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Induced Spermiation of Rainbow Trout, *Oncorhynchus mykiss*, Using a GnRH Analogue

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Abstract: In this study, the benefits of using the first Iranian made GnRHa [D-Ala^6 des Gly^{10}] mGnRH ethylamide, to induce spermiation in male rainbow trout, *Oncorhynchus mykiss*, were evaluated. In addition, its effect on acceleration and synchronization, quality and quantity of milt and the plasma Testosterone (T) fluctuations were examined.

For these purposes, 40 non-spermiating male rainbow trouts were injected with a mammalian gonadotropin releasing hormone analog (GnRHa) preparations of 0 (control), 30, 40 and 80 µg/kg B.W. or vehicle (propylen glycole). Spermiation was very synchronous and accelerated in treated groups. Six days after first injection, the cumulative spermiation rates reached respectively 40, 49 and 79% in injected groups (i.e. 30 to 80 µg/kg B.W.) while none of the control fish was spermiated. GnRHa injections advanced spermiation and reduced the average time to spermiation from 16±3.67 days for control group to14.3±2.2, 9.2±0.75 and 6.6±0.29 days for treated groups, respectively ($p<0.05$).

The average volume of total expressible milt of male fish increased significantly after treatment with GnRHa from 8.29±1.59 ml/kg B.W. for control to 11.42±1.55, 14.39±1.55 and 17.14± 1.55 ml/kg B.W. in groups 2 to 4, respectively ($P<0.05$). The fertilization and survival rates to the eyed stage did not show any significant difference among the groups ($p>0.05$). Circulating levels of testosterone (T) prior to the GnRHa treatment were relatively low in all groups. Treatment with GnRHa

induced significant increase in plasma T after 12 h, increasing it to 43.43±5.82, 38.66±5.63, 39.72±5.07 ng/ml in groups 2 to 4, respectively, which were higher than T levels for control (i.e. 24.58±7.13 ng/ml) $P<0.05$. These levels remained high up until 48 h in treated groups; but after this time, T levels reduced to the basal levels of time 0, except in group 4 which had received its second GnRHa injection at time 48.

KEY WORDS: *Oncorhynchus mykiss*, GnRHa, Induced Spermiation, Testosterone.

Introduction

Modern industrial aquaculture aims at providing a low cost, high quality product based on market demand. Supplying a consumer on-demand product requires a reliable and constant production system that begins with a constant supply of gamet. In a number of species cultured the male spermiates spontaneously; however, in others the captive male fails to spermiate or produces merely a small volume of viscous milt. Salmon have periods of susceptibility whereby the handling of maturing fish may cause a cessation of the maturation process. As well, maturation will occur at different rates in a large population; therefore, fish will spawn at different times. This serves to spread out the spawning season over a period that is both species and strain specific. In some cases, spawning dates may be asynchronous in species of fish that have limited returns to natal streams (Powell, 1997). Many of the methods used to enhance reproductive performance, acceleration and synchronization, spawning require external intervations through environmental manipulations and/or hormonal treatments (Donaldson, 1999). Stable analogues of gonadotropin releasing hormone (GnRH) were shown to have several important advantages over other compounds for inducing spawning in many species, such as common carp, Chinese carp (Zohar, 1989), New Zealand snapper, *Pagrus auratus* (Pankhurst, 1994), Coho salmon *Oncorhynchus kisutch,* and rainbow trout (Powell, 1997).

In salmonids, GnRHa caused acceleration and sychronization of milt production, increasing milt volume (Weil & Crim, 1983 and King & Young, 2001). In mature male landlocked Atlantic salmon, *Salmo salar*, spermiation was induced by intraperitoneal (i.p) injections of 0.75 mg/kg B.W. of mGnRHa D-Ala^6 in saline or propylene glycole (40%). Spermiations commenced in i.p-injected fish on day one post injection and the maximal responses in most groups were noted after 8-12 days. In control group, 75% of fish were spermiating on day 12. The sperm volume produced was correlated with plasma gonadotropin concentration (Weil &

Crim, 1983). Two i.p-injections of 0.05 mg/kg B.W. mGnRHa D-Ala^6 72 h apart could be successfully used to induced spermiation in chum salmon, *Oncorhynchus keta*, (Donaldson & Hunter, 1983). Similar results were observed for sockeye salmon, *O. nerka*, (Slater *et al.*, 1995) and Atlantic salmon (Mylonas *et al.*, 1995 and King & Young, 2001).

In Iran, however, there are no scientific results on using the new products of mGnRHa in order to induce spermiation in fish. Also, some other compounds like LHRH, HCG and pituitary glands were used for inducing spermiation in common and Chinese carps and in sturgeon.

GnRHa treatment did not show any adverse effects on sperm quantity, density, motility and fertilization capability in white bass, *Morone chryops* (Mylonas *et al.*, 1997), yellow tail flounder, *Pleuronentes ferrugineus* (Clearwater and Crim, 1995) and Atlantic salmon, *Salmo salar* (Mylonas *et al.*, 1997 and King & Young, 2001). Also, the increased milt production in treated species reflects itself on an increase in the number of motile sperm and seminal fluid volume (Georgen *et al.*, 1995).

The increase in circulating levels of GnRHa is believed to imitate endogenous changes in hormone levels, such as gonadotropin and sex steroids (Pankhurst, 1994 and King & Young, 2001). GnRHa-induced increase in milt volume were accompained by increased plasma levels of T in New Zealand snapper, *Pagrous auratus*, sparidae (Pankhurst, 1994), white bass, *M. chrysops* (Mylonas *et al.*, 1997) and stellate sturgeon, *Acipenser stellatuse* (Semenkova *et al.*, 2001).

The objectives of the present study were: a) to determine if the new product of GnRHa preparation made in National Research Center of Genetic Engineering and Biotechnology (NRCGEB, Tehran, Iran) can advance and synchronize spermiation in rainbow trout, b) what are the effects of GnRHa injections on expressible milt volume and fertilization capability of sperm? and c) how is the effect of GnRHa injection on plasma levels of sex steroids (T).

Materials and Methods

Stocks:

The experiment was conducted in the onset of spawning season (autumn 2000) in the Shahid Motahari fish farm, Yasouj, Iran. Perior to the experiment, fish were kept in a single concrete raceway (27 x 3 x 1.2 m) during natural photoperiods on 10-15 kg/m^2. Fish were feed 3-5% of B.W. daily with commercial trout food pellet

(Chineh Co. Iran), transferred in separated raceway (12 x 1.5 x 1 m) supplied with a spring water (1 l/s) at ambient temperature during the experiment. Average water temperature was 11.2±0.2°C.

40 non-spermiating male fish, 3 years old (mean weight approx. 1350 g) were selected and anesthesized in MS222 (100 ppm). Fish were individually weighed (±0.1 kg) and marked by placing visible tags in the dorsal fin. Here after, individual fish were randomly assigned to treatment groups.

Hormone Solutions:

Mammalian GnRH [D-Ala ^{6} des Gly ^{10}] mGnRH ethylamide, a gift from NRCGEB, was diluted with 40% propylene glycol to achieve a concentration of 20 µg/kg B.W. at a final injection volume of 0.5ml/kg B.W.

Experiment:

Forty fish (3 years old) were anaesthetized in MS222 (100 ppm) and injected intraperitonally (i.p.) with vehicle (propylene glycol) or 30, 40 and 80 µg/kg B.W. of mGnRHa for group 1 (control), 2, 3 and 4, respectively. Fish in groups 1 and 2 received only one injection, while those in groups 3 and 4 received 2 and 4 injections 72 and 48 h apart respectively in equivalence rate.

Blood samples were collected from injected fish (3 fish in each group) at the time of injection (0 h), 12, 24, 48 and 72 h after the first injection. Then, the brood fish were checked 3 days apart, and spermiated fish were stripped by hand. Milt volume was measured (±0.1 ml) and 0.2 ml of each milt was used to fertilize (by dry methods) approx. 2000 eggs from 5 untreated female fish in three replications according to Billard and Breton (1985). The fertilized eggs were incubated in California trays to the eyed stage under normal hatchery conditions.

Steroid measurements:

Levels of testosterone (T) were measured in plasma using Radio Immune Assay (RIA) with Immonotech kits (French made). Duplicated methods were used for all samples, and the average counts were then recorded.

Statistical analysis:

To detect significant changes in response to GnRHa treatment of total expressible milt, fertilization capability and T levels, data from individual fish

samples were subjected to GLM (General Lenear Models) followed by Duncan's multiple range test at $p<0.05$. Results are presented as means ± SEM. Statistical analyses were done on a personal computer using SAS (1997) software.

Results

In this experiment, the average weight (g) of selected fish were 1330±125, 1400±112, 1300±97.3 and 1380±148 g while their total length (mm) were 472±9.7, 482±11.6, 485±13 & 495±11.6 mm for group 1 to 4 respectively, each group containing 10 fish. However, the difference among the groups for these parameters were not statistically significant ($P>0.05$).

GnRHa injections advanced spermiation and significantly reduced the median days to spawning in all groups from 16±3.67 for the control fish to 14.3±2.22, 9.2±0.75 and 6.6±0.29 days for treated fish in groups 2 to 4, respectively ($p<0.05$) (Fig.1). GnRHa injections also synchronized spermiation in great degree: 6 days after first injection 40, 49 and 79% of fish in respective treated group's spermiated while none of the control fish spermiated (Fig.2). 11 days after the first injection, when the cumulative spermiation reached 62, 100 and 100% in groups 2 to 4 respectively, only 50% of the control fish spermiated (Fig.2).

Although all treatments resulted in higher mean milt volumes relative to the controls (Fig.3), only milt volumes from fish that treated at higher doses, i.e. 40 and 80 μg/kg B.W., were significantly different from the control ($p<0.05$). The total expressible milt volume (ml) per each unit of fish body weight (kg) were 11.41±1.55, 14.39±1.55 and 17.14±1.55 ml/kg compared to 8.29±1.59 ml/kg for the control. There was no significant changes in fertilization capability of sperm and egg survival to the eyed stage among the groups ($p>0.05$), and all were in normal ranges (Fig.4).

The results of T levels of male fish at times 0 (before injections), 12, 24, 48, 72 h post injections are shown in Figure 5. According to these results, circulating levels of Testosterone (T) prior to GnRHa injections were low, but they showed significant differences among the groups. These levels were measured and found to be 32.63±5.82, 25.86±5.64, 35.33±5.82 and 11.58±5.04 ng/ml in groups 1 to 4, respectively. T levels were the lowest in group 4 ($p<0.05$). Treatment with GnRHa induced a significant increase in plasma T after 12h ($p<0.05$) when the T levels reached respectively 43.33±5.82, 38.66±5.63 and 39.72±5.07 ng/ml in groups 2 to

4; however, at this time, T levels decreased to 24.85±7.13 ng/ml for the control. Except for the control group, plasma T remained unchanged and high until 48h after the first injection. 48h post injection, T levels were measured as 43.43 ±5.28, 39.5±5.28 and 26.35±5.04 ng/ml in groups 2 to 4 respectively while it was 27.66±5.28 ng/ml for the control fish. 72h after injection, the circulating levels of T decreased significantly and reached basal levels at time 0 in groups 2 and 3 (i.e., 23.43±5.82 and 24.3±5.79 ng/ml respectively) However, in group 4, which received the second injections at time 48 h., T levels remained significantly high ($p<0.05$) compared to time 0, i.e., 28.5±5.07 vs. 11.58±5.07 ng/ml. Circulating levels of T remained relatively low and unchanged during that time in the control group (Fig.5).

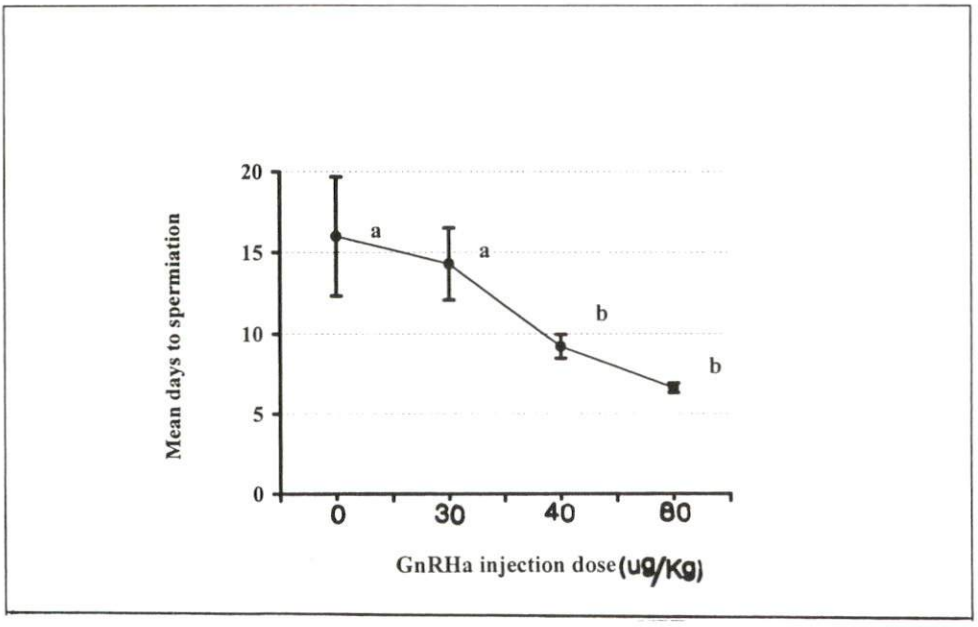

Fig. 1: The effect of GnRHa injections on spermiation of rainbow trout, mean time to spermiation (±s.e.m). Means with different letter superscripts are significantly different ($p<0.05$)

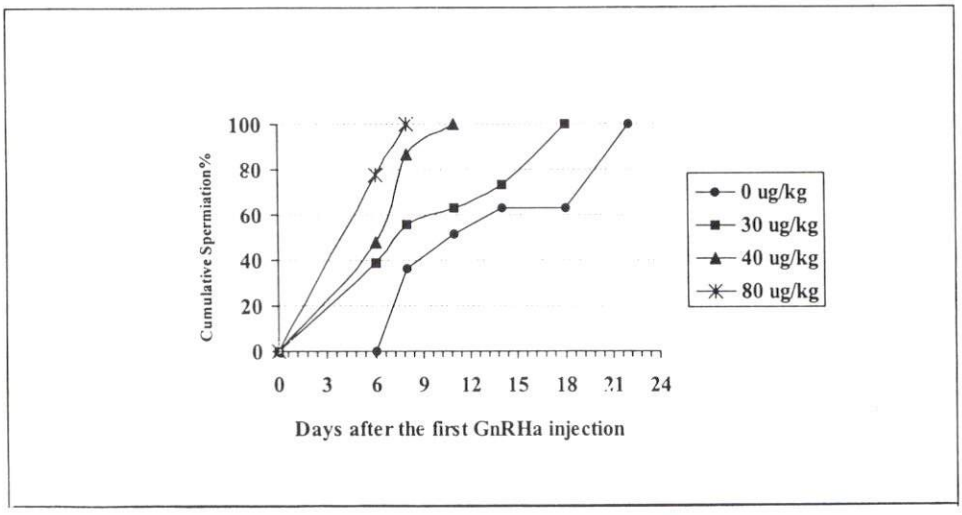

Fig. 2: The effect of GnRHa injections on spermiation of rainbow trout, cumulative percent spermiation

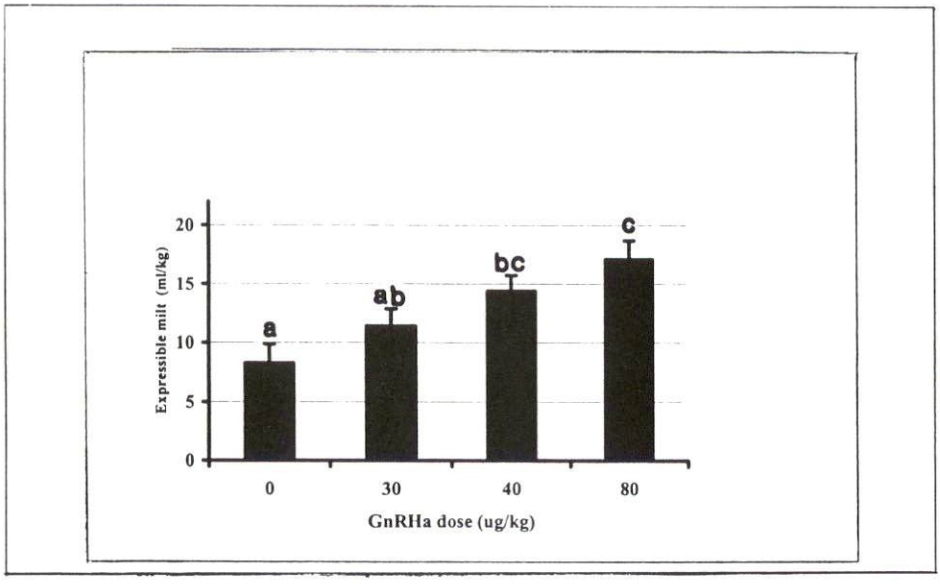

Fig. 3: Mean (±s.e.m) total expressible milt (ml/kg). Means with different letter superscripts are significantly different ($p<0.05$)

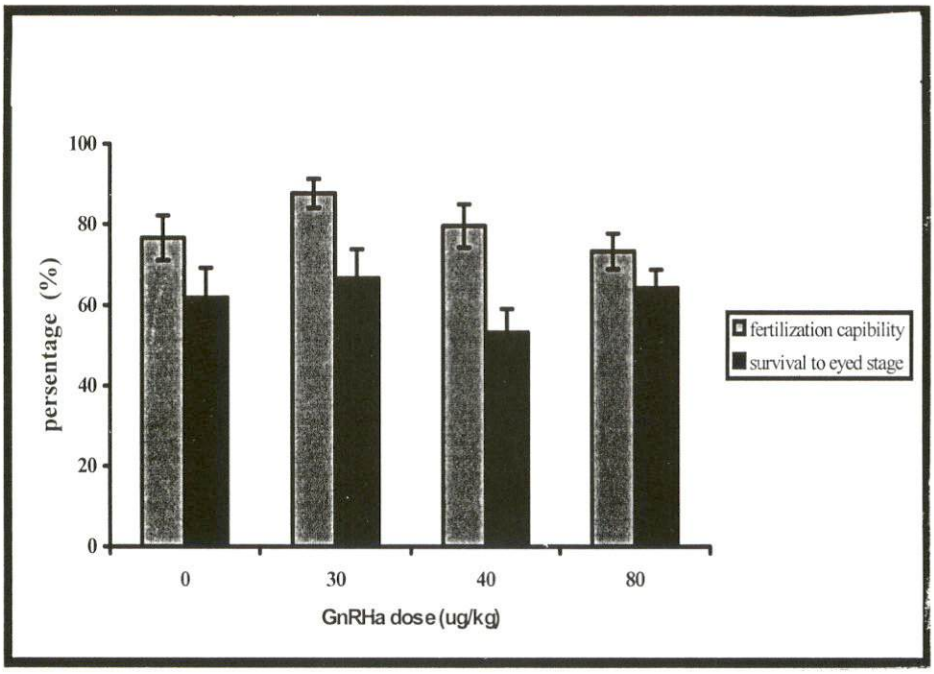

Fig. 4: Mean (±s.e.m) percentage fertilization and survival to eyed stage of milt collected from rainbow trout, there were no significant differences in these subject among groups

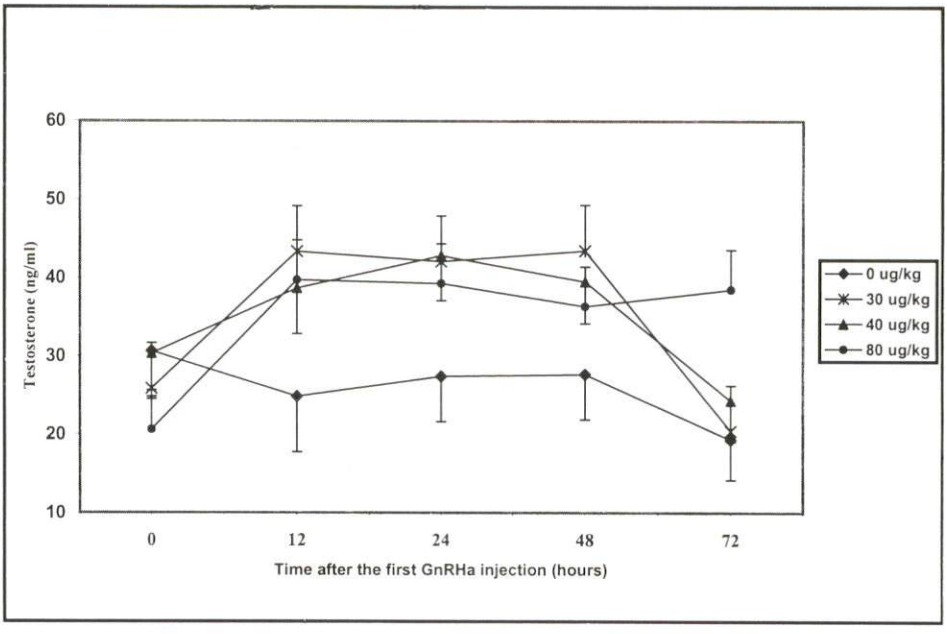

Fig. 5: Mean (±s.e.m) testosterone levels (ng/ml) of rainbow trout (n=3) after treatment with GnRHa

Discussion

In all GnRHa-treated fish, average days to spermiation decreased and spermiation process were very synchronous compared to the control. Fish in group 3, which received 2 injections of GnRHa 72h apart with a total dose of 40μg/kg B.W., demonstrated the best result (Figures 1 and 2). It may be due to the synergism effect of the second injections on the first one, stimulating GtH release from pituitary followed by steroid secretions. Zohar and Mylonas (2001) noted that average activity time of GnRH is too short, being 5 to 23 min for GnRH and GnRH analogues, respectively. However, such a short activity time was not long enough to stimulate GtH release for inducing spawning in salmonid fish. The rapid clearance of GnRH molecules from blood circulations are under effect of cytosolic enzyme activity in pituitary, liver and kidney of fish (Zohar, 1989). Hence, they recommended multiple injections or using sustainable releasing delivery system of GnRH. But, this is important to note that multiple injections and handling can cause stress responses in broodfish and reduce induction responses of treated fish (Campell, 1994, Brooks *et al.*, 1995, Zohar & Mylonas, 2001). This may be the reason for the extended responses of fish in group 4 when compared to group 3 which received lower dose of GnRHa.

Weil and Crim (1983) and Donaldson and Hunter (1983) noted that two injections of (D-Ala 6 des-Gly 10) mGnRH ethylamide 72h apart had a significant effect on accelerating and synchronizing spermiation of *S. salar* and *O. nerka*, respectively. Powell (1997) showed the effectiveness of GnRHa implant on advancement and synchronization of spawning of Coho salmon, *O. kisutch*, and rainbow trout, *O. mykiss*. In Iran, our recent finding showed the same result on induction of spawning by mGnRHa in female rainbow trout (Dorafshan *et al.*, 2002).

GnRHa injections had resulted in significant elevations of milt production in male rainbow trout concomitant with the increase in plasma T levels without any adverse effects on fertilization capability of sperm. In the present study, we demonstrated that GnRHa treatment induced not only seminal fluids production,

but also apparently produced normal spermatozoa. Otherwise, we could not obtain the same fertilization rate in all groups as we used a minimum volume of milt to fertilize the same batch of untreated eggs, i.e. 0.2ml of milt for 2000 eggs (Billard & Breton, 1985). Injections of GnRHa were used to increase milt volume in various fish species, such as common carp (Billard *et al.*, 1983), New Zealand snapper (Pankhurst, 1994), Coho salmon (Powell, 1997) and stellate sturgeon (Semenkova *et al.*, 2001). The effect of single injection is short lived and multiple treatments are necessary for sustained stimulation of milt production in fish (Weil & Crim, 1983 and Powell, 1997).

According to Georgen *et al.* (1995), GnRHa treatment caused an increase both in number of motile sperm and seminal fluid volume in Atlantic salmon. The same results were obtained in common carp (Billard *et al.*, 1983), yellow tail flounder, *Pleuronectes ferrugineus* (Clearwater & Crim 1995), white bass, *Morone saxatilis* (Mylonas *et al.*, 1997) and Atlantic salmon (King & Young, 2001).

These findings noted that using GnRHa-induced sperm to fertilize non treated female eggs did not have any adverse effect on fertilization success and further development process of the fertilized eggs when compared with milt of non treated male.

GnRHa injections caused a marked fluctuation in plasma hormones such as sex steroids. One of the major steroids in male fish which affects spermiation process is T (Zohar, 1989). In this experiment, GnRHa injections caused a marked increase in plasma T levels, which remained high until 48h p.i. and decreased to basal levels after 72h p.i. (except group 4 that received the second hormone injections at time 48h). These results showed that increase in advancements of milt production is in response to the GtH stimulated synthesis of gonadal steroids (Zohar, 1989).
The most important androgens produced by fish testes are testosterone and 11 keto testosterone (11KT). T levels have a common profile in blood showing an increase during spermatogenesis, reaching maximum at the beginning of spermiation in brook trout, rainbow trout and Atlantic salmon (see Zohar, 1989).

Some *In-Vivo* and *In-Vitro* data also suggested that T and 11KT stimulate spermiation (Zohar, 1989). In white bass, *M. saxatilis*, it is believed that androgens were involved not only in spermatogenesis and spermiogenesis but also had a role in milt production (Mylonas *et al.*, 1997). Also, T was used for inducing spermiation in such fish as rainbow trout, green sunfish and northern pike (see Donaldson & Hunter, 1983). In New Zealand snapper, using GnRHa injections (100 μg/kg B.W.) caused significant increase in T and 11KT of plasma at 24h p.i.; increase in plasma T and 11KT levels are associated with increase in milt volume after injection of GnRHa as found in goldfish, common carp and black progy (see Pankhurst, 1994) and stellate sturgeon (Semenkova *et al.*, 2001). Evidence also shoed that there is correlation between plasma androgen levels and milt production in natural population of white sucker, rainbow trout and snapper (see Pankhurst, 1994).

Decrease in plasma T at 72h p.i. may be an indication of the rapid clearance of GnRHa due to the activity of cytosolic enzyme; decrease in GnRHa content caused the reduction in GtH stimulation followed by reduction in gonadal stimulation and T releases (Zohar, 1989). In fish of group 4, which received second injections at 48h after first one, T level remained significantly high compared to time 0, showing the stimulation effect of the second GnRHa injection on GtH and T release. Mylonas *et al.* (2001) noted that injection of hypothalamic peptide (GnRH) can induce short lived elevation of plasma GtH followed by fluctuation in sex steroids. However, for successful induction of gonadal maturation and spawning, multiple injections have to be given over a course of several days in case of trout, salmon and stripped bass or over a several weeks in case of multiple spawners like gilthead sea bream.

Finally, it is necessary to find the minimum effective dose of GnRHa on *O. mykiss* and to examine the effectiveness of this compound in inducing spermiation and in increasing milt volume production in such species as *Salmo trutta caspius*, *Rutiluss frissi kutum* and Sturgeons. This may show some difficulties in the number of available male or induction of spermiation. It is also necessary to

evaluate the sperm quality and the fluctuation of other steroid hormones like 11 KT and 17α 2oβ dihydroxy progesterone.

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