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Sex Steroid Levels and Breeding Performance of F_1 Generation Malaysian Mahseer, *Tor tambroides* (Bleeker, 1854) by Removal of Dopaminergic Inhibition

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ABSTRACT

Induced ovulation of captive Malaysian mahseer (*Tor tambroides*) often encounters low reproductive performances. The present study was conducted to gain insight to problems associated with poor breeding performance of Malaysian mahseer in captivity. In this study, sex steroid hormone levels and ovulation performance in filial 1 (F_1) generation of Malaysian mahseer (*Tor tambroides*) were investigated. Fishes were injected with ovatide, combination of Salmon Gonadotropin Releasing Hormone Analogue (sGnRHa), DOM, sGnRHa+DOM and 0.9% NaCl. A total of 30 matured females *T. tambroides* with weight ranged from 2.01-3.80 kg were used in this study. The females were given ovaplant (39.5-37.3 μg kg^{-1}) pretreatment for 6 weeks prior to hormones injection. Females were given a single intramuscular injection of ovatide (0.5 mL kg^{-1} b.wt.), sGnRHa (10 μg kg^{-1} b.wt.), DOM (5 mg kg^{-1} b.wt.), sGnRHa+DOM (10 μg kg^{-1} b.wt.+5 mg kg^{-1} b.wt.) or 0.9% NaCl. Blood samples were collected at 0, 6, 12 and 24 h (s) after injection. The effect of these treatments on sex steroids level (Testosterone (T) and 17β-Estradiol (E_2) and ovulation performance of F_1 *Tor tambroides* were evaluated. Result showed that higher ovulatory response was observed in the group treated with positive control ovatide (0.5 mL kg^{-1} b.wt.). Interestingly, it was observed that sGnRHa and Domperidone (DOM) produce highest egg and larval qualities compared to ovatide and sGnRHa alone. No ovulation occurred in the group treated with DOM alone. Sex steroids plasma concentration of T and E_2 increased significantly in association with ovulation. The levels fluctuated and reached a peak at 12 h, then dropped dramatically at 24 Post Injection (PI). In contrast the non-ovulated groups, the sex steroids hormone concentration showed little increment after injection and rose at 12 h PI, then slowly dropped at 24 h pi. These results provide evidence for a strong dopamine inhibition on GtH secretion in captive mahseer.

Key words: Dopamine antagonist, F1 generation, 17β-estradiol, testosterone, *Tor tambroides*

INTRODUCTION

In all vertebrate including fish, reproduction is controlled by Gonadotropin (GtH). Production of gonadotropin is under dual control (positive and negative). In the pituitary, Gonadotropin Releasing Hormone (GnRH) stimulates the synthesis of Gonadotropin (GtH). However, the presence of dopamine inhibits the function of this GtH. This leads to ovulation deficiency in cultured fish (Zohar and Mylonas, 2001). Studies have shown that the use of dopamine antagonist such as domperidone and pimozide with GnRHa combination can enhance ovulation and spawning of common carp (Lin *et al*., 1988), nase *Chondrostoma nasus* (Szabo *et al*., 2002), rohu *Labeo rohita* (Dasgupta *et al*., 2009) and *Tor tambroides* (Azuadi *et al*., 2011). Combination of GnRHa with dopamine antagonist induces higher release of luteinizing hormone released than using GnRHa alone (Taufek *et al*., 2009). Combination of dopamine antagonist such as Domperidone (DOM) and Pimozide (PIM) with LHRHa can markedly initiate the response of LHRHa and increase plasma GtH levels (Copeland and Thomas, 1989).

During the seasonal reproductive cycle, dopaminergic activity varies in fish (Senthilkumaran and Joy, 1995). In female fish, 17β-estradiol (E2) likely plays a major positive role in the regulation of Dopamine (DA) activity by increasing the dopaminergic inhibitory tone during vitellogenesis, as reported in several teleost species such as catfish, *Heteropneustes fossilis* and rainbow trout (Senthilkumaran and Joy, 1995; Saligaut *et al*., 1999; Linard *et al*., 1995). Thus, DA inhibitory tone would be maximal at the end of gametogenesis and would drop during spermiation and ovulation induction, under the internal ques control (e.g., a decrease in E2 levels) and/or environmental ques (Dufour *et al*., 2005).

The discovery of DA inhibition has important implication for aquaculture, where environmental conditions in captivity often lead to blockage of oocyte maturation and ovulation (Dufour *et al*., 2005). This phenomenon similar with *T. tambroides* which does not naturally spawn in tank conditions unless environmentally and hormonally induced (Azuadi *et al*., 2011). Artificial propagation method was used to improve production of *T. tambroides* in captivity and it help of natural spawning due to improper condition of the rivers (Yousefian and Mosavi, 2008). Combination of GnRHa and dopamine antagonist such as PIM or DOM was proposed to induced ovulation or spawning in mature females. This method (Linpe method) is widely applied to cyprinid around the world (Dufour *et al*., 2005).

This study highlighted preliminary results of sex steroid hormonal level and breeding performance of *Tor tambroides* induced with sGnRHa combination with DOM in controlled conditions.

MATERIALS AND METHODS

Experimental fish and broodstock selection: Thirty sexually matured filial 1 (F_1) generation of females *Tor tambroides* with total length and body weight ranged from 45-66 cm and 2.01-3.80 kg, respectively were used in this study. The fish were transported from Tarat Indigenous Fisheries Production and Research Center (IFPRC) Serian, Sarawak. Fishes were reared for 4-5 years in concrete tanks equipped with Recirculating Aquaculture System (RAS) at Lu Thian Tack (LTT) Aquaculture Farm, Asajaya, Sarawak. The fishes were fed in the morning with home made pellet (combination of vitamin C, angkabang fruit, emperang fish, wheat flour and squid oil) at 5% b.wt. The feed contained 62.6% crude protein, 17.6% crude lipid, 5.3% carbohydrate, 1.8% crude fibre, 12.7% ash and 4770.5 kcal g^{-1} energy. In the evening, the fish were fed with Tilapia pellet (Cargill, Malaysia) containing 16% protein, 6% crude fibre and 4% fat.

The female broodstock were selected and acclimatized in 20 m^3 concrete tanks before experiment. These fishes were maintained at ambient temperature of 26.5-28.0°C. For identification purposes, all the fish were tagged with microchip [American Veterinary Identification Devices (AVID), Norco, CA, USA]. Six weeks prior to induction activity, all fishes were implanted with slow release sGnRHa pellet, ovaplant (Syndel Laboratories, Vancouver, Canada) (75 and 150 μg pellets, 39.5-37.3 μg kg^{-1}).

The thirty females breeders were separated into five groups and fifteen sexually maturing male F_1 generation of *T. tambroides* were used for breeding activity. No hormone was injected to the male. Five different treatments of ovatide (0.5 mL kg^{-1} b.wt.), sGnRHa (10 μg kg^{-1} b.wt.), sGnRHa+DOM (10 μg kg^{-1} b.wt.+5 mg kg^{-1} b.wt.), DOM mg kg^{-1} b.wt. and 0.9% saline solution were utilised for the experiments. Ovatide was tested as a positive control and 0.9% saline solution was used as a negative control. Plasma sex steroid levels were observed at 0 h (prior to injection), 6, 12 and 24 h after injection.

Hormone preparation: Salmon gonadotropin releasing hormone (sGnRHa/OvaRH), was purchased from Syndel Laboratories, Vancouver, Canada. SGnRHa and DOM were dissolved in sterile physiological saline (0.9% NaCl) solution according to Chang *et al.* (1984) with some modifications before administration. Ovatide (from Hemmo Pharma, Mumbai, India) contained 20 μg of sGnRHa and 10 mg of domperidone in 1.0 mL.

Steroid hormone analysis: The 17β-estradiol (E2) and Testosterone (T) levels were measured in each treated female fish using Enzyme Immunoassay (EIA) kits from cayman chemical company (Ann Arbor, MI, USA). One milliliter of blood was extracted from the caudal vein of individual female fish using heparinized syringe fitted with 22-gauge needle at 0 h (prior to injection), 6, 12 and 24 h Post Injections (PI). The blood samples were centrifuged at 7,000 g for 10 min and plasma samples were collected. The plasma samples were aliquot and stored at -20°C till assayed. E_2 was extracted twice from the aliquot of plasma using methylene chloride (4x the sample, while diethyl ether (5x the sample) was used to extract T. The extracts were evaporated under nitrogen stream. The dry extract was reconstituted in 500 μL Enzyme Immuno-Assay (EIA) buffer by vortexing. E_2 and T were prepared and measured in duplicates. The E_2 and t-levels were quantified by EIA using (Gauthier-Clerc *et al.*, 2006) method with some modifications. The standard and sample absorbances were read at 405 nm by using microplate reader (ELISA, Vantaa, Finland). The absorbance values were analysed by using a computer spreadsheet (by Cayman) and downloaded from the website www.caymanchem.com. The steroid concentrations were expressed as mean and standard error.

Artificial propagation and egg quality determination: Six sexually matured *T. tambroides* were used. The fish were anesthetized with MS222 before implantation or injection to avoid any stress and to ease handling. In the morning, ovulation rate was evaluated as early as 23 h after injection. The eggs from ovulated fish were stripped into volumetric jar and the volume was recorded to estimate the total stripped eggs and fecundity. Dry method of fertilization (Joshi *et al.*, 2002) was used. Fertilization rate was determined at 7 h post fertilization. Hatching rate was determined by placing 100 eggs per petri disk (3 replicates) then the numbers

of hatched larvae were counted. By removing and counting dead larvae from each replicate small hapa, survival rate was determined and live larvae were recorded. Deformed and normal larvae were determined by sub sampling 100 larvae per rearing small hapa (3 replicates). Deformed larvae were determined those having bent notochord, abnormal head, bloated body and bent tail.

Water quality parameters: Water temperature, conductivity, dissolved oxygen, turbidity, pH and total dissolved solid at the hatchery were monitored daily using multiparameter water quality meter, WQC 24-1-2.

Statistical analysis: Differences in the mean for E_2, T, total stripped eggs, stripped fecundity, fertilization rate, hatching rate, survival rate, normal larvae and deformed larvae were tested using one-way Analysis of Variance (ANOVA). Duncan multiple range test at 95% confident level (post-hoc test) using SPSS 16.0 software was used and data were presented as Mean±Standard Error of the mean (SEM).

RESULTS

Daily water temperature, dissolved oxygen, pH, turbidity, conductivity and total dissolved solid ranged from 26.5-28.0°C, 5.50-13.3 mg L^{-1}, 7.20-8.0, 1% FS, 7.0-13.3% FS and 0.12-0.3 g L^{-1}, respectively. Breeding performance of *T. tambroides* treated with ovatide, sGnRHa, sGnRHa with DOM, DOM and saline solution is presented in Table 1. Group receiving DOM (5 mg kg^{-1} b.wt.) and 0.9% saline did not show any ovulation. Highest ovulation rate was observed in the group administrated with positive control ovatide (0.5 mL kg^{-1} b.wt.) followed by sGnRHa+DOM and sGnRHa with values of 50, 33 and 17%, respectively. The total stripped eggs (4560±1) and fecundity (1572±1) were significantly higher ($p<0.05$) in ovatide followed by sGnRHa treatment with total stripped eggs of 1433±1 and fecundity of 515±0.3. The lowest total stripped eggs (838±1) and fecundity (244±1) were observed in sGnRHa+DOM group. Lowest fertilization rate (17%) was shown in the group treated with sGnRHa. Treatment with sGnRHa+DOM produced significantly higher ($p<0.05$) fertilization rate which was 99%. Significant lowest ($p>0.05$) hatching and survival rates were observed in the group treated with sGnRHa alone which was 30 and 43%, respectively. Highest ($p<0.05$) hatching and survival rates were observed in the group treated with sGnRHa+DOM which was 94 and 99%, respectively. Treatment with sGnRHa alone produced

Table 1: Effectiveness of dopamine antagonist on breeding performance of *T. tambroides*

	Treatments				
Parameters	Ovatide (0.5 mL kg^{-1})	sGnRHa (10 µg kg^{-1})	sGnRHa+DOM (10 µg kg^{-1}+5 mg kg^{-1})	DOM (5 mg kg^{-1})	Saline (0.5 mL kg^{-1})
Ovulatory response (N)	3/6	1/6	2/6	0/6	0/6
Total stripped eggs (N)	4560±1.0^{d}	1433±1.00^{c}	838±1.00^{b}	0±0^{a}	0±0^{a}
Fecundity (eggs kg^{-1} b.wt.)	1572±1.0^{d}	515±0.30^{c}	244±1.00^{b}	0±0^{a}	0±0^{a}
Fertilization rate (%)	98±0.4^{b}	17±0.03^{a}	99±0.15^{c}	-	-
Hatching rate (%)	86±1.6^{b}	30±1.20^{a}	94±1.40^{c}	-	-
Survival rate (%)	79±0.4^{b}	43±0.90^{a}	99±0.60^{c}	-	-
Normal larvae (%)	69±0.9^{b}	42±1.60^{a}	95±0.20^{c}	-	-
Deformed larvae (%)	12±0.4^{b}	22±3.20^{c}	2±0.60^{a}	-	-

Mean values with different superscripts were significantly different at $p<0.05$ evaluated using one-way ANOVA followed by Duncan's post-hoc test, The observation are based on n = 3 of six replicates

significantly higher ($p<0.05$) deformed larvae (22%) and lower ($p<0.05$) normal larvae (42%). In contrast, the highest percentage of normal larvae (95%) and lowest deformed larvae (2%) were observed in sGnRHa+DOM treatment.

The plasma T concentration for group treated with ovatide slowly increased and significantly rose ($p<0.05$) to 0.818±0.242 ng mL^{-1} at 12 h Post Injection (PI). However, the concentrations decreased at 24 h PI (0.199±0.075). Similar pattern of plasma steroid concentration changes was observed in the group treated with sGnRHa alone and sGnRHa combination with DOM. Treatment with sGnRHa+DOM showed significant higher fluctuation of plasma T level (0.522±0.277 ng mL^{-1}) as compared to sGnRHa alone at 12 h PI. The levels decreased at 24 h PI (0.17±0.093). Plasma steroid concentrations in the group treated with sGnRHa alone increased to 0.355±0.205 ng mL^{-1} until 12 h PI and slowly decreased to 0.177±0.104 ng mL^{-1} at 24 h PI. Non significant fluctuation of plasma T concentration was observed in the group injected with DOM alone but small increment of plasma T level was noted 24 h PI. Dramatically changes of plasma T level for the saline administered fish were not observed throughout the experiment (Fig. 1). Significant fluctuations of plasma E_2 concentration were not observed in the saline administered fish throughout the experiment. In contrast, significantly higher ($p<0.05$) levels of E_2 was seen in group treated with ovatide, sGnRHa alone and sGnRHa with combination of DOM which peaked at 12 h PI (0.262±0.072, 0.212±0.064, 0.237±0.044 ng mL^{-1}, respectively). The level decreased to (0.055±0.018, 0.074±0.036, 0.048±0.015 ng mL^{-1}, respectively) at 24 h PI. Plasma E_2 level for DOM alone significantly increased at 12 h PI (0.081± 0.007 ng mL^{-1}). The concentration decreased at 24 h PI (0.02±0.004 ng mL^{-1}) (Fig. 2).

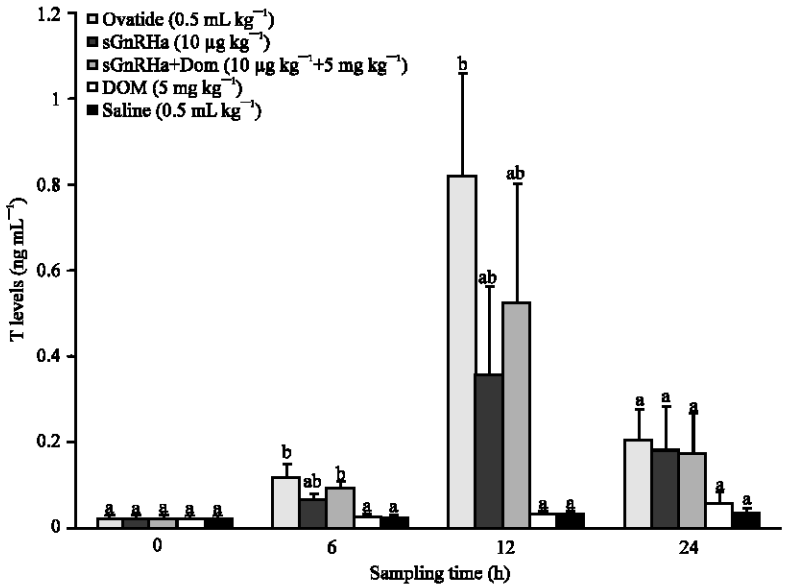

Fig. 1: Plasma levels of T (testosterone) in female Malaysian mahseer (*Tor tambroides*) injected with different treatments of ovatide, sGnRHa and DOM, Values are Mean±SEM, Mean values with different superscripts were significantly different at $p<0.05$ evaluated using one-way ANOVA followed by Duncan's *post hoc* test

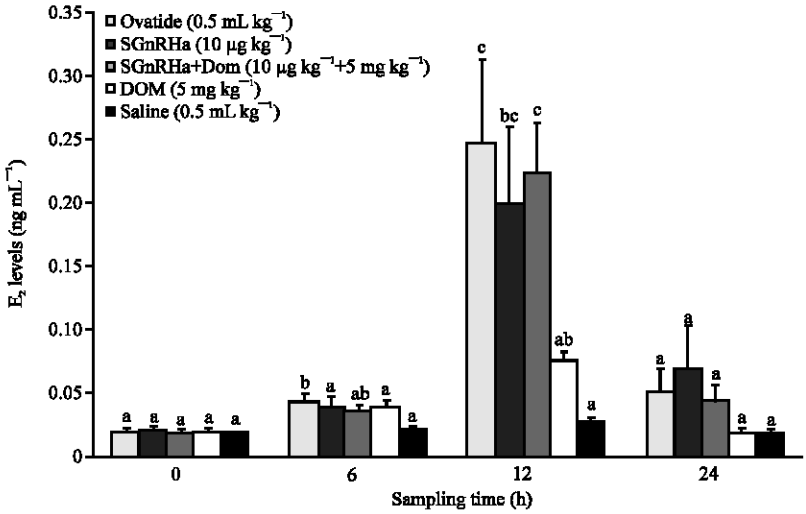

Fig. 2: Plasma levels of E_2 (17β-estradiol) in female Malaysian mahseer (*Tor tambroides*) injected with different combinations of ovatide, sGnRHa and DOM, Values are Mean±SEM, Mean values with different superscripts were significantly different at $p<0.05$) evaluated using one-way ANOVA followed by Duncan's *post hoc* test

DISCUSSION

The present study demonstrated that implantation of slow release GnRHa pellet and Ovaplant improved oocyte maturation and ovulation in female *T. tambroides*. Natural and plant based protein source of feed gave proper diet for the breeders. The plant based protein sources are cheaper and may be the only solution to reduce high feed costs in aquaculture (Hlophe *et al*., 2011). This experiment was conducted during non-spawning season and showed that ovulation can be induced by GnRHa treatment alone or sGnRHa combination with DOM. Gen *et al*. (2001) and Matsuyama *et al*. (1995) reported that post-pubertal female red sea bream was successfully induced with GnRHa treatment during the non-spawning season. Implantation of cholesterol pellet containing GnRHa induced vitellogenesis and ovulation in female red sea bream (Kumakura *et al*., 2003). Implantation of GnRHa caused an up-regulation of GnRH receptor (GnRH-R) and GtH subunit genes expression and stimulated the luteinizing hormone (LH) release from the pituitary (Kumakura *et al*., 2003). Thus, exogenous GnRHa could induce the onset of puberty in female fish (Kumakura *et al*., 2003).

In the present experiment, the highest production of larvae was obtained when treated with ovatide but fertilization, hatching and larval survival rates were highest when treated with sGnRHa+DOM. SGnRHa+DOM treatment. Combination of DOM and GnRHa accelerated oocyte development and increased plasma estradiol levels (Aizen *et al*., 2005). Low effect of sGnRHa alone or DOM alone on final oocyte maturation and ovulation probably because of insufficient and collapse of GtH levels increment in circulation (Szabo *et al*., 2002). In this present experiment, concentration of T was higher than E_2 after several treatments until ovulation. In striped bass, *Morone saxatilis* L. (King *et al*., 1994) concentration of T was higher than E_2 after administration until ovulation and level of T was higher than E_2 in sGnRHa+DOM. Combination of sGnRHa and DOM enhanced T and E_2 levels significantly in female Malaysian mahseer. These steroids dramatically increase at 12 h PI, while injection of DOM alone did not stimulate significant increase

in T and E_2 and thereby unsuccessful ovulation. These present results are fully supported by Wen and Lin (2004), who reported that combination of LHRHa and DOM enhanced serum GtH levels significantly in female catfish and DOM alone did not produced significant increased in serum GtH thus no ovulation.

Administration of sGnRHa alone stimulated only a modest increase of T and E_2 levels and ineffective in inducing ovulation (Wen and Lin, 2004). Similar situation occurred in induced ovulation of goldfish and carp (Chang *et al.*, 1984; Peter *et al.*, 1988). Injection of LHRHa or sGnRHa alone in wild catfish stimulated only modest increase in the serum GtH levels (Wen and Lin, 2004). The injection of DOM greatly assist the action of sGnRHa on T and E_2 release and combinations of them are highly effective in inducing ovulation in *T. tambroides*. These results demonstrated that in *T. tambroides*, similar to other cyprinids, dopamine acted as inhibitory factor on GnRH-stimulated GtH release. The effect of sGnRHa+DOM treatment dramatically improved ovulation and reproductive performance of Malaysian mahseer under captivity.

CONCLUSION

This study provided an insight into the variations of sex hormone steroids (T and E_2) during ovulation induction and suggests the possible application as a parameter to predict the success of hormonally-induced ovulation. Inhibitory effect of dopamine on GtH release led to the reduction of plasma sex steroids production. However, Dopamine antagonist (DOM) can enhance sGnRHa action and increase the plasma sex steroids production during vitellogenesis. This action can lead to higher ovulation success and improve reproductive performance of Malaysian mahseer *T. tambroides*.

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