

## Effects of cortisol treatment and ovaprim treatment on sex steroid hormones, plasma and oocyte cortisol content and ovulation induction in Caspian kutum, "*Rutilus frisii* (Kamansky, 1901)" broodstocks

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Received: November 2016

Accepted: September 2017

### Abstract

Caspian kutum fish (*Rutilus frisii*) wild broodstocks were caught by gillnet during their upstream migration and subjected to short-time confinement stress and their physiological responses have been examined. Thirty fish (average weight of 1217.6 g) were divided into three experimental groups CO (control), OV (ovaprim) and CO+OV (cortisol+ovaprim) and placed into rearing tanks (10 fish tank<sup>-1</sup>; 2 m<sup>3</sup> each) and kept for 10 days. Fish in CO+OV group received a single cortisol injection (20 µg kg<sup>-1</sup> B.W.) before being transferred in tank. After 10 days of confinement, all fish in OV and CO+OV groups were injected by ovaprim (20 µg kg<sup>-1</sup> B.W.) to induce maturation and ovulation. Plasma cortisol in CO+OV fish have showed a gradual decrease during confinement and reached to the lowest level after maturation (ovulation) while the highest values for glucose has been found in OV group ( $p < 0.05$ ). Testosterone and 17β-estradiol levels declined significantly in all experimental groups following cortisol treatment and maturation, while no significant difference had been found in the content of plasma progesterone among brood-stocks ( $p < 0.05$ ). The concentration of oocyte cortisol in CO+OV was 2-fold higher than that of CO and OV after maturation. These results indicated that cortisol treatment with subsequent ovaprim injection decreased plasma sex steroids and increased oocyte cortisol content in confined broodstocks but had no effect on oocyte histological characteristics.

**Keywords:** Cortisol treatment, Ovaprim, Sex steroids, Oocyte, Broodstocks, *Rutilus frisii*

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## Introduction

Fish brood-stocks in hatcheries are typically encountered by stresses resulting from aquaculture practices such as capture, handling, transportation and stocking density. This may influence animal's reproduction success and welfare (Bayunova *et al.*, 2002; Nikoo and Falahatkar, 2012). Greenburg and Wingfield (1987) and Schreck (2009) have been stated that both corticosteroids and sex steroids can be affected by stressors in teleost fishes. In animals subjected to stressful conditions, animal's body immediately responds the stress recognized as primary and secondary response. During stress, altered conditions are percept by the animal central nervous system, leading to release of stress hormones, cortisol and catecholamine into the blood circulation by endocrine system. This event is regarded as the primary stress responses (Martinez-Porchas *et al.*, 2009). Primary stress response itself triggers the sequential secondary stress response in teleost such as the increase in plasma glucose, lactate and hematocrit as well as the decrease in plasma ions (Barton, 2002; Nikoo *et al.*, 2010). Thus, changes in blood biochemical parameters have been determined in several aquatics faced stressful conditions to monitor their physiological status (Barcellos *et al.*, 2004; Patterson *et al.*, 2004; Gbore *et al.*, 2006; Caipang *et al.*, 2009). In general, two cortisol hormones and glucose have been considered as reliable indicators of stress responses in aquaculture species. Several studies

have been demonstrated that concentration of plasma cortisol and glucose are increased in animals subjected to stressful conditions (Barton, 2002; Martinez-Porchas *et al.*, 2009).

Stressors affect reproductive hormones in fishes, where the stressed fish have elevated circulatory adrenocorticotrophic hormone, cortisol and decreased reproductive hormones such as testosterone and 17 $\beta$ -estradiol (Schreck, 2009). Information regarding the effects of acute stresses on subsequent gametes quality of fish brood-stocks is scarce and generally depends on the severity and duration of the stress (Bobe and Lobbé, 2010). Several studies have been demonstrated that in matured fish, acute stressful conditions lowered the content of plasma sex steroids. These kind of brood-stocks, produced fewer oocytes when stripped and the quality of eggs such as their fertilization rate, further embryonic cleavages and hatching success were negatively reduced (Soso *et al.*, 2008; Yasemi and Nikoo, 2010).

For restocking larvae to the natural environment, every year up to 200 million of 1 g juveniles released into southern Caspian Sea (Abdoli and Naderi, 2009; Nikoo and Falahatkar, 2012). Hundreds of kutum fish brood-stocks have been captured from the coastal waters and kept for a short period in confinement to reach their final maturity and oocyte ovulation. It is necessary to assess the response of brood-stocks to short time confinement. Understanding the stress responses and managing the stress is necessary for

optimal performance in brood-stocks holding (Carneiro and Urbinati, 2002). Assessments of physiological responses of kutum fish brood-stocks to short time confinement stress will be useful in providing basic information for the development of restocking program.

One of the new methods for controlling and improving fish propagation is using hormonal treatment. In Cyprinidae, generally hypophyse hormone have been used and in kutum female brood-stocks as well, especially by using Ovaprim hormone (sGnRH + Domperidon) (Sodagar *et al.*, 2015).

The objective of this study was to assess physiological responses of kutum female's brood-stocks in response to cortisol treatment with subsequent maturation (ovulation) induction by using ovaprim injection in confined brood-stocks with the aim to provide a better understanding of the brood-stock's responses to acute stresses and which may lead to improvements in efficacy of artificial propagation.

## Materials and methods

### *Brood-stocks and hormonal treatments*

Fish were caught from the coastal waters of Anzali city (Guilan province, Iran) by gillnet during their upstream migration. Thirty females which hold oocytes attached tightly to the body cavity have been caught and transported to Siahkal hatchery (Siahkal, Guilan province, Iran). Fish average weight was 1217.6 g. Brood-stocks were adapted to experimental conditions for 3 days. After this adaptation period, brood-stocks were divided into 3

experimental groups as follow: Co (control; injected by saline solution only after adaptation), OV (injected with ovaprim at  $20 \mu \text{ kg}^{-1}$  B.W. after 10 days rearing in tanks), and CO+OV (injected with cortisol at  $20 \mu \text{g kg}^{-1}$  B.W. after adaptation and ovaprim at  $20 \mu \text{g kg}^{-1}$  B.W. after 10 days rearing). Each experimental group was consisted of 10 female brood-stocks. Each group was held in one 2-m<sup>3</sup> fiberglass tank. During the experiment, water temperature was 14-18 °C; pH was approximately 7.6, dissolved oxygen was above  $6.0 \text{ mg L}^{-1}$  and photoperiod controlled to 12 h light and 12 h dark.

### *Blood Sampling*

Blood samplings did at three different steps, 30 seconds at each step with a non-heparinized 2-ml plastic syringe from the caudal vein of each fish. Then 3 days after adaptation, after 10 days raring in tanks and finally after maturation. Samples centrifuged (model 1-15PK, Sigma Co., USA) at 1600 g for 10 min to obtain serum which was stored at -20 °C until analysis (Pottinger and Carrick, 2001; Mingist *et al.*, 2007). Serum cortisol level measured using the radioimmunoassay (RIA) method according to Rottlant *et al.* (2001) and expressed as  $\text{ng ml}^{-1}$ . Serum glucose levels were determined by colorimetric method using a commercial kit (Greiner, Bahlingen, Germany) according to Bayunova *et al.* (2002). lactate measured by enzymatic colorimetric method according to Barton *et al.* (2005) and expressed as  $\text{mg dl}^{-1}$ . Progesterone, testosterone and  $17\beta$ -estradiol contents ( $\text{ng ml}^{-1}$ )

measured by RIA method with the use of Immunotech kit (France). All samples analyzed in duplicate.

#### *Histological analysis*

For histological analysis, oocytes kept in Bouin's solution. Bouin's-fixed samples were sectioned and routinely processed for histology including dehydration in graded ethanol and equilibration in xylene (Hinton., 1990). Previously fixed eggs were paraffin embedded and cut about 5-6 $\mu$ m thick by rotary microtome (Leica RM 2125RT, China). The sections were mounted on glass slides and stained with haematoxylin and eosin (HE). Two glass slides were prepared from each sample. All slides read under light microscope (Olympus BX51, Japan) by multiple magnifications (32 $\times$ , 80 $\times$  and 160 $\times$ ). Images are captured by using on-board camera (Zeiss, Cyber-Shot, Japan).

#### *Measurement of egg cortisol content*

Extraction of eggs cortisol performed according to the method of Hiroi *et al.* (1997). Approximately 200-300 mg of eggs was stored in 2 ml micro-centrifuge tubes at  $-70^{\circ}\text{C}$  until analysis. Samples homogenized in five volume of cold 10 m phosphatebuffer (pH 7.2) by using an Ultra Turax homogenizer (IKA T18 digital Ultra Turax, Staufen, Germany). 3 ml of diethyl ether added to 300  $\mu$ l of the homogenized sample, and shaken vigorously for 2 min. After freezing at  $-70^{\circ}\text{C}$ , the top layer containing diethyl ether was collected by decantation and dried at room

temperature. 300  $\mu$ l of carbon tetrachloride added to the dry residue, and vortexed for 4 min. to the solution, 300  $\mu$ l of phosphatebuffer containing 0.1% gelatin was added and mixed for 10 min. After centrifuging at 1600 g (10 min), the defatted aqueous phase containing cortisol was stored at  $-70^{\circ}\text{C}$ . Cortisol levels measured by ELISA.

#### *Statistical analysis*

Data normality was determined using Kolmogorov-Smirnov and statistical differences were analyzed using the two-way ANOVA. When a significant difference was observed ( $p < 0.05$ ), Tukey's post hoc test was used to compare the means. All analyses were performed by SPSS 16.0 software package for Windows (SPSS, 2016). The levels of significance were taken as  $p < 0.05$ . All values are presented as Mean  $\pm$  SD.

#### **Results**

Initial mean level (AA) of plasma cortisol was 979.6 ng ml $^{-1}$ . After 10 days rearing, cortisol contents in CO, OV and CO + OV groups were 748.8, 1055.8 and 742.8 ng ml $^{-1}$ . After maturation, the lowest level of plasma cortisol was found in CO + OV ( $p < 0.05$ ). Comparison of mean cortisol levels after 10 days rearing in tanks and after maturation in three groups indicated that only in the CO group mean cortisol content was significantly different between the two stages ( $p < 0.05$ ) while no differences were observed for OV and CO + OV groups ( $p > 0.05$ ) (Fig.1). The initial amount (AA) of glucose was 144.8 mg dl $^{-1}$ . The

mean level of glucose in OV showed a significantly increase after 10 days rearing in tanks and after maturation when compared to other groups (CO and CO + OV) ( $p < 0.05$ ) whereas it did not show considerable elevation after maturation in CO and CO + OV. After 10 days of rearing brood-stocks in tanks, the contents of plasma lactate in the CO, OV and CO + OV were 74.2,

64.4 and 52.4 mg dl<sup>-1</sup> (Fig.1). Lactate levels increased slightly in OV and CO following maturation and the increase was significant ( $p < 0.05$ ). In contrast, its level in the CO group showed a significant reduction after maturation ( $p < 0.05$ ) (Fig.2).

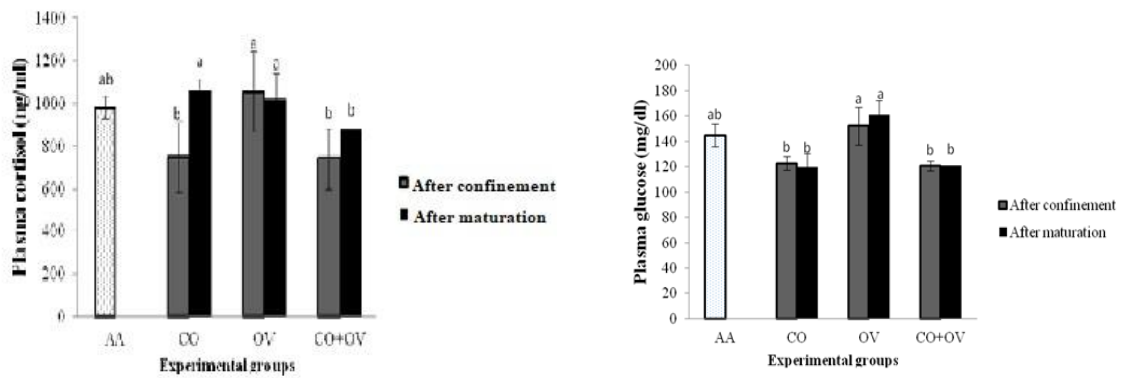


Figure 1: Changes in plasma cortisol (ng ml<sup>-1</sup>) and glucose (mg dl<sup>-1</sup>) in wild Caspian kutum (*Rutilus frisii*) broodstocks subjected to hormonal treatments and confinement. Values are Mean ± SD from ten fish with n=2 per fish. Bars accompanied by different letters are significantly different ( $p < 0.05$ ) from others within each treatment. AA: after adaptation; CO: control; OV: ovaprim; CO+OV: cortisol+ovaprim.

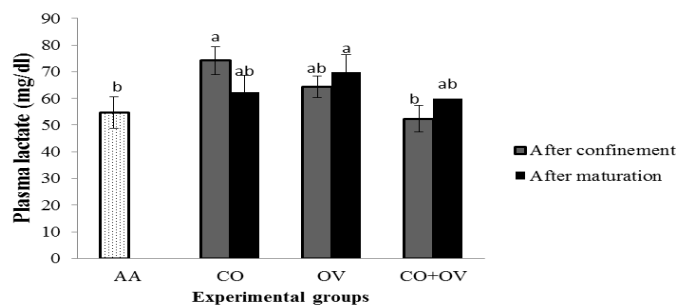


Figure 2: Changes in plasma lactate (mg dl<sup>-1</sup>) in wild Caspian kutum (*Rutilus frisii*) broodstocks subjected to hormonal treatments and confinement. Values are Mean±SD from ten fish with n=2 per fish. Bars accompanied by different letters are significantly different ( $p < 0.05$ ) from others with in each treatment. AA: after adaptation; CO: control; OV: ovaprim; CO+OV: cortisol+ovaprim.

The changes in plasma sex steroid levels in kutum brood-stocks subjected to hormonal treatment and confinement (Fig.3).

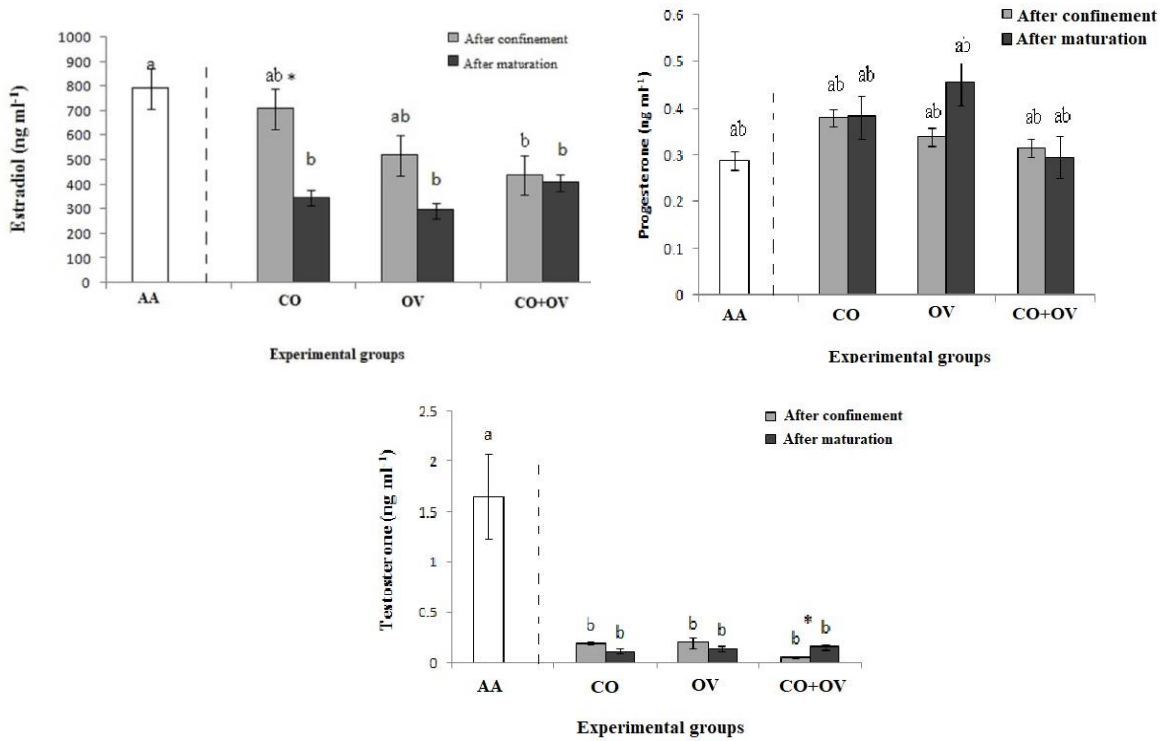


Figure 3: Changes in plasma 17β-estradiol (ng ml<sup>-1</sup>), testosterone (ng ml<sup>-1</sup>) and progesterone (ng/ml) in wild Caspian kutum (*Rutilus frisii*) broodstocks subjected to hormonal treatments and confinement. Values are Mean±SD from ten fish with n = 2 per fish. Bars accompanied by different letters are significantly different (p < 0.05) from others within each treatment. AA: after adaptation; CO: control; OV: ovaprim; CO+OV: cortisol+ovaprim.

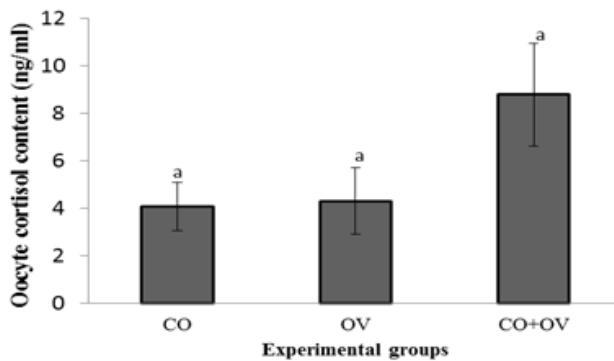
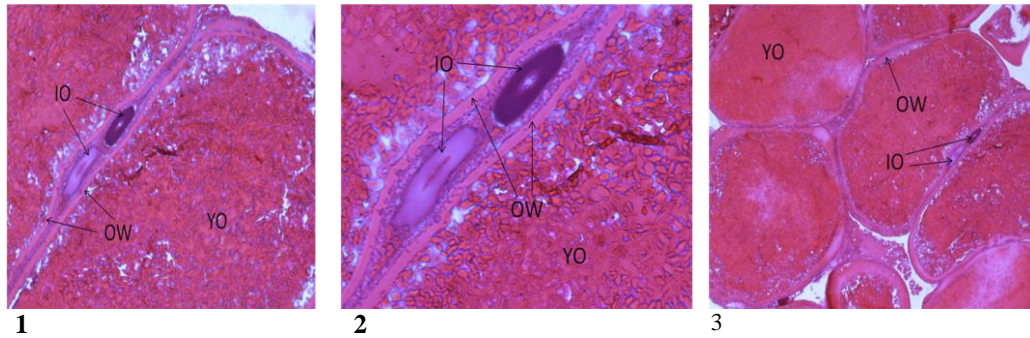


Figure 4: Changes in oocyte cortisol content (ng ml<sup>-1</sup>) in wild Caspian kutum (*Rutilus frisii*) brood stocks subjected to hormonal treatments and confinement. Values are Mean±SD from ten fish with n=2 per fish. Bars accompanied by different letters are significantly different (p < 0.05) from others within each treatment. AA: after adaptation; CO: control; OV: ovaprim; CO+OV: cortisol+ovaprim.

The mean initial level (AA) of  $17\beta$ -estradiol was  $793.4 \text{ ng ml}^{-1}$ .  $17\beta$ -estradiol content in all groups was reduced compared to the initial content after short time rearing in tanks; however, this reduction was significant only in CO+OV group ( $485 \text{ ng ml}^{-1}$ ).

The content of plasma  $17\beta$ -estradiol showed a considerable decline after maturation in the CO and OV ( $p < 0.05$ ) but this decrease was not significant in CO+OV group ( $p > 0.05$ ).



**Figure 5: Histomorphological characteristics of Caspian kutum (*Rutilus frisii*) brood stocks oocytes. YO: egg yolk; OW: oocyte wall; IO: immature oocyte [H&E, X32(1), X80(2), X160(3)], All images are captured under 2.4 optical zoon provided by camera.**

Regarding to the changes in plasma testosterone levels, it was observed that in all groups its levels declined significantly ( $0.07$  to  $0.20 \text{ ng ml}^{-1}$ ) from the mean initial value of  $2.32 \text{ ng ml}^{-1}$  ( $p < 0.05$ ). However, the difference among three experimental groups was not significant ( $p > 0.05$ ). Plasma progesterone level at the beginning of the experiment was  $0.30 \text{ ng ml}^{-1}$  and did not change in response to hormonal treatment and confinement (min:  $0.29$  to max:  $0.45 \text{ ng ml}^{-1}$ ), this differences were not significant ( $p > 0.05$ ).

The content of oocyte cortisol in matured brood-stock was measured. (Fig.4). The content of eggs cortisol in CO + OVgroup ( $8.79 \text{ ng ml}^{-1}$ ) was 2-fold higher than CO ( $4.08 \text{ ng ml}^{-1}$ ) and OV ( $4.30 \text{ ng ml}^{-1}$ ) groups. However, the difference was not statistically different ( $p > 0.05$ ). Histological analysis of oocytes in the experimental groups in response to cortisol treatment with

subsequent ovaprim injection performed (Fig.5). Results indicated the presence of matured oocytes with normal oocyte wall (OW) filled with yolk (YO) in all groups. Furthermore, immature oocytes (IO) were also observed in the ovary of all brood-stocks regardless of the hormonal treatment. No effect of hormonal treatment on oocyte histological characteristics, and microscopic observation did not show considerable differences between oocyte yolks, walls and other parameters, and the number of oocyte per gram had the same condition (between  $257.33$  and  $262.66$ ). So for those parameters no significant differences have been observed ( $p > 0.05$ ).

**Discussion**

In this study slight increase in plasma cortisol levels in OV groups after 10 days captivity has been observed but its

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level in the CO and CO + OV groups have been decreased. After maturation, the content of cortisol significantly increased in all groups but the increase was not significant for the other two groups. A similar result has been observed in Coho salmon, *Oncorhynchus kisutch*, subjected to stressors in which plasma cortisol levels increased following captivity while eggs viability and gonadosomatic index has not been affected (Andersen *et al.*, 1991). Stress is an energy-demanding process, and the production of glucose provides energy to cope with that demand (Iwama *et al.*, 2006). Glucose levels in kutum brood-stocks were declined in the CO and CO + OV groups after captivity and hormonal treatments when compared to initial value while it showed a significant increase in OV groups. The increase in glucose level in OV groups was confidential with an increase in cortisol levels. It is reported that hyperglycemia after stress results from the release of catecholamines (Andersen *et al.*, 1991; Davis and McEntire, 2006) which is used by the fish to satisfy the increasing demand for energy during environment disturbances (Umminger, 1977). Cortisol and glucose are accepted to be the main stress indicators in aquatics, however due to their high variability, they must be complemented with other stress indicators (e.g. lactate) in order to have a more complete profile about the stress status of aquatic animals (Martinez-Porchas *et al.*, 2009). Lactate levels are generally enhanced following stressful conditions (Grutter and Pankhurst, 2000). In this study, plasma

lactate increased in fish after captivity and hormonal treatments, which was in accordance with other studies that was showed even short time captivity had increasable effect on kutum's plasma lactate (Nikoo *et al.*, 2010).

Cortisol and ovaprim injections in confined brood-stocks had a significant stress effects as evidenced by decreasing in sex steroid  $17\beta$ -estradiol. This result indicated that Caspian kutum fish is sensitive to acute stress resulting from confinement during the breeding season. The lowest content of plasma  $17\beta$ -estradiol has been found in OV group received a single ovaprim injection. In snapper (*Pagrus auratus*), serum testosterone and estradiol levels were declined following confinement stress (Cleary *et al.*, 2007). In catfish (*Rhamdia quelen*) subjected to handling stress, the levels of plasma  $17\beta$ -estradiol was decreased (Soso *et al.*, 2008) and a similar result has been reported for black bream (Haddy and Pankhurst, 2000). In contrast to changes in  $17\beta$ -estradiol, plasma progesterone level did not change in response to hormonal treatment and confinement, which is in agreement with the findings on black bream (Haddy and Pankhurst, 2000) and *Oncorhynchus nerka* (Kubokawa *et al.*, 1999).

In the present study, the number of eggs per gram was not influenced by hormonal treatment and confinement, and stated that gamete quality parameters such as fecundity and number of eggs per gram are set a month prior to freshwater re-entry, thus stress might not affect fecundity at spawning time. Similarly (Bayunova *et*



al. 2002) reported that acute and low severity associated stress did not affect gamete quality of stellate sturgeon (*Acipenser stellatus*) and Russian sturgeon (*Acipenser guldenstaedtii*). Furthermore, histological analysis indicated the presence of matured oocytes along with some immature oocytes in the ovarian tissue of all brood-stocks. Cortisol and ovaprim injections and confinement exerted no effect on histological characteristics of oocytes.

In conclusion, results have been indicated that kutum brood-stocks subjected to cortisol treatment with subsequent ovaprim injection to induce maturation and ovulation typically practiced in hatcheries responded to stress associated with these treatments as evidenced by the decline in sex steroid levels and an increase in oocyte cortisol content. The effects of cortisol treatment with subsequent ovaprim injection in confined wild brood-stocks on artificial breeding success in terms of fertilization rate, cell cleavages, embryonic and fry survival need to be further investigated.

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