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Effects of dietary supplementation with astaxanthin and β-carotene on the semen quality of goldfish (*Carassius auratus*)

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ABSTRACT

This study was conducted to investigate the effects of two carotenoids (astaxanthin and β-carotene) on the sperm quality of goldfish *Carassius auratus* (Linnaeus, 1758). For this purpose, six diets containing concentrations of 50, 100, and 150 mg/kg of synthetic astaxanthin and β-carotene were added to a basic carp diet. One group of fish was also fed with a control diet (no added carotenoids). Osmolality, spermatocrit value, and sperm concentration significantly increased in the treatment supplemented with 150 mg/kg of astaxanthin (296.6 ± 1.1 mOsm/kg; 29.2 ± 0.6%; 17.2 ± 0.4 × 10^9 cells/mL, respectively) and β-carotene (295.2 ± 2.1 mOsm/kg; 32.5 ± 1.6%; 17.9 ± 0.5 × 10^9 cells/mL, respectively). The highest concentration of astaxanthin (10.4 ± 1.4 mg/kg) was recorded in the treatment of A_{150} ($P < 0.05$) and did not differ between β-carotene treatments. The highest motility was observed in the A_{150} and B_{150} treatments, and the lowest was observed in the control group ($P < 0.05$). The artificial fertilization of the treated males with the similar females (fed with the control diet) showed that the fertilization rate in the A_{150} treatments was higher than in the other treatments ($P < 0.05$). In conclusion, dietary supplementation with 150 mg/kg of astaxanthin improves osmolality, motility, fertilization rate, and sperm concentration.

1. Introduction

Goldfish (*Carassius auratus*) are one of the most valuable fishes in the world. These fish propagate annually at high numbers [1]. The manipulation of environmental parameters, hormone treatments, and high-quality diets can improve the efficiency of reproductive performance and productivity [2]. The improvement of fish reproduction is possible through dietary supplementation with antioxidants, such as vitamin C [3], α-tocopherol [4], and carotenoids [5].

Carotenoids are lipid-soluble pigments that are produced by plants and other photosynthetic organisms [6] and consumed by other animals. To date, there has been a great deal of valuable information regarding the functions of astaxanthin and other xanthophyll carotenoids, such as canthaxanthin and zeaxanthin, in aquaculture. These functions include the improvement of antioxidant activity for vitamin A, the improvement of larval development [7], an increase in fecundity and egg quality in goldfish, and an improvement in fetal development in goldfish [1]. In many aquaculture activities, pigments are used to improve meat color [8] or for ornamental fishes [9]; however, there are currently more important roles for carotenoids: as provitamin

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A [10], antioxidants [11], O_2 quenchers or free radical scavengers [12], skin coloration during sexual maturation, and signal substances in reproduction [13]. The application of pigments in aquaculture is conducted in salmonids primarily at the commercial level; however, as the cost of pigments has risen by a factor of 2.2, alternative sources are being explored to reduce costs [14,15].

One of the reported roles of carotenoids is their antioxidant role in animals. Trace amounts of reactive oxygen species can decrease fertility in organisms [14,16]. Fish sperm is sensitive to oxidant agents that destroy the fat layer of sperm [17]. In many aquaculture farms, there has been an attempt to improve egg and larvae quality, but sperm quality has been ignored. Blount et al. [18] found that male fishes transport carotenoids linked to lipoproteins into testes to inhibit damage due to reactive oxygen species. Other studies have shown that there are significant positive correlations between carotenoid levels in the bodies of male fish regarding sperm concentration and motility [19]. These sperm enter into the ovule more rapidly than other sperm during mating [20], resulting in increased fertilization efficiency [21]. The efficiency of dietary carotenoid supplementation on the growth, skin coloration, and immune response of fancy carp was investigated using locally common agricultural plant materials as natural carotenoid sources [22].

However, few reports exist that evaluate the performance of male goldfish fed diets differing in astaxanthin and β-carotene [1]. The objective of this study was to determine the effect of different dietary levels of astaxanthin and β-carotene on the reproductive performance of male goldfishes. In this experiment, the role of astaxanthin and β-carotene in goldfish sperm and their effects on reproductive efficiency are studied.

2. Materials and methods

2.1. Fish

The freshwater fish species examined in this study is the goldfish *C auratus* (Linnaeus, 1758) of the cyprinidae family. The female and male goldfish broodstock used in this study were produced at the Rasht Bony Fish Hatchery Complex in Rasht, Iran; the fish-conditioning experiment and egg incubation were also performed here. The experiment was conducted between December and April. All fish were handled in compliance with published guidelines for animal experimentation [23].

2.2. Composition of the diets

To prepare the carotenoid-enriched diets, each carotenoid source was weighed, dissolved in water at 35 °C, and added to the diet according to each tested level [24]. All the diets were prepared with fish oil and water and processed in a California Pelleting Machine at a final pellet size of

Table 1
Composition of experimental diets fed to goldfish (*Carassius auratus*) for 150 days.

Description	Unit	Treatments[a]						
		A_{50}	A_{100}	A_{150}	Control	B_{50}	B_{100}	B_{150}
Ingredient								
Fish meal	g/kg	290	290	290	290	290	290	290
Soybean meal	g/kg	290	290	290	290	290	290	290
Corn meal	g/kg	200	200	200	200	200	200	200
Clupen fish oil	g/kg	20	20	20	20	20	20	20
Wheat flour	g/kg	164	164	164	164	164	164	164
Lime	g/kg	4.5	4.0	3.5	5.0	4.5	4.0	3.5
Methionine	g/kg	2	2	2	2	2	2	2
Lysine	g/kg	2	2	2	2	2	2	2
Kavilamycine[b]	g/kg	2	2	2	2	2	2	2
Dicalcium phosphate	g/kg	5	5	5	5	5	5	5
Salt	g/kg	5	5	5	5	5	5	5
Vitamin premix[c]	g/kg	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Mineral premix[d]	g/kg	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Astaxanthin[e]	g/kg	0.5	1	1.5	—	—	—	—
β-Carotene[f]	g/kg	—	—	—	—	0.5	1	1.5
Dietary chemical analysis								
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

The nutritional values are expressed as the mean ± standard error of the mean.

[a] A_{50}, A_{100}, and A_{150} = 50, 100, and 150 mg astaxanthin/kg feed, respectively; B_{50}, B_{100}, and B_{150} = 50, 100, and 150 β-carotene/kg feed, respectively; control diet (control) did not contain an added carotenoid source.

[b] Kavilamycine: contains 10,000 mg/kg of avilamycin.

[c] Vitamin premix (in 10 g): ρ-aminobenzoic acid (10.0 mg); biotin (0.40 mg); inositol (400.0 mg); nicotinic acid (40.0 mg); calcium pantothenate (60.0 mg); pyridoxine HCl (12.0 mg); riboflavin (8.0 mg); thiamin HCl (4.0 mg); menadione (4.0 mg); cyanocobalamin (0.08 mg); calciferol (1.20 mg); folic acid (0.80 mg); choline chloride (120.0 mg).

[d] Mineral premix (in 100 g): k_2HPO_4 (2.0 g); $Ca_3(PO_3)_2$ (2.72 g); $MgSO_4.7H_2O$ (3.04 g).

[e] Astaxanthin: Carophyll Pink 10% (DSM).

[f] β-Carotene: β-carotene 10% (CWS).

2 × 3 mm in diameter. Next, the pellets were dried in a drying machine (Hobart manufacturing Company Ltd., London, UK) for 24 hours at 50 °C. The amount of total protein, fat, carbohydrates, ash, and moisture in each diet was measured according to the Association of Official Analytical Chemists [25] (Table 1).

2.3. Experimental design

A group of 1050 male goldfish measuring 51.20 ± 2.21 g and 14.22 ± 1.37 cm was used for the broodstock–conditioning experiment with the carotenoid-enriched diet. The experiment lasted for 150 days, and it compared the diets enriched with synthetic astaxanthin (Carophyll Pink 10%; DSM Nutritional Products Philippines, Inc.—Bright Science, Brighter Living, the Netherlands) and β-carotene (β-carotene 10% CWS; Direct Food Ingredients Ltd., England) with a control diet for a total of seven treatments run in triplicate.

For the fish-conditioning experiment, 21 circular fiberglass tanks (3.77 m^3; 2 m in diameter and 1.2 m in height) were used. The freshwater parameters of temperature, pH, dissolved oxygen, nitrite, and ammonia were measured daily during the experimental period.

One month before the end of the experiment, a group of 300 female broodstock of 6-month-old goldfish (47.21 ± 2.19 g) from a commercial hatchery was matured for 30 days in a fiberglass tank (2 m^3) with the control diet to achieve egg fertilization. The freshwater parameters of temperature, pH, dissolved oxygen, nitrite, and ammonia were measured daily during the 30 days of female maturation.

2.4. Artificial propagation

In mid-April, when the water temperature was 21 °C and 24 hours before stripping, the male fish were injected intraperitoneally with a single dose with Ovaprim hormone injections at 0.1 mg/kg of their body weight. After 5 hours of injection, the water was wiped out of their body with a soft cotton cloth. To observe the free oozing of semen flow, the fish were held head up and tail down in an inclined position. During sperm collection, care was taken to prevent contamination by fecal matter, blood, scales, and urine, and so forth. Sperm were provided with sufficient oxygen by maintaining sufficient headspace in the collection tubes (15 mL) and keeping collected milt at 4 °C. Milt was inserted into 10-mL syringes and transferred into collection tubes.

Similar to male injections, the female fish were also injected intraperitoneally with the Ovaprim hormone at the same time at 0.2 mg/kg of their body weight [26]. Eggs were collected in glass dishes and kept at room temperature (20.0 ± 1.0 °C) until fertilization trials started (within <1 hour).

The male/female ratio was 3:1, and 12 male broodstock were selected for each replication at each step of propagation. In this experiment, semen from 36 males per treatment was used (12 per tank). The average water temperature was 19 °C at the time of artificial fertilization. The fertilization rates were calculated 2 hours after fertilization. Fertilized eggs were incubated in 1-L vase incubators. After incubation and complete larva emergence, the survival rate at the incubation stage was calculated as the following formula:

$$\textit{Survival rate during incubation} = (\textit{number of produced larvae}/\textit{number of fertilized eggs}) \times 100$$

2.5. Carotenoid extraction

Two fish per replication were sampled at random, and astaxanthin, β-carotene, and total carotenoid were measured using the following method: the semen of each sample was collected and frozen using N_2 gas, and the samples were then placed inside an amber flask under an N_2 gas environment at −18 °C. The procedure of carotenoid extraction from the samples was conducted using pure acetone specific to high performance liquid chromatography (HPLC) and n-hexane (Hex, analytical grade) based on a hexane/acetone ratio of 1:3. Afterward, 50 mL was stored in a glass bottle and wrapped with aluminum foil, and 5 mL of the 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 °C under the dim lighting conditions during the extraction of carotenoid from the sample [27].

2.6. Measurement of carotenoid

To measure the astaxanthin and β-carotene concentrations, their authentic standards were used for the experiment. The standard solutions were prepared with 2.5 to 540 μg/L concentrations (Sigma Aldrich and Fluka). All the production and extraction procedures for the samples were conducted under dim light and an ambient temperature of 22 °C. To extract carotenoid from the semen, a 50-mL wet sample was homogenized in 3 mL of acetone and vortexed for 30 seconds followed by 5 minutes of centrifugation (1500 rpm). A mixture of solvents, including 2 mL of hexane and 0.5 mL of water, was added to a 2.5-mL volume of the upper layer. Next, it was vortexed for 30 seconds followed by 5 minutes of centrifugation (1500 rpm), and the hexane layer was dried in a clean tube under a stream of nitrogen. Next, 250 μL of methanol was added to the residue, and 70 μL of this solution was injected into the HPLC (a Younglin HPLC system equipped with a pump [SP930D], ultraviolet detector [730D], Rheodyne injector, and Autochro 2000 integrator software). The HPLC includes a separation condition: 1.4 mL/min; wavelength: 470 nm; temperature: 25 °C; and column: C18, Inertsil ODS-3V: 250 × 4.6 mm. Mobile phase A included methanol and water (97:3), and mobile phase B comprised methanol, tetrahydrofuran, and water (37:60:3) [27].

2.7. Spermatological analysis

Spermatological analysis was performed at the Dadman International Sturgeon Research Institute, Rasht, Iran. For each treatment, 32 male broodstock were selected. The semen was prepared 22 hours after hormone injection.

During semen collection, the anal fins of the broodstock were dried completely. The semen was stripped by abdominal massage, collected into glass vials, and stored on ice until ready for use. Fresh sperm was immediately maintained at 4 °C, and the sperm concentration was measured using a Neubauer chamber hemocytometer and the following method: duplicate samples were prepared for each semen sample, and a triplicate count was made on a hemocytometer for each dilution.

The mean of six counts was calculated and provided as the actual sperm concentration given in numbers per mL [28]. The counts and measurements were conducted using a Nikon Eclipse E600 microscope equipped with a Sony Exwave HAD camera and Biocom Visual 2000 software. The spermatocrit was determined using semen collected into standard microhematocrit tubes (75 mm length and 1.1–1.2 mm inner Ø) and centrifuged at 3000 rpm for 10 minutes [29].

The activity of spermatozoa was measured by using the computer-assisted sperm motion analysis system. Microscope stage and chambers were chilled at 15 ± 2.0 °C. Using a 50-Hz video camera (Panasonic wv-BL600) connected to an S-VHS-video recorder (Panasonic AG-7350), sperm activity was recorded and later analyzed. The total magnification was 320-fold. For digital image analysis, a PC with appropriate hardware and Mika motion analyzer software were used. One microliter of diluted sperm (2 μL of semen in 25 μL of a nonactivating solution [300 mOsm/kg sucrose, pH = 7.4]) was used for each measurement. Motility was recorded at 15 and 30 seconds after activation. Only sperm samples showing 80% motility or higher were used for the experiments [30]. Each sample was analyzed three times. The number of cells exceeding the previously established minimum motile speed was used to determine percent motility, curvilinear velocity, and straight-line velocity, which were estimated on this motile fraction.

The analyses of density, velocity, and osmolality were performed immediately after semen collection according to the methodology used for carp by Warnecke and Pluta [31]. Sperm concentration (109 cells/mL) was measured according to the methods described [32]. Semen was diluted by a factor of 1000 in a physiologically saline solution, a droplet was placed on a Burker cell hemocytometer (depth 0.1 mm) and left for a few seconds for sedimentation, and then, 16 cells of the hemocytometer were counted at × 200 magnification. For the osmolality assays, a digital freezing osmometer at 45 AV (Roebling) was used.

2.8. Statistical analysis

The results were expressed as the mean ± standard error of mean. A one-way ANOVA, followed by Tukey's test post hoc test. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using SPSS 11.5 for Windows (SPSS Worldwide Headquarters, Chicago, IL, USA).

3. Results

The temperature, pH, dissolved oxygen, nitrite, and ammonia during the 5 months of male maturation were 17.0 ± 1.35 °C, 7.6 ± 0.12, 8.1 ± 1.2 mg/L, 0.0 ± 0.00 mg/L, and 0.01 ± 0.01 mg/L, respectively. No differences ($P > 0.05$) in the concentration of the parameters of water quality were found during the first month of maturity of the females. Only the temperature changed.

The spermatological parameters in different treatments are shown in Table 2. Osmolality, spermatocrit value, and sperm concentration increased ($P < 0.05$) in the treatment supplemented with 150 mg/kg of astaxanthin (296.6 ± 1.1 mOsm/kg, 29.2 ± 0.6%, 17.2 ± 0.4 × 10^9 cells/mL, respectively) and β-carotene (295.2 ± 2.1 mOsm/kg, 32.5 ± 1.6%, 17.9 ± 0.5 × 10^9 cells/mL, respectively).

The male fish fed diets with varying degrees of astaxanthin concentrations were found to have higher levels of the substance in their semen (Table 2), whereas fish semen did not differ in the β-carotene treatment. The results also indicated that astaxanthin concentration in the fish semen of the control was lower than both astaxanthin and β-carotene treatments ($P < 0.05$). These findings showed that fish semen did not differ for β-carotene concentration in both A_{100} to A_{150} and B_{50} to B_{150} treatments. The concentrations of astaxanthin and carotenoids were higher ($P < 0.05$) than those in the control. The highest concentration of astaxanthin (10.4 ± 1.4 mg/kg) was recorded in the treatment of A_{150} ($P < 0.05$).

Table 2
Effects of astaxanthin, β-carotene, and the total carotenoids in the sperm and fertility parameters (mean ± standard error of the mean) of goldfish (*Carassius auratus*) in different treatments (n = 252), for 150 days[e].

Parameter	A_{50}	A_{100}	A_{150}	B_{50}	B_{100}	B_{150}	Control
Osmolality (mOsm/kg)	291.8 ± 1.0^{ab}	289.3 ± 0.7^{b}	296.6 ± 1.1^{a}	292.6 ± 2.7^{ab}	287.2 ± 3.5^{b}	295.2 ± 2.1^{a}	289.8 ± 1.0^{b}
Spermatocrit (%)	22.0 ± 1.4^{c}	24.6 ± 1.9^{c}	29.2 ± 0.6^{b}	32.0 ± 1.3^{a}	29.9 ± 0.9^{b}	32.5 ± 1.6^{a}	20.0 ± 0.5^{d}
Motility (%)	90.0 ± 1.0^{b}	89.3 ± 1.0^{b}	92.1 ± 0.6^{a}	90.3 ± 0.8^{b}	89.7 ± 0.7^{b}	91.9 ± 1.0^{a}	89.0 ± 0.7^{b}
Motility duration (s)	119.2 ± 4.4^{b}	124.0 ± 2.7^{a}	128.8 ± 6.1^{a}	118.8 ± 4.3^{b}	114.5 ± 5.1^{bc}	122.7 ± 5.4^{a}	111.8 ± 3.0^{c}
Sperm concentration (×10^9 cells/mL)	13.8 ± 0.4^{c}	15.1 ± 0.5^{c}	17.2 ± 0.4^{a}	16.3 ± 0.4^{b}	16.0 ± 0.3^{b}	17.9 ± 0.5^{a}	11.4 ± 0.3^{d}
Fertilization rate (%)	90.6 ± 0.6^{c}	91.0 ± 0.5^{bc}	96.0 ± 0.6^{a}	91.0 ± 0.7^{bc}	90.5 ± 0.8^{c}	93.6 ± 1.0^{b}	90.0 ± 0.8^{c}
Survival during incubation (%)	74.9 ± 0.8^{b}	73.8 ± 0.7^{b}	74.9 ± 0.9^{b}	75.1 ± 1.0^{ab}	76.2 ± 0.7^{a}	78.3 ± 0.8^{a}	74.3 ± 0.6^{b}
Astaxanthin (mg/kg)	7.8 ± 0.7^{bc}	8.6 ± 0.5^{b}	10.4 ± 1.4^{a}	7.1 ± 0.5^{c}	7.9 ± 0.5^{bc}	8.7 ± 0.2^{b}	6.1 ± 0.3^{d}
β-Carotene (mg/kg)	51.3 ± 3.2^{b}	66.6 ± 3.7^{a}	64.9 ± 5.8^{a}	62.2 ± 4.5^{a}	61.1 ± 5.3^{a}	62.1 ± 5.7^{a}	38.6 ± 2.0^{c}
Total carotenoids (mg/kg)	10.8 ± 0.6^{b}	12.3 ± 0.7^{a}	12.8 ± 1.0^{a}	10.3 ± 0.3^{b}	11.7 ± 1.1^{a}	12.9 ± 0.8^{a}	10.0 ± 0.3^{b}

[a,b,c,d]Data within each row of dietary treatments with no common superscript differ significantly at $P < 0.05$.

[e] The carotenoid-enriched diets were tested at three concentrations 50, 100, and 150 mg astaxanthin/kg feed (treatments: A_{50}, A_{100}, and A_{150}, respectively) and 50, 100, and 150 β-carotene/kg feed (treatments: B_{50}, B_{100}, and B_{150}, respectively).

The seminal osmolality value within groups receiving astaxanthin and β-carotene did not differ. Indeed, it was equal to the osmolality rate in the control. Generally, the spermatocrit in β-carotene treatments was higher than in astaxanthin and control treatments. However, the spermatocrit percentage in the semen, sampled from groups treated by the B_{50} to B_{150} treatments, was higher than in the A_{50} to A_{150} treatments.

The highest motility was observed in the A_{150} and B_{150} treatments, and the lowest was observed in the control group ($P < 0.05$). At this level, the duration of motility had no significant difference among the studied treatments (Table 2). The sperm concentrations in A_{150} and B_{150} were higher than those in the other treatments ($P < 0.05$). However, sperm concentration in the astaxanthin and β-carotene treatments showed a significant difference in comparison with the control treatment ($P < 0.05$).

The artificial fertilization of the treated males with the similar females (fed with the control diet) showed that the fertilization rate in the A_{150} treatment (Table 2) was higher than that in the other treatments ($P < 0.05$). However, survival was higher in the treatment of β-carotene to astaxanthin at the end of the incubation stage.

4. Discussion

In fish farms and hatcheries, the biotic and abiotic factors that affect sperm quality are diverse and are dependent on complex interactions between genetic, physiological, and environmental factors [20]. Improvements in broodstock nutrition and feeding greatly improve gamete quality and seed production [4]. The importance of the dietary antioxidant function of vitamin C on male fish fertility has been reported in rainbow trout [33]. This provides protection for the sperm cells by reducing the risk of lipid peroxidation and ascorbic acid deficiency which reduces both sperm concentration and motility (consequently, the fertility) [34].

In the case of astaxanthin, a synergistic antioxidant effect was found with α-tocopherol [35–37] protecting against the early stages of lipid peroxidation.

This is the first study to examine the effect of dietary supplementation with astaxanthin and β-carotene on the quality of goldfish semen. Nutrition provided by the breeder can play an important role as an enhancement factor of sperm quality.

Generally, motility is the most commonly used parameter to evaluate sperm quality [38], whereas fertilizing capacity is the most conclusive test of sperm quality [38,39]. In this work, the fertilization rate was higher (90%–96%) than that obtained in other studies (60%–80%) on carp [40] and Atlantic halibut (54.6%–58.3%) [41], and it was similar (>95%) to the records for brown trout [42,43]. It has been found that some nutrients have improved semen quality and fertilization in fish [44], which agrees with the findings in this work for the pigments (astaxanthin and β-carotene). Fertilization increased by the effect of the pigments was not noticeable because of the high fertilization rate obtained with this species.

Fish spermatozoa show species differences in the initiation [45,46], duration [47,48], and pattern of motility [49,50]. In this study, the mean motility in goldfish was higher (89.3%–93.1%) than the values obtained for rainbow trout *Oncorhynchus mykiss* [44] broodstock (88.83%–89.32%) and the 65% motility at 30 seconds after activation in *Perca fluviatilis* [51]; this indicates that the appropriate pH (9.0) and the ionic composition of activation solution (osmolality of about 200 mOsm/kg) had conditions [52]. Nevertheless, it is clear that spermatozoa that are highly motile will have a greater chance of fertilization. In most farmed fish species, the motility life span is brief (approximately 1 minute), and accurate evaluation of the motility characteristics can only be made using rapid and sensitive methods. However, the duration of motility was high in goldfish (111.8–128.8 seconds). In most freshwater species, spermatozoa usually move for less than 2 minutes, and in many cases, they are only highly active for less than 30 seconds [53]. This agrees with the values found in this work for goldfish.

Although spermatocrit, viability, and motility are scored relatively easily, the usefulness of motility measurements has long been questioned because subjective scoring methods are used and have produced variable results [20]. Therefore, measures of sperm quality should be validated against successful egg fertilization. In this work, we found that higher osmolality, motility, duration of motility, and sperm concentration were associated with the highest rate of fertilization in the treatment with the highest concentration of astaxanthin (A_{150}). Similar correlations between sperm motility and fertilization capacity have already been reported in carp [54], turbot [55], and rainbow trout [56]. If sperm quality can be related to fertilizing ability, then, it is possible to use computer-assisted sperm analysis to routinely assess a very wide range of freezing protocols without the requirement of females and without the variability inherent in the use of eggs from different female goldfish.

In this study, the mean sperm concentration in goldfish was higher than the values obtained for European hake *Merluccius merluccius* [57] and lower than the values obtained for perch [58] and rainbow trout [59], but similar to those obtained for *C carassius* [60]. In carp, osmolality-dependent permeabilization and structural changes are induced in the sperm membrane by hyposmolality, and the reorganization of the lipid structure has been proposed as a possible mechanism [61]. The difference in K^+ ion concentration (in salmonids) or osmotic pressure (in cyprinids, clariids, and other families) between the seminal plasma and water triggers the initiation of movement [62,63]. The osmotic pressure seems to be the major controlling factor in cyprinids [64].

In conclusion, dietary supplementation with 150 mg/kg of astaxanthin improves osmolality, motility, fertilization rate, and sperm concentration. This work provides novel and informative data that could be used to devise the best rearing conditions for male broodstock and the optimal handling and storage of spermatozoa before fertilization.

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Competing Interests

None of the authors have any conflicts of interest to declare.

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