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Effects of different spawning agents on serum levels of reproductive steroid hormones and cortisol level in adult female *Barbus sharpeyi* (Gunther, 1874)

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Abstract The question of whether, as hormone therapies, spawning agents differ from each other to induce physiological pathways of gametogenesis and oocyte maturation in fish remains important, because it could modify undesirable changes, regulated by endocrine systems of individual fish. A series of experimental treatments were applied to investigate the underlying mechanism(s) in which female bunnei (*Barbus sharpeyi*) fish respond differently to hormone therapies. Female broodstocks were injected twice (with 12 h interval) by three different treatments namely A, B and C. The treatment A received carp pituitary extract (CPE) + luteinizing hormone-releasing hormone analogs (LHRHα_2) (0.5 mg CPE kg^{-1} BW for first injection and 2 mg CPE kg^{-1} BW + 10 μg LHRHα_2 kg^{-1} for second injection), treatment B received CPE (0.5 and 3.5 mg kg^{-1} BW), and treatment C received ovaprim (0.1 and 0.15 ml kg^{-1} BW). Blood samples were collected at four different time intervals, including prior to injections, 6 h after first injection, 6 h after second injection and at the time of spawning, and serum steroid hormones, including testosterone, progesterone and estradiol-*17β* as well as cortisol, were measured. Results showed significant increases in serum estradiol-*17β* following all treatments, but the most profound response was found in treatments A and B. Testosterone was higher in larger broodfish than in small-sized broodfish (>1.5 vs. <1.5 kg) in all treatments. CPE led to higher concentration of testosterone rather than two other treatments. CPE also increases the progesterone following first injection and approximately remains unchanged till the end of experiment. Change in progesterone level was only significant after second injection of ovaprim as well as after spawning compared with previous time. Linear regression analyses indicated that cortisol had adverse effects on progesterone and testosterone levels of weight group <1.5 kg. These results suggest that among inducing agents, applied here, CPE can provide more reasonable response in reproduction of female *B. sharpeyi*.

Keywords *Barbus sharpeyi* · Cortisol · Testosterone · Progesterone · Estradiol-*17β* · Ovaprim

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Introduction

Bunnei fish (*Barbus sharpeyi*, Gunther, 1874), which belongs to Cyprinidae family, are widely distributed in slow-running rivers and wetlands of Iran, Iraq, Turkey, Egypt and Syria. This species' populations are faced with undesirable changes in aquatic ecosystems since several years ago and hence identified as an endangered species in some Iraqi wetlands (UNEP 2001). To combat this problem, artificial spawning and larval rearing of this species has been commenced in Iran, Khuzestan province. During 1999 up to 2012, more than 150 million bunnei larvae were produced for restocking program of the population in the nature as well as for aquaculture purposes with other cyprinid fish (poly-culture system) (Basak Kahkesh et al. 2010).

Adult bunnei spawn in their habitat during January–April when water temperature reaches 20 °C, while there is no evidence for spawning of captive fish in aquaculture systems (Nikpey 1996). Since reproduction of fish is basically dependent on environmental stimuli, it is not, however, possible to provide some required conditions that are appropriate to species ecobiology, like spawning migration in captivity (i.e., performance of natural reproduction in this species will be initiated by small-sized migration (Al Mukhtar et al. 2006). In these instances, steroidogenesis, which in turn, triggers gametogenesis, final oocyte maturation (FOM) and ovulation, will be ceased due to absence of environmental stimuli (Podhorec and Kouril 2009). Therefore, exogenous hormone therapies are an inevitable way in gamete maturation and spawning procedures of such captive fish (Mylonas et al. 2010).

Regulation of steroid hormones will occur at different levels, including changes in the expression or activity of steroidogenic enzymes, changes in affinity of each steroid to their specified receptors and finally, at the level of responsible enzyme to transform other androgens into estradiol-*17β* (E2) (Mazzeo et al. 2014). By measuring the amount of E2 in serum due to the role of that steroid for vitellogenin production, the level of vitellogenesis could be estimated indirectly (Hobby and Pankhurst 1997). Changes in testosterone (T) and progesterone (P4) in serum of female fish due to their precursor role for biosynthesis of other steroid hormones can be used as indicators for spawning status of teleost fish (Henningsen et al. 2008).

Hormonal manipulations in fish species are employed in order to either enhance female fish fecundity or elevate the chance of fertilization as the only way for spawning induction in some species (Mylonas et al. 2010). Moreover, there has been controversy over whether different hormonal injections cause similar reproductive success. Previously, Rinchard et al. (2002) proved that among different inducing agents, the only luteinizing hormone-releasing hormone analogs (LHRHα_2) could enhance the ovulation and increase the level of some steroid hormones in plasma of yellow perch, *Perca flavescens*. In another study, human chorionic gonadotropin (hCG) has been recommended for induction of FOM and ovulation in pikeperch, *Sander lucioperca* (Żarski et al. 2013). Moreover, the use of carp pituitary extract (CPE) and hCG were determined to be effective for FOM and ovulation in Brazilian catfish, *Pseudoplatystoma fasciatum* (Leonardo et al. 2004). Kahkesh et al. (2011) examined the optimal hormonal induction for shirbut, *Barbus* and *grypus* bloodstock and concluded that LHRHα_2 induction in combination with CPE accounted as a more effective method for this species elsewhere. Additionally, commercial products containing LHRH analogs with dopamine antagonist, i.e., ovaprim, has more efficacy in another cyprinid species, *Aspius aspius* (Targońska et al. 2010). However, the selection of hormone therapy for FOM in different female species might also be based on management programs and whether the fish is synchronous or asynchronous spawner (i.e., the biology of fish) (Mylonas et al. 2010). Therefore, different manipulations tools, which contain low potency, high required dose and multiple injections, might engender less success in captive broodstocks, and subsequently justify application of an alternative inducing agent. Furthermore, application of GnRH + domperidone was accounted as an effective hormone therapy in the case of bunnei fish, which shows high fecundity with low latency period (Mohammadian et al. 2009). Additionally, Kahkesh et al. (2011) determined the efficacy of different hormones, including ovaprim, ovatide, HCG, CPE and LHRH + CPE on final maturation of this species and concluded that treatments received CPE and LHRH + CPE show more ovulation.

There are many stressors during culturing and artificial reproduction that fish face (Gennotte et al. 2012). It has been well documented that chronic and acute stress conditions disturb reproduction success in most fish by alteration of hypothalamic–pituitary–gonadal (HPG) axis hormones (Chabbi and Ganesh 2012). Different manipulations during captive reproduction practice might, however, be a particular problem by depressing the sex steroid hormones and led to further inability in fish to response to hormone therapies (Haddy and Pankhurst 2000). Therefore, measurement of serum stress indicator such as cortisol can lead us not only to the level of stress that each fish probably faced but also to judging the success of fish reproduction.

Information regarding the quality of artificial spawning of this species such as fertilization, hatching rate as well as survival rate of larvae is already available (Kahkesh et al. 2011) as long as researchers have supposed that is necessary to improve our entire breeding program. The decision to select best spawning agents as well as manipulation procedure might be achieved by performing such field and laboratory experiments, while the physiological basis of gametogenesis and FOM remains obscure due to unpredictable changes, induced by endocrine systems of individual fish.

Up to our knowledge, there is no significant study on determination of any possible change in the patterns of sex steroid hormones in the serum of matured bunnei fish when artificially stimulated by either hormone or other stimulating agents. In this respect, this study was performed to identify physiological basis of process that regulates gametogenesis among routine commercially methods applying for artificial spawning of broodstocks. Trends in serum steroid hormones, including E2, P4 and T as well as cortisol level following administration of different inducing agents, were examined at the present study.

Materials and methods

Experimental animals

Female bunnei fish (*B. sharpeyi*) were randomly selected from a local fish breeding center, Susangerd, Khuzestan province, Iran. The selected female fish were divided into two separated groups, including <1.5 kg body weight (weight range = 450–1170 g) namely size 1 and higher than 1.5 kg (1500–2625 g) namely size 2 and then transferred to holding tanks to acclimate for future treatments. The spawner fish were fed twice daily with normal carp commercial diet (3 % of body mass). During this study, water quality parameters, including pH (8.31–8.9), electrical conductivity (230–270 μs m^{-2}), total dissolve solid (102–155 mg l^{-1}), dissolve oxygen (7.6–8.8 mg l^{-1}), hardness (441–478 mg $CaCo_3$ l^{-1}), salinity (0.385 mg l^{-1}) and alkalinity (289–298 mg $CaCo_3$ l^{-1}), were measured. The natural photoperiod was also maintained in a constant range during the experiment.

Spawning stimulation

Six separated groups containing forty female fish (totally 240 fish) were assigned to different treatments namely CPE + luteinizing hormone-releasing hormone analogs (LHRHα_2), CPE and ovaprim (Salmon GnRHa (20 μg ml^{-1}) + domperidone (10 μg ml^{-1} as an anti-dopamine treatments). The experiment was carried out in April when water temperature reached 20 °C. Prior to injection, each fish was anesthetized using clove oil (60 mg l^{-1}) and then weighed and measured for total length and randomly assigned to duplicate inter-muscular (IM) injections as follow:

Treatment A received CPE + LHRHα_2 (first injection: 0.5 mg kg^{-1} of CPE; second injection: 10 μg LHRHα_2 kg^{-1}BW + 2 mg kg^{-1} CPE BW), treatment B received CPE with first injection of 0.5 mg kg^{-1} BW and second injection of 3.5 mg kg^{-1} BW), and treatment C received ovaprim (0.1 ml kg^{-1} BW and 0.15 ml kg^{-1}BW) (Fig. 1).

Each IM injection was prepared by dissolving aforementioned agent in 1 ml of normal saline (0.9 % NaCl) to provide exact amount required per kg BW and was then injected to fish at appropriate time period.

Sampling and physiological parameters measurement

In order to investigate the effects of different spawning agents on serum sex steroid hormones, including E2, P4 and T as well as cortisol level, four time intervals were designed namely before injection (i.e., control group without any injection), 6 h after first injection,

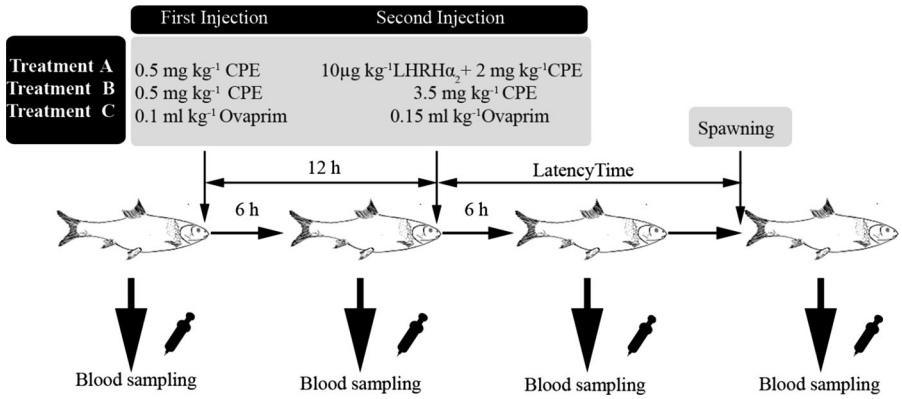

Fig. 1 Schematic representation of the experimental protocol

6 h after second injection and immediately after spawning or egg stripping (i.e., it takes 22–24 h following second injection) (Fig. 1). The sampling was included two separated groups, i.e., <1.5 kg (size 1) and higher than 1.5 kg (size 2) in each specific time period. At each specific time of experiment, ten fish were withdrawn from each tank and anesthetized with clove oil (60 mg l^{-1}). Blood sample (3 ml) was collected from caudal vein to acid pre-washed tube and then centrifuged at 2500 rpm for 10 min to separate blood cells from serum. Serum samples were stored until hormonal assay was performed.

The cortisol and sex steroid hormones were measured in serum using enzyme-linked immunosorbent assay (ELISA). To do this, separated serum was purified by diethyl ether to withdraw lipid and protein contents from serum if required. ELISA Kits (Neogen Corporation, China) as a solid phase based on the principle of competitive binding were used in this study. Absorbance of each well was detected by using Dynatech MR5000 ELISA reader at 450 ± 10 nm within 10 min following addition of stop solution. Finally, data were calculated in the presence of control and standard.

Statistical analyses

Normality (Kolmogorov–Smirnov) of data and homogeneity for variance (Leven's test) were used to check all treatments. If normality and homogeneity were achieved, general linear model univariate/multivariate ANOVA was used. When interaction effects between different independent parameters were not significant, one-way ANOVA was performed to compare data by further complementary post hoc test of Duncan for comparisons of different treatments as well as different sampling times. Independent sample *t test* was also applied to compare weight group (size 1 vs. size 2). Linear regression analyses were applied to determine the probable relationships between serum cortisol and other steroid hormones. Analyses were performed in SPSS for Windows, version 18. Data are presented as mean ± standard deviation (SD) for all cases.

Results

In this study, two separated female groups, including <1.5 kg (size 1) and higher than 1.5 kg (size 2), were selected based on repeated observations in which the latter group shows much reasonable response to hormone therapies for artificial spawning in Iran hatchery sites. Throughout the experiment, there is no mortality observed among female fish that were injected with CPE, LHRHα_2 or ovaprim. Working fecundity was measured for each group to achieve better conclusion by comparing with their related steroid hormone concentrations. Regarding that, the working fecundity of treatment A was achieved as 34,289 ± 1213 and 41,152 ± 1752 for <1.5 and >1.5 kg, respectively, and that of treatment B showed 20,097 ± 934 and 300,533 ± 3407, respectively, for <1.5 and >1.5 kg groups. The fecundity of treatment C was not measureable due to its high fluctuation. Table 1 summarizes the output of MANOVA for either simple or interactive effects of each parameter.

Table 1 Multivariate analysis of variance (MANOVA) performed for each parameter with its exact *P* value

Parameters	*P* value			
	Cortisol	*Testosterone*	*Progesterone*	*Estradiol*
Weight groups	**0.008**	**<0.0001**	**<0.0001**	**0.001**
Time	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Treatments	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Weight groups × Time	0.053	**<0.0001**	**<0.0001**	**<0.0001**
Weight groups × Treatments	**<0.0001**	**<0.0001**	**<0.0001**	**0.002**
Time × Treatments	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Weight groups × Time × Treatments	**<0.0001**	0.032	**<0.0001**	**<0.0001**

P value with bold-faced type indicated significant differences for the tested parameter

Trends in steroid hormones

E2 changes

In both weight groups of treatment A, the amounts of E2 have been elevated either significantly or insignificantly following stimulation. Before first injection, the levels of E2 were lower than other sampling times (Fig. 2). The mean levels of E2 rise significantly ($P < 0.001$) for both weight groups (6 h later). At the end of the experiment, serum E2 concentrations were gradually increased by 5.3 ($P = 0.514$) and 14.25 % ($P = 0.077$), respectively, for size 1 and size 2 groups when compared to previous measurement time (i.e., after spawning vs. 6 h after second injection).

Significant increases in E2 level were observed for both weight groups in treatment B as well. The initial concentrations of E2 (as a control without any injection) were 260 ± 16.40 and 273 ± 26.17 pg ml^{-1}, respectively, for size 1 and size 2 groups. Administration with CPE elevated acutely ($P < 0.001$) the serum levels of E2 up to 2439.80 ± 308.10 and 2455.00 ± 349.26 pg ml^{-1}, respectively, on 6 h after injection (Fig. 2).

Following treatment C, E2 concentrations were increased gradually from 258.01 ± 13.84 to 327.61 ± 81.68 pg ml^{-1} ($P = 0.016$) and from 264.72 ± 34.31 to 424.38 ± 233.82 pg ml^{-1} ($P = 0.002$) for size 1 and size 2 groups, respectively. Final concentrations of E2, measured immediately after spawning, were observed as 490.80 ± 65.51 pg ml^{-1} ($P = 0.025$) and 1373.30 ± 228.94 pg ml^{-1} ($P < 0.001$) for the two weight groups, respectively (Fig. 2).

Progesterone

There was a fluctuation in P4 level of female fish when stimulated by treatment A. This hormone reaches a peak after first injection by amounts of 39.37 ± 12.19 ng ml^{-1} ($P < 0.001$) and 38.43 ± 7.49 ng ml^{-1} ($P < 0.001$) for the two weight groups as mentioned previously. Sharp declines ($P < 0.001$) were observed for size 1 and size 2 groups for nearly 6 h after second injection with CPE + LHRHα_2. These lessening patterns were much clear for size 1 rather than size 2 groups (5.82 ± 1.69 vs. 19.47 ± 5.41 ng ml^{-1}). Afterward, serum P4 rose by 38.5 ($P = 0.172$) and 57.9 % ($P = 0.02$) in comparison with previous measurement time (i.e., 6 h after second injection) for size 1 and size 2 groups, respectively (Fig. 3).

When fish were injected with CPE, significant increase in P4 was observed [amount of 30.25 ± 7.47 ng ml^{-1} ($P < 0.001$) and 32.35 ± 12.36 ng ml^{-1} ($P < 0.001$) for two different weight groups], since then, serum P4 has begun to level out and a little fluctuation for size 1 and size 2 groups (Fig. 3).

As it can be seen from Fig. 3, treatment C did not able to change significantly ($P = 0.334$ and $P = 0.496$) P4 level of female fish in comparison with earlier time in both weight groups, but there were rapid increases ($P < 0.001$ and $P = 0.006$) in this hormone following second injection of ovaprim by 0.15 ml kg^{-1} BW (Fig. 3).

Testosterone

The mean level of testosterone in treatment A rose significantly ($P < 0.001$) following 6 h from first

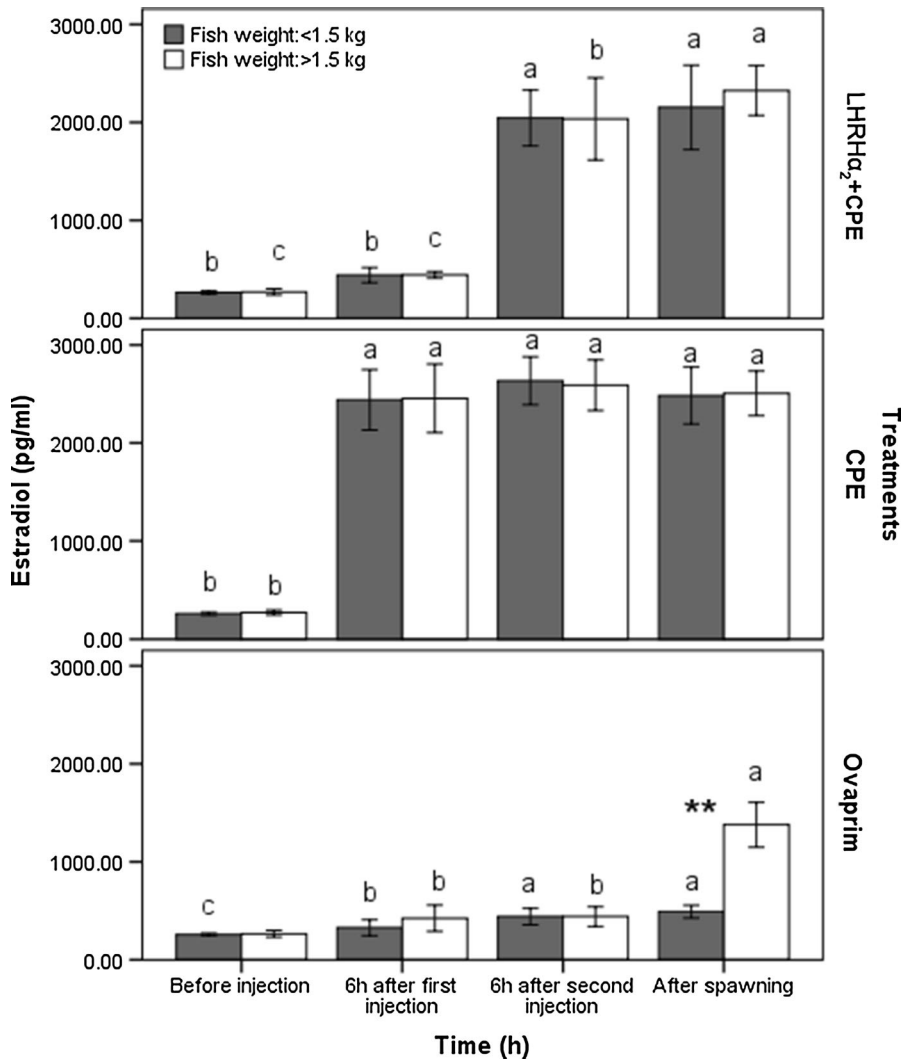

Fig. 2 Serum E2 level of female bunnei fish following different hormone therapies, including LHRHα_2 + CPE, CPE and ovaprim. The E2 concentrations were illustrated at four different time courses, namely before injection, 6 h after first injection, 6 h after second injection and after spawning. Data presented as mean ± SDs ($n = 10$). *Alphabetic letters* denote significant difference between sampling time, and * and ** denote significant difference between two weight groups, respectively, for $P < 0.05$, $P < 0.01$

injection. For size 1 group, a lessening ($P < 0.001$) pattern was observed by 2.26-fold following second injection (CPE + LHRHα_2), but a 32 % increase ($P = 0.153$) in this androgen was observed at the end of experiment in which female fish were spawned. Similar but lesser decrease (22 %, $P = 0.05$) was observed in high-weight group when injected by CPE + LHRHα_2. On the attainment of ovulation, the T value was increased ($P = 0.097$) by 30 % for this group (Fig. 4).

Female bunnei fish revealed significant elevations ($P < 0.001$) in T levels of serum following treatment B. Since then, a significant reduction (38 %, $P = 0.094$) was observed in T of size 1 group, and there was a corresponding increase (77 %, $P = 0.001$) in this androgen for high-weight group. These fluctuations were seen even after spawning time (Fig. 4).

Treatment C resulted in a significant increase ($P < 0.001$) in T both in size 1 (5.6-fold) and size 2 (3.9-fold) groups. Thereafter, a significant decrease was observed in two aforementioned weight groups, which reached 4.50 ± 2.43 ng ml^{-1} ($P = 0.038$) and 7.52 ± 3.58 ng ml^{-1} ($P = 0.089$), respectively (Fig. 4).

Cortisol

There was a fluctuation in serum cortisol of female bunnei fish among different sampling times, when treatment A was applied. Initial concentrations for

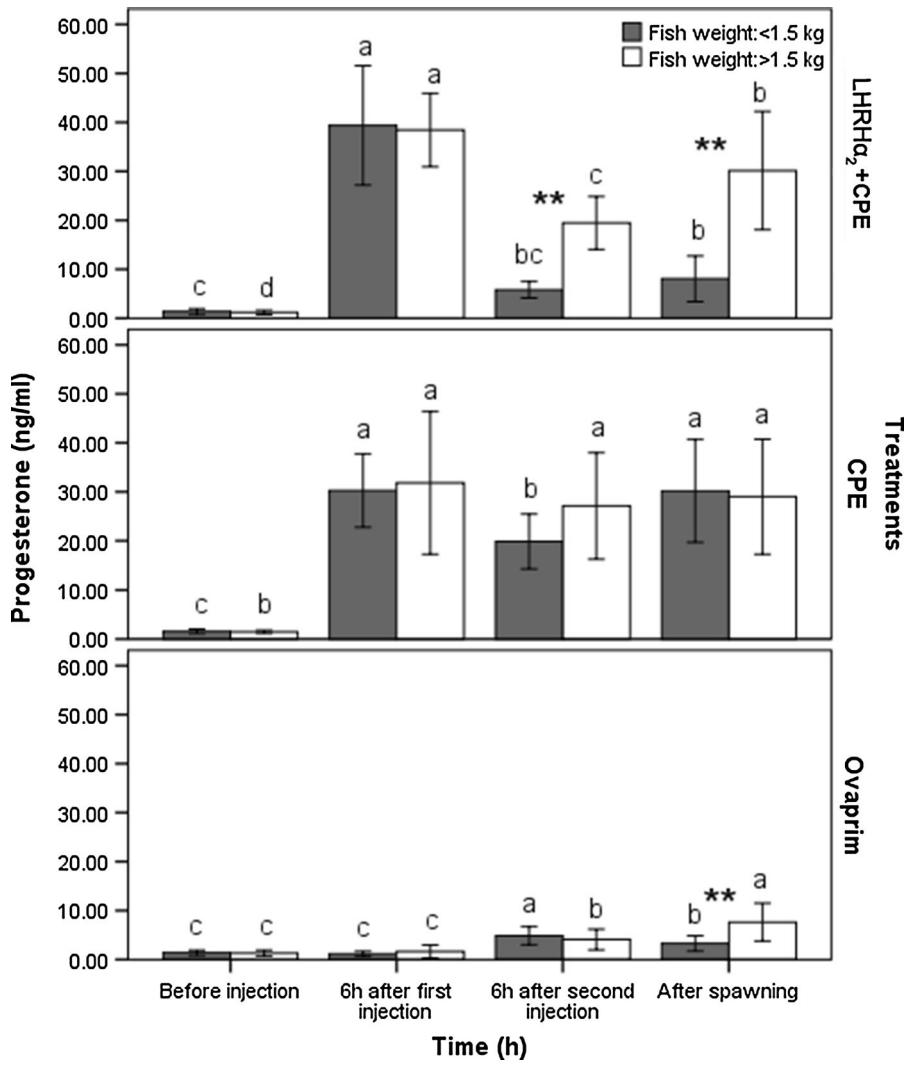

Fig. 3 Time-course changes in testosterone level of female bunnei fish under treatment of different spawning agents. All data were expressed as mean ± SDs ($n = 10$). Comparisons have been done in different times (one-way ANOVA) as well as weight groups (*t test*) within each treatment. *Different alphabetic letters* indicate significant differences ($P < 0.05$) between different sampling time, and *asterisks* ($*P < 0.05$, $**P < 0.01$) were applied to show any possible significance differences between two weight groups (<1.5 vs. >1.5 kg)

cortisol were found about 534.30 ± 55.51 and 498.53 ± 67.80 ng ml^{-1}, respectively, for size 1 and size 2 groups. Post-spawning concentrations for this hormone were measured as 503.12 ± 91.98 ng ml^{-1} for size 1 group and 437.55 ± 40.90 ng ml^{-1} for high-weight group. In the case of treatment B, over the period of before injection to 6 h after second injection as a whole, there was not any significant change in cortisol for both weight groups. At the end of experiment, when each individual fish spawned, marginal increases were observed in cortisol hormone, especially for high-weight group. Similar upward trend was observed for ovaprim injection but with more obvious increase, which shows significant change during injection (Fig. 5).

Parameters relationships

No significant relationship between cortisol and sex steroid hormones was observed except for sampling time, namely before injection. Serum cortisol in treatment A shows a linear regression ($n = 10$, $P = 0.003$, $r^2 = 0.677$) with P4 in size 1 group at before injection (Fig. 6). In treatment B, a linear relationship between cortisol and T ($n = 10$, $P = 0.049$, $r^2 = 0.402$) and P4 ($n = 10$, $P < 0.0001$, $r^2 = 0.799$) was also found before injection (Fig. 7). Initial cortisol level in treatment C indicates significant relationships with both T ($n = 10$, $P = 0.041$, $r^2 = 0.424$) and P4 ($n = 10$, $P = 0.022$, $r^2 = 0.499$) (Fig. 8).

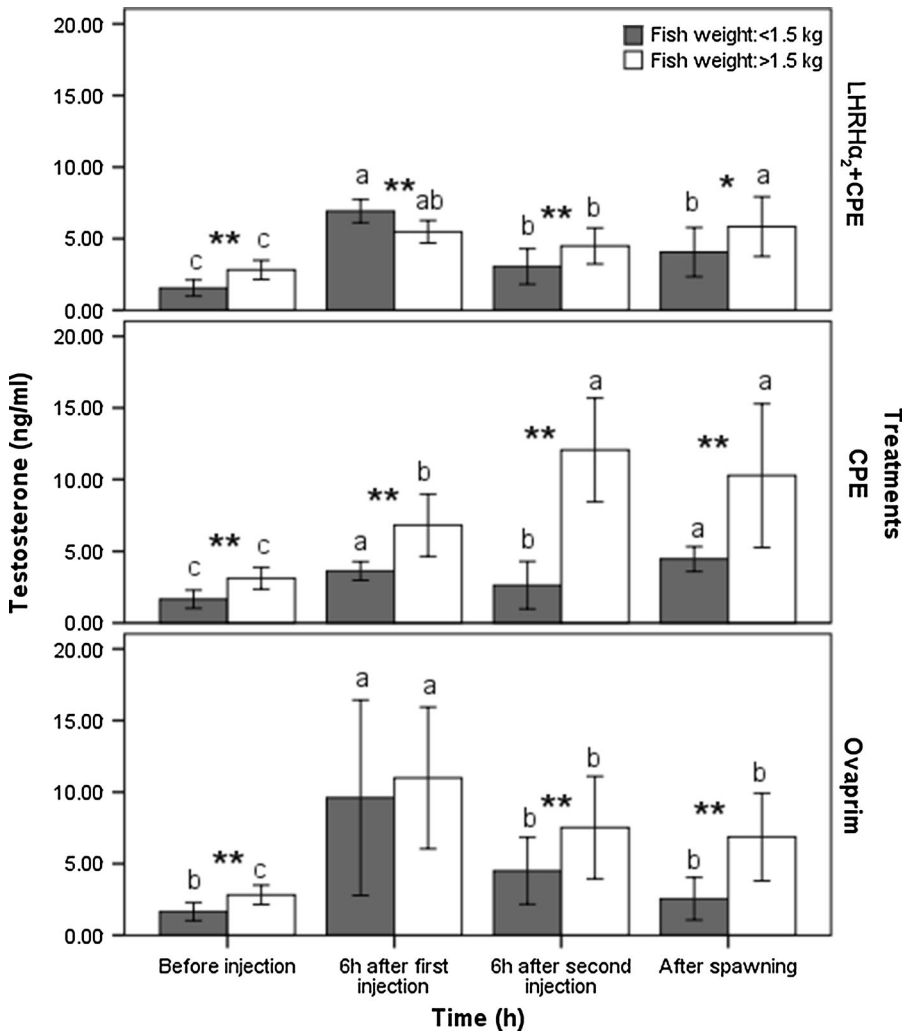

Fig. 4 Serum E2 level of female bunnei fish following different hormone. All data were expressed as mean ± SDs ($n = 10$). Comparisons have been done in different times (one-way ANOVA) as well as weight groups (*t test*) within each treatment. *Different alphabetic letters* indicate significant differences ($P < 0.05$) between different sampling time, and *asterisks* (*$P < 0.05$, **$P < 0.01$) were applied to show any possible significance differences between two weight groups (<1.5 vs. >1.5 kg)

Weight groups comparison (<1.5 vs. >1.5 kg)

The response of female bunnei to either CPE or LHRHα_2 revealed no significant difference in E2 between all sampling time. Ovaprim-injected females indicated higher ($P < 0.05$) amount of E2 in size 2 group in comparison with size 1 group after spawning (Fig. 2).

The concentrations of P4 in two different weight groups were similar before injection and after first injection with CPE, while following second injection in which LHRHα_2 applied, higher ($P < 0.05$) level of this hormone could be found in high-weight group. This trend lasted even after spawning (Fig. 3). There were no significant differences between the two weigh groups when injected by CPE. Treatment C resulted in higher ($P < 0.05$) P4 in serum of bunnei fish only when spawning occurred (Fig. 3).

T levels were higher either significantly or insignificantly (i.e., treatment C, 6 h after first injection) in size 2 group rather than that in size 1 group (Fig. 4).

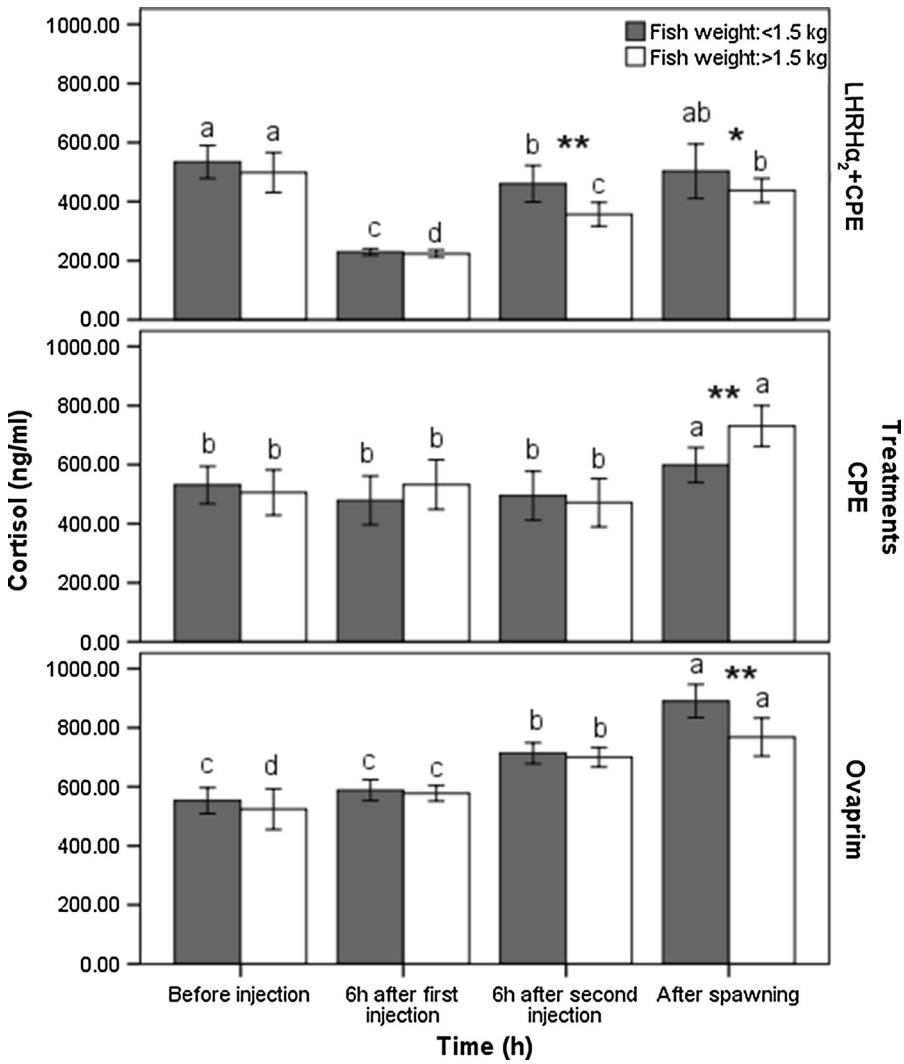

Fig. 5 Time-course changes in cortisol level of female bunnei fish under treatment of different spawning agents. All data were expressed as mean ± SDs ($n = 10$). Comparisons have been done in different times (one-way ANOVA) as well as weight groups (*t test*) within each treatment. *Different alphabetic letters* indicate significant differences ($P < 0.05$) between different sampling time, and *asterisks* ($*P < 0.05$, $**P < 0.01$) were applied to show any possible significance differences between two weight groups (<1.5 vs. >1.5 kg)

Cortisol level did not show significant differences in most cases, but after spawning we found higher content of cortisol in size 1 group (Fig. 5).

Treatment comparison

Comparisons between different hormone therapies have been shown that initial levels of all steroid hormones did not significantly differ. Higher level of cortisol was found in fish treated with treatment C rather than in fish treated with two other treatments ($P = 0.622$ and $P = 0.712$, respectively, for size 1 and size 2 groups). T response of fish to ovaprim was more obvious initially rather than two other treatments ($P = 0.009$ and $P = 0.002$). This pattern did not continue longer due to more content of this hormone in treatment B fish, especially for high-weight group. P4 levels in female broodstock fish ranged from 1.18 ± 0.44 to 1.58 ± 0.43 ng ml^{-1} before injection of any stimulating agents, which were not observed significant among different treatments. The lowest E2 and P4 were seen in treatment C during the experiment, and the most profound response can be seen in CPE-injected female broodstock (Table 2). When the weight groups ignored, the results of statistically analyses were nearly similar, but the uniformity of variance will be loose. Anyway, the results of treatment comparisons are shown in Table 3.

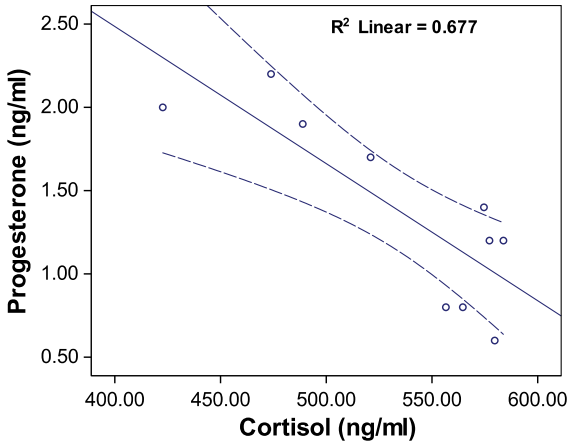

Fig. 6 Relationship between serum progesterone (ng ml^{-1}) and cortisol (ng ml^{-1}) in CPE + LHRHα_2 treatment at sampling time namely before injection. Significant negative correlation was observed between these two measured parameters. Linear relationship ($n = 10$, $P = 0.003$, $r^2 = 0.677$) with 95 % confidence interval (*dash line*) has been shown

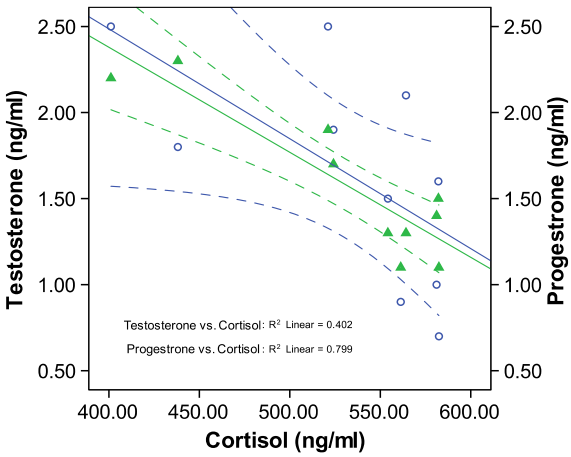

Fig. 7 Linear relationship between testosterone and cortisol (*blue line*) and between progesterone and cortisol (*green line*) before injection of CPE. 95 % confidence interval was expressed by *dash line* for each regression. (Color figure online)

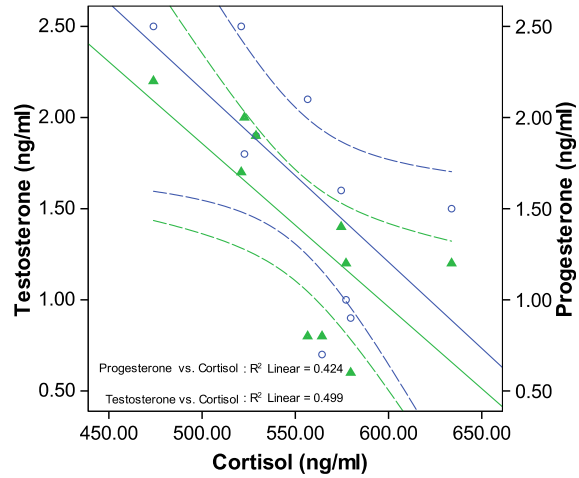

Fig. 8 Linear relationship between testosterone and cortisol (*blue line*) and between progesterone and cortisol (*green line*) before injection of Ovaprim. 95 % confidence interval was expressed by *dash line* for each regression. (Color figure online)

Discussion

General concept

This study examined difference in serum steroid hormones obtained from female bunnei fish when injected with three different inducing agents (CPE + LHRHα_2, CPE and ovaprim). Additionally, trends in serum steroid hormones and their relationships with serum cortisol were also analyzed at different sampling times following injections. If a breeder did not contribute to ovulation, suppose that this fish did not respond to hormone injection, and consequently, related data were ignored; i.e., analyses have been conducted only with collected data from breeders that respond positively and avoid further injection (Marte 1989).

Similar to other carp and catfish species, which did not undergo FOM and ovulation in captivity (Adebiyi et al. 2013), this species are not able to complete oogenesis unless hormone therapies have been applied. It has been observed that application of CPE and CPE + LHRHα_2 injections in female bunnei fish showed more reasonable outcomes when compared to ovaprim injection (we did not aim to measure related parameters because it has been done previously by Kahkesh et al. 2010), and we only focus on comparing some important parameters visually to check each treatments' efficiency. Although obtained results indicate differences in androgen T between different treatments, the most conspicuous differences were observed in the case of P4 and E2 between ovaprim and two other treatments, i.e., CPE + LHRHα_2 and CPE alone (Table 2).

Table 2 Effects of different hormonal agents on cortisol and serum steroids at each sampling time, including before injection, 6 h after first injection, 6 h after second injection and immediately after spawning

Time	Weight groups (kg)	CPE + LHRHα_2	CPE	Ovaprim	*P* value
Cortisol(ng/ml)					
Before injection	<1.5	534.30 ± 55.51	530.97 ± 63.22	553.27 ± 43.73	0.622
	>1.5	498.53 ± 67.80	505.53 ± 76.75	524.13 ± 69.03	0.712
6 h after first injection	<1.5	229.28 ± 11.30^{c}	478.98 ± 82.53^{b}	588.45 ± 35.22^{a}	**<0.0001**
	>1.5	223.97 ± 12.37^{b}	532.39 ± 83.79^{a}	578.07 ± 26.39^{a}	**<0.0001**
6 h after second injection	<1.5	460.50 ± 61.45^{b}	494.88 ± 82.65^{b}	713.83 ± 35.39^{a}	**<0.0001**
	>1.5	356.90 ± 40.27^{c}	471.40 ± 81.78^{b}	699.89 ± 33.05^{a}	**<0.0001**
After spawning	<1.5	503.12 ± 91.98^{c}	598.66 ± 59.35^{b}	890.28 ± 56.20^{a}	**<0.0001**
	>1.5	437.55 ± 40.90^{b}	730.76 ± 69.10^{a}	768.09 ± 64.40^{a}	**<0.0001**
Testosterone (ng/ml)					
Before injection	<1.5	1.55 ± 0.56	1.65 ± 0.64	1.65 ± 0.64	0.915
	>1.5	2.82 ± 0.67	3.12 ± 0.76	2.82 ± 0.67	0.551
6 h after first injection	<1.5	6.92 ± 0.81^{ab}	3.63 ± 0.65^{b}	9.60 ± 6.82^{a}	**0.009**
	>1.5	5.48 ± 0.79^{b}	6.81 ± 2.16^{b}	10.99 ± 4.94^{a}	**0.002**
6 h after second injection	<1.5	3.06 ± 1.24^{ab}	2.63 ± 1.66^{b}	4.50 ± 2.43^{a}	**0.070**
	>1.5	4.49 ± 1.26^{c}	12.05 ± 3.63^{a}	7.52 ± 3.58^{b}	**<0.0001**
After spawning	<1.5	4.06 ± 1.72^{a}	4.47 ± 0.87^{a}	2.55 ± 1.48^{b}	**0.012**
	>1.5	5.84 ± 2.08^{b}	10.27 ± 5.01^{a}	6.86 ± 3.05^{b}	**0.027**
Progesterone (ng/ml)					
Before injection	<1.5	1.38 ± 0.55	1.58 ± 0.43	1.38 ± 0.55	0.613
	>1.5	1.18 ± 0.44	1.46 ± 0.32	1.32 ± 0.59	0.416
6 h after first injection	<1.5	39.37 ± 12.19^{a}	30.25 ± 7.47^{b}	1.14 ± 0.52^{c}	**<0.0001**
	>1.5	38.43 ± 7.49^{a}	32.35 ± 12.36^{b}	1.64 ± 1.33^{c}	**<0.0001**
6 h after second injection	<1.5	5.82 ± 1.69^{b}	19.87 ± 5.56^{a}	4.86 ± 1.85^{b}	**<0.0001**
	>1.5	19.47 ± 5.41^{b}	27.14 ± 10.82^{a}	4.09 ± 2.11^{c}	**<0.0001**
After spawning	<1.5	8.06 ± 4.68^{b}	30.18 ± 10.49^{a}	3.28 ± 1.57^{b}	**<0.0001**
	>1.5	30.15 ± 12.09^{a}	29.04 ± 11.71^{a}	7.62 ± 3.87^{b}	**<0.0001**
E2 (pg/ml)					
Before injection	<1.5	264.01 ± 16.67	260 ± 16.40	258.01 ± 13.84	0.688
	>1.5	269 ± 31.60	273 ± 26.17	264.72 ± 34.31	0.825
6 h after first injection	<1.5	1750.69 ± 37.39^{b}	2439.80 ± 308.10^{a}	327.61 ± 81.68^{c}	**<0.0001**
	>1.5	1943.4 ± 33.44^{b}	2455.00 ± 349.26^{a}	424.38 ± 233.82^{c}	**<0.0001**
6 h after second injection	<1.5	2044.50 ± 284.90^{b}	2634.30 ± 243.91^{a}	440.97 ± 85.15^{c}	**<0.0001**
	>1.5	2035.10 ± 419.52^{b}	2589.72 ± 258.48^{a}	441.06 ± 101.47^{c}	**<0.0001**
After spawning	<1.5	2153.10 ± 429.78^{b}	2483.50 ± 290.27^{a}	490.80 ± 65.51^{c}	**<0.0001**
	>1.5	2325.80 ± 254.41^{a}	2506.00 ± 226.81^{a}	1373.30 ± 228.94^{b}	**<0.0001**

Comparisons were performed at each time course and within each weight group, namely <1.5 kg and higher than 1.5 kg. Letters express significant differences with exact *P* value (Bold faced type)

Table 3 Effects of different hormonal agents on cortisol and serum steroid hormones at each sampling time, including before injection, 6 h after first injection, 6 h after second injection and immediately after spawning

Time	CPE + LHRHα_2	CPE	Ovaprim	*P* value
Cortisol (ng/ml)				
Before injection	516.41 ± 63.039	518.25 ± 69.66	538.70 ± 58.19	0.476
6 h after first injection	226.62 ± 11.84^{c}	505.68 ± 85.45^{b}	583.26 ± 30.75^{a}	**<0.0001**
6 h after second injection	408.70 ± 73.35^{c}	483.14 ± 80.92^{b}	706.86 ± 34.08^{a}	**<0.0001**
After spawning	470.33 ± 77.01^{c}	664.71 ± 92.31^{b}	829.18 ± 85.96^{a}	**<0.0001**
Testosterone (ng/ml)				
Before injection	2.18 ± 0.88	2.38 ± 1.01	2.23 ± 0.87	0.779
6 h after first injection	6.20 ± 1.07^{b}	5.22 ± 2.25^{b}	10.29 ± 5.84^{a}	**<0.0001**
6 h after second injection	3.77 ± 1.41^{b}	7.34 ± 5.55^{a}	6.01 ± 3.32^{a}	**0.016**
After spawning	4.95 ± 2.07	7.37 ± 4.59	4.70 ± 3.21	0.053
Progesterone (ng/ml)				
Before injection	1.28 ± 0.49	1.52 ± 0.37	1.35 ± 0.56	0.280
6 h after first injection	38.90 ± 9.86^{a}	31.48 ± 10.39^{a}	1.39 ± 1.01^{b}	**<0.0001**
6 h after second injection	12.64 ± 8.01^{b}	23.50 ± 9.17^{a}	4.47 ± 1.97^{b}	**<0.0001**
After spawning	19.10 ± 14.42^{b}	29.61 ± 10.83^{a}	5.45 ± 3.64^{c}	**<0.0001**
E2 (pg/ml)				
Before injection	266.55 ± 24.72	266.66 ± 22.32	261.36 ± 25.69	0.734
6 h after first injection	1840.61 ± 57.63^{b}	2447.40 ± 320.63^{a}	375.98 ± 118.77^{c}	**<0.0001**
6 h after second injection	2041.51 ± 411.85^{b}	2604.30 ± 249.91^{a}	441.15 ± 96.15^{c}	**<0.0001**
After spawning	2239.45 ± 354.96^{b}	2494.95 ± 253.81^{a}	934.55 ± 483.87^{c}	**<0.0001**

Comparisons were performed at each time course by pooling two weight groups. Statistical differences were analyzed by using Games–Howell since homogeneity of variance was not achieved. Letters express significant differences with exact *P* value (Bold faced type)

Trends in steroid hormones

Changes in sex steroid hormones from the beginning of experiment (i.e., before injection) to the injection period have been measured in this study. Serum E2 levels of female fish were surged following injection of CPE or CPE + LHRHα_2 and remained high even after spawning. This increasing pattern was also observed in ovaprim treatment, but with light elevation rather than those reported for two other treatments. T was also increased following CPE or CPE + LHRHα_2 injections with a light fluctuation over the experimental period. LHRH injection in black bream, *Acanthopagrus butcheri*, indicated elevations in plasma E2 and T on day 1 and 2 post-injection when compared to saline-injected fish (Haddy and Pankhurst 2000). The rise of E2 might be related to elevation of ovarian production of E2, which is likely due to the presence of GTH II to stimulate aromatization of T to E2 in granulosa layer of mature oocytes in the (Adebiyi et al. 2013; Barcellos et al. 2001). The concentrations of both E2 and T rose following LHRH injection in yellow perch, *Perca flavescens*, while it decreased once eggs released (Rinchard et al. 2002). As suggested by Rinchard et al. (2002), this kind of decline is associated with the ovulatory gonadotropin (GtH II) surge. Similar pattern was only observed in ovaprim treatment but not in CPE or CPE + LHRHα_2 here. Higher levels of T rather than E2 are indeed related to the change in steroidogenesis pattern of mature follicle, which produces more T when compared to that produced in vitellogenesis period (Kagawa et al. 1984). Serum P4 level responds to CPE injection in two first treatments and remains unchanged afterward only in CPE treatment. LHRHα_2 as second injection could not maintain this steroid hormone at peak especially in the case of size 1 group. In the contrary, ovaprim does not provide such rapid change even after spawning.

Comparison of treatments

CPE-inducing program has become commonplace to induce FOM, ovulation and spawning in cyprinid species such as bunnei broodstocks. This homogenate contains several hormones, including GtHs, prolactin, growth hormone, thyroid stimulating hormone, which stimulate gonadal steroidogenesis and vitellogenesis (Berlinsky et al. 1997). The inability of ovaprim to induce changes in serum steroid hormones may be related to either the absence of additional hormones found in CPE or a dose-related phenomenon. Similar hypothesis has been previously suggested for hCG (Berlinsky et al. 1997). On the other hand, the high suitability of ovaprim to induce ovulation in other Barbus species, i.e., *Barbus barbus*, was recently approved (Targońska et al. 2011). In another study, injection of ovaprim in yellowfin bream, *Acanthopagrus australis*, was not effective as seen for LHRH treatment alone (Cowden 1995). Previously, it has been documented that single injection of ovaprim (0.5 ml kg^{-1}) did not cause a reasonable stimulation in female bunnei fish (Kahkesh et al. 2010). Investigation by DiMaggio et al. (2013) indicated that single lower dose of ovaprim (0.25 and 0.5 ml kg^{-1}) was superior to the higher amount of that (i.e., 1 or 2 ml kg^{-1}) in pinfish, *Lagodon rhomboides*. In addition, the authors found more dose efficacy for ovaprim rather than hCG (0.25 vs. 4000 IU kg^{-1}), suggesting that this divergence may attribute their probable difference in mechanism of action to other hormones. In spite of anti-dopamine portion, which is prepared in ovaprim, it could not trigger the endocrine procedure for FOM in this species. Dopamine inhibitory factors have been well evidenced in most carp species (Podhorec and Kouril 2009). However, the more efficacy of GnRH in combination with domperidone was approved for this species (Mohammadian et al. 2009).This discrepancy might be, however, related to applied dose of ovaprim, which was higher in such aforementioned study rather than present. Our results suggest that larger dose of ovaprim rather than that used here might be required to induce endocrine system for FOM and ovulation in female bunnei fish. Furthermore, it seems that double high-ovaprim injections could diminish its efficacy. Similarly, 1 ml kg^{-1} ovaprim induced optimum fecundity in *Clarias batrachus* as compared to either higher or lower injected doses (Sahoo et al. 2007). The possible mechanism underlying this phenomenon might indeed be associated with high level of GnRH, which resulted in low LH secretion as anti-gonadotropic effects on pituitary (DiMaggio et al. 2013). Interestingly, this hypothesis can be supported by lower amounts of E2, T and P4 which were observed following double injections of ovaprim (0.1 and 0.15 ml kg^{-1}) here. These opposing results may be partially attributable to the mechanism of action for each of the hormones in question.

Relationship with cortisol

Plasma cortisol in black bream, *A. butcheri*, was observed high following LHRH and hCG treatments that were similar to saline-injected fish. Therefore, the authors suggested that hormone treatments had no effect on plasma cortisol levels (Haddy and Pankhurst 2000). Obtained data from the present study indicated that injection of different stimulating agents did not provoke the increase in serum cortisol level, especially in the case of CPE and CPE + LHRHα_2 injections until volitional spawning or manual stripping has been occurred. Previously, it has been observed that handling stress in wild-caught fish causes suppressions in T and E2 which is likely to be in coincident with higher amount of plasma cortisol (Clearwater and Pankhurst 1997; Haddy and Pankhurst 1999). This kind of stress can impair endocrine response to hormone therapy on day following capture (Haddy and Pankhurst 2000). It is noteworthy that ovaprim treatment led to higher amount of serum cortisol in comparison with CPE and CPE + LHRHα_2 treatments. Therefore, it might imply the lower response of female bunnei, treated with ovaprim due to lower concentration of measured steroids. In the present study, the significant reverse relationship between cortisol and T was observed in all treatments prior to injection except for high-weight groups. In addition, P4 shows similar relationship with cortisol only in CPE and ovaprim treatments. The higher serum cortisol was measured following spawning or striping stress, which was similar to previous findings in cyprinid and salmonid species (Schreck et al. 2001; Stratholt et al. 1997). It is likely due to either a physiological change such as swelling of body cavity, which is related to gonadal volume (Kubokawa et al. 1999), or egg stripping stress.

Weight groups

Based on current situation, it is better to select breeders weighing more than 1.5 kg for reproduction purpose due to more satisfactory results in commercial hatcheries. Present study indicated that female fish weighing more than 1.5 kg have higher amount of T in serum rather than size 1 group. This difference was higher when CPE treatment was applied. T in female fish plays an important role for FOM and ovulation (Kime 1993), and therefore, undesirable responses of fish in size 1 group are likely supposed to be due to lower amount of this hormone in serum. Similarly, Almeida et al. (2013) suggested selection of female breeder of Nile tilapia, *Oreochromis niloticus*, weighing around 600 g due to better egg quality value and fecundity.

Conclusion

In summary, this study reveals that measurement of sex steroids hormones is a valuable tool to predict fecundity and gonadal maturation of *B. sharpeyi*. Although several environmental factors could effect on reproductive efficiency of fish, we aimed these parameters (as mentioned in "Materials and methods" section) to be under control during the study to avoid any probable discrepancies in reproduction outcomes. However, the most profound oocyte development was observed in CPE and, to some extent, in CPE + LHRHα_2 treatments, which were associated with higher serum E2 level in comparison with ovaprim. Thus, this can likely confirm the role of E2 for vitellogenesis and FOM (Adebiyi et al. 2013) and indicates that ovaprim did not impel enough the pituitary to release GTHs and sequentially trigger steroidogenesis in female bunnei fish.

The relationship between serum cortisol and sex steroid hormones indicate that female bunnei fish are probably susceptible to be stress-induced by hormone therapy, especially in the case of weight group <1.5 kg.

Although the CPE treatment showed the best performance in most reproductive steroid hormones, the criteria used for selection of hormone therapy will always depend on the desired goals, e.g., in light of aquaculturists' purposes; improvement of egg production would certainly be the most appropriate plan. However, this kind of decision making can contribute to conservation programs and aquaculture.

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