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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (8): 1486-1491, 2006
ISSN 1028-8880

The Effects of [D-Ala^6 Pro^9 NEt]-LHRHa and LHRHa + Pimozide on Plasma Sex Steroid Profiles in Adult Female Seabream (*Sparus aurata*)

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Abstract: The present study examined changes in the concentrations of plasma testosterone (T), progesterone (P4) and estradiol-17β (E_2) to determine changes in serum hormone profiles during the functional female phases in *Sparus aurata*. The fish were treated with [D-Ala^{6}Pro^9 NEt]- luteinizing hormone releasing hormone analogue (LHRHa) alone, LHRHa plus pimozide (PIM) and Physiological Saline (PS) alone to stimulate gonadal development and sexual maturation. All fish were sampled and plasma levels of oestradiol (E_2) Testosterone (T) and progesterone (P4) were measured by radioimmuno assay. LHRHa treatment alone, or in combination with PIM, elevated serum E_2 and T concentrations ($p<0.05$) at 6, 24, 48 h after treatments while plasma P4 concentrations were unaffected by the treatments ($p>0.05$). Vitellogenesis was also stimulated by a combined LHRHa and PIM treatment. Responses to treatment with LHRHa plus PIM were comparable to those treated with LHRHa alone ($p>0.05$), suggesting that dopamine receptor antagonist, pimozide may not inhibit dopamine secretion in *S. aurata*.

Key words: *Sparus aurata*, LHRHa, pimozide, sex sterids

INTRODUCTION

Gilthead sea bream, *Sparus aurata*, a marine protandrous hermaphrodite fish, is important for aquaculture of Mediterranean countries, including Turkey (Tekelliolu, 1998; Arabacı, 2000; García *et al*., 2003). It has an annual reproductive cycle with a multiple spawning pattern occurring from December to May, Kadmon *et al.* (1984, 1985). Males reach sexual maturity during the first year of life. Under certain environmental conditions all fish remain males during their second year but some undergo testicular regression and ovarian recrudescence and become functional females, during which ovaries contain oocytes at varying stages of development Zohar *et al.* (1995). However, these fish do not spawn spontaneously in captivity and ovulation is not synchronized within a population. In fish culture, spawning can be induced by exogenous hormone administration (Prat *et al*., 2001; Garciá *et al*., 2003; Firat *et al*., 2005). Hormonal induction of spawning is necessary for species that do not spawn spontaneously in captivity. However, this technique may also be very useful to synchronise spawning in those species, which spawn normally in captivity.

Fish pituitary extract and mammalian gonadotropins or super active analogues of mammalian Luteinizing Hormone Releasing Hormone (LHRHa) alone or in combination with dopamine receptor antagonists have been widely used to induce ovulation in fish, including a number of sparids Zohar *et al.* (1986), Crim *et al.* (1987), Pankhurst and Carragher (1992), Ventling and Pankhurst (1995) and Firat *et al.* (2005).

There are several studies investigating effects of LHRHa and LHRHa+PIM treatments on maturation and ovulation in sea bream but, to our best knowledge, no study on effects of the gonodal sex steroids has been reported. Sex steroids secreted by gonads are believed to have a role and affect on the vitellogenesis and maturation in fish (Cornish, 1998; Prat *et al*., 2001). Although profiles of steroids during the spawning season have also been reported in *Sparus aurata* Eckstein *et al.* (1978), João *et al.* (1999), *Acanthopagrus butcheri* Haddy and Pankhurst (2000), *Acanthopagrus schlegeli* Chang and Yueh (1990) and seabass *Dicentrarchus labrax* Prat *et al.* (2001). Little is known about the plasma and gonadal changes in steroid hormone concentrations during the reproductive cycle in *S. aurata*.

The objective of this study is two fold. First, we examined the changes in plasma estradiol-17 β (E_2), testosterone (T) and progesterone (P4) concentrations after injection with [D-Ala^6 Pro^9 NEt]-LHRHa alone or together with the dopamine receptor antagonist pimozide

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(PIM), during the functional female phases in *S. aurata*. Second, the study investigated interaction and effects of the steroid hormones LHRHa and PIM known to be involved in the regulation of maturation and gonodal development in teleosts and other vertebrates, on the ovarian development in *S. aurata*.

MATERIALS AND METHODS

Experimental fish: Sea bream used in this experiment were obtained from a commercial fish farm in Antalya, Turkey. Experiment was conducted at the Beymelek Aquaculture Research Centre in Antalya in outdoor rectangular Fiber glass tanks under natural conditions of photoperiod and temperature. Fish were fed to satiation three times per day, once with moist paste made at two-days intervals by mixing a commercial sea bream dry pellet, (Pınar Yem, Izmir) frozen anchovy flesh and fish oil and twice with a commercial sea bream pellet, (Korkuteli Yem, Korkuteli). Prior to experiment fish were sexed by cannulation Ross (1984) and marked using an Indian dye (Mysore Paints and Varnish Ltd., Karnataka, India). Experiments were conducted on 2 to 3 year-old, reproductively functional female sea bream. After 1 week of acclimation to the conditions, females were then identified by biopsy as described previously Tao *et al.* (1993). Fish were anaesthetized with 2-phenoxyethalon at a concentration of 1:20000 in water (Sigma Chem, Co., Dorset, UK). Gonads were removed through a 1 to 1.5 cm incision in ventral body wall, fixed in buffered formaldehyde and embedded in paraffin for subsequent histological examination Bancroft and Stevens (1990). The incision was then sutured and the fish returned to the tank. Two weeks after biopsy, the fish received an intramuscular injection of either fish saline (control) or [D-Ala^{6}Pro^9 NEt]-LHRHa (Sigma Chemical Co. St Louis, MO) dissolved in fish saline.

LHRHa and LHRHa+pimozide administration and sampling procedure: Twenty one fish were randomly divided into three groups of seven fish each. [D-Ala^6 Pro^9 NEt]-LHRHa solution was administered to the first group, as a series of intramuscular injections on alternate sides at a dose of 2.5 μg kg^{-1} BW. In the second group, fish were injected with PIM (10 mg kg^{-1} BW) followed 3 h by a single injection of [D-Ala^6 Pro^9 NEt]-LHRHa. As controls, fish in the third group were injected with 0.5 mL kg^{-1} Physiological saline (0.7% NaCl; 0.5 mL kg^{-1}) alone Pankhurst and Carragher (1992). Injections in each group were administered three times at 6, 24 and 48 h. Blood was sampled for determination of the plasma E_2, T and P4 concentrations from all treated fish prior to initial injection and 6, 24 and 48 h after the first, second and third injection. All fish from each experimental group were sacrificed following the final blood sampling and their gonads were fixed in formaldehyde solution for histological examination. The stage of ovaries development was determined for each fish. Classification was based on the histological criteria adapted from Bromaga and Cumaranatunga (1987).

Assays: A 3 mL sample of blood was collected from the caudal vasculature using a 10 mL heparinized vacutainer with a 21 gauge needle. Plasma was separated by centrifuging at 4000 rpm for 10 min at 4°C and stored at-20°C until assayed. Plasma E_2 and T concentrations were measured in duplicate 50 and 20 μL aliquots, respectively using a commercially available kit (RIA_S Diagnostic Products, Los Angeles, CA.) Extractions of E_2 and T were performed according to the protocol, described by Pankhurst and Conroy (1987). Both assays use iodinated tracer ligands. Standards were prepared in charcoal-stripped *S. aurata* serum. The anti-17β-esradiol-6 (o-carboxymethyl)-oxima cross-reacts with estradiol (100%), anti-testosteron-17 α-carboxyl-thioether cross-reacts with testosterone (100%). The log^{10} steroid values were compared using one-way analysis of variance and Duncan's Multiple Range Test, Zar (1984).

RESULTS

Ovarian follicles at very early developmental stages were detected in *S. aurata* early on the first day of the experiment. Histological examination and macroscopic observations of the ovaries at all stages studied showed the presence of previtellogenic oocytes, but not vitellogenic oocytes in the gonad of fish (Fig. 1). Nucleus contained numerous small nucleoli, which were often arranged at the periphery of the germinal vesicle. *S. aurata* contained ovarian follicles at various stages of development at the end of this study. Status of oocyte maturation exhibited slight differences: oocytes of different sizes were common while some fish were in the advanced stage of development. Cytoplasm was filled with yolk and micropyle clearly visible. The nuclear membrane was disintegrated and the nucleus present was small (Fig. 2). During the disintegration the nucleoli migrated from the nucleus membrane towards the centre of the nucleus. The nucleoli were then decreased in the number and diameter and became unapparent in broken down nucleus.

Experiments were also conducted to investigate the effects of [D-Ala^6 Pro^9 NEt]-LHRHa alone and together with the pimozide on ovulation and sex steroids. LHRHa

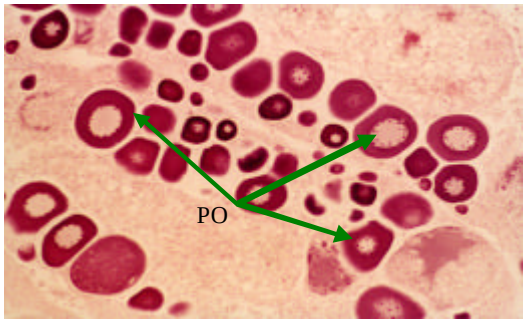

Fig. 1: Biopsy sample of the gonad from a female *S. aurata*, showing oocytes at previtellogenic stage, PO, previtellogenic oocyetes (Haematoxylin and Eosinx 400)

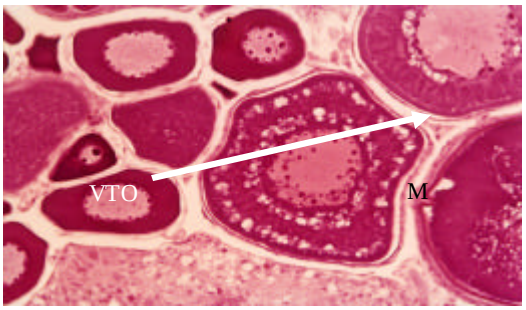

Fig. 2: Cross-section of gonad from a female *S. aurata*, after injections of [D-Ala^6 Pro^9 NEt]-LHRHa (2.5 μg kg^{-1}) and Pimozide (10 mg kg^{-1}), VTO, vitellogenic oocyetes; M, micropyle (H and Ex40)

and PIM treatment, alone or in combination, did not significantly induce ovulation. Although the oocytes at maturation stage were clearly detected; diameters of oocytes in treatment groups tended to be greater than those in the corresponding controls. In most samples obvious micropyle and yolk platelets was observed (Fig. 2). Figure 3 and 4 show plasma 17 β-estradiol and testosterone concentrations measured 6, 24 and 48 h after injections. A significant increase in plasma E_2 and T concentrations was observed 6 h after injections (Fig. 3 and 4; $p<0.05$).

Plasma P4 concentration remained unchanged 6, 24 and 48 h after injections (Fig. 5 and $p>0.05$). The fish in LHRHa and LHRHa+PIM treated groups had significantly higher E_2 and T values than control fish at 6 h after injections ($p<0.05$, Fig. 3 and 4). E_2, T and P4 were measured as 0.334±0.145, 0.033±0.030 and 0.338±0.056, respectively in plasma amples 24 h before injections. After injections, serum E_2 and T concentrations in LHRHa and

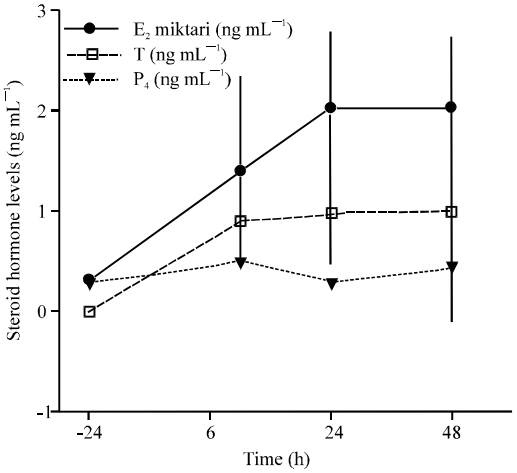

Fig. 3: Serum E_2, T and P4 levels in female *S. aurata* 24, 6, 24 and 48 h were following intraperitoneal injections of LHRHa. Values are reported at the mean±SE, n = 5

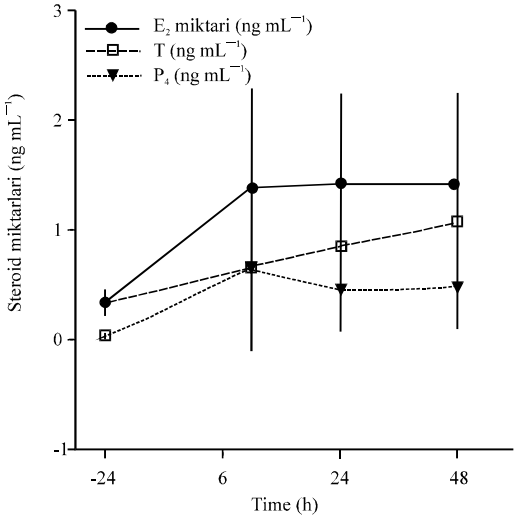

Fig. 4: Serum E_2, T and P4 levels in female *S. aurata* 24, 6, 24 and 48 h following intraperitoneal injections of LHRHa+PIM. Values are reported at the mean±SE n = 5

LHRHa+PIM treated groups were 3.3 times higher than those in control group, but the P4 concentration was remained low similar to that of the control group. Plasma P4 concentrations were comparable among the groups. On the other hand, all groups (controls and treatments) had similar plasma T and E_2 concentrations. Although plasma T level varied concomitantly with plasma E_2 concentration, the plasma level of T was lower than that of measured for plasma E_2 during the experiment.

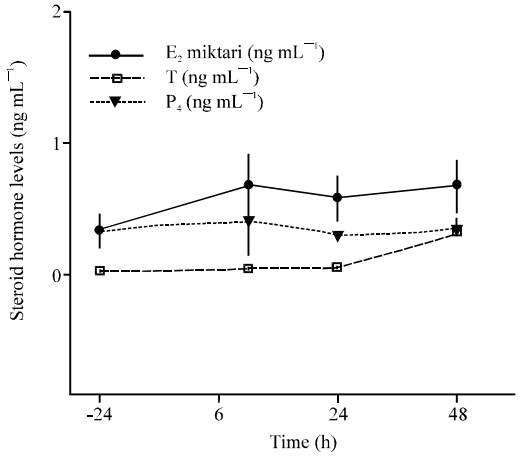

Fig. 5: Serum E_2, T and P4 levels in female *S. aurata* 24, 6, 24 and 48 h following intraperitoneal injections of 0.07% physiological saline (Control group) Values are reported at the mean±SE n = 5

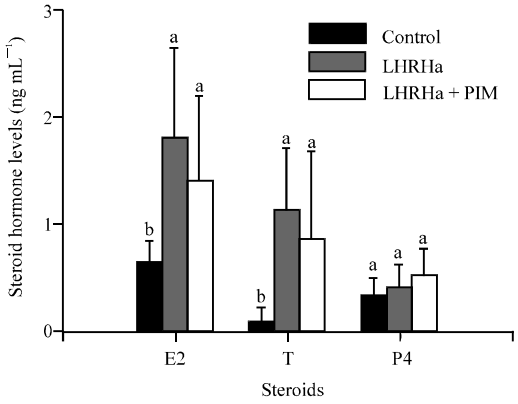

Fig. 6: Serum E_2, T and P4 levels in 3+ years old female *S. aurata* after injections of LHRHa, LHRHa+PIM and physiological saline. Values are reported at the mean±SE n = 5

Treatment with LHRHa resulted in a significant increase in Plasma T and E_2 concentrations in females compared with control(s). By contrast, plasma T and E_2 concentrations were lower during the combined LHRH/PIM treatment than during LHRHa alone. Although treatment with LHRHa and LHRHa+PIM resulted in higher concentrations of E_2 and T in comparison with PS-injected groups (controls), combining LHRHa with PIM did not induced a greater increase in plasma steroid concentrations than did LHRHa alone (Fig. 6, $p>0.05$).

DISCUSSION

The results of the present study demonstrate that treatment with LHRHa, alone or in combination with PIM could increase the amount of sex steroids and subsequent ovarian development in the *S. aurata*. Treatment with LHRHa presumably stimulates the secretion of gonadotropic hormone(s), which in turn may increase the sex steroids and induce the development of ovarian lobules. It is well established that GnRH stimulates the release of GtH in *Sparus aurata*, Kadmon *et al.* (1985), Pankhurst and Conroy (1987) and Zohar *et al.* (1995). In the present study, treatment with LHRHa found to promote sex steroid production, elevate serum E_2 and T levels and stimulate ovarian growth. These *in vivo* results are in agreement with those previously reported for black bream, *Acanthopagrus butcheri by* Haddy and Pankhurst (2000), snapper, *Pagrus auratus*, by Pankhurst and Carragher (1992), *Dicentrarchus labrax* by Prat *et al.* (2001) where the treatment of GnRHa induced an increase of plasma sex steroids. The sharp increase in plasma E_2 and T levels could occur as a consequence of a high aromatase activity in the ovary at the moment of the LHRHa administration. However, present results are in contrast with the situation in salmonids where the treatment of GnRHa induced a decrease of plasma sex steroids (Sower *et al.*, 1984; Van Der Kraak *et al.*, 1984).

Morehead *et al.* (1998) showed that multiple ovulations in fish, treated with LHRHa were dependent on recruitment of previtellogenic oocytes into vitellogenesis and were associated with elevated plasma T and E_2 levels. Based on these findings they suggested that multiple ovulations in *S. aurata* might also have been supported by serial recruitment of follicles from various stages of vitellogenesis.

In this study, *in vivo* administration of LHRHa and LHRHa+PIM resulted in an increased serum T and E_2 levels in sea bream, during which previtellogenic oocytes developed into vitellogenic oocytes. Although responses to both treatments (LHRHa alone or with PIM) exhibited similar patterns, the serum oestrogen levels in all groups before and after the treatments were constantly higher than those of testosterone. The patterns of responsiveness of see bream to the administrations observed in this study are similar to patterns previously described in other females of sparids Kadmon *et al.* (1984), Ventling and Pankhurst (1995) and they support the widely held hypothesis that E_2 plays an important role in initiating and maintaining vitellogenesis. Previous studies have shown that GnRH stimulates GtH secretion in the *S. aurata* and that dopamine may not inhibit GnRH-stimulated GtH release, Kadmon *et al.* (1985). This is consistent with the present study where injection of LHRHa+PIM induced greater responses to serum steroid levels and vitellogenesis, comparing fish treated with LHRHa alone. Therefore, dopamine-inhibition of GtH-

secretion appears to be non-existent in *S. aurata*. However, in some other teleost species, Yaron (1995), Haddy and Pankhurst (1998) and Morehead *et al.* (1998), blocking the actions of dopamine by the administration of dopamine antagonists such as pimozide/domperidone has been shown to greatly potentiate the actions of GnRH peptides on GtH release and steroids Peter *et al.* (1988) and Murthy *et al.* (1994). GnRH has also been shown to act directly on ovarian follicles to modulate vitellogenesis and steroidogenesis Morehead *et al.* (1998), which is similar to the present study.

In conclusion, the results of the present study indicate that the initial signal for induction of sex steroids and vitellogenesis is might be neuroendocrine and that GnRH is a key factor in *S. aurata*.

ACKNOWLEDGEMENTS

We wish to thank Prof. Niall Bromage and Haydar Fersoy for their kind suggestions and we are also grateful to Erdal Saban who helped us to establish steroid radioimmuno assays.

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