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The Relation Between Egg Viability, Selected Aspects of Egg and Ovarian Fluid Composition and Time of Stripping in Endangered Caspian Brown Trout (*Salmo trutta caspius*)

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Abstract: The effects of egg retention time in the abdominal cavity after ovulation on egg viability were studied in Caspian brown trout (*Salmo trutta caspius*). Eggs were retained in the parental abdominal cavity for 40 days post ovulation. Partial volumes of eggs stripped from 10 individually identified females and fertilized with fresh semen obtained from 8 males at 10 days intervals for 4 stages. The biochemistry of the eggs and ovarian fluid were studied to investigate possible links with post-ovulatory oocyte aging. The eyeing and hatching rate of the eggs declined with over-ripening time: that is, the expected amounts (90.60±6.28% for eyeing and 86.33±6.82% for hatching) in newly ovulated eggs (0-10 days post ovulation) decreased to 0.67±1.34% and 0.49±0.98%, respectively, in over-ripened eggs (30-40 days post ovulation). However, larval abnormalities remained constant for 30 days after ovulation. Over the course of post-ovulation oocyte aging, 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 also changed. 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 days post ovulation. Also, egg viability was related to both ovarian fluid parameters (e.g., pH, protein, aspartate aminotransferase, glucose, cholesterol, triglycerides, iron, calcium) and egg parameters (e.g., cholesterol, triglycerides, iron, aspartate aminotransferase) which can be used to detect egg quality defects associated with oocyte post-ovulatory aging.

Key words: Oocyte, ovulation, over-ripening, ovarian fluid, caspian brown trout

INTRODUCTION

Post-ovulation oocyte aging in the ovary or coelomic cavity of fish results in over-ripening of eggs, which always diminishes eggs quality. This degradation has been demonstrated in the form of

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decreased fertilization, eyeing and hatching rates and as increases in morphological abnormalities or in the appearance of various ploidy anomalies in larvae (Craik and Harvey, 1984; Lahnsteiner, 2000; Aegerter and Jalabert, 2004). Thus, over-ripening is one of the limiting factors in successful artificial spawning. This issue is particularly important among salmonids, because relative to other cultivated fish they have a limited number of eggs and their ovulation is spontaneous and occurs without administration of hormones. Moreover, predicting egg quality decrease from post-ovulatory aging time in salmonids is difficult because simple and reliable tests are lacking. Therefore, a study of the 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 (Lahnsteiner, 2000).

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 (Springate *et al*., 1984; Gaudemar and Beall, 1998; Azuma *et al*., 2003; Bonnet *et al*., 2003; Mohagheghi *et al*., 2008). Also, some information about the biochemical changes that occur in the egg and ovarian fluid during the over-ripening period is available. Craik and Harvey (1984) reported that post-ovulatory aging time 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 among the studied biochemical parameters of the rainbow trout egg and ovarian fluid, such as esterified and non-esterified fatty acids, protein, acid phosphatase and aspartate aminotransferase, only biochemical parameters and the pH of the ovarian fluid are suitable parameters for predicting egg quality. 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 a salmonid indigenous to Iranian waters and its population in Caspian Sea is decreasing strikingly. In 1999 the IUCN registered this fish as a species in danger of extinction, so the Iranian Fisheries Organization has been conducting artificial reproduction of the brood stocks that enter rivers south of the lake for spawning. This organization also has been growing fry to the smolt level and releasing them (Mojazi *et al*., 2005). Although, the over-ripening of egg has been studied in rainbow trout, because of the importance of Caspian brown trout especially from restocking aim, we investigated the effect of post-ovulatory oocyte aging on egg developmental success and the biochemical changes that occur in the egg and ovarian fluid after ovulation. In particular, t he aim of this study was answer to the following questions: How does post-ovulation oocyte aging (taking into account temperature) affect egg quality and when do the limits of the threshold of egg quality decrease change? It can determine the best time interval for examine of females for ovulation. How do the selected biochemical parameters of the egg and ovarian fluid change during over-ripening and are these changes related to egg quality decrease due to over-ripening? These parameters can be used as egg quality biomarkers. Factors related to the metabolism of carbohydrates (e.g., glucose), lipids (e.g., cholesterol and triglycerides) and total protein were studied because they provide information about the energy resources of eggs (Lahnsteiner *et al*., 1999); calcium and iron were studied because they are important ions for metabolic activities of the embryo and play roles in skeleton and blood construction (Evans and Claiborne, 2005) and aspartate aminotransferase activity was studied because this cytoplasmic enzyme is an indicator of coherence of the plasma membrane (Lahnsteiner *et al*., 1999; Lahnsteiner, 2000). Moreover, these factors were studied in ovarian fluid as the medium maintaining ovulated eggs and any change in egg can affect its characteristics, particularly its pH (Lahnsteiner, 2000).

MATERIALS AND METHODS

Fish

In this study, we used 4-year-old cultured brood stocks of Caspian brown trout in their first spawning season in December, 2 007. The fish were held in 5 m^3 outdoor ponds at a flow rate of 0.5 L sec^{-1} at the Kelardasht cold-water Fish Reproduction and Culturing Center in Mazandaran, Iran, under a natural photoperiod. The fish density was 3 kg m^{-3}. The water in the ponds was a mixture of water from the nearby river and spring and its temperature throughout the experiment was 7±0.6°C. The fish were fed a commercial diet (46% protein) until satiation once per day, except for a starvation period 1 month before the expected time of ovulation and spermiation.

Experimental Plan and Sampling

To reduce the stress created by examination, the brood stocks were anaesthetized using 100 ppm MS222. Based on the examination, females that had already ovulated were extracted from the population and 62 of non-ovulated females were selected for the experiment because they were expected to ovulate soon based on their appearance (abdominal and anal form, 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±2.66 cm, body weight: 452.77±69.68 g). A partial volume (25 g) of eggs of these females, individually identified by color tags, were stripped and fertilized with mixed fresh milt repeatedly every 10 for 40 days. The sperm used for fertilization was taken from 8 males and was mixed slowly in equal proportions and maintained cold until used. Spermatozoa density was 248.55×10^6 cell mL^{-1}. Indeed to study the biochemical makeup of the eggs and ovarian fluid, 5 g of eggs and 5 mL 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 in Science and Research Branch of Azad university laboratory. An additional 2.5 mL of ovarian fluid were collected to determine pH.

Fertilization Assays and Evaluation of Success of Egg Development

From each female, 25 g egg (500.7±136.22 eggs) were obtained and gently mixed with 1 mL sperm in a plastic container. Afterwards, 10 mL of water from the hatchery were added to it to stimulate the sperm movement and the solution was gently mixed for 2 min so that fertilization could take place. 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, respectively, in comparison with the total number of fertilized eggs were obtained. In addition, the number of larval abnormalities relative to the number of alevins was assessed at each hatching stage.

Determination of Metabolites and Enzyme of Egg and Ovarian Fluid

From each female, 5 g egg (100±27.38 eggs) were homogenized completely using a manual glass homogenizer for 10 min (Lahnsteiner *et al*., 2001). Sorensen's phosphate buffer (pH 7.38) was used as dilution buffer (McPheron and Pincus, 2007). The sample then was centrifuged for 10 min at 4000×g and the supernatant phase was used for biochemical analysis (Aegerter and Jalabert, 2004). The photometric methods were used to determine biochemical factors of the egg and ovarian fluid (Lahnsteiner, 2000). Calcium was measured following the Arsenazo method (Bauer, 1981), iron with the Ferrozine method (Burtis *et al*., 1996), aspartate aminotransferase enzyme activity with the IFCC method (Bergmeyer *et al*., 1986), protein with the Biuret method (Tietz, 1986) cholesterol with the CHOD-PAP method (Rifai *et al*., 1997), triglycerides with the GPO method and glucose with the glucose oxidase method (Burtis *et al*., 1996).

Collection and Determination of Ovarian Fluid pH

At the time of stripping, 2.5 mL 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 x g to separate the blood cells. Afterwards, the pH of the supernatant was measured using a micro electrode (Aegerter and Jalabert, 2004).

Statistical Analysis

In this study, we used completely randomized design for brood stocks selection. The concentration of metabolites and enzyme activity of ovarian fluid were expressed as units per volume of ovarian fluid. For the eggs, the concentrations were expressed in any egg and every mg of egg (Lahnsteiner, 2000). In order to compare the means of the obtained data, we used a one-way Analysis of Variance (ANOVA) and homogeneity of variances was investigated 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, we used the scatterplots. Finally, to examine the relationship between egg and ovarian fluid parameters and egg viability, we used multiple regression (Lahnsteiner *et al*., 1999; Lahnsteiner, 2000). A significance level of 0.05 was used for all analyses.

RESULTS

Effects of Post-Ovulation Oocyte Aging on Egg Viability

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 ($p>0.05$), but after that it decreased significantly. Indeed, 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 days post ovulation (DPO) ($p>0.05$). Because of the total annihilation of all eggs for nine of the 10 females in the fourth treatment, it was not possible to measure their larval abnormality rate (Table 1).

Effects of Post-Ovulation Oocyte Aging on Ovarian Fluid Parameters

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

Effects of Post-Ovulation Oocyte Aging on Egg Parameters

The biochemical parameters of eggs differed among the sampling periods. The concentration of glucose, cholesterol, calcium and iron showed no significant changes ($p>0.05$). 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, the aspartate aminotransferase activity both per egg and per mg of egg increased over time (Table 3).

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

	Time of post-ovulatory aging (days post-ovulation)			
Parameter	0-10	20-Oct	20-30	30-40
Eyed eggs (%)	90.65±6.28^{a}	69.80±10.98^{a}	4.54±4.32^{b}	0.67±1.34^{b}
Hatched alevins (%)	86.33±6.82^{a}	49.88±11.87^{b}	2.92±2.87^{c}	0.49±0.98^{c}
Deformed alevins (%)	1.19±1.2^{a}	2.40±1.92^{a}	4.08±1.02^{a}	-

Values with the same letter(s) 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

	Time of post-ovulatory aging (days post-ovulation)			
Parameter	0-10	20-Oct	20-30	30-40
Glucose (mg 100 mL^{-1})	38.07±4.90^{a}	62.00±6.05^{b}	66.33±12.01^{b}	72.67±8.50^{b}
Cholesterol (mg 100 mL^{-1})	11.13±3.20^{a}	16.75±2.75^{a}	31.67±4.04^{a,b}	46.00±21.28^{b}
Triglycerides (mg 100 mL^{-1})	9.90±0.66^{a}	20.00±6.98^{a}	26.30±13.75^{a}	29.33±25.69^{a}
Protein (mg 100 mL^{-1})	223.30±45.09^{a}	277.50±55.60^{a}	520.00±85.44^{a,b}	650.00±274.04^{b}
Calcium (mg 100 mL^{-1})	5.30±0.62^{a}	9.32±0.60^{b}	9.83±1.10^{b}	10.63±0.98^{b}
Iron (mg 100 mL^{-1})	64.33±25.00^{a}	502.30±303.05^{a,b}	708.70±205.01^{a,b}	971.70±413.89^{b}
Aspartate aminotransferase (IU L^{-1})	10.67±5.13^{a}	76.00±19.76^{a,b}	92.00±33.00^{b}	166.70±42.12^{c}
pH	8.32±0.12^{a}	8.22±0.44^{a}	7.88±0.47^{b}	7.72±0.13^{b}

Values with the same letter(s) 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

	Time of post–ovulatory aging (days post–ovulation)			
Parameter	0-10	20-Oct	20-30	30-40
Glucose (μg egg^{-1})	36.47±4.32^{a}	18.15±5.27^{a}	18.81±8.00^{a}	31.37±15.28^{a}
(μg mg^{-1} egg)	(0.61±0.18)^{a}	(0.34±0.99)^{a}	(0.31±0.17)^{a}	(0.66±0.29)^{a}
Cholesterol (μg egg^{-1})	301.70±53.85^{a}	279.58±35.72^{a}	297.37±85.63^{a}	244.60±111.44^{a}
(μg mg^{-1} egg)	(5.69±1.23)-^{a}	(5.28±0.67)^{a}	(4.75±1.12)^{a}	(4.9±1.68)^{a}
Triglycerides (μg egg^{-1})	755.55±48.58^{a}	646.60±41.51^{a,b}	728.20±91.53^{a}	545.60±88.19^{b}
(μg mg^{-1} egg)	(14.20±1.46)^{a}	(12.2±0.78)^{a}	(11.67±0.46)^{a}	(11.40±2.22)^{a}
Protein (μg egg^{-1})	17080±508.62^{a}	13581±952.40^{a,b}	17094±523.86^{a}	11541±2576.63^{b}
(μg mg^{-1} egg)	(320.83±22.33)^{a}	(256.25±17.97)^{a}	(275.6±21.13)^{a}	(241.50±59.36)^{a}
Calcium (μg egg^{-1})	58.19±18.88^{a}	64.66±6.24^{a}	76.34±12.94^{a}	56.46±4.23^{a}
(μg mg^{-1} egg)	(1.1±0.36)^{a}	(1.22±0.12)^{a}	(1.22±0.10)^{a}	(1.19±0.26)^{a}
Iron (μg egg^{-1})	1493.4±491.13^{a}	1094.5±208.5^{a}	1064.1±71.64^{a}	891.70±163.04^{a}
(μg mg^{-1} 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^{-1})	0.011±0.003^{a}	0.014±0.010^{a}	0.042±0.030^{a,b}	0.063±0.018^{b}
(IU mg^{-1} egg)	(0.0002±0.0005)^{a}	(0.0003±0.0002)^{a}	(0.0007±0.0005)^{a,b}	(0.0013±0.0005)^{b}

Values with the same letter(s) superscript are not significantly different (i.e., p>0.05)

Table 4: Regression models for the prediction of the egg eyeing rate using ovarian fluid and egg composition parameters

Parameter	R	Regression model	R^2	F-value
Ovariaon fluid parameters				
Cholesterol	0.750	y = -1.62x+86.73	0.562	8.99
Protein	0.904	y = -0.52x+0.0003x^{2}+182.35	0.818	13.487
Calcium	0.674	y = -14.76x+180.11	0.454	5.821
Aspartate aminotransferase	0.756	y = -0.49x+91.77	0.571	9.325
pH	0.975	y = -2813.48x+185.07x^{2}+10691.25	0.95	48.018
Egg parameters				
Cholesterol	0.861	y = -9.80x+0.04x^{2}-5.79x^{3}+678.75	0.742	6.694
Iron	0.659	y = 0.11x-82.98	0.435	6.925
	(0.885)	(y = 26.35x+13.44x^{2}-0.01x^{3}-339.95)	(0.783)	(14.424)
Aspartate aminotransferase	0.681	y = -0.95x+68.32	0.464	7.792
	(0.652)	(y = -41.97x+64.13)	(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^{-1} and data in parenthesis are in units mg^{-1} egg. N = 40. Statistical significance was set at p<0.05 for all regression models

The Relationship Between Egg and Ovarian Fluid Parameters and Egg Viability

Multiple regression analysis showed that eyeing rate was correlated with the concentration of cholesterol, protein, calcium and with the activity of the aspartate aminotransferase enzyme and pH of ovarian fluid. Among the biochemical parameters of egg that were studied, eyeing rate was correlated with the concentration of cholesterol, iron and with aspartate aminotransferase activity (Table 4). For ovarian fluid, hatching rate was correlated with the concentration of glucose, cholesterol, protein, calcium and with aspartate aminotransferase activity and pH. Furthermore, hatching rate was correlated with the concentration of cholesterol, iron and with aspartate aminotransferase activity in egg

(Table 5). Indeed the larval anomaly rate was correlated with the concentration of cholesterol, triglycerides, protein, iron and aspartate aminotransferase activity and pH in ovarian fluid. For egg, larval anomaly rate was correlated with the concentration of cholesterol, triglycerides and with aspartate aminotransferase activity (Table 6).

The Relationship Between the Studied Variables in Egg and Ovarian Fluid

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.

Table 5: Regression models for the prediction of the egg hatching rate using ovarian fluid and egg composition parameters

Parameter	R	Regression model	R^2	F-value
Ovariaon fluid parameters				
Glucose	0.793	$y = -2.14x+168.54$	0.629	11.844
Cholesterol	0.926	$y = -5.95x+0.59x^2+134.12$	0.858	18.133
Protein	0.901	$y = -0.44x+0.0002x^2+150.97$	0.812	12.960
Calcium	0.779	$y = -14.09x+164.44$	0.606	10.784
Aspartate aminotransferase	0.831	$y = -0.82x+0.001x^2+91.50$	0.69	6.705
pH	0.998	$y = -3269.49x+212.29x2+12586.20$	0.997	90.278
Egg parameters				
Cholesterol	0.882	$y = -7.58x+0.034x^2-4.5x^3+523.80$	0.676	4.872
Iron	0.676	$y = 0.09x-72.11$	0.457	7.588
	(0.838)	$(y = 20.24x+18.28x^2-0.011x^3-262.14)$	(0.704)	(9.5)
Aspartate aminotransferase	0.667	$y = -0.76x+53.83$	0.445	7.215
	(0.636)	$(y = -33.54x+50.36)$	(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^{-1} and data in parenthesis are in units mg^{-1} egg. N= 40. Statistical significance was set at $p<0.05$ for all regression models

Table 6: Regression models for the prediction of the larval anomaly rate using ovarian fluid and egg composition parameters

Parameter	R	Regression model	R^2	F-value
Ovariaon fluid parameters				
Cholesterol	0.936	$y = 0.19x-1.03$	0.877	35.567
Triglycerides	0.961	$y = 0.24x-2.2$	0.923	59.997
Protein	0.914	$y = 0.01x-1.84$	0.836	25.456
Iron	0.957	$y = 0.03x-6.48x^2+4.09x3-0.16$	0.916	11.023
Aspartate aminotransferase	0.922	$y = -0.02x+0.0003x^2+2.14$	0.851	11.403
pH	0.927	$y = -13.76x+114.79$	0.86	24.538
Egg parameters				
Cholesterol	(0.883)	$(y = -19.55x+1.93x^2+51.04)$	(0.779)	(8.851)
Triglycerides	(0.707)	$(y = -2.76x+37.63)$	(0.5)	(6.013)
	0.952	$y = -2.16x+0.09x^2-0.001x^3+14.16$	(0.907)	13.048
Aspartate aminotransferase	(0.836)	$(y = -30.003x+38.21x^2+6.45)$	(0.698)	(5.801)

In the regression model, the percent of deformed alevins was the dependent variable and analyzed parameters were the independent variables. Egg parameter data without parenthesis are in units egg^{-1} and data in parenthesis are in units mg^{-1} egg. N = 31. Statistical significance was set at $p<0.05$ for all regression models

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 $p<0.05$

DISCUSSION

The results of this study of Caspian brown trout showed that post-ovulation oocyte aging and over-ripening led to severely decreased egg quality. Other studies of other species (Craik and Harvey, 1984; Lahnsteiner, 2000; Aegerter and Jalabert, 2004; Mohagheghi *et al*., 2008) support this finding. Changes in egg quality result in many biochemical changes in the egg and ovarian fluid, some of which can be used to detect egg quality defects associated with oocyte post-ovulatory aging. In this study, stripping was done at 10 day intervals because in Mohagheghi *et al*. (2008), study on rainbow trout, at approximately similar condition, egg quality decrease arising from post-ovulatory aging time occurred in 28-35 DPO. Another reason was the sensitivity of Caspian brown trout females and possibility of death of them duo to successive handing. 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 and Jalabert, 2004; Mohagheghi *et al*., 2008). 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 8 °C eggs can be maintained in the coelomic cavity of females for at least 2 weeks (Mohagheghi *et al*., 2008) and at 10-12°C for at least 1 week without any decrease in egg quality (Springate *et al*., 1984; Aegerter and Jalabert, 2004). In Caspian brown trout kept at 7°C, eyeing rate up to 20 DPO and hatching rate up to 10 DPO were high, but then they decreased severely. Thus the best time to take Caspian brown trout egg after ovulation was up to 10 DPO. Also, this result showed that the examination of females for ovulation can be done every 10 days. The larval anomaly rate up to 30 DPO showed no significant change, which supports previous reports about the consistency of the larval anomaly rate in rainbow trout up to 14 DPO (Azuma *et al*., 2003). In contrast, Aegerter and Jalabert's study (2004) of rainbow trout showed that the percentages of morphological anomalies significantly increased as early as 7 DPO and remained constant between 7 and 14 DPO. 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 about the changes of glucose, cholesterol, triglycerides, calcium and iron amount in ovarian fluid during over-ripening which next studies can compare with it. These findings agree to some extent with Lansteiner's (2000) findings in rainbow trout, which showed that post-ovulation oocyte aging 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 17°C throughout the over-ripening period remained constant; only at 12°C did density of ovarian fluid increase and pH decrease. The results of this study of Caspian brown trout also verified Fauvel *et al*. (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 egg degeneration. Lahnsteiner *et al*. (1997) studies on bleak (*Alburnus alburnus*) illustrate the role of the ovarian epithelial layer in discharging glucose, proteins, acid phosphatase, protease and β-D glucuronidase into ovarian fluid, which creates an ionic slope in the fluid. Ovarian fluid in salmonids includes Na^+, K^+ and Ca^{+2} ions as well as glucose, fructose, cholesterol, phospholipids, proteins and free amino acids (Lahnsteiner *et al*., 1995). Lahnsteiner (2000) reported that of the 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 present study, the increase in protein and aspartate aminotransferase activity in ovarian fluid during the over-ripening period led to a severe decrease of pH. There also was 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 aminotransferase activity and pH in rainbow trout (Lahnsteiner, 2000) also have been reported.

Among the investigated factors in ovarian fluid of Caspian brown trout, the concentration of glucose, cholesterol, triglycerides, protein, iron and calcium, aspartate aminotransferase activity and pH were significantly correlated with eyeing, hatching and larval anomaly rate which can be used as egg quality biomarkers. The relationships between pH, protein and aspartate aminotransferase activity of ovarian fluid with eyeing rate of rainbow trout (Lahnsteiner, 2000) and pH of ovarian fluid and fertilization rate in turbot (Fauvel *et al*., 1993) were previously reported. 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 0.67 and 0.49%, 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 concentration of protein and triglycerides for each egg changed significantly during the over-ripening period. Aspartate aminotransferase activity both in egg and in mg of egg increased, but the other parameters remained constant. 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 concentration of these two factors did not change significantly during the over-ripening process in Caspian brown trout. Although, low sample numbers in Craik and Harvey's (1984) study allowed no general conclusions. 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 but confirm Lansteiner's (2001), report about constancy of glucose amount during over-ripening in *Cyprinus carpio* and *Ctenopharyngodon idella*. Also, it was the first report about triglycerides and cholesterol amount in egg. In salmonids, yolk proteins, which are derived from vitellogenin, compose 80-90% of the dry weight of ovulated eggs (Mommsen and Moon, 2005). In present study, protein composed the highest portion of egg weight and among the lipids triglycerides weighed more than cholesterol. In rainbow trout eggs, triglycerides and phosphatidylcholine are the main lipids, with cholesterol and free fatty acids contributing less (Lahnsteiner, 2000). Moreover, in this study of Caspian brown trout, the eyeing, hatching and abnormality rates were related to cholesterol, triglycerides and iron content and the aspartate aminotransferase activity of the egg which can be used as egg quality biomarkers. 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.

In conclusion, the best time to take Caspian brown trout eggs after ovulation at 7±0.6°C was up to 10 DPO. Egg viability decrease due to over-ripening was related to both ovarian fluid parameters

(e.g., pH, protein, aspartate aminotransferase, glucose, cholesterol, triglycerides, iron, calcium) and egg parameters (e.g., cholesterol, triglycerides, iron, aspartate aminotransferase) which can be used to detect egg quality defects associated with oocyte post-ovulatory aging. However, due to abundant biochemical changes that occur in the egg, ovarian fluid would be more appropriate for this purpose.

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