Biochemical and histological studies of over-ripened oocyte in the Caspian brown trout (*Salmo trutta caspius*) to determine biomarkers for egg quality

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Abstract

The aims of the present study were to determine the best time for egg stripping after ovulation and to study oocyte over-ripening in the Caspian brown trout (*Salmo trutta caspius*). Eggs were retained in the female abdominal cavity for 40 days post ovulation (DPO). Partial volumes of eggs stripped from 10 individually identified females at 10 days intervals and fertilized with a pool of semen obtained from 8 males. The biochemistry and histology of the eggs and the biochemistry of the ovarian fluid were studied. The eyeing and hatching rate of the eggs declined with over-ripening time, which decreased from 90.60±6.28% for eyeing and 86.33±6.82% for hatching in newly ovulated eggs (0–10 DPO) to 1.34±0.67% for eyeing and 0.98±0.49% for hatching in over-ripened eggs (30–40 DPO). However, larval abnormalities remained constant for 30 days after ovulation. During the course of oocyte over-ripening, the pH of the ovarian fluid significantly decreased and the concentration of glucose, protein, calcium, iron, and aspartate aminotransferase activity significantly increased. Moreover, the concentration of protein, triglycerides, and aspartate aminotransferase activity in the eggs changed with over-ripening. In the newly ovulated eggs, the yolk consisted of homogenous tissue and its perivitelline space diameter had no considerable differences. With over-ripening, the yolk became heterogeneous, and while chorion diameter did not change, the perivitelline space diameter varied among different areas. The present study demonstrated that the best time to take Caspian brown trout eggs after ovulation at 7±0.6°C was up to 10 DPO. Among the studied parameters of the egg and ovarian fluid, egg quality was related to both ovarian fluid parameters (pH, protein, aspartate aminotransferase, glucose, cholesterol, triglycerides, calcium) and egg parameters (iron, aspartate aminotransferase), suggesting that these parameters can be used as egg quality biomarkers for Caspian brown trout.

Keywords: Oocyte, Ovulation, Over-ripening, Ovarian fluid, Caspian brown trout

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Introduction

Post-ovulation oocyte aging in the ovary or coelomic cavity of fish causes over-ripening of eggs, which always diminishes eggs quality (Lahnsteiner, 2000). This degradation has been demonstrated in the form of decreased fertilization, eyeing, and hatching rates and as increases in morphological abnormalities or in the appearance of various ploidy abnormalities in larvae (Sakai *et al*., 1975; Craik & Harvey, 1984; Lahnsteiner, 2000; Aegerter & Jalabert, 2004). Thus, over-ripening is one of the limiting factors in successful artificial spawning. This issue is particularly important among salmonids, because their ovulation is spontaneous and occurs without administration of hormones. Moreover, predicting egg quality in salmonids is difficult because simple and reliable tests are lacking. To solve this problem, a study of the physiological and biochemical changes that gradually appear in the eggs and ovarian fluid of salmonids during the post-ovulation period would be useful, as would determination of the threshold for egg quality degradation.

The influence of aging of eggs in the coelomic cavity of salmonids on egg quality for different time intervals and under different temperature conditions has been well studied (Sakai *et al*., 1975; Springate *et al*., 1984; Gaudemar & Beall, 1998; Azuma *et al*., 2003; Bonnet *et al*., 2003; Mohagheghi Samarin *et al*., 2008). Also, some information is available about the morphological, physiological, and biochemical changes that occur in the egg and ovarian fluid of rainbow trout during the over-ripening period. Craik and Harvey (1984) reported that over-ripening in rainbow trout (*Oncorhynchus mykiss*) is accompanied by changes in water content of the egg, chorion weight, and quantities of lipid, protein, iron and calcium in the egg. Lahnsteiner (2000) showed that only the biochemical parameters of the ovarian fluid such as esterified and non-esterified fatty acids, protein, acid phosphatase, aspartate aminotransferase and the pH are suitable parameters for predicting egg quality in rainbow trout. Also for rainbow trout, Aegerter and Jalabert (2004) demonstrated the relationship between egg quality and female weight, pH, and osmolality of the ovarian fluid, and Rime *et al*. (2004) showed that changes in protein content in the ovarian fluid can be used to predict egg quality during the over-ripening period.

Caspian brown trout (*Salmo trutta caspius*) is an indigenous salmonid to Iranian waters, and its population in the Caspian Sea is decreasing strikingly. Since 1985, the Iranian Fisheries Organization has been conducting artificial reproduction of the brood stocks and growing fry to the smolt level of this species and releasing them (Bahramian, 2001). The present study was performed to examine the effects of egg retention time on egg viability and to determine biomarkers to predict the egg quality of the Caspian brown trout. In particular, we addressed the following questions: How does post-ovulation oocyte aging (taking into account temperature) affect egg quality? Does the structure of the

egg (e.g., cortical vesicles, perivitelline space, and yolk) change during the course of over-ripening? How do the main biochemical parameters of the egg and ovarian fluid (e.g. glucose, cholesterol, triglycerides, protein, calcium, iron, aspartat amino-transferase enzyme activity and pH) change during over-ripening and are these changes related to egg quality?

Materials and methods

In this study we used 4-year-old brood stocks of cultivated Caspian brown trout in their first spawning season in December, 2007. Three hundred female and male fish with the sexual ratio (2:1) were held in $5m^3$ outdoor ponds in Kelardasht Cold-water Fish Reproduction and Culturing Center, Mazandaran, Iran, under a natural photoperiod. The water in the ponds was a mixture of water from the nearby river and spring, and the water temperature throughout the experiment was 7±0.6°C. During the months preceding experiment, the brood stocks were fed a commercial diet (Biomar) composed of 46% protein and 22% fat once a day until satiation, except for a starvation period 1 month before the expected time of ovulation and spermiation.

To reduce the stress created by examination, the brood stocks were anaesthetized using 100ppm MS222 (Mohagheghi Samarin *et al*., 2008). Based on the examination, females that had already ovulated were removed from the population, and 62 of them were selected for the experiment because they were expected to ovulate soon based on their appearance (abdominal and anal forms, body color). These fish were examined and checked for ovulation after 10 days and ten fish were selected randomly to be bred for the first time (total length: 37.62±2.66cm; body weight: 452.77±69.68g). A partial volume (25g) of eggs of these females were stripped and fertilized with mixed milt repeatedly every 10 days for 40 days. The sperm used for fertilization was taken from 8 males mixed slowly in equal proportions. Spermatozoa density was 248.55 × 10^6cell/ml. To study the biochemical parameters of the eggs and ovarian fluid, 5g of eggs and 5ml of ovarian fluid were collected from each female. The eggs and ovarian fluid were frozen in liquid nitrogen and stored at –20°C until analysis. An additional 2.5ml of ovarian fluid were collected to determine pH. Furthermore, to investigate histological changes during the over-ripening process, samples of eggs were collected at the first and fourth examination periods.

From each female, 25g egg (500.7± 136.22) were obtained and gently mixed with 1ml sperm in a plastic container. 10ml of water from the hatchery were added to stimulate the sperm movement and the solution was gently mixed for 2 min. To wash and rinse the excess sperm, the eggs were placed in the flowing water of the hatchery for 30 min, and then the eggs of each female were separately put into Californian incubators with similar density and flow of water at 7±0.2°C. Dead eggs and embryos were regularly removed. At 30 and 60 days after fertilization, values for eyeing

and hatching rate were obtained in comparison with the total number of fertilized eggs. In addition, the number of larval abnormalities relative to the number of alevins was assessed at each hatching stage.

From each female, 5g egg (100±27.38 eggs) were homogenized completely using a manual glass homogenizer for 10 min. Sorensen's phosphate buffer (pH 7.38) was used as dilution buffer (McPheron & Pincus, 2007). The sample then was centrifuged for 10 min at 4000 ×g and the supernatant phase was used for biochemical analysis. The photometric methods were used to determine biochemical factors of the egg and ovarian fluid. Calcium was measured following the Arsenazo method (Bauer, 1981); iron with the Ferrozine method (Burtis & Ashwood, 1996); aspartate aminotransferase activity with the IFCC method (Bergmeyer *et al*., 1986); protein with the Biuret method (Tietz, 1986); cholesterol with the CHOD-PAP method (Artiss & Zak, 1997); triglycerides with the GPO method (Tietz, 1986), and glucose with the Glucose Oxidase method (Burtis & Ashwood, 1996).

At the time of stripping, 2.5ml of ovarian fluid were obtained from each female; to minimize the sample's contact with air, it was collected in a capped micro tube. It was centrifuged for 10 min at 4000 × g to separate the blood cells. Afterwards, the pH of the supernatant was measured using a micro electrode.

For each female, immediately after stripping, 10 eggs were fixed in 10% buffered formaldehyde. These samples were dehydrated in a series of ethanol solutions and embedded in paraffin, and then 2-3µm thick sections were cut and stained with haematoxylin-eosin (Drury & Wallington, 1980).

In order to compare the means, one-way analysis of variance (ANOVA) was used and homogeneity of variances was tested using the Leven test. The non-parametric Welch test was used to compare means and the Tukey test was applied for multiple comparisons of means. To determine the relationship between the investigated parameters, the Pearson correlation coefficient was used. Finally, to examine the relationship between egg and ovarian fluid parameters and egg quality, simple linear regression was used. A significance level of 0.05 was used for all analyses.

Results

Over the course of the four sampling periods, egg viability significantly decreased. The difference in the eyeing rate between the first and second sampling times was not significant but after that it decreased significantly ($P<0.05$).

Throughout the over-ripening period, hatching rate decreased significantly. Although the larval anomaly rate increased gradually throughout the over-ripening period, this increase was not significant up to 20–30 DPO (Table 1). Because of the total annihilation of all eggs for nine of the ten females in the fourth treatment, it was not possible to measure their larval abnormality rate.

Among the investigated parameters of ovarian fluid, the concentration of triglycerides did not significantly change over the sampling period. However, the concentration of glucose, cholesterol, protein, calcium, and iron and the aspartate aminotransferase activity increased significantly, whereas pH significantly decreased (P<0.05) (Table 2).

The biochemical parameters of eggs differed among the sampling periods. The concentration of glucose, cholesterol, calcium, and iron showed no significant changes. The concentration of protein and triglycerides remained constant for each mg of egg, but because of unequal egg sizes, the change for each egg in some of the treatments was significant. In addition, as showed in table 3, the aspartate aminotransferase activity both per egg and per mg of egg increased over time (P<0.05).

Table 1: Effect of post-ovulation oocyte aging (days post-ovulation: DPO) on the egg viability

Parameter	Time of post–ovulatory aging (days post – ovulation)			
	0-10	**10-20**	**20-30**	**30-40**
Eyed eggs (%)	90.65±6.28[a]	69.80±16.98[a]	4.54±4.32[b]	1.34±0.67[b]
Hatched alevins (%)	86.33±6.82[a]	49.88±11.87[b]	2.92±2.87[c]	0.98±0.49[c]
Deformed alevins (%)	1.19±1.2[a]	2.40±1.92[a]	4.08±1.02[a]	-

Within column values with the same letter superscript are not significantly different (i.e., P> 0.05).

Table 2: Effect of post-ovulation oocyte aging (days post-ovulation: DPO) on the ovarian fluid parameters

Parameter	Time of post – ovulatory aging (days post – ovulation)			
	0-10	**10-20**	**20-30**	**30-40**
Glucose (mg/100ml)	38.07±4.90[a]	62.00±6.05[b]	66.33±12.01[b]	72.67±8.50[b]
Cholesterol (mg/100ml)	11.13±3.20[a]	16.75±2.75[a]	31.67±4.04[a,b]	46.00±21.28[b]
Triglycerides (mg/100ml)	9.90±0.66[a]	20.00±6.98[a]	26.30±13.75[a]	29.33±25.69[a]
Protein (mg/100ml)	223.3±45.09[a]	277.5±55.60[a]	520.0±85.44[a,b]	650.0±274.04[b]
Calcium (mg/100ml)	5.30±0.62[a]	9.32±0.60[b]	9.83±1.10[b]	10.63±0.98[b]
Iron (mg/100ml)	164.33±25.00[a]	502.3±303.05[a,b]	708.7±205.01[a,b]	971.7±413.89[b]
Aspartate aminotransferase (IU/lit)	10.67±5.13[a]	76.00±19.76[a,b]	92.00±33.00[b]	166.7±42.12[c]
pH	8.32±0.12[a]	8.22±0.44[a]	7.88±0.47[b]	7.72±0.13[b]

Within column values with the same letter superscript are not significantly different (i.e., P> 0.05).

Table 3: Effect of post-ovulation oocyte aging (days post-ovulation: DPO) on egg parameters

Parameter	Time of post – ovulatory aging (days post – ovulation)			
	0-10	10-20	20-30	30-40
Glucose (µg/egg)	36.47±4.32[a]	18.15±5.27[a]	18.81±8.00[a]	31.37±15.28[a]
(µg/mg egg)	(0.61±0.18)[a]	(0.34±0.99)[a]	(0.31±0.17)[a]	(0.66±0.29)[a]
Cholesterol (µg/egg)	301.70±53.85[a]	279.58±35.72[a]	297.37±85.63[a]	244.60±111.44[a]
(µg/mg egg)	(5.69±1.23)[a]	(5.28±0.67)[a]	(4.75±1.12)[a]	(4.9±1.68)[a]
Triglycerides (µg/egg)	755.55±48.58[a]	646.60±41.51[a,b]	728.20±91.53[a]	545.60±88.19[b]
(µg/mg egg)	(14.20±1.46)[a]	(12.2±0.78)[a]	(11.67±0.46)[a]	(11.40±2.22)[a]
Protein (µg/egg)	17080±508.62[a]	13581±952.40[a,b]	17094±523.86[a]	11541±2576.63[b]
(µg/mg egg)	(320.83±22.33)[a]	(256.25±17.97)[a]	(275.6±21.13)[a]	(241.50±59.36)[a]
Calcium (µg/egg)	58.19±18.88[a]	64.66±6.24[a]	76.34±12.94[a]	56.46±4.23[a]
(µg/mg egg)	(1.1±0.36)[a]	(1.22±0.12)[a]	(1.22±0.10)[a]	(1.19±0.26)[a]
Iron (µg/egg)	1493.4±491.13[a]	1094.5±208.5[a]	1064.1±71.64[a]	891.70±163.04[a]
(µg/mg egg)	(28.26±10.36)[a]	(20.65±3.93)[a]	(17.27±2.95)[a]	(19.55±8.92)[a]
Aspartate aminotransferase (IU/egg)	0.011±0.003[a]	0.014±0.010[a]	0.042±0.030[a,b]	0.063±0.018[b]
(IU/mg egg)	(0.0002±0.0005)[a]	(0.0003±0.0002)[a]	(0.0007±0.0005)[a,b]	(0.0013±0.0005)[b]

Within column values with the same letter superscript are not significantly different (i.e., P>0.05).

Linear regression analysis showed that eyeing rate was negatively correlated with the concentration of cholesterol, protein, and calcium and with the activity of the aspartate aminotransferase enzyme of ovarian fluid. It was positively correlated with the pH of ovarian fluid so that the relationship between eyeing rate and pH was highly significant ($P<0.05$, $r = 0.939$). Among the biochemical parameters of egg that were studied, eyeing rate was positively correlated with iron concentration and negatively correlated with aspartate aminotransferase activity (Table 4). For ovarian fluid, hatching rate was negatively correlated with the concentration of glucose, cholesterol, protein, and calcium and with aspartate aminotransferase activity and positively correlated with pH. The relationship between hatching rate and pH ($P<0.05$, $r = 0.931$) and aspartate amino-transferase activity ($P<0.05$, $r = 0.801$) was highly significant. Furthermore, hatching rate was positively correlated with the concentration of iron in egg and negatively correlated with aspartate aminotransferase activity in egg (Table 5). Although the larval anomaly rate was not significantly related to any of the biochemical factors of the egg, linear regression showed a positive correlation between this rate and the concentration of cholesterol, triglycerides, and protein and aspartate aminotransferase activity in ovarian fluid and a negative correlation with pH of ovarian fluid (Table 6).

Table 4: Statistical models predicting the quality of Caspian brown trout eggs during over-ripening. R is the Pearson correlation coefficient between analyzed parameters and eyed eggs

Parameter	R	Regression model	R^2	F-Value
Ovariaon fluid parameters				
Cholesterol	0.750	y= 86.729-1.616x	0.562	8.990
Protein	0.758	y=95.888-1.26x	0.575	9.457
Calcium	0.674	y=180.113-14.756x	0.454	5.821
Aspartate aminotransferase	0.756	y=91.774-0.493x	0.571	9.325
pH	0.939	y= -994.561+129.695x	0.882	45.029
Egg parameters				
Iron	0.659	y= -82.983+0.113x	0.435	6.925
Aspartate aminotransferase	0.681	y= 68.325-0.948x	0.464	7.792
	(0.652)	(y= 64.131-41.966x)	(0.425)	(6.649)

In the regression model, the percent of eyed eggs was the dependent variable and analyzed parameters were the independent variables. Egg parameter data without parenthesis are in units/egg and data in parenthesis are in units/mg egg. N=40. Statistical significance was set at P< 0.05 for all regression models.

Table 5: Statistical models predicting the quality of Caspian brown trout eggs during over-ripening. R is the Pearson correlation coefficient between analyzed parameters and hatched alevins

Parameter	R	Regression model	R^2	F-Value
Ovariaon fluid parameters				
Glucose	0.793	y=168.546-2.138x	0.629	11.844
Cholesterol	0.730	y=68.569-1.3x	0.533	7.982
Protein	0.737	y=75.889-0.102x	0.543	8.327
Calcium	0.779	y=164.44-14.094x	0.606	10.784
Aspartate aminotransferase	0.801	y=76.164-0.432x	0.642	12.547
pH	0.931	y=-818.042+106.49x	0.867	39.023
Egg parameters				
Iron	0.676	y=-72.113+0.95x	0.457	7.588
Aspartate aminotransferase	0.667	y=53.828-0.761x	0.445	7.215
	(0.636)	(y=50.361-33.539x)	(0.404)	(6.098)

In the regression model, the percent of hatched alevins was the dependent variable and analyzed parameters were the independent variables. Egg parameter data without parenthesis are in units/egg and data in parenthesis are in units/mg egg. N= 40. Statistical significance was set at P<0.05 for all regression models.

Table 6: Statistical models predicting the quality of Caspian brown trout eggs during over-ripening. R is the Pearson correlation coefficient between analyzed parameters and deformed alevins

Ovariaon fluid parameters	R	Regression model	R^2	F-Value
Cholesterol	0.936	y=-1.027+0.190x	0.877	35.567
Triglycerides	0.961	y=-2.202+0.240x	0.923	59.997
Protein	0.914	y=-1.838+0.014x	0.836	25.456
Aspartate aminotransferase	0.826	y=-1.030+0.056x	0.682	10.712
pH	0.927	y=114.792-13.757x	0.860	24.538

In the regression model, the percent of deformed alevins was the dependent variable and analyzed parameters were the independent variables. N= 31. Statistical significance was set at P<0.05 for all regression models.

The Pearson correlation coefficient ($P<0.05$) revealed a positive significant correlation between eyeing rate and hatching rate and a negative correlation between eyeing rate and larval anomaly rate. The relationship between hatching rate and larval anomaly rate was not significant. Table 7 shows the main physiological relationships between egg and ovarian fluid parameters.

In newly ovulated eggs (0–10 DPO), the chorion diameter was 23.21±3.45µm and the perivitelline space diameter was 20.83±8.01µm. Cortical vesicles, which are located at the margin of the egg in the plasma membrane, were of various sizes; their average diameter was 48.35±19.52µm. Moreover, the yolk consisted of quite homogenous tissue. In over-ripe eggs (30–40 DPO), chorion diameter was almost constant (22.98±2.88µm), and the diameter of cortical vesicles was 63.23±13.45µm. The diameter of the perivitelline space varied from 17.3 to 91.2µm. The yolk became heterogeneous; most of it contained vesicular inclusions with diameters ranging from 12.69 to 97.74µm (Fig. 1).

Table 7: Physiologically important correlations between egg and ovarian fluid parameters

Correlations	R
Between egg quality parameters	
Eyed egg – Hatched alevins	0.975
Eyed egg – deformed alevins	-0.737
Between ovarian fluid parameters	
pH - protein	-0.898
pH - Aspartate aminotransferase	-0.917
Protein - Aspartate aminotransferase	0.871

R is the pearson correlation coefficient. Statistical significance was set at $P<0.05$. Egg parameter data without parenthesis are in units/egg and data in parenthesis are in units/mg egg.

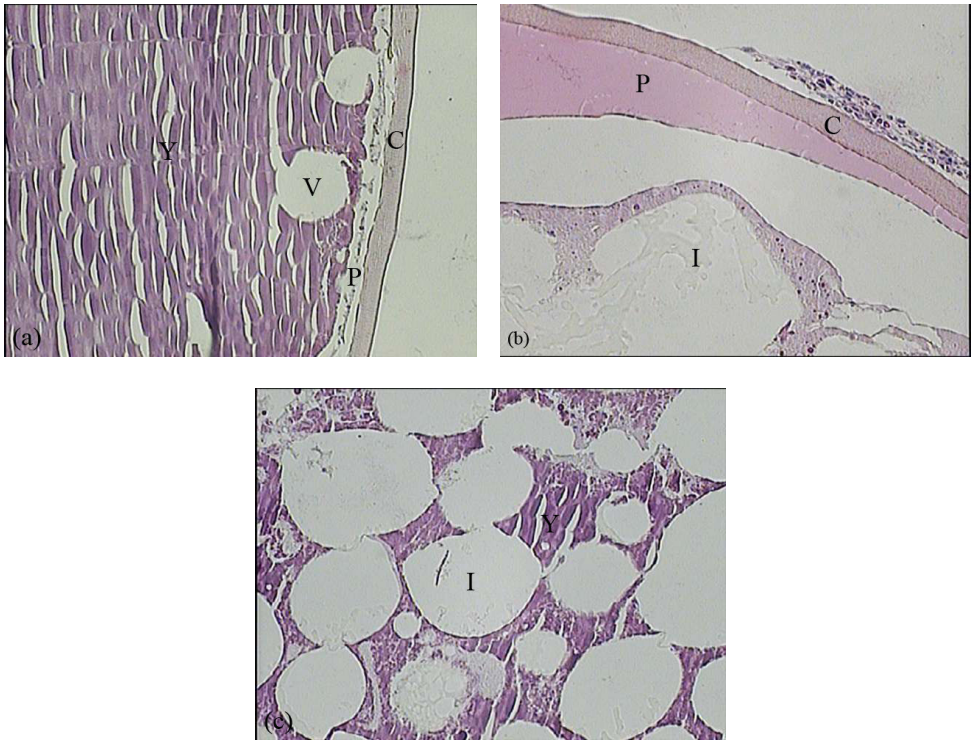

Figure 1: Histological changes of Caspian brown trout eggs during over-ripening. C: chorion; P: perivitelline space; V: cortical vesicle; Y: yolk; I: vesicular inclusion. (a) Freshly ovulated egg (H & E ×450). (b) Over-ripened egg. Note: the enlargement of the perivitelline space and the non-homogenous composition of the yolk (H & E ×450). (c) The yolk of an over-ripened egg. Note the numerous vesicular inclusions with different diameters (H & E ×450).

Discussion

The present study demonstrated that egg viability of Caspian brown trout significantly reduced during over-ripening in the coelomic cavity. Changes in egg quality, result in many biochemical changes in the egg and ovarian fluid, some of which are useful in determining egg quality. In the present study, stripping was carried out at 10 day intervals, since according to Mohagheghi *et al*. (2008), egg quality can lower arising from post-ovulatory aging time, which occurred in 28-35 DPO. Another reason was the sensitivity of Caspian brown trout females and possibility of occurred of death in them due to successive handling. Also, mixed sperm were obtained from 8 male fish, thereby keeping differences in fertilization success due to variable sperm quality in different treatments to a minimum (Aegerter & Jalabert, 2004; Mohagheghi Samarin *et al*., 2008). We used eyeing rate, hatching rate, and the rate of larval abnormalities as indices of egg quality and not fertilization

rate. According to the study of Azuma *et al*. (2003) on rainbow trout, a delay in cell division caused by post-ovulation oocyte aging causes embroying to occur at a slower pace, which can lead to mistakes in measuring fertilization rate. In addition in our study successive stripping of eggs according to Lahnsteiner's report (2000) had no influence on the quality of ovarian fluid.

In rainbow trout, at 8ºC, eggs can be maintained in the coelomic cavity of females for at least 2 weeks (Mohagheghi Samarin *et al*., 2008) and at 10–12ºC, for at least 1 week without any decrease in egg quality (Springate *et al*., 1984; Aegerter & Jalabert, 2004). In Caspian brown trout kept at 7ºC, eyeing rate up to 20 DPO and hatching rate up to 10 DPO were high, but then they decreased severely. The larval anomaly rate up to 30 DPO showed no significant change, which supports previous reports on the consistency of the larval anomaly rate in rainbow trout (Azuma *et al*., 2003). In contrast, Sakai *et al*. (1975), who studied rainbow trout, and Rizzo *et al*. (2003), who studied *Prochilodus marggravii* reported that the larval anomaly rate increased with duration of post-ovulation oocyte aging. Aegerter and Jalabert's study (2004) of rainbow trout also showed clear changes in the larval anomaly rate at 12ºC and 17ºC. In Caspian brown trout, the larval anomaly rate was significantly correlated only with eyeing rate; its relationship with hatching rate was not significant, which agrees with part of Aegerter and Jalabert's (2004) findings about the weak relationship between the larval anomaly rate and eyeing and hatching rate in rainbow trout.

Over the course of post-ovulation oocyte aging, the pH of ovarian fluid of Caspian brown trout drastically decreased, but the concentration of glucose, cholesterol, protein, calcium, and iron and aspartate aminotransferase activity increased. The quantity of triglycerides did not considerably change. This was the first report on the changes of glucose, cholesterol, triglycerides, calcium and iron amount in ovarian fluid during over-ripening stage. These findings agree to some extent with Lansteiner's (2000) findings in rainbow trout, which showed that post-ovulation oocyte aging of up to 21 DPO led to decreases in the pH and increases in the concentration of protein and aspartate aminotransferase activity in ovarian fluid. In Aegerter and Jalabert's (2004) study of rainbow trout, protein density in ovarian fluid kept at 17ºC throughout the over-ripening period remained constant; only at 12ºC density of ovarian fluid increase and pH decrease. The results of this study of Caspian brown trout also verified Fauvel *et al*.'s (1993) report that pH decreased in ovarian fluid due to over-ripening in turbot (*Scophthalmus maximus*). The source of particles in ovarian fluid can be epithelial cells of the ovary or degenerate eggs. Lahnsteiner *et al*. (1997) illustrated the role of the ovarian epithelial layer in discharging glucose, proteins, acid phosphatase, protease, and β-D glucuronidase into ovarian fluid in bleak (*Alburunus alburunus*), which creates an ionic slope in the fluid. Ovarian fluid in salmonids consists of Na^{+}, K^{+}, and Ca^{+2} ions,

glucose, fructose, cholesterol, phospholipids, proteins, and free amino acids (Satia *et al*., 1974; Lahnsteiner *et al*., 1995). Lahnsteiner (2000) reported that from nine proteins found in ovarian fluid of rainbow trout, three were abundant in the egg. In salmonids, although some proteins in ovarian fluid are derived from blood (Matsubara *et al*., 1985), most are produced by discharging activities of the ovary after ovulation and enter the coelomic cavity (Rime *et al*., 2004). In our study, the increase in protein and aspartate amino-transferase activity in ovarian fluid during the over-ripening period led to a severe decrease of pH. There was also a significant positive correlation between protein concentration and aspartate aminotransferase activity ($P<0.05$, $r = 0.871$). The protein and aspartate aminotransferase being discharged into ovarian fluid via degeneration of the egg likely results in the drastic reduction of ovarian fluid pH. A negative correlation between the protein concentration of ovarian fluid and pH in turbot (Fauvel *et al*., 1993) and rainbow trout (Lahnsteiner, 2000) and a negative correlation between aspartate amino-transferase activity and pH in rainbow trout (Lahnsteiner, 2000) have been also reported.

Among the investigated factors in ovarian fluid of Caspian brown trout, the concentrations of glucose, cholesterol, triglycerides, protein, calcium, aspartate aminotransferase activity, and pH value were significantly correlated with eyeing, hatching, and larval anomaly rates. The relationships among pH, protein, and aspartate aminotransferase activity of ovarian fluid with the eyeing rate of rainbow trout (Lahnsteiner, 2000) and between pH of ovarian fluid and fertilization rate in turbot were previously reported (Fauvel *et al*., 1993). Aegerter and Jalabert (2004) found a significant correlation only between pH of ovarian fluid and eyeing rate; no relationship between protein content and egg quality was observed. In all studies conducted to date, including the one presented herein, low pH (particularly<8) has been accompanied by a significant decrease in egg quality. Therefore, in Caspian brown trout, pH reduction from 8.32 to 7.72 resulted in the reduction of eyeing and hatching rates from 90.65 and 86.33% to 1.34 and 0.98%, respectively. Furthermore, due to the relationship between protein and pH of ovarian fluid ($P<0.05$, $r = -0.898$) it seems that protein affects egg quality by affecting pH. However, aspartate aminotransferase, which is soluble in the cytoplasm, can be discharged from damaged eggs and indicated alterations in the permeability of the oolemma (Lahnsteiner *et al*., 1999).

Among the analyzed biochemical parameters in Caspian brown trout egg, only the concentrations of protein and triglycerides for each egg changed significantly during the over-ripening period. Aspartate amino-transferase activity both in egg and in mg of egg increased, but the other parameters remained constant. This was the first report about the changes of glucose, cholesterol and triglycerides level in egg. In contrast to Craik and Harvey's (1984) results for rainbow trout, which described an increase in iron and calcium in over-ripened eggs,

the concentrations of these two factors did not change significantly during the over-ripening process in Caspian brown trout. Our results also conflict with those of Lansteiner (2000), who reported consistency in the level of protein and the aspartate aminotransferase activity of rainbow trout eggs. In salmonids, yolk proteins, which are derived from vitellogenin, compose 80–90% of the dry weight of ovulated eggs (Mommsen & Moon, 2005). In our study, protein composed the highest portion of egg weight, and among the lipids triglycerides weighed more than cholesterol. In rainbow trout eggs, triglycerides and phosphatidyl-choline are the main lipids, with cholesterol and free fatty acids contributing less (Lahnsteiner, 2000). Moreover, in this study of Caspian brown trout, the eyeing and hatching rates were related only to iron content and the aspartate aminotransferase activity of the egg. The relationship between iron content of the egg and hatching rate in rainbow trout was previously reported by Hirao *et al*. (1954), whereas Lahnsteiner (2000) reported no relation between the studied biochemical factors in the egg and its quality. The reason for such similarities and differences wanted further studies. However, variables fish species, physiological conditions, water quality variables and nutritional conditions may cause the obtained results on egg and ovarian fluid composition. For instance, some nutritional elements including ascorbic acid, tocopherol, essential amino acids and fatty acids can significantly affect the progress of fish reproduction system, particularly use of diet with high quality can significantly improve the egg quality, it's fertilization and the produced larvae (De Silva & Anderson, 1994).

Histologically, the chorion diameter and cortical vesicles did not differ considerably between newly ovulated eggs and over-ripened eggs. The most significance difference found pertains to yolk mass and perivitelline space. In the over-ripened egg, the yolk became heterogeneous and contained many vesicles composed of polysaccharide acids and lipids generated by catabolic and analytic processes (Lahnsteiner, 2000). Irregularity of the perivitelline space diameter can be attributed to the osmolality misbalance between the egg and ovarian fluid (Lahnsteiner, 2000). The histological findings of this study are similar to those of Lansteiner (2000) in rainbow trout eggs, but they differ from those of Formacion *et al.* (1993) in gold fish (*Carassius auratus*) and Rizzo *et al.* (2003) in *Prochilodus marggravii*.

In conclusion, the best time to take Caspian brown trout eggs after ovulation at 7±0.6ºC is up to 10 DPO. Parameters such as glucose, cholesterol, triglycerides, protein, calcium, and pH of ovarian fluid, iron of egg, and aspartate aminotransferase activity in the egg and ovarian fluid can be used as biomarkers to predict egg quality in this species. However, due to abundant biochemical changes that occur in the egg, ovarian fluid would be more appropriate for this purpose.

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مطالعات بيوشيميايي و بافتشناسي فوق رسيدگي تخمك در ماهي آزاد درياي خزر (***Salmo trutta caspius***) براي تعيين نشانگرهاي زيستي كيفيت تخم

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تاريخ دريافت:بهمن ۱۳۸۷ تاريخ پذيرش: شهريور ۱۳۸۸

چكيده

به منظور تعيين بهترين زمان تخم‌كشي پس از تخمك‌گذاري و بررسي فوق رسيدگي تخمك در ماهي آزاد درياي خزر، تخمك‌ها به مدت ۴۰ روز پس از تخمك‌گذاري در حفره شكمي مولدين باقي ماندند. مقداري تخمك از هر مولد (n=۱۰) با فواصل ۱۰ روز استحصال شده و با ذخيره اسپرمي ۸ مولد نر لقاح داده شدند. تخمك‌ها از نظر بيوشيميايي و بافت‌شناسي مطالعه شده و مايع تخمداني نيز از نظر بيوشيميايي بررسي گرديد. فوق رسيدگي باعث كاهش درصد چشم‌زدگي و تفريخ تخمك شد بطوريكه درصد چشم‌زدگي و تفريخ از ۶/۲۸±۹۰/۶۰ و ۶/۸۲±۸۶/۳۳ درصد برآورد شده در تخمك‌هاي تازه اوولـه شده (۱۰-۰DPO) به ۰/۶۷±۱/۳۴ و ۰/۴۹±۰/۹۸ درصد در تخمك‌هاي فوق رسيده (۴۰-۳۰DPO) كاهش يافت. درصد ناهنجاري لاروي تا ۳۰ روز پس از تخمك‌گذاري ثابت بود. در طول دوره فوق رسيدگي تخمك، pH مايع تخمداني به شدت كاهش و مقادير گلوكز، پروتئين، كلسيم، آهن و فعاليت آنزيم آسپارتات آمينوترانسفراز به شدت افزايش يافت. همچنين ميزان پروتئين، تري گليسيريد و فعاليت آنزيم آسپارتات آمينوترانسفراز در تخمك بر اثر فوق رسيدگي تغيير كرد. در تخمك تازه اوولـه شده زرده داراي بافت يكنواخت بود و قطر فضاي پري ويتلين تفاوت چنداني نداشت اما با ايجاد فوق رسيدگي، زرده ناهمگن شد و در حاليكه تغييري در قطر كوريون حاصل نشد، قطر فضاي پري ويتلين آن در نقاط مختلف متفاوت گرديد. اين مطالعه نشان داد كه بهترين زمان استحصال تخمك در ماهي آزاد درياي خزر در دماي ۰/۶±۷ درجه سانتيگراد تا ۱۰ روز پس از تخمك‌گذاري مي‌باشد. از بين فاكتورهاي مورد بررسي در تخمك و مايع تخمداني، كيفيت تخمك هم با فاكتورهاي مايع تخمداني (pH، پروتئين، آسپارتات آمينوترانسفراز، گلوكز، كلسترول، تري‌گليسيريد و كلسيم) و هم فاكتورهاي تخمك (آهن و آسپارتات آمينوترانسفراز) مرتبط بود كه نشان مي‌دهد مي‌توان از اين فاكتورها بعنوان نشانگرهاي زيستي كيفيت تخمك ماهي آزاد درياي خزر استفاده نمود.

كلمات كليدي: تخمك، تخمك‌گذاري، فوق رسيدگي، مايع تخمداني، ماهي آزاد درياي خزر

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