing carotenoid was greater than the rest of treatments (Table 4). Such a finding goes parallel with the results of earlier research suggesting higher fertilization rate in the eggs of trout (*Oncorhynchus mykiss*) breeder's which were fed with diets containing carotenoid (Hartmann, 1947, Quantz, 1980).

Such findings were not, of course confirmed by Vasslalo-Agius et al (2001) in the case of Pseudo caranx dentex. However researchers such as Tsushima and Matsuno (1998) confirmed this to be the case in trout fish, while others believe that the greater fertilization rate of carotenoid loaded eggs is because these eggs become more receptive to spermatozoa.

Some researchers like Christiansen et al (1995) and Quantz (1980) and Tveranger (1986) while confirming the reinforcing effects of carotenoid on the reproductive functions of trout, showed that diets containing carotenoid do not have any positive impact on egg fertilization rate as compared to carotenoid free diets. Sawanboonchun and Williams (2008) illustrated the positive effects of carotenoid on egg fertilization rate of cod.

The results of the present study (Table 4) indicated that the average egg fertilization showed no significant differences in treatments containing various amounts of astaxanthin and β-caroten pigments. Nevertheless, it was revealed that these treatments have higher fertilization rate compared to the control group. This is indicative of the fact that the carotenoid present in the diets of goldfish broodstock improves fertilization during propagation process.

The ratio of egg fertilization to the broodstock' body weight in the astaxanthin treated groups was greater than in the control group. Considering the lack of any significant difference between the relative and working fecundity across various treatments, it may be hypothesized that the greater ratio of fertilized eggs to breeder's weight reflects the higher egg fertilization rate in carotenoid, and more specifically in astaxanthin treatments. Such results show that the average number of fertilized eggs per gram of breeder's body weight in diets containing astaxanthin was higher than that of control group as well as in groups with β-caroten diets. All three different astaxanthin levels resulted in greater fertilized egg:fish weight ratios than that in the control, B_{50} and B_{100} treatments. Such a conclusion was confirmed by Sawanboonchun and Williams (2008). Egg number:gram broodstock weight is an important and economical trait in aquaculture. Based on their studies on cod broodstock (*Gadus morua*), they concluded that astaxanthin loaded diets induced a greater ratio of fertilized eggs to broodstock' body weight by 37% as compared to the control diet. In addition, a 47% increase was also detected in the ratio of egg number to body weight. The inclusion of 50mg of astaxanthin per each kilogram of tiger shrimp (*P. monodon*) feed resulted in higher proportion of egg number to broodstock' weight (Huang et al., 2008).

During the incubation phase, there was a rise in the mean value of egg weight within the time interval between egg fertilization and gastrolation. Such a weight gain was associated with an increase in their volume which can be attributed to water absorption during post fertilization and cellular divisions.

Similar to the fertilization phase, the broodstock' egg weight in A_{150} showed to be higher than other treatments in the gastrolation phase. The relatively greater weight gain of eggs in A_{150} and B_{150} may be

due to the greater volume of absorbed water (since their volume was higher than that other treatments) or the higher cellular density of the embryos formed in these groups of eggs.

Gastrolation is an embryonic stage which is related to survival rate of broodstock. Many researchers detected a significant correlation between the carotenoid contents of eggs and egg survival rate during the incubation process (Harris, 1984; Craik, 1985, Palace et al., 1998; Bromage et al 1995, Pettersson and Lignell, 1999; Ahmadi et al., 2006; Tyndale et al., 2008). In the present research eggs with greater amount of carotenoid showed a higher survival rate up to the gastrolation phase. In other words, A_{150} and B_{150} experienced higher survival rates than the control group and other treatments. Similar results were reported by Ahmadi et al (2006) who examined rainbow trout and concluded that diets with higher astaxanthin content brought about greater survival in eggs up to the eyed-ova phase. Furthermore, this result has already been confirmed by Tyndale et al (2008) who focused on Chinook salmon (*Oncorhynchus tshawytscha*).

The results of this study indicated that the end of the incubation phase was associated with a significant difference in terms of egg hatching rate across different treatments. In this respect, A_{150} treatment revealed a higher egg survival rate than the other treatments. Although egg survival failed to show any significant difference between A_{150} and B_{150} at the end of gastrolation, a significant difference were recorded in per egg hatching rate which were higher in A_{150} than in B_{150}.

All experimental groups excluding B_{50}, displayed a far higher hatching rate than the control group, showing the efficacy of varying carotenoid resources on the developmental stages of goldfish embryos, as well as the improvement that occurred in the quality of their eggs ($P \leq 0.05$). Similar results were reported by Tsushima and Matsuno (1998) in *Aliptis discus*. Nevertheless, the present research findings are inconsistent with those of Chubert et al (2006) on rainbow trout and Christiansen et al (1995) on Atlantic salmon who believed that carotenoids have no effect on the developmental stages of embryos.

Since available carotenoid in fish eggs can reduce lipid oxidation during incubation (Young and Lowe, 2001), the egg sensitivity and vulnerability to lipid oxidation risks may be decreased and thus egg mortality can be prevented during incubation phase. High carotenoid concentrations may help alleviate such sensitivities in fish eggs (Graw et al., 2005). Furthermore, the superior egg hatching level and total survival in the carotenoid treated groups compared to the control together with the higher overall survival in A_{150} and B_{150} compared to treatments with lower carotenoid contents it can be concluded that such differences might have been due to better performance of carotenoid particularly carotenoid containing oxygen, like astaxanthin, which tended to eliminate free oxygen radicals caused by lipid oxidation produced during embryonic development (Britton et al., 1997, Woodall et al., 1997, Pavlvov et al., 2004). On the other hand, some researchers stated that carotenoid present in finfish eggs and mollusks might have a role in egg respiratory functions (Craik, 1985). However, these researchers consider a more crucial role for carotenoid containing oxygen (Apoxi carotenoid), like astaxanthin in the oxygen production process within the eggs as compared to β-caroten (Karnaukhov, 1971, 1973, 1979). The results obtained in this research revealed that the superior egg fertilization of broodstock in A_{150} and B_{150} compared to the rest of treatments and their higher egg survival rates in the developmental stages of embryos are somehow related to the size of eggs prior to fertilization. Such a relationship was also mentioned by Mikulin (2003). These functions could have vital implications for goldfish that mainly spawn in stagnant, low oxygen waters.

There are various carotenoids in goldfish egg which some of these carotenoids have a positive role in embryonic development (Hata and Hata, 1971). Lower levels of astaxanthin in egg results inreducing survival rate in trout, *Oncorhynchus mykiss* (Craik, 1985). Salze et al., (2005) also reported that a reduction in astaxanthin amounts will result in reproduction problems in Cod (*Gadus morhua*). Our

findings showed a positive correlation between astaxanthin amounts of egg and fertilization rate, and also increased fertilization rate along with increase in astaxanthin concentrations. In contrast, there was no correlation between β-caroten and fertilization rate which was similar to the results of Miki et al., (1984).

Based on the results of the present study, it can be concluded that high concentrations of astaxanthin improve reproduction performance in goldfish. Thus, it is recommended to use astaxanthin in broodstock diets for economical improvement.

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تاثیر منابع آستازانتین و بتاکاروتن جیره بر عملکرد تولیدمثلی و کیفیت تخم ماهی طلایی ماده (*Carassius auratus*)

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چکیده

پژوهش حاضر با هدف بررسی اثرات منابع مختلف کاروتنوئیدی و غلظت‌های مختلف آنها روی عملکرد تولیدمثلی ماهی گلدفیش در قالب هفت تیمار و سه تکرار در هر تیمار در مجتمع تکثیر ماهیان استخوانی رشت از دسامبر 2011 تا می 2012 انجام شد. در این آزمایش، شش جیره با مقادیر 50، 100، و 150 میلی‌گرم در کیلوگرم آستازانتین و بتا- کاروتن در جیره پایه کپور ماهیان و یک جیره بدون کاروتنوئید افزودنی ساخته شد. ماهیان مولد گلدفیش به مدت چهار ماه با جیره‌های آزمایشی تغذیه شدند. در ماه می، مولدین با نرهای یکسان (که با جیره شاهد تغذیه شده بودند) مورد تکثیر مصنوعی قرار گرفتند و سپس میزان هماوری کل، کاری، نسبی، نرخ لقاح، و نرخ بازماندگی آنها در طول مدت انکوباسیون در تیمارهای مختلف اندازه‌گیری شد. نتایج نشان داد که نرخ هماوری در تیمارهای مختلف، اختلاف معنی‌داری ندارد ($P > 0.05$). لیکن قطر تخم و وزن انفرادی تخم تلقیح شده، در تیمارهای A_{150}، بیشتر از تیمارهای دیگر بود ($P \leq 0.05$). ضمنا تیمارA_{150} نرخ بازماندگی بیشتری را درمرحله انکوباسیون نشان داد ($P \leq 0.05$). همبستگی بین میزان آستازانتین تخم با نرخ لقاح و نرخ ماندگاری تخم در دوره انکوباسیون معنی‌دار بود ($P \leq 0.05$). همچنین همبستگی معنی‌داری بین میزان بتا- کاروتن تخم با نرخ ماندگاری تخم در دوره انکوباسیون مشاهده گردید ($P \leq 0.05$).

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