Sexual hormones profiles and some immune parameters in the cage-reared female rainbow trout (*Oncorhynchus mykiss*) from the Caspian Sea

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Received: January 2022 Accepted: July 2022

Abstract

The aim of the present study was to investigate the sexual maturation process and changes in some immune parameters of female rainbow trout reared in a cage in the Caspian Sea. All-female fish with an average weight of 122±5 g were prepared from a local farm and released into a cage. Fish were fed daily. Blood samples were collected from 5 fish randomly once a month from January 2015 to May 2016. The levels of 17 β-estradiol, testosterone, progesterone, melatonin, vitellogenin, calcium, lysozyme, and immunoglobulin M (IgM) were measured. Water quality parameters were also measured daily. The results showed that the levels of sex hormones gradually increased over time. Also, an increase in the amount of vitellogenin (from 382.40±55.26 $ng.ml^{-1}$ in January to 686.40±86.34 $ng.ml^{-1}$ in May) and calcium ion (from 12.84±1.42 mg. dl^{-1} in January to 19.04±1.55 mg. dl^{-1} in May) was observed (p<0.01). However, melatonin levels decreased from January (294.20±16.89 ngl^{-1}) to May (175.00±17.54 ng. l^{-1}) (p<0.01). Immune parameters (Lysozyme and IgM) changes were similar and the highest levels were observed in March, which was significantly higher than other months (p<0.01). The findings suggest that female Rainbow trout are under normal sexual maturity process in the Caspian Sea like freshwater resources.

Keywords: Female Rainbow trout, Cage culture, Immune parameters, Sexual Hormones, Melatonin, Vitellogenin

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[DOI: 10.61186/injbir.1.1.51]

Introduction

Rainbow trout (*Oncorhynchus mykiss*) is one of the valuable fish species belonging to the Salmonidae, which is rapidly spread all over the world (Farahani *et al*., 2015). Rainbow trout is mainly cultured in freshwater and ponds. However, policies of the Fisheries organization of Iran in order to increase fish production as well as freshwater productivity have led to the use of brackish water sources such as the Caspian Sea for fish production. Therefore, Rainbow trout culture in the cage in the Caspian Sea was on the agenda of the government and investors as an appropriate option for Rainbow trout production. Fish culture in the cage is one of the most common methods in the world for optimal use of water resources in order to fish farming (Kumar and Karnatak, 2014). Cage culture is an aquaculture production system where fish are held in floating net pens located in water resources such as the sea, lake, water behind a dam, etc. In fact, fish are in an enclosed cage or basket and water passes freely between the fish and the pond permitting water exchange and waste removal into the surrounding water (Soltan, 2016). Currently, more than 62 countries are involved in cage aquaculture. It is predicted that marine fish production will reach about 10 million tons by 2025. Malaysia, Thailand, Vietnam, China, Norway, and Iceland are the top countries in fish cage culture (Halwart *et al.,* 2007). Cage culture in Iran is a quick-return investment. It is the main issue in terms of optimal use of resources and fish production and also in terms of employment and sustainable development. Therefore, aquaculture development in the Sea is a necessity in Iran (Azari, 2017). Rainbow trout culture in the cage in the Caspian Sea has unknown aspects that are important in evaluating the fish reproductive process, such as the possibility of sexual maturation in the Sea (cages), time of sexual maturation, changes in blood parameters such as sex hormonal levels (17-β estradiol, testosterone, progesterone), concentrations of vitellogenin, melatonin, calcium ion, and immune parameters. However, a few studies have been conducted to examine the sexual maturation of rainbow trout in marine and brackish waters in Iran and in the world (Falahati Marvast *et al.,* 2002; Mohammadi *et al.,* 2009; Golshahi *et al.,* 2012; Rehulka and Adamec, 2004). Yet, the sexual maturation process and some blood changes of rainbow trout reared in the cage in the Caspian Sea have not been studied till now. The economic aspect of aquaculture is one of the most important aspects of industrial sustainability and fish growth in different culture systems is the main factor of production efficiency, so somatic growth has always been a higher priority for aquaculture than sex maturation. Knowing the process of fish reproduction and maturity in the cage can be effective in planning and policies in order to fish production. Therefore, the present study was conducted to investigate sexual

[DOI: 10.61186/injbir.1.1.51]

maturation process of rainbow trout in the Caspian Sea considering hormonal profiles of sex hormones as well as immune parameters.

Material and methods

Animal and sampling

All-female fish with an average weight of 122±5 g were prepared from a local farm and released into a cage (located in Nowshahr, Mazandaran province) in December. Fish density was 40 $Kg.m^{-2}$ which was kept in a cage with a capacity of 25 tons. The cage was located at depth of 20 m, with a distance of 3.5 km from the coast of the Caspian Sea, at longitude "11 '46 51 and latitude "91 '66 36. Fish were sampled monthly and randomly for examinations. Sampling was done from January to May. Feeding was done daily by commercial food. The amount and size of the food were determined according to the water temperature and fish biomass (Falahati Marvast *et al.*, 2002). Water quality parameters such as temperature, dissolved oxygen, salinity, and pH were also measured daily using a thermometer, oxygen meter, salinity meter, and pH meter, respectively (Table 2).

Plasma collection

Blood samples were collected from 5 fish randomly once a month. To facilitate blood sampling as well as bioethics, fish was first anesthetized with MS222 (1 $g.L^{-1}$) and then blood samples were collected using a syringe from the caudal vein and immediately transferred to the laboratory in containers containing ice powder. Blood plasma was isolated by centrifuge at 3,000 rpm for 15 minutes, and kept at -20°C until biochemical analysis (Heidari *et al.*, 2010).

Hormonal and immune analyses

Measured parameters were including 17 β-estradiol, testosterone, progesterone, melatonin, vitellogenin, calcium, lysozyme, and IgM. 17 β-estradiol, testosterone, and progesterone levels were measured using hormonal kits and Gama counter by Immunoassay method (Good *et al.*, 2014). Vitlogenin level was measured using the ELISA kit and through Biotin double antibody sandwich technology. calcium ion was measured by a colorimetric method (Methyl thymol Blue) and two-solution method using auto analyzer at 37°C (Dehghanzadeh *et al.*, 2014); immunoglobulin M and melatonin levels were measured using ELISA kit and double_ antibody sandwich technique and lysozyme enzyme activity was measured by the turbid metric measurement method (Ellis, 1990).

Statistical analysis

Data were analyzed using SPSS 19 software. All the data were examined for normality using the Shapiro-Wilk test. Statistical analysis for parametric data was subjected to one-way ANOVA with Tukey's post-hoc test with a *p-value*<0.01.

[DOI: 10.61186/injbir.1.1.51]

Results

Sex hormone levels are shown in Figures 1 to 3. The concentrations of immune parameters, vitellogenin, melatonin, and calcium ion are presented in Table 1. An increasing trend was observed in sex hormones (17 β-estradiol, testosterone, and progesterone), vitellogenin, and calcium ion from January to May. A reduction trend was observed inleveltonin levels over the months. Immune parameters (Lysozyme and IgM) changes were similar so that, the highest amount was observed in March and after that in January (p<0.01), but in February, April and May were lower than other months with no significant difference (p>0.01) (Table 2).

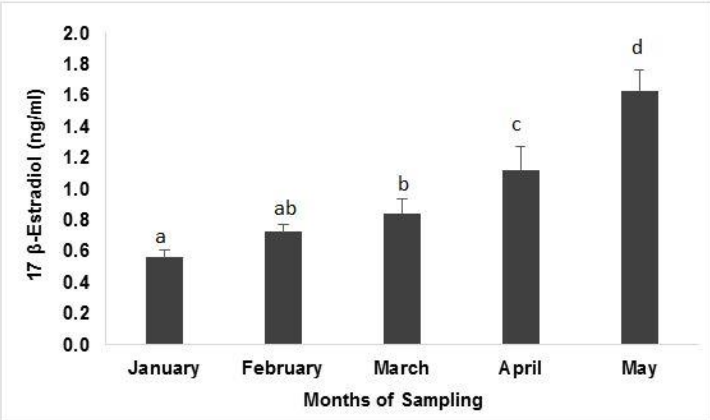

Figure 1: Changes in plasma 17 β-Estradiol levels in female rainbow trout from January to May (n=). Statistics should be indicated.

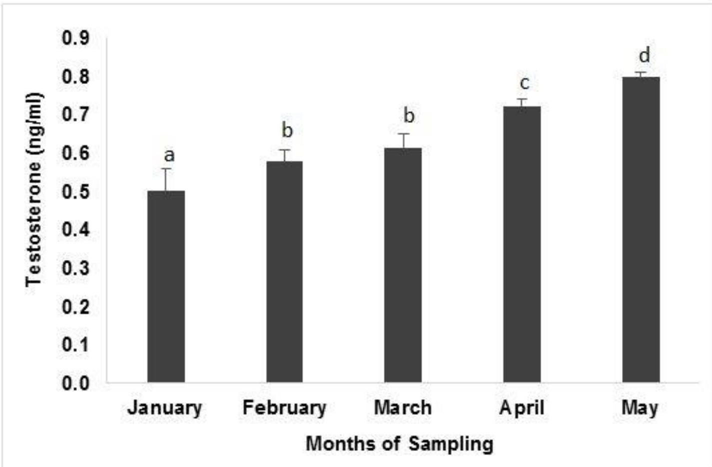

Figure 2: Changes in plasma Testosterone levels in female rainbow trout from January to May. (n=). Statistics should be indicated.

[DOI: 10.61186/injbir.1.1.51]

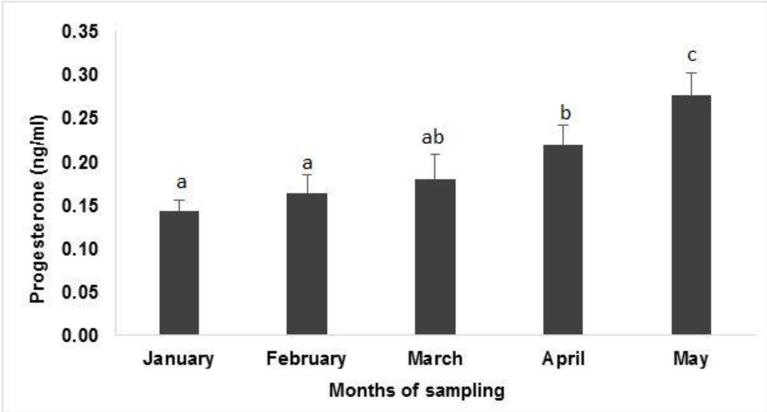

Figure 3: Changes in plasma progesterone levels in female rainbow trout from January to May. (n=). Statistics should be indicated.

Table 1: Vitellogenin, melatonin, calcium ion, lysozyme, and IgM levels in the cage-reared female rainbow trout in the Caspian Sea from January 2015 to May 2016.

Parameter	Month				
	January	February	March	April	May
Vitellogenin ($ngmL^{-1}$)	328.40 ± 55.26^{ab}	338.40 ± 73.53^{a}	473.60 ± 69.25^{b}	466.60 ± 10.26^{b}	651.40 ± 86.34^{c}
Melatonin (ngL^{-1})	294.20 ± 16.89^{d}	256.40 ± 24.13^{c}	226.40 ± 12.70^{bc}	193.20 ± 15.20^{ab}	175.00 ± 17.54^{a}
Calcium ion ($mgdL^{-1}$)	12.84 ± 1.42^{a}	14.20 ± 1.99^{a}	14.70 ± 1.05^{a}	18.94 ± 1.18^{b}	19.04 ± 1.55^{b}
Lysozyme ($umL^{-1}min^{-1}$)	28.00 ± 5.24^{b}	18.40 ± 3.97^{a}	36.60 ± 4.98^{c}	14.60 ± 1.82^{a}	21.80 ± 4.97^{ab}
IgM ($mgdL^{-1}$)	26.60 ± 2.70^{b}	14.90 ± 2.40^{a}	35.60 ± 2.30^{c}	14.60 ± 2.07^{a}	12.60 ± 1.52^{a}

Data with different superscripts in a row have a statistically significant difference (p<0.01).

Table 2: The amount of Caspian Sea water parameters from January 2015 to May 2016.

Month	Parameter			
	Temperature (°C)	Dissolved O_2 (mgL^{-1})	pH	Salinity (ppt)
January	13	12.2	7.8	13
February	12	12.4	6.9	13
March	12	13	6.5	12.1
April	10	12.7	7.3	11.9
May	11	13.3	7.1	11.5

Discussion

Hormonal assessment and physiological studies of blood serum along with the environmental factors are important for the examination of the fish reproductive cycle. Humoral and hormonal evaluations are useful for the examination of the fish's physiological condition, since blood is directly involved in many metabolic processes and shows the alterations in the fish body (Sattari, 2006).

In the present study, sex hormone fluctuations had been increased from January to May and the most levels of 17 β-estradiol, testosterone, and progesterone were observed in May (p<0.01). In teleost fish, 17 β-estradiol, testosterone, and progesterone hormones cause the development and maturation of oocytes (Bosak Kahkesh *et al.*, 2010). Gonadotropins stimulate the ovary during oocyte development and cause the production of 17 β-estradiol. In addition, Testosterone act as 17 β-estradiol precursor (Vazirzade *et al.*, 2011). 17 β-estradiol stimulates the synthesis and secretion of vitellogenin in the liver. Vitellogenin accumulation in oocytes and changes in

[DOI: 10.61186/injbir.1.1.51]

17 β-estradiol levels are associated with the growth of oocytes in the ovaries and GSI increase (Lee and Yang, 2002). Progesterone is also responsible for the final maturation of the immature oocyte in most female fishes (Aghili *et al.*, 2018).

According to Bohemen and Lambert (1981), the annual reproductive cycle of female rainbow trout can be divided into 4 physiological stages: previtellogenesis, endogenous vitellogenesis, exogenous vitellogenesis, and ovulation – spawning. During previtellogenesis, low levels of plasma sex steroids appear and 17 β-estradiol and testosterone start to increase during endogenous vitellogenesis and reach the highest levels in exogenous vitellogenesis stage and there is a feedback inhibition mechanism between sex steroids and gonadotropin, during oocyte maturation and ovulation (Pavlidis *et al.,* 1994). Although in the present study, the trend of 17 β-estradiol and testosterone changes from January to May has been increasing, the maximum amount of 17 β-estradiol and testosterone (1.63±0.13 $ngml^{-1}$ and 0.80±0.01, respectively) was much lower than the values reported in Rainbow trout in the final stages of sexual maturation. Maximum levels of 17 β-estradiol in the normal reproduction cycle of rainbow trout reported by Scott *et al.* (1980) and Estay *et al.,* (2012) were 50, and 30 $ngml^{-1}$ respectively.

Lower levels of sex hormones observed in the present study show that female rainbow trout started maturation and they are in the first stages of sexual maturation however the sex hormones levels are very low but an increase in all sex hormones was accrued over time. Therefore, considering that the fish was less than one-year-old at the beginning of the study, they reached the endogenous vitellogenesis stage until the end of the study period (May) and have not yet reached the peak of sex hormones nor the final stage of maturation. Other findings in different fish species showed that the levels of 17 β-estradiol will increase with vitellogenin activity and reach their maximum in the third stage of vitellogenesis (Kagawa *et al.*, 1982; Lee andYang 2002; Mehrpoosh *et al.*, 2013).

Testosterone is present in the blood plasma of female fish (Fostier and Jalabert., 1983) and acts as a precursor to the production of 17 β-estradiol (Kagawa *et al.*, 1982); this is justifying the increase in the levels of 17 β-estradiol and testosterone in rainbow trout in the present study. On the other hand, the gradual increase in testosterone is due to changes in the activity of key enzymes in steroid formation, so that the follicles are ready to synthesize progesterone (Nagahama, 1994). In fact, the concentration of progesterone increases in the final stages of oocyte maturation (the oocytes have not yet reached that stage in the present study) (Mylonas *et al.,* 2010) and the follicles are ready to synthesize progesterone.

Although the trend of progesterone alterations has been increasing in the

[DOI: 10.61186/injbir.1.1.51]

present study and the maximum amount of progesterone in May was 0.28±0.03 ng mL^{-1}, a greater increase in progesterone level is expected with the sexual maturation development in the next months. It is reported that 17 β-estradiol and testosterone levels in *Rutilus frisii kutum* were increased during the oocyte development stage till the end of vitellogenesis in the Sea and then decreased, but progesterone level was increased until spawning (Heidari *et al.,* 2014). In the annual reproductive cycle in *salivary fontinalis Mitchill*, the levels of the 17 β-estradiol and testosterone hormones began to rise in January and peaked in June (Fatima *et al.,* 2015). Consequently, increase in all three sex hormones in rainbow trout during this study indicates the gradual onset of the sexual maturation and hormones levels will increase more in the next months because a sharp rise in hormone levels is expected in the final stages of maturation and oocyte development (Pavlidis *et al.,* 1994; Estay *et al.,* 2012).

Vitellogenin concentration gradually increased and the highest amount was observed in May (651.40±86.34 ng mL^{-1}). Vitellogenin is made in liver cells during vitellogenesis in response to 17 β-estradiol. It releases into the blood and is transported to the ovaries (Verslycke *et al.,* 2002). Vitellogenesis will increase followed by an increase of 17 β-estradiol (Aghili *et al.,* 2018), this procedure was also observed in the present study and reported by Estay *et al.,* (2012) in rainbow trout and by Akhoundian (2014) in Caspian roach (*Rutilus rutilus*). Bohemen and Lambert (1981) also reported a positive relationship between hormones during vitellogenesis in the ovaries of rainbow trout.

Melatonin levels decreased from January (294.20±16.89 ngL^{-1}) to May (175.00±17.54 ng L^{-1}) (p<0.01) but no significant differences were observed in melatonin levels in February, March, and April (p>0.01). The sequence of reproductive procedures in an annual cycle is mainly under the control of a species-specific endogenous timing system, which essentially depends on a well-equipped physiological response mechanism to changing environmental cues. The duration of solar light or photoperiod is one of the most predictable environmental signals used by fish to coordinate their seasonal reproduction (Maitra and Nurul Hasan, 2016), and the melatonin is also an effective endogenous hormone in determining the temporal pattern of spawning in fish (Bromage *et al.,* 2001; Lima- Cabello *et al.,* 2014). Melatonin shows the rhythm of the daily cycle and the most melatonin level is produced during the dark period of the photoperiod (Esteban *et al.,* 2013). Therefore, due to the longer period of darkness than the light in winter and vice versa in spring, the observation of the highest amount of melatonin in January and the lowest in May, in the present study, is fully justified. A gradual decrease in melatonin along with the increase of daylight (from winter to spring), in female rainbow trout, reared in the cage supports its

[DOI: 10.61186/injbir.1.1.51]

physiological role in regulating the fish's seasonal reproduction. Melatonin by acting directly on gonads may regulate the transcription of genes whose proteins are involved in the synthesis of gonadal steroids to control oocyte competence and maturation (Maitra and Nurul Hasan, 2016). Effective role of melatonin in fish reproduction was reported in different fish species such as Atlantic salmon (*Salmo salar*) (Falcon *et al.*, 2010), rainbow trout (Falcon *et al.* 2010; Migaud *et al.* 2010), and Atlantic Cod (*Gadus morhua*) (Porter *et al.*, 2000).

Blood serum parameters are specific-species indicators that are affected by various factors such as disease, environmental factors, and reproduction (Rafati *et al.*, 2015). Lysozyme and IgM levels changes were similar and the highest levels were observed in March, which was significantly higher than the other months (p<0.01) (Table 1).

Lysozyme, as one of the components of the non-specific defense mechanism, is produced by white blood cells (neutrophils and monocytes and to a lesser extent macrophages) and secreted in various tissues and blood (Kim *et al.*, 2012). Factors affecting the level of lysozyme are including season, sexual maturity, fish sex, temperature, antigen stimulation that increases it, and immune stimulants consumption (Rafati *et al*., 2015). There are many reports of seasonal changes in fish immune responses which are affected by changes in temperature and length of the day during different seasons. For example, in *Dicentrarchus labrax*, *Limanda limanda*, *Hippoglossus hippoglossus*, and *Clarias batrachus* (Bowden *et al.*, 2004; Kumari *et al.*, 2006; Pascoli *et al.*, 2011; Valero *et al.*, 2014). In most studies, it has been found that lysozyme concentration decreases in winter and cold months (Farzadfar *et al.*, 2013; Pascoli *et al.*, 2011). Also, in our study, a decreasing trend from March to May was observed. The temperature of sea water in January was higher than other months (13°C) and then decreased, and the lowest temperature was measured in April and May (10 and 11°C, respectively). In addition, lysozyme level was significantly lower during February, April, and May. The reason for the lysozyme decrease, along with the decrease in water temperature, can be related to the decrease in fish activity as well as the reduction of pathogens in the cold water during late winter and early spring. On the other hand, sexual maturation and gonadal growth stages have been studied as one of the factors affecting the activity of the non-specific immune system (Abbasi *et al.*, 2009) and it was stated that sex steroid hormones in rainbow trout act as an immune suppressor (Hou *et al.*, 1999).

In fish, there is only one isotope of immunoglobulins called IgM. Cells that produce IgM are in the skin mucosa, gills, lymph, thymus, kidneys, intestines, and gallbladder (Altinok *and Grizell*, 2004). In the present study, IgM levels decreased since March. It shows that as sexual maturation progresses, IgM levels like lysozyme decline in female rainbow trout caged in the Caspian Sea. Similar to our finding, Hou *et al.* (1999) stated that sex steroid hormones act as a suppressor of the immune system in rainbow trout, and by increasing the hormones, the IgM levels will decrease. Finally, it seems that both water temperature (as an external factor) and sexual maturation of rainbow trout (as internal factors) affect lysozyme and IgM levels in female rainbow trout.

When steroid hormones induce the synthesis of vitellogenin, large amounts of calcium ions are involved in the synthesis of this protein and bind to the negative phosphate groups of this molecule. The vitellogenin molecule, which is the precursor of yolk, is structurally a protein enriched with phospholipids and calcium (Akhundian, 2014). Calcium binding to negative phosphate groups in vitellogenin-producing amino acid units continues until the maturation of the oocytes to store the minerals needed for early embryonic growth in the yolk molecule (Patino and Sullivan, 2002). Therefore, it appears that changes in calcium ion in the plasma of the female rainbow is due to the presence of yolk precursor (vitellogenin). On the other hand, it has been shown that the increase in total plasma calcium during vitellogenesis in rainbow trout is related to its binding to vitellogenin (Bjornsson and Haux, 1985). Increase in vitellogenin and calcium ion levels was observed over time in the present study. Therefore, it is emphasized that changes in calcium ions are due to the presence of vitellogenin in the female rainbow trout. Calcium ion changes in this study were increased (from 12.84±1.42 $mgdL^{1-}$ in January to 19.04±1.55 $mgdL^{-1}$), but the highest calcium ion concentration will be observed in the oocyte maturation stage (Wallace and Selman., 1985; Watanabe and Kuo, 1986). Calcium ion concentration was reported about 12-13 $mgdL^{-1}$ in previtellogenesis and vitellogenesis stages in rainbow trout reared in brackish water (Falahati marvast *et al.*, 2014) and about 10 $mgdL^{-1}$ in rainbow trout reared in the Aegean Sea in Turkey (Guner *et al.*, 2006). It should also be noted that the presence of calcium ion and the formation of calcium messages is one of the important metabolic pathways for processes such as meiosis restart, cell dehydration, vitellogenin integration, and germinal vesicle breakdown (Tosti, 2006).

Finally, increases in sex hormones, vitellogenin, and calcium ion, along with a decrease in melatonin levels and immune parameters in female rainbow trout reared in the cage in the Caspian Sea, indicate the gradual beginning of the fish normal sexual maturation in the Caspian Sea similar to other water resources and culture system of rainbow trout.

Acknowledgment

We acknowledge from Inland waters aquaculture research center- Anzali, Kheiroud research station- Nowshahr and cage culture center located in Nowshar for their valuable help to do

[DOI: 10.61186/injbir.1.1.51]

the study and carrying out the laboratory examinations.

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[DOI: 10.61186/injbir.1.1.51]

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