www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 13: 95-100 (2013) DOI: 10.4194/1303-2712-v13_1_12

Effect of Synthetic Luteinizing Hormone - Releasing Hormone (LHRH-A2) Plus Pimozide and Chlorpromazine on Ovarian Development and Levels of Gonad Steroid Hormones in Female Kutum *Rutilus frisii kutum*

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Received 25 September 2012
Accepted 10 October 2012

Abstract

To assess changes of gonad development and sex steroids hormones of *R. frisii kutum*, broodfish were captured during spawning season 2010 (March-April) and induced intramuscularly with following hormone treatments: single injections of 1 μg kg^{-1} b.w. LHRH-A2, combination of LHRH-A2 with two dopamine antagonists as follows: 1 μg kg^{-1} b.w. LHRH-A2 + 5 mg kg-1 b.w. PIM, 1 μg kg^{-1} b.w. LHRH-A2 + 2.5 mg kg-1 b.w. chlorpromazine, 1 μg kg^{-1} b.w. LHRH-A2 + 5 mg kg^{-1} b.w. PIM + 2.5 mg kg^{-1} b.w. chlorpromazine, single injections of 5 mg kg^{-1} b.w. PIM and 2.5 mg kg^{-1} b.w. chlorpromazine, carp pitatury extract (CPE), saline solution (NaCl 0.7%) and control group (without injection) were used, respectively. The number of ovulated fish was different in each treatment. Ovaries were more progressed in ovulated rather than non-ovulated fish. Also, sex hormones such as testosterone (T) and estradiol (E2) were changed in different hormonal treatments. In ovulated fish, histological analysis revealed six stages in ovaries, while ovaries in non-ovulated ones were developed only in fourth stage

Keywords: Spawning season, Histology, Steroid profiles, *Rutilus frisii kutum*.

Introduction

Reproduction in fish is under hormonal regulation by the hypothalamus–pituitary–gonadal axis. The main factors involved in the control of reproduction are pituitary gonadotropin and gonadal steroids. In teleosts testosterone (T) and oestradiol-17β (E2) regulate a number of reproductive processes (Fostier *et al*., 1983). Correlations between changes in plasma levels of gonad steroids and oocyte development have been well documented in a number of freshwater species including Salmon forms (Whitehead *et al*., 1983; Truscott *et al*., 1986), Cyprinids (Kobayashi *et al*., 1987), catfish *Heteropneustes fossilis* (Lamba *et al*., 1983), goldeye *Hiodon alosoides* (Pankhurst *et al*., 1986), walleye *Stizostedion vitrum* (Malison *et al*., 1994), and marine species including orange roughly *Hoplostethus atlanticus* (Pankhurst and Conroy, 1988), Japanese whiting *Sillago japonica* (Matsuyama *et al*., 1990), Japanese sardine *Sardinops melanostictus* (Matsuyama *et al*., 1991) and Common snook *Centropomus undecimalis* (Roberts *et al*., 1999).

In many teleost species, maturation and ovulation have been induced by combined injections of luteinizing hormone-releasing hormone analogue (LHRHa; Des-Gly" [D-Ala'] LHRHethylamide) and pimozide (PIM) (De Leeuw *et al*., 1985a, 1985b). The LHRH acts by stimulating the release of gonadotropin from the pituitary, and the pimozide by suppressing the action of a natural hypothalamic gonadotropin release-inhibiting factor (GRIF) which has been identified as dopamine (DA) (Peter *et al*., 1978; Chang and Peter, 1983; Chang *et al*., 1984). The same combination of compounds had been shown to induce oocyte maturation and ovulation in the goldfish, *Carussius aurutus* (Chang and Peter, 1983; Sokolowska *et al*., 1984). The kutum, *Rutilus frisii kutum*, is an anadromous species that has been considered for a biological conservation programme in the southern part of the Caspian Sea (Emadi, 1995).

In recent years, because of dramatic declines in broodstock capture and subsequently insufficient availability to sexual materials (ova), the aquaculturists have produced brooders from fertilised eggs in artificial reproduction in the hatchery. Several researchers have investigated fish maturation by histological and hormonal observations during artificial breeding to the evaluation of the efficiency of different hormones (Matsuyama *et al*., 1991; Kozul

et al., 2001; Dahle *et al*., 2003). Dahle *et al*. (2003) investigated the gonadal development and associated changes in sex steroids during the reproductive cycle of captive Atlantic cod, *Gadus morhua*. Reliable indicators of reproductive status are essential for good fisheries management and they are also important to evaluate the effects of different hormonal treatments on sexual maturation in fish farming. The aim of the present study was to investigate changes in sex steroid hormones (T and E2) and ovarian development in *R. frisii kutum* under hormonal treatments.

Materials and Methods

Brooders

The experiments were carried out on kutum broodfish at Shahid Ansari Cyprinid Fish Complex, Rasht, Iran. Kutums were captured from the Sefid Rood River inlets to the Caspian Sea during spawning migration (water temperature 9-12ºC). Eighty one mature female fish in the 500-1300 g body weight (b.w.) were selected, on the basis of soft rounded abdomen. Before injections, fish were individually weighed and marked by placing tags on the dorsal fin and randomly assigned to eight treatment groups.

Hormones and Drugs Preparation

Luteinizing hormone-releasing hormone analogue (Des-Gly10, [D-Ala 6] LH-RH Ethylamide) or LHRH-A_2 is a peptide that is similar in structure to native luteinizing hormone (LHRH). The LRH-A_2 available on the market is a white powder and is combined with mannite as filler (made in China). White powder was dissolved by adding saline solution (0.7% NaCl) to adjust volume injection to 1 ml kg^{-1}b.w. PIM known as a trade name "Orap" which is available as 5 gram tablets. These tablets were dissolved in 0.7% NaCl to adjust volume injection to 5 mg kg^{-1}b.w. Chlorpromazine was purchased in the soluble form (2 ml syringe containing 50 mg of chlorpromazine) from Iranian drug stores. Two milligram per kilogram body weight of CPE diluted in 0.7% saline was used for the induction of spawning as positive control and injected intramuscularly (injections were performed by penetration of the dorsal muscles at the base of the dorsal fin) in a single injection according to hatchery practice.

Gonads Histology and Blood Sampling

Eighty one broodfish were divided into 9 treatments (n= 9) and injected as follows: single injection of 1 µg kg^{-1} b.w. LHRH-A_2. Single injections of 5 mg kg^{-1} b.w. PIM and 2.5 mg kg^{-1} b.w. chlorpromazine and following combination of LHRH-A_2 with two dopamine antagonists: 1 µg kg^{-1} b.w. LHRH-A_2 + 5 mg kg^{-1} b.w. PIM, 1 µg kg^{-1} b.w. LHRH-A_2 + 2.5 mg kg^{-1} b.w. chlorpromazine and 1 µg kg^{-1} b.w. LHRH-A_2 + 5 mg kg^{-1} b.w. PIM + 2.5 mg/kg b.w. chlorpromazine. CPE (2 mg kg^{-1} b.w) and saline solution (0.7% NaCl) were injected as positive and negative treatments respectively. All treatments were carried out intramuscularly with a single injection. After injection fish were placed in indoor holding tanks with running water and a temperature of 10-13°C. Then fish were checked to detect the onset of ovulation by hand stripping 24 h post-injection and repeated every 8-10 h for 24 h. Blood samples were taken from the caudal vessels by using heparinized disposable syringes both ovulated and non-ovulated fish. Sample was centrifuged to separate the serum and this was stored at -20ºC until hormonal analysis. Plasma levels of E2 and T were measured by radioimmunoassay using the procedure described by Rinchard *et al*. (1993). Ovaries collected fish were fixed in Bouin's solution, embedded in paraffin wax, sectioned at 6 mm thick, and stained with hematoxylin and eosin. The slides of ovarian tissues were examined and classified into stages of ovariogenic development based on criteria of Kesteven (1960).

Statistical Calculation

The average of data among treatments was analyzed by one way ANOVA followed by Tukey test using SPSS software (Version 13). Results are presented as means ± standard error of the mean (SEM).

Results

Among injected fish, only 33 specimens were ovulated successfully and rest of brooders did not respond to hormonal treatments. In ovulated fish histological observations showed six stages in ovaries, whereas in non-ovulated ones ovaries just developed in fourth stage. The cross section of ovaries in spawned fish showed in Figure 1 (Hematoxylin-eosin ×40). As can be seen in this figure follicles after spawning and ovulated oocyte were clearly observed, also a class 111 oocyte was visible. Histological picture, cross section showed compressed yolk granules, pressed membrane yolk vesicles, thick zona radiata and weak follicular and theca layers in non-ovulated fish (Figure 2). Injection of LHRH-A_2 (1 µg kg^{-1} b.w) and PIM (5 mg kg^{-1} b.w) caused a significant increase in serum T levels compared to other treatments in spawned fish ($P<0.05$), although their values were not statistically significant (Table 1). E_2 level in ovulated kutum did not changed in all treatments (Table 2). Higher level of T was observed in Control group and saline solution treatments of non-ovulated fish (Table 3). Injection of LHRH-A_2 plus PIM (1 µg kg^{-1} b.w+(5 mg kg^{-1} b.w) and PIM (5 mg kg^{-1} b.w) caused a significant increase in serum E_2 levels compared with other treatments in non-ovulated fish (Table 4).

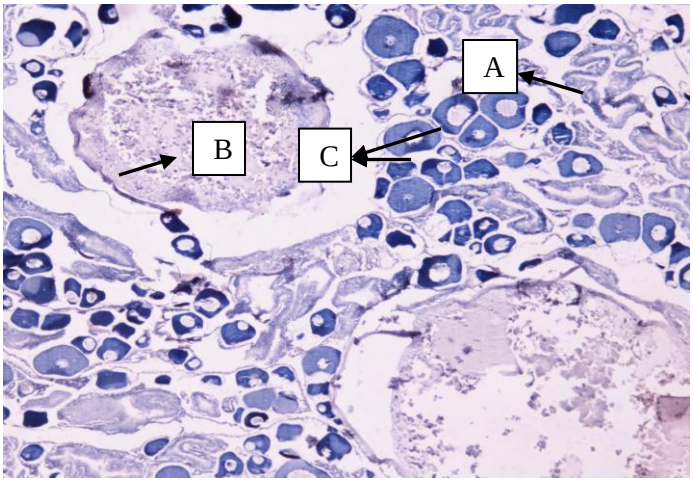

Figure 1. Histological picture, cross section of ovulated kutum: A- follicles after spawning B-ovulated oocyte, C- class 11 1oocyte

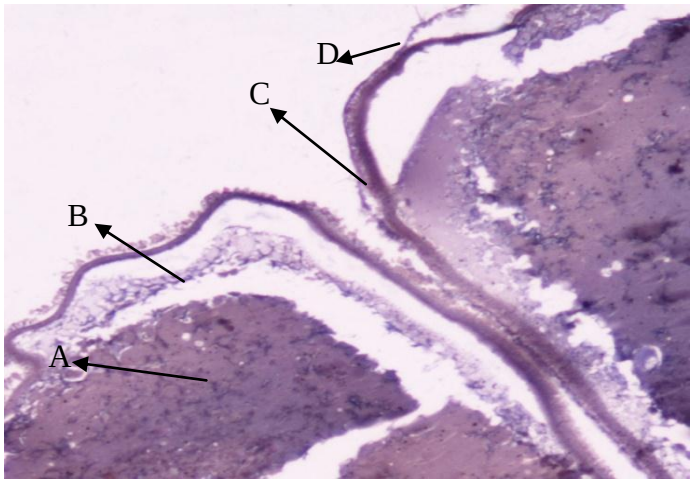

Figure 2. Histological picture, cross section of non-ovulated kutum- A- Compressed yolk granules, B- compressed membrane yolk vesicles, C- thick zona radiata and D- weak follicular and theca layers.

Table 1. Testosterone concentration in ovulated kutum after inducing with hormonal treatments

Treatment	Dosage	Number of ovulated fish	T (ng ml^{-1})
LHRH-A_2	1 µg	6	0.046 ± 0.009
LHRH-A_2 + PIM	1+5	7	0.027 ± 0.009
LHRH-A_2 + Chlorpromazine	1+2.5	3	0.027 ± 0.009
LHRH-A_2 + PIM + Chlorpromazine	1+5+2.5	4	0.06 ± 0.023
PIM	5	4	0.045 ± 0.014
Chlorpromazine	2.5	2	0.013 ± 0.001
CPE	2	7	0.019 ± 0.007
Saline solution (0.7% NaCl)	1 cc	0	-
Control group (without injection)	-	0	-

First number indicates the dose of LHRH-A_2 in µg kg^{-1} b.w and the second in mg kg^{-1} b.w CPE, carp pituitary The extract; PIM, pimozide.

Discussion

Hormonal manipulations for induction of ovulation and spawning have made possible the control of reproductive processes in fish, and have contributed significantly to the sophistication and expansion of the aquaculture industry. In this experiment, fish on those treatments that induced successfully, gonadal development passed all phases to made spawning. In contrast, gonad development just proceeds in fourth phase in non-ovulated fish. A possible explanation for this difference between groups is that synthetic luteinizing hormone - releasing hormone (LHRH-A_2) administration could

Table 2. Estradiol level in ovulated kutum after inducing with hormonal treatments

Treatments	Dosage	Number of ovulated fish	E_2 (ng ml^{-1})
LHRH-A_2	1 µg	6	14.3 ± 1.8
LHRH-A_2 + PIM	1 + 5	7	17.3 ± 2.2
LHRH-A_2 + Chlorpromazine	1 + 2.5	3	18 ± 1.7
LHRH-A_2 + PIM + Chlorpromazine	1 + 5 + 2.5	4	17.3 ± 2.4
PIM	5	4	17.5 ± 2.5
Chlorpromazine	2.5	2	18.5 ± 3.5
CPE	2	7	16.7 ± 2.7
Saline solution (0.7% NaCl)	1 cc	0	-
Control group (without injection)	-	0	-

The first number indicates the dose of LHRH-A_2 in µg kg^{-1} b.w and the second in mg kg^{-1} b.w CPE, carp pituitary extract; PIM, pimozide.

Table 3. Testosterone concentration in non-ovulated kutum after inducing with hormonal treatments

Treatment	Dosage	Number of non-ovulated fish	T (ng ml^{-1})
LHRH-A_2	1 µg	3	0.014 ±0.005
LHRH-A_2 + PIM	1 + 5	2	0.146 ±0.085
LHRH-A_2 + PIM + Chlorpromazine	1 + 5 + 2.5	5	0.017 ±0.005
PIM	5	5	0.066 ±0.034
Chlorpromazine	2.5	7	0.028 ±0.007
CPE	2	2	0.025 ± 0.007
Saline solution (0.7% NaCl)	1 cc	0	0.032 ± 0.008
Control group (without injection)	-	0	0.036 ± 0.009

The first number indicates the dose of LHRH-A_2 in µg kg^{-1} b.w and the second in mg kg^{-1} b.w CPE, carp pituitary extract; PIM, pimozide.

Table 4. Estradiol level in non-ovulated kutum after inducing with hormonal treatments

Treatment	Dosage	Number of non-ovulated fish	E_2 (ng ml^{-1})
LHRH-A_2	1 µg	3	19.7 ± 6.7
LHRH-A_2 + PIM	1 + 5	2	22.5 ± 11.5
LHRH-A_2 + PIM + Chlorpromazine	1 + 5 + 2.5	5	20.8 ± 4.6
PIM	5	5	27.3 ± 6.4
Chlorpromazine	2.5	7	19.6 ± 2.5
CPE	2	2	20.5 ± 8.5
Saline solution (0.7% NaCl)	1 cc	0	18.6 ± 2.5
Control group (without injection)	-	0	17.2 ± 2

The first number indicates the dose of LHRH-A_2 in µg kg^{-1} b.w and the second in mg kg^{-1} b.w CPE, carp pituitary extract; PIM, pimozide.

not stimulate substantial surge of gonadotropin to induce ovulation. However, other factors such as environmental (for example photoperiod and water temperature) provide necessary cues that are perceived by the central nervous system that initiate the oocyte developmental processes. In response to hormonal stimulation, gonadotropin-releasing hormone (GnRH) is secreted from hypothalamus, which in turn stimulates the release of FSH and LH from the pituitary. Studies demonstrated that injection of pimozide caused a marked potentiation of the GtH-II release response to GnRH-A, and combined injections of pimozide and GnRH-A were highly effective in inducing ovulation (Chang and peter 1983; Sokolowska *et al.,* 1984). During the breeding season, an increase in the blood levels of several different hormones can be seen due to the activities of gonadotropic hormones, especially the LH (Schulz and Miura, 2002). While FSH is mainly involved in the vitellogenic process, LH plays a role in final oocyte maturation and ovulation (Nagahama, 1994; Pham *et al*., 2010). In fish, the co-ordination between environmental stimuli and gonadotropin stimulate the secretion of FSH and LH, which regulate hormonal responses that are important for successful reproduction. These two factors are most important, because they can act, directly or through sense organs, on the glands that produce hormones, which in turn produce the appropriate physiological or behavioral responses that ultimately control the timing of spawning in fish. Therefore, understanding the manipulation of this reproductive status is an integral aspect of sustainable fisheries management. These parameters are important for an accurate evaluation of the effects of different treatments on sexual maturation in fish farming. The effects of synthetic hormone on gonadal steroid production is in accordance with the increasing evidence that GtHs may regulate several aspects of ovarian development and function by several steroid-dependent and

independent actions (Planas *et al*., 2000; Pham *et al.*, 2010). During vitellogenesis, there is a gradual increase in plasma E_2 levels in females with matching patterns of T. Plasma E_2 levels peak towards the end of vitellogenesis and they decline rapidly in the maturation phase. In the present study, greatest number of ovulated fish was observed in treatments receiving 1 µg kg^{-1} b.w. LHRH-A_2, 5 mg kg^{-1} b.w. PIM and CPE. In above mentioned treatments T level was higher compared with other treatments. E_2 level in spawned individuals showed almost equal level in all treatments. In female teleosts, sexual maturation is stimulated by a gonadotropin-induced increase in plasma E_2 levels. E_2 stimulates the liver production of vitellogenin (VTG) which is a yolk protein precursor that is released to the circulation, producing an increase of total plasma. VTG is subsequently sequestered by the oocytes, processed and stored for the nutrition of the embryo (Nagahama, 1987; Persson *et al.*, 1998). In carp has shown that plasma E_2 levels increased significantly after 12 h, in response to GnRHa injection, and peaked after 24 h (Levavi-Zermonsky and Yaron, 1986). A similar increase in E_2 levels from postvitellogenic oocytes before final oocyte maturation was also reported in longfinned eels, *Anguilla dieffenbachia* (Lokman *et al.*, 1998) and gilthead seabream, *Sparus aurata* (Gothilf *et al.*, 1997). The development of methods using hypothalamic factors was only possible when both stimulation and inhibition mechanisms of neuroendocrinne LH regulation were known and understand in detail. Further research needed to identification and synthesis of more potent GnRHa for the reasons of reproductive dysfunction should contribute to future progress in the area of artificial stimulation of final oocyte maturation and ovulation in Cyprinidae. The present study shows that the injections of LHRH-A_2 plus pimozide had higher efficiency than in combination with chlorpromazine. It can be conclude that using of LHRHa with PIM is more effective in spawning induction of female kutum in hatchery operation.

Acknowledgments

The authors express their sincere gratitude to the people who spent their time, advice and support to this study, including the manager (Mr. Darvishi) and staff of the Shahid Ansari Cyprinid Fish Complex.

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