Indian Journal of Geo Marine Sciences
Vol. 51 (03), March 2022, pp. 249-254

Effects of GnRH-a on the ovarian maturation of blue tang fish (*Paracanthurus hepatus* Linnaeus, 1776)

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Received 21 August 2020; revised 27 February 2022

The effects of gonadotropin-releasing hormone – analogue (GnRH-a) injection on the ovarian maturation and steroid hormone level of blue tang (*Paracanthurus hepatus*) females were investigated. Pre-matured blue tang females were injected with GnRH-a at dosages of 150, 200 and 250 µg/kg body weight and were then observed for fluctuation of steroid hormone levels, including testosterone (T), estradiol (E2) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in plasma and ovary after 2 and 7 days. Plasma T level was not significantly different among the treated groups at sampling times. However, the ovarian T levels in the fish injected with 200 and 250 µg/kg GnRH-a were higher than that of the non-administered GnRH-a and fish injected with 150 µg/kg after two days ($P < 0.05$). After seven days of the administration, the ovarian T level was lowest in the fish without GnRH-a injection and highest in the fish injected with 250 µg/kg ($P < 0.05$). The plasma and ovarian E2 levels were higher ($P < 0.05$) in the fish injected with 200 and 250 µg/kg of GnRH-a than in the control fish after 2 and 7 days of injection. Both the plasma and ovarian DHP levels in GnRH-a injected fish were significantly ($P < 0.05$) increased after day 2 and was highest in the fish injected with 250 µg/kg ($P < 0.05$). After 7 days of injection, the plasma and ovarian DHP levels remained higher in the GnRH-a injected fish than in the controlled group ($P < 0.05$). There was no difference in DHP levels among the GnRH-a-treated groups. The results indicate that the dosage of 200 µg/kg of GnRH-a is appropriate for inducing maturation of the blue tang fish.

[**Keywords**: Blue tang female, GnRH-a, Ovarian maturation, Plasma and ovarian steroids]

Introduction

Gonadotropin-releasing hormones (GnRHs), gonadotropins (GTHs) and steroids play an important role in the regulation of reproduction in teleost. GnRH stimulates the release of GTH from the pituitary, and in turn, GTH in circulation induces the synthesis and release of steroids regulating oocyte growth, maturation and ovulation in females[1]. Among eight GnRH forms found in the teleost, two or three GnRHs coexisted in the same species[2]. Synthetic GnRH-analogue (GnRH-a) has been used as an exogenous hormone to induce maturation in some fish species, for example, sea bass, *Lates calcarifer*[3], winter flounder *Pseudopleuronectes americanus*[4], gilt-head sea bream *Sparus aurata*[5], American shad, *Alosa sapidissima*[6], yellowtail flounder *Pleuronectes ferrugineus*[7], rainbow trout *Oncorhynchus mykiss*[8], striped bass (*Morone saxatilis*)[9], and starry flounder *Platichthys stellatus*[10]. Several studies demonstrated that injection of GnRH-a alone or in combination with pimozide caused GTH-II release in inducing ovulation[11-13]. Doses used for fish species are different, even for individuals of the same species[14]. Luteinizing hormone-releasing hormone analogues *D-Ala6*-des-*Gly10*-LHRH Ethylamide (LHRH-a) and Follicle-Stimulating Hormone-Releasing Hormone (*FSH*-RH) have been successfully used to induce final maturation and synchronize ovulation in many commercially cultured teleosts[14-17]. GnRH-a administration also increased the level of some steroids relating to maturation and ovulation in marine female fish[18-19]. The short-term induction of ovulation and changes in plasma testosterone (T), 17β-estradiol (E_2), *17α,20β*-dihydroxy-4-*pregnen-3-one* (DHP) levels in fish injected with exogenous hormones were observed in wild black bream *Acanthopagrus butcheri*[21], yellow perch *Perca flavescens*[22], *Kutumrutilus frisiikutum*[18], and in Waigieu seaperch *Psammoperca waigiensis*[22].

Blue tang fish *Paracanthurus hepatus* is found distributed throughout the Indo-Pacific. In wild, the fish at 9 – 12 months of age[24] and 149.2 mm in total length[25] can reach sexual maturity. The exploitation of blue tang used for the ornamental aquarium has been

depleting the natural resources of this species in Vietnam. Thus, it is necessary to implement management in the protection of the natural resource of the fish, including a strategy for its breeding to support the ornamental fish industry. One of the effective strategies to compensate for diminishing natural resources is to promote breeding under captive conditions. Wherein, management of broodstock for artificial breeding is an important aspect. Several studies showed that maintenance of blue tang broodstock was successful under capture conditions. However, to date, no success has been achieved in breeding stimulation of the species[26]. Therefore, in the present study, we investigate the effects of GnRH-a on the ovarian maturation of blue tang fish (*Paracanthurus hepatus*).

Materials and Methods

Experimental fish

Broodstocks of blue tang with a total length from 13.5 to 15.5 mm and weight from 153 to 183 g were collected from wild from the Khanh Hoa and Binh Thuan seawaters in Vietnam. The fishes were transported to the aquaculture station of the Institute of Oceanography, Vietnam and were then reared under the cultured condition for 10 days before the commencement of the experiment- when the fish resumed their normal behaviour. During the acclimation, the fishes were fed to satiation twice a day (8:00 and 16:00 h). The diet containing shrimp, squid, vitamin and mineral, fish oil, squid oil, cow liver, chicken egg and algae spirulina was used to feed the fish. Crude protein and lipid in the diet were about 55 % and 14 %, respectively.

Culture system and experimental design

The culture system comprised 16 tanks representing four treatments, and each treatment was quadrupled. The composite rectangular tanks (500 × 800 × 800 mm, filled with 300 L of natural seawater) were used as one unit for the experiment. Each tank was filled with re-circulating seawater and aerated at a rate of approximately 3 L min^{-1}. The seawater in the tank was filtered through a Protein skimmer. During the experiments, the seawater temperature, pH, salinity and dissolved oxygen were measured by Hana instruments. The seawater temperature ranged from 26.0 – 28.6 °C, pH from 7.8 – 8.0; salinity from 32.5 – 33.0 ppt, and dissolved oxygen from 6.0 – 6.3 ppm. The photoperiod was maintained naturally similar to tropical regions (approximated as 12 h of light and 12 h of dark). After acclimation for ten days, each tank was stocked with 10 broodstocks of blue tang fish (5 males and 5 females, random selection). During the culture period, the fishes were fed the above processing diet twice a day until satiation. Every day, the excess food and faeces were removed by siphoning, and an equal amount of seawater was added to maintain the constant volume of water. After three months of culture, the ovaries of the females were pre-mature (at stage III), and the fish in the tanks were injected with GnRH-a (Sigma-Aldrich, Germany) at different dosages of 0 µg/kg (control); 150 µg/kg; 200 µg/kg and 250 µg/kg of fish[26] at the base of the pectoral fin using 0.5 ml hypodermic syringes.

Sample collection

Two female fish from each treatment was sampled on day 2 and 7 to analyze the steroid reproductive hormones (T, E2 and DHP) in the blood and gonads and the gonadal development stages were analyzed. Blood was collected from the caudal arteries with heparinized syringes and was then centrifuged at 4 °C for 15 min at 1000×g to separate the plasma. The plasma was stored at -30 °C to extract steroid hormones. Ovaries were collected to determine the gonadal development stages and to extract steroid hormones.

Histological analysis

Ovarian processing and sectioning were done according to Sang *et al.*[25]. Hematoxylin-eosin was used to stain the ovary sections. The sections were then observed under a microscope (Olympus BX50).

Sample analysis

Ovarian development stages:

After 2 and 7 days of injection, all the fishes were determined for ovarian development stages according to the method by Sakun & Buskaa[27].

Extraction of plasma steroid hormones:

To obtain steroid extracts, 200 µl plasma was extracted twice using 1 ml of diethyl ether with snap freezing at -30 °C. The dried extracts were reconstituted by adding the assay buffer supplied by the manufacturer along with the ELISA kit (MyBioSource Inc., San Diego, USA) and then frozen at -30 °C.

Extraction of ovarian steroid hormones:

The extraction was performed following the manufacturer protocol as referred by Sang *et al.*[29].

Determination of steroid hormone levels:

Steroid hormones were analyzed using the commercial ELISA kits supplied by the manufacturer as referred by Sang *et al.*[29].

Data analysis

Data were analyzed by ANOVA with the significance level of $P < 0.05$[30] using the SPSS version 18 for Windows.

Results

Maturation of the fish injected by the different dosages of GnRH-a

Before the injection of GnRH-a, the ovaries of all the female fishes were at stage III. After the GnRH-a injection, the proportion of the female having ovary at stage IV was the highest ($P < 0.05$) at the dosage of 250 µg/kg than the fish injected at a concentration of 150 µg/kg of GnRH-a after the 2nd and 7th day of injection. In the case of the control group, in which the females were not subjected to injection with GnRH-a, no fish at stage IV of the ovary was observed (Table 1).

Changes in steroid hormone level

Injection of GnRH-a at different doses of 150, 200 and 250 µg/kg body weight resulted in similar changes in steroid hormones both in plasma and the ovary. No significant difference ($P > 0.05$) in plasma testosterone level was observed among the fish treated with different dosages of GnRH-a after the 2nd and 7th day of injection. However, after the 2nd day post-injection, the ovarian testosterone levels in the fish injected with 200 and 250 µg/kg body weight of GnRH-a were higher than in the fish injected with 150 µg/kg body weight and the fish without any injection. After 7 days post-injection, the ovarian testosterone level in the fish was significantly different ($P < 0.05$) among different treated fishes. The testosterone level of the fish without GnRH-a injection was the lowest and the highest ($P < 0.05$) was in the fish injected with 250 µg/kg body weight of GnRH-a (Fig. 1).

At 2 days of post-injection, the plasma and ovarian DHP levels in GnRH-a injected fish at different dosages were remarkably elevated ($P < 0.05$) and the highest was in the fish injected at 250 µg/kg body weight ($P < 0.05$). At 7-days post-injection, the plasma and ovarian DHP levels remained higher ($P < 0.05$) in the GnRH-a injected fishes than in the fish without GnRH-a injection. However, there was no difference in the plasma and ovarian DHP levels among the fish injected with different dosages of GnRH-a (Fig. 2).

At 2nd and 7th days of post-injection of the GnRH-a, the plasma and ovarian estradiol levels in the fish injected with 200 and 250 µg/kg body weight were higher ($P < 0.05$) than of the control (Fig. 3).

Table 1 — Percentage (%) of ovary at stage IV of the blue tang injected with different dosages of GnRH-a

Day after injection	GnRH-a injection dosage (µg/kg)			
	0	**150**	**200**	**250**
2	0^a	25.48 ± 6.61^b	51.78 ± 8.91^c	53.21 ± 8.36^c
7	0^a	43.21 ± 4.72^b	69.52 ± 8.40^c	74.52 ± 6.61^c

Discussion

As described earlier, broodstock of blue tang could be successfully maintained in captive conditions but the female could not breed. The ovary of the female reached a pre-maturational level but degenerated at a certain time during reared condition[26]. This observation is similar to that of some other marine

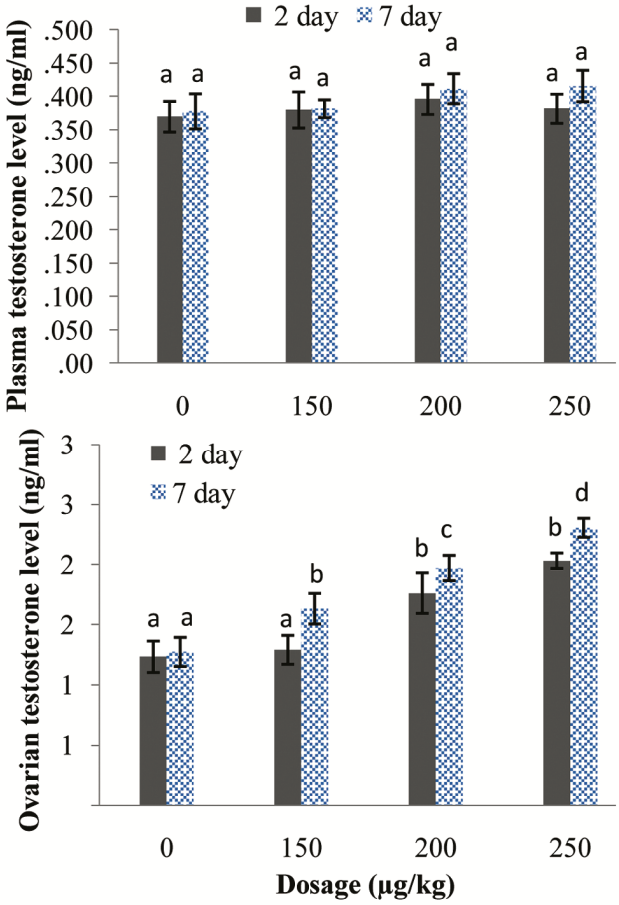

Fig. 1 — Changes in testosterone level in plasma and ovary of the fish injected with different dosages of GnRH-a (Different letters indicate significant difference at $P < 0.05$)

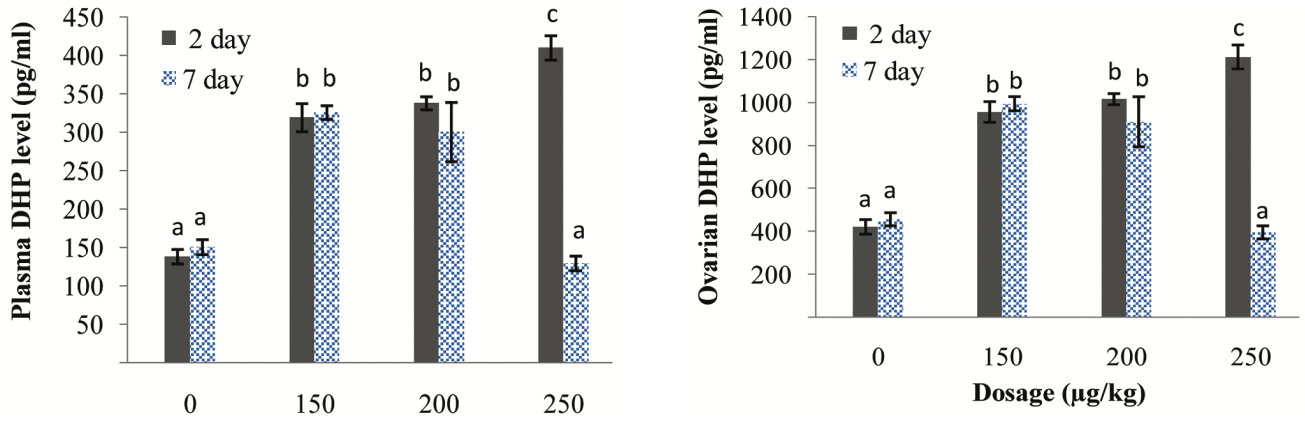

Fig. 2 — Changes in DHP level in plasma and ovary of the fish injected with different dosages of GnRH-a (Different letters indicate significant difference at $P < 0.05$)

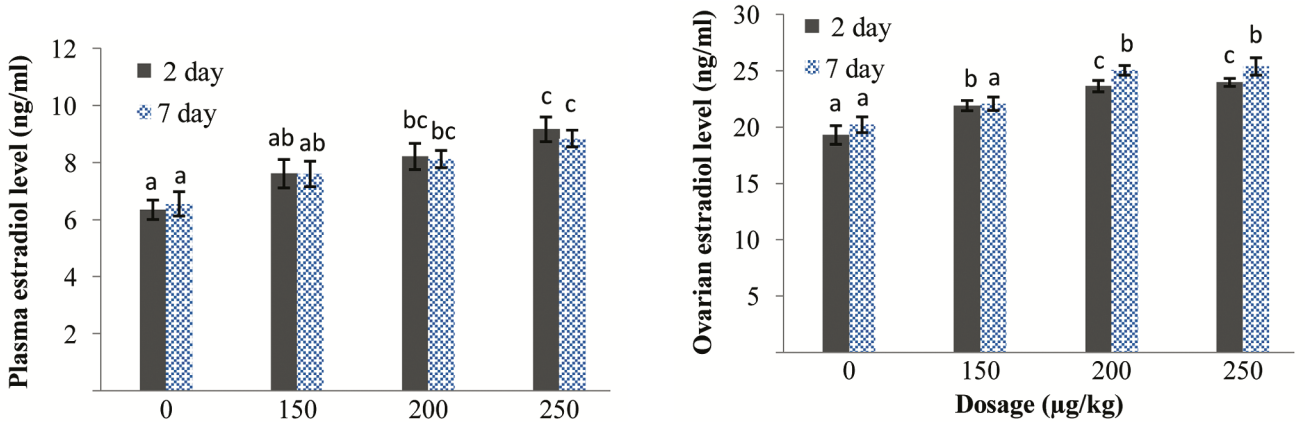

Fig. 3 — Changes in estradiol level in plasma and ovary of the fish injected with different dosages of GnRH-a (Different letters indicate significant difference at $P < 0.05$).

fish species which cannot mature or spawn in captivity. This might be due to malfunctioning of the endocrine system. The results of this study indicated the stimulation of the GnRH-a injection on the maturation of the pre-maturated blue tang fish. The difference in the proportion of maturation in the fish was also dosage-dependent. The appropriate dosage of GnRH-a 200 µg/kg is suggested for the application of this reproductive induction hormone in blue tang fish. The obtained results are consistent with several previous studies on the role of GnRH-a in the induction of maturation and ovulation in the fish. These studies demonstrate that exogenous hormones are effective manipulation for induction of maturation, ovulation, spawning and enhancing the egg quality and fertility in fish. Among them, GnRH-analogue (GnRH-a) has been successfully used to promote maturation and ovulation in hatcheries of teleosts[4,19,31,31]. In fish, the optimal effective dose of GnRH-a on maturation and ovulation is species-specific and varies between species and the degree of maturity[33,34]. In this study, the injection of GnRH-a at different doses caused maturation in blue tang females. However, it is necessary to investigate the optimal dose of GnRH-a, the timing of injection, egg quality and fertility rate in this species.

In blue tang, steroid profile relating to ovarian maturation in wild females was documented[29]. However, in the capture conditions, the knowledge related to the steroid profile and the effects of exogenous hormones on steroid levels is still unknown. Thus, this study is the first attempt to elucidate the roles of GnRH-a on changes in the steroid hormone including T, E2 and DHP in the blue tang fish and on induction of the maturation of the fish. In the present study, the injection of GnRH-a resulted in changes in plasma and ovarian steroid levels in the blue tang females, relating to successful maturation in experimental fishes. As observed in current study, there was no increase in T levels in the

plasma of fish upon exogenous hormone treatment. This may be because of the rapid aromatization and/or stress as reported in many species[34]. The increases in E2 levels in the plasma and ovary of the GnRH-a injected fish in this study could result from the steroidogenesis in the ovarian follicles of blue tang[29]. The elevation of DHP levels is in association with maturation in captured blue tang females, which is similar to greenback flounder (*Rhombosolea tapirina*)[36], and starry flounder *Platichthys stellatus* (Han, 2016). Several studies have indicated that DHP plays the role of a Maturation-Inducing Steroid (MIS), regulating spermiation and maturation in most teleosts[37]. In combination with the results of previous study, in which DHP levels increased the spawning of wild blue tang females[29], DHP as a candidate MIH as suggested for blue tang.

However, the potency of this hormone in inducing oocyte maturation *in-vivo* and an injection of this steroid into the fish further needs to be tested. Moreover, the coordination between environmental factors and efficacy of GnRH-a treatment regulating hormonal responses for successful reproduction should be studied.

In summary, GnRH-a injection caused changes in the levels of some steroids and GnRH-a was effective in inducing maturation in blue tang female fish. The use of a dosage of GnRH-a at about 200 µg/kg body weight was appropriate in an artificial hatchery in this species.

Acknowledgements

The authors especially thank Mrs Le Ho Khanh Hy for her technical support in steroid hormone analysis. This research was a part of project number 106-NN.02-2016.05 by the Vietnamese National Foundation for Science & Technology Development (NAFOSTED). This paper is a contribution to celebrate the 100th anniversary of the Institute of Oceanography, Vietnam Academy of Science and Technology.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Ethical Statement

The Vietnamese National Foundation for Science & Technology Development (NAFOSTED) committee have approved for this research under project number 106-NN.02-2016.05. In this research, the fishes were anesthetized using MS-222 (tricaine methanesulfonate; Sigma, USA) before blood sampling and ovary sampling.

Author Contributions

Conceptualization, funding acquisition, writing - review & editing, and investigation: HMS. Investigation: HSL. Investigation, writing - review & editing: PXK.

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