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EFFECTS OF DECAFFEINATED GREEN TEA (*CAMELLIA SINENSIS*) ON REPRODUCTIVE CHARACTERISTICS AND EGG QUALITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

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This study investigated the effects of dietary decaffeinated green tea (*Camellia sinensis*) in female broodstock of rainbow trout on their reproductive performance and subsequent egg quality. Commercial diet with 100 mg.kg^{-1} feed decaffeinated green tea extract was prepared. Sixty rainbow trout (2475.5 ± 64.4 g) were randomly allocated to 2 groups in triplicates and fed diet containing 100 mg kg^{-1} decaffeinated green tea or control diet for 30 days. On days 20 and 30 during feeding trial, mature fish were weighed and sampled for stripping. Results showed that reproductive performance in terms of total egg weight, egg number per gramme and fecundity were not affected in treatment group. Level of glucose increased markedly on days 20 and 30 while on day 20 of feeding trial, triglyceride content in fish eggs showed a significant decrease. Lipid peroxidation products, indicated by malondialdehyde (MDA), significantly decreased in fish egg with 100 mg kg^{-1} feed administration, indicating elevated antioxidant status in treatment group. Slight increase in superoxide dismutase activity in treatment group was also revealed on day 20 of trial. These results demonstrated significant benefits of decaffeinated green tea extract supplementation of rainbow trout broodstock feed in terms of improved egg quality.

Key words: broodstock nutrition, egg quality, decaffeinated green tea, rainbow trout, reproductive performance

INTRODUCTION

Farmed fish populations are dependent upon the production of good quality eggs. Poor egg quality is one of the major constraints in the expansion of aquaculture of fish species (Brooks *et al.*, 1997). Factors affecting egg quality are determined by the environment in which the egg is fertilised and subsequently incubated and the intrinsic properties of the egg itself. Components affecting egg quality include the physicochemical conditions of the water in which the eggs are subsequently incubated, the endocrine status of the female during the growth of the oocyte in the ovary, the diet of the broodfish and the complement of nutrients deposited into the oocyte (Brooks *et al.*, 1997). A review of broodstock nutrition

by Izquierdo *et al.* (2001) outlined major nutrients such as protein, lipid, fatty acids, vitamins E and C and carotenoids influencing various reproduction processes such as fecundity, fertilisation, hatching and larval development.

Green tea which is produced from leaves of an evergreen shrub, *Camellia sinensis*, is initially used as medicine and later as beverage. Main compositions of green tea are tea polyphenols, vitamins, nitrogenous compounds, caffeine, inorganic elements, lipids and carbohydrates (Chu & Juneja, 1997). Several studies in humans and laboratory animals suggest a beneficial impact of green 'non-fermented' tea on bone density, cognitive function, dental caries and kidney stones, among other effects (Crespy & Williamson, 2004; Cabrera *et al.*, 2006). Some studies have shown that green tea could be an useful supplement to fish diets improving disease resistance, survival rate, growth rate, antioxidant and immune system functions. For example, green tea polyphenois feeding was effective against lipid peroxidation, deterioration of flesh color and microbial growth in yellowtails (*Seriola quinqueradiata*) (Ishihara *et al.*, 2002). Dietary inclusion of green tea extract improved growth and feed utilisation and lowered serum LDL cholesterol in the olive flounder, *Paralichthys olivaceus* (Cho *et al.*, 2007). Green tea could also improve performance, health and prevent aermoniosis in Nile tilapia, *Oreochromis niloticus* (Abdel-Tawwab *et al.*, 2010). Epigallocatechin-3-gallate, a very potent antioxidant derived from green tea, was found to be an antioxidant and an immunostimulant for rainbow trouts, at least at inclusion level of 32 $mg.kg^{-1}$ diet (Thawonsuwan *et al.*, 2010). Meanwhile, dietary green tea supplementation positively enhanced the non-specific humoral and cellular immune responses and disease resistance of kelp grouper (*Epinephelus bruneus*) to *Vibrio carchariae* (Harikishnan *et al.*, 2011).

In some species of fish, few studies have also reported reduction of growth and body lipid accumulation in yellowtail (*Seriola quinqueradiata*) and ayu (*Plecoglossus altivelis*) by green tea extracts and ground green tea (Kono *et al.*, 2000). Previously, it was concluded that the presence of some components in green tea such as caffeine can decrease the immune potency of fish species. Our previous findings showed that decaffeinated green tea in lower doses of administration (20 and 100 $mg.kg^{-1}$) could be optimal to enhance the immunity of rainbow trout (Sheikhzadeh *et al.,* 2011).

The present study was undertaken to investigate the effects of 100 $mg.kg^{-1}$ decaffeinated green tea in female rainbow trout diet on reproductive performance of the rainbow trout through investigation of some aspects eggs metabolites and antioxidant system.

MATERIALS AND METHODS

Method of green tea leaves decaffeination

In order to prepare decaffeinated tea extract, the procedure described in a previous article was followed (Sheikhzadeh *et al.,* 2011). Green tea leaves were first soaked in hot distilled water (1:5 w/v) for 15 min. The supernatant was then removed from the hot water by ethyl acetate in equal volume. Then, by the maceration method the remaining part from tea leaves was extracted with 70% ethanol. The extract was filtered and the solvent was evaporated in a rotary evaporator under reduced pressure at 40 °C. The resulting

extract (decaffeinated tea extract) was frozen at –20 ºC until use.

Fish husbandry and feeding

The experiment was performed in a fish farm in Tabriz, Iran with three-year old female rainbow trout broodstock (2475.5± 64.6 g) from September 2010 until March 2011. All fish were checked for ovulation by applying a manual pressure onto the abdomen and fish in running stage were removed from tanks. The broodstock were kept under natural photoperiod (11L:13D) and fed a commercial diet (Chineh Company, Iran) (Table 1) at the rate of 0.85% body weight once daily. Fish were kept in cement tanks (1.8×1.3×1.3 m) with aerated free-flowing river water and flow rate set at 1.5 $L.s^{-1}$ and water temperature of 10–12 ºC.

Table 1. Analysis of the commercial feed for rainbow trout broodstock.

Analytes	% of the commercial feed
Moisture	5.50
Crude protein	41.68
Total lipid	16.20
Ash	7.70
Starch	23.68
Fibre	5.24

Fish were allocated into 2 groups (30 fish/group) in triplicates with 10 fish per tank. Fish from the experimental group received diets containing 100 $mg.kg^{-1}$ decaffeinated green tea extract for 30 days continuously. Cornmeal oil (10 mL. kg^{-1} feed) was used to bind the powdered green tea to the fish feed. In control group, oil was also added to fish feed for making the situation the same as for the treatment group. Feed was mixed with the decaffeinated tea extract and maintained at 4 ºC for further use during 2 weeks.

Propagation and sample preparation

All fish from both groups were anaesthetised into clove oil bath (50 $\mu L.L^{-1}$) and examined individually for sexual maturity at days 20 and 30 of the feeding trial. After removing the over ripped females, mature ones were weighted and stripped individually. Harvested eggs from each broodstock were collected in separate fine sieves and weighted. The number of eggs in 1 g was determined. By dividing the total weight of egg collected by number of eggs per gram, mean number of eggs spawned by each broodstock (total fecundity) was calculated. Meanwhile, mean number of eggs spawned per kg body weight (relative fecundity) was estimated by dividing the total fecundity by total weight of each female.

From each broodstock, 10 g eggs were put in separate sterile tubes, rinsed in distilled water and blotted on filter paper before being frozen in –20 °C. For the preparation of egg homogenate, 1 g of egg was homogenized with 10 mL of 0.1 M phosphate buffer, pH 7.2, in a Waring blender for 5 min. The homogenate was rapidly centrifuged at 4000×*g* for 15 min at 4 °C. A transparent supernatant was obtained and used for further studies.

Biochemical analysis

Lipid peroxidation assay. The malondialdehyde (MDA) level in the egg homogenate was determined using the thiobarbituric acid (TBA) method (Buege & Aust, 1978). After preparing 20% w/v egg homogenate in a cold 0.1M phosphate buffer (pH 7.2), 200 µL of the homogenate was added to a 1 mL trichloroacetic acid (TCA)-TBA solution (0.67% TBA in 2% TCA). Samples were shaken and incubated for 20 min in a boiling water bath. After cooling and centrifugation for 10 min at 1500×*g*, the absorbance of the

supernatant was measured colorimetrically at 531 nm on a Unico UV-2100 PC spectrophotometer.

Antioxidant enzyme assays. Samples of eggs were homogenized in a cold 0.1M phosphate buffer (pH 7.2) and centrifuged at 2500×*g* for 10 min at 4 ºC. The resultant supernatants were directly used for enzyme assays.

Superoxide dismutase (SOD) activity was assayed by reduction of nitro blue tetrazolium (NBT) with NADH mediated by phenazine methosulfate (PMS) which is inhibited upon addition of SOD (Nishikimi *et al.*, 1972). In a cuvette, 2.6 mL of phosphate buffer (0.017 M, pH 8.3) was mixed with 0.1 mL each of PMS (0.093 mM), NBT (1.5 mM) and supernatant prepared as above at 25 °C. After addition of 0.1 ml of NADH (2.34 mM), the reaction was started, and an increase in absorbance was recorded at 560 nm for 3.5 min at 30 s intervals. A unit of SOD was defined as the activity of enzyme required for suppressing the increase in absorbance by 50%.

Glutathione peroxidase (GPx) activity was measured by the method of Paglia & Valentine (1967). The assay was based on a nicotinamide adenine dinucleotide phosphate (NADPH)-coupled reaction where oxidised glutathione, produced upon reduction of organic peroxide (tert-butyl hydroperoxide) by GPx, was recycled to its reduced state by utilising the enzymes GR and NADPH. The oxidation of NADPH to $NADP^+$ was associated with a decrease in absorbance at 340 nm, directly proportional to the GPx activity.

Determination of metabolites. Total protein content in egg supernatants was determined by the method of Lowery *et al.* (1951). Glucose concentration was measured by the enzymatic colorimetric method of Lott & Turner (1975). Triglycerides were estimated according to the enzymatic colorimetric method of Fossati & Prencipe (1982).

Statistical analysis

Data were analysed by Student *t* test between control and treatment groups using the SPSS 15. $P<0.05$ was accepted as levels of statistical significance.

RESULTS

During this study, no mortality of fish was observed in different groups. Data on reproductive performance observed after the feeding trial are presented in Table 2.

Table 2. Effect of 30-day decaffeinated green tea (100 $mg.kg^{-1}$) supplementation to female rainbow trout diet on reproductive performance. Data are mean±SEM (n=30)

		Control group	Experimental group
Total weight of eggs collected (g)	day 20	296.07 ± 26.17	326.39 ± 28.94
	day 30	350.0 ± 32.93	368.13 ± 42.29
Egg number per 1 g	day 20	11.44 ± 0.41	10.68 ± 0.25
	day 30	12.31 ± 0.44	12.31 ± 0.16
Total fecundity (mean number of eggs spawned)	day 20	3407.51 ± 316.90	3478.89 ± 311.71
	day 30	4372.22 ± 511.85	4525.78 ± 522.70
Relative fecundity (mean number of eggs spawned per 1 kg body weight)	day 20	1373.57 ± 115.85	1277.07 ± 111.44
	day 30	1565.03 ± 156.92	1664.58 ± 139.15

Table 3. Effect of 30-day decaffeinated green tea (100 mg.kg^{-1}) supplementation to female rainbow trout diet on metabolites status of eggs. Data are presented as mean±SEM (n=30)

		Control group	Experimental group
Triglyceride (mmol L^{-1})	day 20	3.25 ± 0.11	2.92 ± 0.08*
	day 30	3.13 ± 0.10	3.01 ± 0.11
Total protein (g L^{-1})	day 20	6.98 ± 0.34	6.60 ± 0.15
	day 30	6.29 ± 0.29	6.75 ± 0.28
Glucose (mmol L^{-1})	day 20	0.79 ± 0.09	0.94 ± 0.05*
	day 30	0.53 ± 0.04	1.04 ± 0.05*

* statistically significantly differences between control and experimental (supplemented) group (P<0.05).

Table 4. Effect of 30-day decaffeinated green tea (100 mg.kg^{-1}) supplementation to female rainbow trout diet on antioxidant activity in eggs. Data are presented as mean±SEM (n=30)

		Control group	Experimental group
Lipid peroxidation products (μmol.L^{-1})	day 20	0.22 ± 0.02	0.09 ± 0.01*
	day 30	0.15 ± 0.03	0.16 ± 0.03
Superoxide dismutase (U.L^{-1})	day 20	162.08 ± 9.47	209.88 ± 19.87
	day 30	222.90 ± 37.86	222.15 ± 28.60
Glutathione peroxidase (U.L^{-1})	day 20	748.06 ± 48.50	693.99 ± 54.32
	day 30	771.10 ± 15.99	672.96 ± 36.65

* statistically significantly differences between control and experimental (supplemented) group (P<0.05).

Total weight of eggs collected, egg number per gramme, total fecundity and relative fecundity did not differ significantly between groups. Table 3 presents data on the effects of decaffeinated green tea on egg metabolite status. Significant decrease in triglyceride was observed in fish supplemented with decaffeinated green tea on day 20 while on day 30, differences were not statistically significant. On both days of sampling, total protein content did not show significant differences (P>0.05) between groups. A significantly higher glucose content in fish eggs was established on days 20 and 30 of the feeding trial.

Table 4 shows data on lipid peroxidation and antioxidant enzymes activity in rainbow trout eggs after feeding decaffeinated green tea. Lipid peroxidation products, indicated by MDA, were significantly affected by addition of decaffeinated green tea to diet (P<0.05) on day 20. On the other hand, no significant changes were shown on day 30 of feeding. Antioxidant enzyme activities did not show any significant changes during this study despite the minor increase in superoxide dismutase activity on day 20 of feeding compared with control group.

DISCUSSION

In the current study, decaffeinated green tea could not improve the reproductive performance in female broodstock in terms of total egg weight, egg size and broodstock fecundity. There are no available data on the effects of green tea or its derivatives on fish reproductive performance but there are lots of studies regarding the effects of different dietary supplements with important roles in fish reproduction (Izquierdo *et al.*, 2001). It seems that the increase in the number of oocytes happens before the onset of salmonid vitellogenesis that is nearly 9 months before ovulation. Another factor with key role in fish fecundity is oocyte atresia that is widespread during vitellogenesis which is mainly related to fish condition and nutrition (Tyler *et al.*, 1990). Meanwhile, during vitellogenesis the increase in oocytes size happen (Mañanós *et al.*, 2009) which shows the importance of broodstock management during this time. Therefore, in salmonids with up to 6 months of vitellogenesis, broodstock must be fed a good quality diet for several months before the spawning season to improve the reproductive performance (Izquierdo *et al.*, 2001). It can be assumed that longer duration of decaffeinated green tea administration could improve the reproductive performance in terms of fecundity and egg size but this has to be validated in clinical trials.

The present study showed a significant decrease in triglyceride value in rainbow trout eggs on day 20. Even though in previous studies, no effects of green tea on lipid values were observed in fish species (Cho *et al.*, 2007; Abdel-Tawwab *et al.* 2010), it was revealed that in laboratory animals green tea intake decreased the absorption of triglycerides and cholesterol from digestive tract and this finding is in accordance with the increase in fat excretion (Crespy & Williamson, 2004; Shrestha *et al.*, 2009). Although in this study, total protein remained unaffected, fish egg glucose level was increased significantly in broodstock administered with decaffeinated green tea. Our finding corroborates study by Abdel-Tawwab *et al.* (2010) which showed significant increase of serum glucose when fish received 0.5–2.0 $g.kg^{-1}$ of green tea. Significant changes in egg metabolites level reflect their contents in maternal serum. Therefore, it seems that administration of decaffeinated green tea in female broodstock resulted in significant decrease in blood triglyceride and higher glucose level. Since nutrients stored in the egg must satisfy nutritional demands for embryonic development and growth, it needs more consideration to understand the effects of such changes in metabolites level in fish eggs on embryonic development. Meanwhile, evaluating these biochemical parameters in larval stage should be interesting to understand whether the same positive effects would also be noted in fish larvae.

According to the present study, malondialdehyde, a marker of oxidative stress, decreased after green tea intake. Intake of green tea also increased the activity of superoxide dismutase in rainbow trout eggs. Thawonsuwan *et al.* (2010) also showed that epigallocatechin-3-gallate (EGCG), a component derived from green tea, increased the availability of antioxidant vitamin E. Meanwhile, lower levels of lipid hydroperoxide in liver of fish administrated with EGCG were shown. Abdel-Tawwab *et al.* (2010) also observed the stimulation of superoxide anion production in fish blood after green tea supplementation. Reactive oxygen species

(ROS) are continuously produced during basal metabolism, but there are several situations including cold water temperature, exposure to contaminants and pathogenic agents, as well as rapid tissue growth when ROS production is enhanced. Therefore, the early development stages of fish would be expected to increase the production of ROS. The antioxidant systems in the liver and other tissues of adult fish are not triggered until late in the embryonic development of larval fish. This makes early antioxidant protection by maternally derived antioxidants essential (Palace & Werner, 2006). This point shows the importance of enhancing broodstock antioxidant system for improving egg quality.

In conclusion, addition of decaffeinated green tea extract to female broodstock diet improved the egg quality of rainbow trout, but had no effects on broodstock fecundity and egg size. More research effort is needed to determine the convenient duration of decaffeinated green tea supplementation for optimum response. Meanwhile, investigating the potential of different derivates of green tea besides the caffeinated and crude green tea on broodstock performance might be of research interest too.

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