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The Effect of Algal and Synthetic Astaxanthin (*Heamatococcus pluvialis*) on Egg Quality of Rainbow Trout Broodstock (*Oncorhynchus mykiss*)

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Abstract: This research was done in order to compare the effect of two astaxanthin sources (synthetic and algal) on egg quality of rainbow trout. It was considered 7 groups consisting 6 treatments by two different astaxanthin sources and a control (without astaxanthin). So, algal astaxanthin (H. pluvialis) in three levels of 2.67, 3.55 and 8 gm/kg food (T1, T2, T3) and synthetic source in three levels of 40, 80 and 120 mg/kg food in diet (T4, T5, T6) examined on 140 trout broods (3-4years) for 4 months, before the spawning season. Egg quality analyzed through indices such as egg diameter, fecundity, fertilization, eyed egg and hatching rates. Significant difference between treatments in terms of fecundity, eyed eggs rate and the number of eggs per gram was observed ($P<0.05$), although in some factors such as diameter, egg weight per body and fertilization rate, no significant differences was observed. By increasing level of astaxanthin in both sources of algal and synthetic, hatching and eyed egg rates increased, but the effect of alga source on these indices was more perfect. Considering the results, the best result related to treatment of 8 gm/kg alga (T3). Observations during this research indicated no disease and mortality and also proper coloration and freshness of fishes. Overall the results asserted that the application of astaxanthin improves egg quality of rainbow trout. It also concluded that natural astaxanthin (*Heamatococcus pluvialis*) for the reason that contains supplementary nutritious, is extraordinary preferable than synthetic astaxanthin to improve reproductions indices and egg quality of rainbow trout.

Key words: Astaxanthin · *Heamatococcus pluvialis* · Egg quality · Synthetic · Rainbow trout

INTRODUCTION

Carotenoids are liposoluble pigments that are responsible for many animal colors in the range of yellow to red. They are biosynthesized by plants and certain bacteria and fungi and may be absorbed and metabolized by animals such as fishes. However, fish and other animals are unable to biosynthesize carotenoids de novo and must obtain them from their diets; for this reason the carotenoids are added in the diet. Several source materials such as yeast *Phaffia rhodozyma*, alga *Dunaliella salina* and blue green alga *Spirulina maxima* and synthetic h-carotene, canthaxanthin and astaxanthin have been used for pigmentation of aquatic animals [1-6]. Efficiency variation in pigmentation from the above sources can be attributed to the type, composition and concentration of the pigments contained [1-3, 7, 8], digestibility of the material itself [2] and possibly the presence of cofactors in the material involved in absorption and deposition [9]. The green unicellular freshwater alga, *Haematococcus pluvialis*, a potent producer of astaxanthin [10-13] has been demonstrated to enhance the pigmentation of rainbow trout [14-16], gilthead Seabream [17] and ornamental fish [18].

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is one of the major carotenoids in aquatic animals including salmonid [19]. The colour of salmonids is an important criterion of quality. The typical red to pink muscle color of salmonids is due to astaxanthin, a carotenoid from dietary origin that fish cannot synthesize.

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Today Astaxanthin is the preferred pigment added to salmon feed. This pigment is present in nature; nevertheless it can also be produced by chemical synthesis [20]. The ingested carotenoids are deposited in muscle, liver and skin along with their metabolites. Known or suspected functions of astaxanthin in aquatic animals include: improved provitamin A activity, antioxidant, improved embryonic and larval development, Cellular protection from photodynamic damage, enhanced growth and maturation and formation of in-chain epoxides that act as oxygen reserves under anoxic condition [21].

The iflnuence of nutrient availability on reproductive physiology and broodstock performance in fish has been reviewed previously [22-24]. These studies have investigated the effects of a number of nutrient supplements including polyunsaturated fatty acids, vitamins C and E and the carotenoid pigment astaxanthin. The quality of eggs is important because poor quality eggs result in increased larval mortality and deformities during egg and larval rearing which results in reduced production effciency as well as fish health and welfare problems. At present it is generally accepted that the best source of eggs comes from wild caught fish, as these fish tend to produce better quality eggs and larvae than farmed broodstock. In cod, differences in carotenoid pigment concentration have previously been identified between wild and farmed cod broodstock [25]. A number of studies have been carried out on other species of farmed fish and numerous parameters have been reported to influence egg quality such as broodstock nutrition, environmental conditions and husbandry practices [23, 26, 27]. If nutritional factors are responsible for quality problems, then manipulation of broodstock diets should provide a practical means of improving egg quality via supplementation with essential nutrients.

Numerous functions have been proposed for carotenoids in fish eggs include UV protection, provitamin A activity, improved respiratory function [28, 29] and antioxidant protection against free radical damage [30]. These findings suggest that carotenoids are important in ensuring normal embryonic development and could also affect hatching rates and larval survival [28, 31, 32]. Caretonoids are also a source of pigmentation in the embryo [33] and may be involved in photoreception processes [34]. Supplementation of broodstock diets with astaxanthin has also been shown to improve egg quality in red sea bream and yellow tail [35, 36]. Dietary carotenoid supplements have also shown a positive relationship between egg pigmentation and fertilization as well as survival of rainbow trout eggs [28, 36], while Svensson *et al.* [38] found the coloration of female *G. flavescens* was strongly related to the carotenoid content of the eggs.

This study was thus designed to systematically compare various pigment sources including natural (*Heamatococcus pluvialis*) and synthetic in different levels in diets, for their effects on some indices related to reproduction efficiency of rainbow trout.

MATERIALS AND METHODS

Experimental Design and Diets: The experiment carried out in seven cement ponds (5×1×1) at a trout hatchery in Zagros of Iran. Twenty randomly tagged 3-4 years-old broods of rainbow trout female in average weight of 2800 gm were stocked in each pond. Six parts of BFT diets were supplemented by 2 sources of astaxanthin consisting algal (*H. pluvialis*) and synthetic astaxanthin; each at three concentrations, respectively: 2.67, 3.55 and 8 gmkg^{-1} alga (T1-T2-T3) and 40, 80 and 120 mgkg $^{-1}$ (T4-T5-T6). Group C was considered as control (without astaxanthin) (Table 1). Except for the differences in source and concentration of astaxanthin, the other feed characteristics in all seven diets were similar (Table 1).

Fish were fed with prepared foods two times a day. Daily Food quantity until spawning season was 0.6-0.7% of biomass and then decreased to 0.3-0.4% during spawning season. The feeding period lasted for 120 days. Due to better dissolution of carotenoids in oil, soybean oil was used as algae and synthetic astaxanthin solvents (30 ml/kg food). The supplemented oil sprayed slowly onto the food, while food stirred simultaneously until all the pellets were well coated with oil and pigment.

Table 1: Treatments supplemented by different levels of astaxanthin in two sources

Treatment	Astaxanthin source	Concentration
T_1	Algal astaxanthin (*H.pluvialis*)	2.67g kg^{-1} food
T_2	"	5.33g kg^{-1} food
T_3	"	8g kg^{-1} food
T_4	Synthetic astaxanthin	40 mg kg^{-1} food
T_5	"	80 mg kg^{-1} food
T_6	"	120 mg kg^{-1} food
C (control)	Without ASTA	-
Diet composition of used food		
Crude protein (%)		38.0
Crude fat (%)		12.0
Ash (%)		10.0
Fiber (%)		3.5
Phosphorus (%)		1.0
Moisture (%)		11.0

Some environmental factors such as water temperature, pH and dissolved oxygen were measured daily by a digital apparatus.

Egg Quality Assessment: The spawning period was about three weeks so during this period, all of fish were checked individually for their ripening stage once every ten days so ready fishes were stripped and the obtained eggs from each female were mixed by milt from at least 2 matured males. For this purpose, one group of selected male was holed in a distinct pond by using usual BFT food during experiment. In each stripping, about 10 gm eggs were collected to measure indices including egg diameter and number in gm. Other egg quality indices involving fecundity, fertilization, eyed egg and hatching rates were calculated during artificially propagation stages.

Egg samples were counted and weighted and then number in grams was calculated as number of eggs/ egg weight. The diameter of these eggs was determined by a caliper. The total weight of eggs obtained from each fish was measured and fecundity rate was calculated as weight of obtained eggs to body weight of fish.

The fertilized eggs were placed into troughs and incubated at temperature of 10.5±0.5°C. Eight days post fertilization (formation of the neural streak), white eggs were counted to determine the fertilization rate. It was calculated as the eggs number fertilized/ total egg. At the eyed egg stage at 18-20 days after fertilization, all dead eggs were counted and eyed egg rate measured as the number of eyed egg / total eggs fertilized. After hatching (35 days after fertilization), the fry was counted. Hatching rate was calculated as the number of hatched larvae/ total incubated eggs.

Statistical Analysis: Data were processed using the SPSS (Version 11.5) software. The results are presented as mean± SE of two replicates of grouped samples. Homogeneity of variance test was conducted before applying the parametric statistical analysis and when necessary the data were transformed. The effect of the treatments on measured indices was evaluated with a one-way analysis of variance (ANOVA), considering as factors the experimental diets and the samples. Tukey's HSD test was applied to determine the significant differences between diets and samples.

RESULTS

Variation in some egg quality indices for the feeding trial is shown in Table (2). The overall weight of eggs obtained from a broodstock ranged between 365.5 (T4) to 481.25g (T3) with non- significantly different between diets. By increasing in level of astaxanthin in both sources, the weight of obtained eggs increased. There was a significant difference between treatments among egg diameter ($P<0.05$) as broods fed diet containing 8 $gmkg^{-1}$alga (T3) had maximum egg diameter (5.42 mm) while control diet (C) was lowest (4.72) (Fig. 1).

Significant difference in number of egg in gram was found among all treatments ($P<0.05$). Broods fed control diets had significantly greater number of eggs ($P<0.05$) than those fed diets containing pigments. No significant difference was found in diets containing different level of algal astaxanthin while by increasing in synthetic astaxanthin concentration, the number of eggs per gram decreased significantly.

Treatments significantly influenced fecundity rate in this study (Fig. 2); T3 containing algal astaxanthin produced highest rate of fecundity (14.4). However, no significant difference was found between three levels of astaxanthin in both synthetic and algal sources. Fertilization percentages were similar between treatments. The highest rate of fertilization obtained in T5 (96.05%) and control treatment (C) produced lowest rate (82.7)

Table 2: Effect of different levels of synthetic and algal astaxanthin on some egg quality indices of rainbow trout (Mean ± SE)*

	Treatments						
Parameter	Control (C)	T1	T2	T3	T4	T5	T6
Brood weight (gm)	3287.5±70.16^{a}	3317.5±307.17^{a}	3147.5±289.8^{a}	3322.5±121.95^{a}	3280.0±286.12^{a}	3315.5±295.57^{a}	3071.5±165.04^{a}
Egg weight (gm)	452.0±31.71^{a}	380.75±62.28^{a}	398.25±33.77^{a}	481.75±24.98^{a}	365.5±37.21^{a}	438.75±70.14^{a}	455.25±21.21^{a}
Egg diameter (mm)	481.75±24.98^{a}	5.22±0.11^{abc}	5.2±0.04^{abc}	5.42±0.01^{a}	4.92±0.01^{cd}	4.96±0.08^{bcd}	5.27±0.01^{ab}
Egg number in gram	16.1±0.22^{a}	12.0±0.50^{d}	13.22±0.04^{cd}	12.02±0.38^{d}	15.37±0.35^{ab}	14.4±0.28^{ab}	13.67±0.68^{c}
Fecundity (%)	11.44±0.89^{bc}	14.78±0.34^{a}	13.24±0.55^{ab}	14.64±0.31^{a}	10.27±0.88^{c}	12.62±0.65^{abc}	12.41±0.58^{abc}
Fertilization (%)	90.74±0.96^{b}	93.13±0.56^{ab}	95.3±0.60^{a}	95.75±0.55^{a}	92.84±0.86^{ab}	96.17±0.12^{a}	96.54±0.98^{a}
Hatching (%)	75.52±0.42^{d}	82.35±0.47^{c}	84.00±0.64^{bc}	88.82±0.47^{a}	78.10±0.66^{d}	83.3±0.64^{c}	85.77±0.45^{b}
Eyed egg (%)	72.02±0.40^{f}	89.12±0.40^{c}	93.90±0.64^{b}	98.12±0.41^{a}	78.27±0.65^{e}	85.55±0.85^{d}	85.65±0.44^{d}

*Results represent means ± SE of 20 fish per treatment. Means not bearing the same superscript letters are significantly different ($P<0.05$)

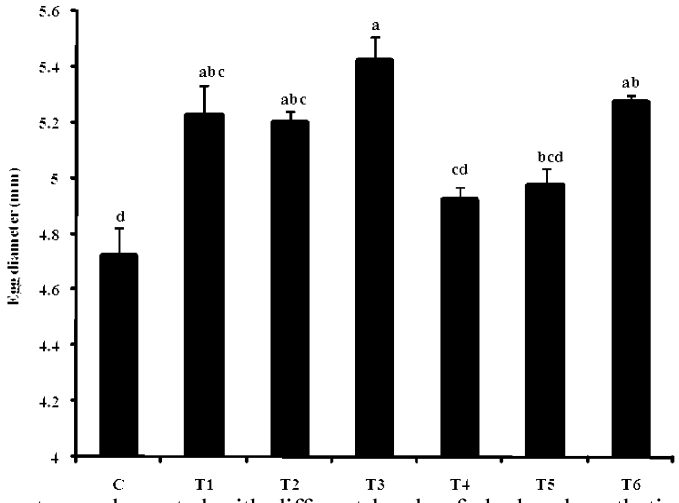

Fig. 1: Egg diameter in treatments supplemented with different levels of algal and synthetic astaxanthin. Values are means ± SE (n=20 fish). Values with different letters are significantly different ($p<0.05$)

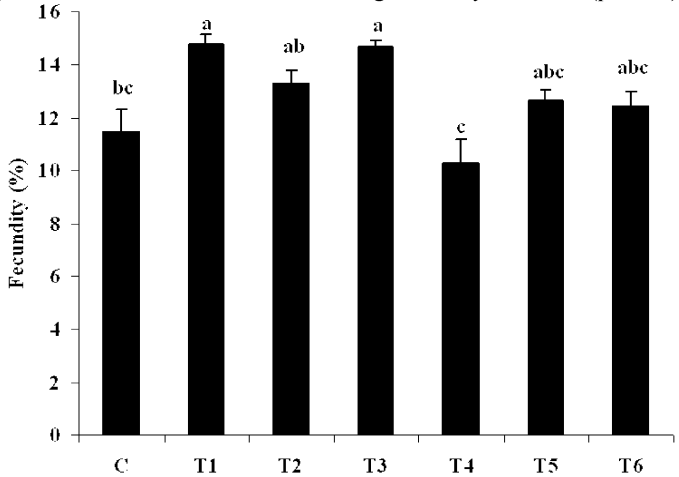

Fig. 2: Fecundity rate analysis of eggs collected from the treatments. Values are means ± SE (n=20fish). Bars with different letters are significantly different ($p<0.05$)

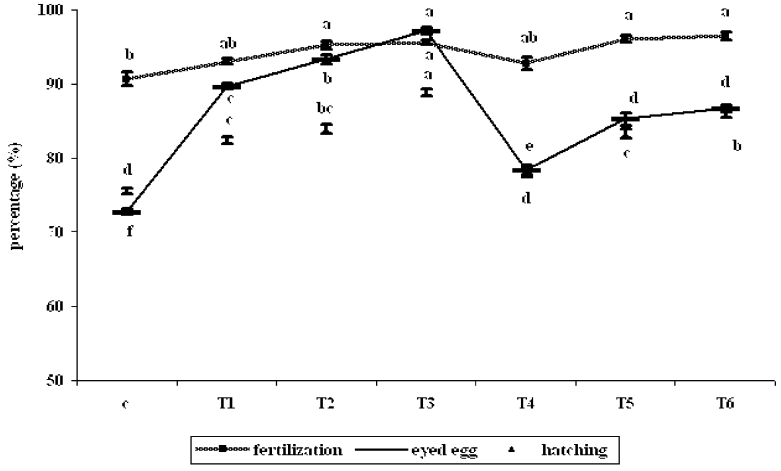

Fig. 3: Egg quality parameters (fertilization, hatching and eyed egg rates) affected by dietary astaxanthin levels from algal and synthetic resources. Values are means ± SE (n=20 fish). The different superscripts within each group at the top of each line are significantly different, $P<0.05$

(Fig. 3). Treatments (T2 & T3) and also (T5 & T6), with same levels of astaxanthin in utilized resources, showed significant difference with control treatment in fertilization rate.

Significant differences were found among eyed egg rate between treatments. Highest eyed egg rates obtained in T3 (Fig. 3). Broods fed diets containing algal astaxanthin had greater percentage of eyed eggs than those fed synthetic ($P<0.05$), as T1 supplemented with lowest level of algal astaxanthin produced greater value than T6 which supplemented with highest level of synthetic one.

About eyed egg rate, a significant difference observed among treatments in term of astaxanthin levels. Figure (3) shows the highest hatching rate in T3 (88.82) and lowest in control treatment (75.52). In overall, diets containing algal astaxanthin had significantly greater hatching rate than synthetic one.

DISCUSSION

Egg quality and fecundity of broodstock are very important for hatchery operators. Problems with egg quality can affect the larvae and lead to later production problems such as slow growth, high mortality and deformities. This can affect the economics and profitability of production. Carotenoids have a wide range of functions in fish including reproduction, egg respiration, cell growth and proliferation, as a precursor of vitamin A, in vision, as a source of pigment and as an antioxidant [21, 24, 28, 39]. Its role in reproduction may be as a substance which increases fertilization rates [40].

Along with the salmon growth, the concentration of cantaxanthin and astaxanthin increases in muscle [41], But during the development of the ovaries, carotenoids from muscle and liver moving toward to the growing ovarian and accumulate in ovule [42-44]. Carotenoid content of eggs and its color is full depended on amount of carotenoid stored in egg during yolk formation [45, 46]. Such species with large eggs require higher amounts of pigment than those with small eggs and it is correlated between the time of embryonic development and the concentration of carotenoids in eggs [47]. In rainbow trout, high levels of astaxanthin and canthaxanthin in eggs were found to improve fertilization rates [31]. In addition, Ahmadi *et al.* [19] showed that dietary supplementation with astaxanthin in rainbow trout broodstock gave a significant improvement in egg quality. Mikulin and Soin [48] found that astaxanthin may help improve egg quality during embryonic development. It has also been suggested that astaxanthin functions as an antioxidant and protects against light induced free radical production.

Many researchers have demonstrated the importance of dietary supplementation with astaxanthin for a variety of species including striped jack (*Pseucocaranx dentex*) [49], rainbow trout (*Oncorhynchus mykiss*) [19] and Atlantic Cod (*Gadus morhua L.*) [50]. It contributes to the fertility and egg quality of females and positively affects the growth and survival of young salmon [51].

In present study quality indices of eggs produced by broods fed dietary astaxanthin especially natural astaxanthin were desirable. The highest egg weight obtained in T3 containing highest level of natural astaxanthin. Egg diameter obtained in diets supplemented with algal astaxanthin was more than synthetic ones in size which asserted better performance of natural astaxanthin. Similar results were reported by Watanabe *et al.* [52] on sea bream and striped jack brood stock.

The number of eggs per gram was significantly affected by astaxanthin especially algal astaxanthin as maximum number obtained in control group (without astaxanthin) and minimum number in T3.

The fecundity was increased with increased dietary astaxanthin levels and maximum value obtained in broods fed diet containing highest level (8 $gmkg^{-1}$ alga) of algal astaxanthin. Previous studies also showed astaxanthin improved egg quality and increased fecundity in broods of cod [53] and in *Penaeus monodon* [54]. Pangantihon-Kuhlmann *et al.* [55] and Ribeiro *et al.* [56] also reported that broods of *Penaeus monodon* fed diets containing high level of astaxanhin produced more egg and spermatozoa.

Previous findings suggest that carotenoids are important in ensuring normal embryonic development and could also affect hatching rates and larval survival [28, 31]. Because of the importance of astaxanthin in embryo growth and development and in increasing fertilization rates [28, 48], the egg concentrations of this compound might also be an indicator of egg quality [57]. In present study, although no significant differences was found among different sources and levels of astaxanthin on fertilization rates, but levels of 80 and 120 $mgkg^{-1}$ astaxanthin (in both algal and synthetic source) were significantly difference with control group (without astaxanthin), which was indicating effect of astaxanthin

on fertilization rate improvement. This is in agreement with previous findings [58, 59]. As well as, a lack of carotenoids in the diet of broodstock has been shown to result in low fertility and deformities in marine fish larvae [24].

Results of hatching and eyed egg rates in this study showed that algal astaxanthin in level of 8 $gmkg^{-1}$ alga (T3) significantly affected on these factors as broods fed control diets (without astaxanthin) had lowest hatching rate. On the other hand, diets containing algal astaxanthin produced higher rates of hatching rates other synthetic astaxanthin, which demonstrated more effective influence of algal astaxanthin than synthetic ones in egg quality improvement as highest eyed egg rate obtained in T3. In present study, eyed egg rate quite impressed by natural astaxanthin in sofar as even in T1 containing lowest level of algal astaxanthin, eyed egg rates was more than T6 containing highest level of synthetic astaxanthin.

Observations during this research indicated no disease and mortality and also proper coloration and freshness of fishes which asserted the effect of astaxanthin on stress resistance and immunity to diseases improvement. Recent studies showed that enhancement of resistance to oxygen depletion stress [60], salinity and thermal stress [61], ammonia stress [62] and pathological stress [63] in penaeid post larvae was associated with an increase in dietary and body astaxanthin. It was also demonstrated that formulated diets which carry the microbial carotenoids in the feed resulted in recovery of skin carotenoids to the fishes subjected to stress [64]. So, astaxanthin in addition to pigmentation of tissue and sexual cells, adversely affected the efficacy of astaxanthin supplementation in stress reduction and enhancement of immune function.

In overall, this study showed that supplementation of astaxanthin in broodstock diets and also the type of pigment resource was an effective factor on egg quality enhancement and reproduction efficiency improvement. Although among some factors such as diameter, weight and fertilization rate was found no significant differences between treatments but totally, best performance of trial obtained in T3 (8 $gmkg^{-1}$ alga). According to this study and recent findings [52, 65, 66] natural astaxanthin have proven far superior to synthetic astaxanthin in broods feeding and improved breeding and increased both the quantity of eggs as well as the quality of the eggs. Some aquaculture companies are beginning to use natural astaxanthin instead of synthetic even though it costs more. Aquaculture is a highly competitive industry, so paying more for a feed ingredient is only done when there is a clear reason why it makes economic sense. Similar to all the research going on with natural astaxanthin for human nutrition, researchers and companies are sponsoring feed trials for animals with natural astaxanthin.

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