Asian Fisheries Science **28** (2015): 26-36

ISSN 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2015.28.1.003

Asian Fisheries Science
The Journal of the Asian Fisheries Society

Profiles of Sex Steroids in Wild-caught Carp *Cyprinus carpio carpio* Linnaeus 1758 During Ovulation Induction by Acute Versus Sustained Delivery Methods of Different GnRHa Analogues

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Abstract

The effects of acute versus sustained delivery methods of GnRHa on sex steroid profiles of wild-caught female carp *Cyprinus carpio carpio* Linnaeus 1758 (Cyprinidae) were studied in fish receiving 20 $\mu g \cdot kg^{-1}$ mammalian or salmon GnRHa in acute (Linpe method) or sustained delivery methods, i.e., cholesterol pellets (Chol) and emulsion of GnRHa (GnRHa-FIA). Negative and positive control fish were injected by physiological saline (control) and 4 $mg \cdot kg^{-1}$ carp pituitary extract (CP), respectively. Plasma steroid levels of testosterone (T), 17β-estradiol (E2) and 17α, 20β-dihydroxy, 4-pregnone, 3-one (DHP) were measured by RIA at 0, 6, 12, 24 and 48 h after final injection. Plasma level of T decreased after final injection in almost all treatment groups and gradually increased thereafter, and peaked at ovulation time (from 0.56±0.12 in sChol to 1.50±0.56 $ng \cdot mL^{-1}$ in CP). E2 levels showed similar changes in almost all treatments with an increase at ovulation time (from 0.46±0.14 in sChol to 1.69±0.93 $ng \cdot mL^{-1}$ in CP) and a decrease afterwards except for Chol groups in which the levels of E2 decreased at ovulation time. Plasma DHP levels showed a surge at the ovulation time (from 14.27±3.27 in mlinpe to 21.80±17.44 $ng \cdot mL^{-1}$ in sLinpe) before a rapid decrease in all groups except for Chol groups in which the levels of DHP gradually increased during ovulation induction and remained high even after 48 h (from 7.92±1.4 in mChol to 9.37±0.76 $ng \cdot mL^{-1}$ in sChol).

Introduction

Changes in plasma levels of sex steroid hormones have been reported during natural or induced ovulation of teleost fish (Fostier et al. 1983; Kime 1993; Nagahama 1994; Yaron 1995; Peter and Yu 1997; Zohar and Mylonas 2001). In females, testosterone (T) acts as a precursor of 17β-estradiol (E2), although its role in maintaining the surge of plasma gonadotropin during final oocyte maturation and ovulation was also reported (Fostier et al. 1983; Santos et al. 1986; Aida 1988; Kobayashi et al. 1988; Kime 1993). Recently, the role of androgens in primary growth of oocyte has also been reported in some fish species (Lokman et al. 2007; Kortner et al. 2009a; Kortner et al. 2009b).

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Although the main role of E2 is in vitellogenin synthesis in liver during vitellogenesis, many cyprinids and non-cyprinid fishes showed a change of E2 during ovulation (Fostier et al. 1983; Weil and Crim 1983; Fostier and Jalabert 1986; Levavi-Zermonsky and Yaron 1986; Santos et al. 1986; Kime 1993; Yaron 1995; Aizen et al. 2012). 17α-20β, dihydroxy, 4-pregnen-3-one (DHP) is known as maturation inducing hormone (MIH) in most of the teleost fish and acts as a main triggering hormone of the final oocyte maturation (Nagahama 1997). Changes had also been reported for circulating DHP level during induction of ovulation and spawning in domestic strains of carp (Levavi-Zermonsky and Yaron 1986; Drori et al. 1994; Yaron 1995; Aizen et al. 2012).

Wild carp *C*yprinus *carpio carpio* Linnaeus 1758 (Cyprinidae) is one of the most economically important fish in the Caspian Sea basin and European region (Barus et al. 2001; Vazirzadeh et al. 2011). Populations of wild carps in natural ecosystems are vulnerable or endangered in many places due to anthropogenic activities (Balon 2004; Vazirzadeh et al. 2011). Artificial and semi-artificial breeding of wild carp has been started since two decades ago in the southeastern coastal waters of the Caspian Sea for restocking natural populations, but the output is low because of traditional spawning methods. Our previous studies pointed out that wild carp is a multiple spawner in this region (Vazirzadeh et al. 2014) and sustained releasing delivery systems of GnRHa are more effective than acute delivery methods in enhancement of its reproduction performance (Vazirzadeh et al. 2011).

The effects of acute injection of GnRHa and other spawning stimulating agents such as carp pituitary extract (CP) and hCG on sex steroid profiles were widely studied in domestic strains of common carp (Breton and Weil 1973; Weil et al. 1980; Levavi-Zermonsky and Yaron 1986; Santos et al. 1986; Drori et al. 1994; Yaron 1995; Yaron et al. 2009; Aizen et al. 2012). Also profiles of steroid hormones were studied in some species during ovulation induction by sustained delivery methods of GnRHa (Mylonas and Zohar 2001; Guzman et al. 2009), but the effects of GnRHa administration in the form of sustained delivery methods on sex steroids have yet to be determined in wild carp with asynchronous ovarian development pattern (Vazirzadeh et al. 2014). Also, many studies reported that different strains of carp showed various reproduction behaviours in each local environments (Levavi-Zermonsky and Yaron 1986; Balon 1995; Yaron 1995; Barus et al. 2001; Sivakumaran et al. 2003; Smith and Walker 2004) or in captive conditions even among domesticated lines (Levavi-Zermonsky and Yaron 1986; Yaron 1995; Brzuska 2005; Yaron et al. 2009). Thus, the basic reproductive features of different populations could differ from each other which need to be studied before any spawning induction programme.

This research aimed to study the effects of acute versus sustained delivery systems of GnRHa on the plasma concentrations of T, E2 and DHP, in wild carp brooders from the Caspian Sea during induction of ovulation.

Materials and Methods

Fish maintenance and hormone treatments

Cholesterol pellets and GnRHa-FIA emulsion using Freud's incomplete adjuvant were made according to the methods described in Vazirzadeh et al. (2008; 2011). Fish for hormonal therapy were selected from those in Sijowal Center for Fish Propagation and Culture in Golestan province, Iran. These fish were originally caught by beach seine net from Golestan coastal waters and transferred to the propagation centre in a tank with oxygenated water.

The following day, 24 h after transfer, brooders were checked again and those without any injury were separated and stored in 1,700 L indoor rectangular tanks with running fresh water with temperature ranging from 21-23 ºC. The weight of fish was 2,535±546 g (mean±S.D.)

Hormonal therapies were carried out in 8 treatments, each containing 10 individuals (except treatment 8 with 6 individuals due to lack of sufficient fish with GV stage of 2 or more) as follows:

T1: fish injected intraperitoneally with 0.7% saline solution combined with Freud's incomplete adjuvant (control).

T2: fish received 4 $mg \cdot kg^{-1}$ CP in 2 steps with 12 h interval (CP).

T3 and 4: fish were injected intraperitoneally with 20 $\mu g \cdot kg^{-1}$ mGnRHa (mLinpe) and sGnRHa (sLinpe) dissolved in saline solution, respectively (Peter et al. 1988).

T5 and 6: fish received intraperitoneal injections of GnRHa-FIA emulsion containing 20 $\mu g \cdot kg^{-1}$ mGnRHa (mGnRHa-FIA) and sGnRHa (sGnRHa-FIA), respectively.

T7 and 8: fish were implanted with cholesterol pellets containing 20 $\mu g \cdot kg^{-1}$mGnRHa (mChol) and sGnRHA (sChol), respectively.

All GnRHa treated groups received 20 $mg \cdot kg^{-1}$ metoclopramide as dopamine D2 receptor antagonist intraperitoneally. To avoid over ripening, fish were checked regularly 6 h after final injection and ova from ripened brooders were stripped artificially. The effects of hormonal therapies on the reproduction performance were studied as previously described in Vazirzadeh et al. (2011).

Steroid hormones measurements

To measure the steroid hormones (T, E2 and DHP), blood samples were taken from fish in all treatments, at 0, 6, 12, 24 and 48 h after final injection using heparinised syringes. Steroid hormones were measured by radioimmunoassay (RIA) using the reagents and protocol as previously described (Fostier et al. 1983; Fostier and Jalabert 1986). Briefly, 500 µL of plasma were extracted with 2 mL ethyl acetate: cyclohexane (50:50) and 200 mL extract were used for each assay tube. Samples were measured in duplicate and all samples were measured in a single

assay. The cross-reactivities of various steroids with the antisera used in the radioimmunoassay have already been described (Fostier and Jalabert 1986).

Data analysis

Normal distribution of data was verified by Kolmogorov-Smirnov test. Variance homogeneity of data was tested using Leven test. The effects of hormonal treatments on plasma levels of steroids were analysed using two-way ANOVA looking at time and treatment as factors followed by Tukey's test to compare means. This analysis was based on the individuals in which spawning occurred to remove the effects of non-ovulated fish. Data analysis was carried out in SAS 9.3.1 portable. Statistical significance was accepted at $P \leq 0.05$

Results

Effects of treatments on plasma testosterone levels

The details of testosterone fluctuations in different treatments over sampling times are shown in Fig. 1. Variation in levels of plasma testosterone in different hormonal therapy groups did not follow an orderly pattern. In T2 (pituitary extract), T3 and T4 (Linpe methods), testosterone decreased at 6 h post-injection but increased at 12 h (ovulation time) and decreased again afterwards. In T6 and T7 (GnRHa-FIAs) testosterone decreased until 12 h post-injection, then increased at 24 h (ovulation time) and finally decreased after that. In T7 and T8 (cholesterol pellets), the hormone level showed a decrease at 6 h post-injection and gradually increased afterwards but did not reach the level observed at time zero. Plasma steroid levels of testosterone showed no significant differences between ovulation time and time zero in all groups (except in fish receiving GnRHa-FIAs).

Effects of treatments on plasma E2 levels

The details of E2 changes in different treatments over sampling times are shown in Fig. 2. The level of E2 increased at spawning time and decreased thereafter in all hormonal treated groups except for cholesterol groups in which E2 peaked at 24 h post-injection but decreased at spawning time. The levels of E2 at spawning time were significantly higher than control group and time zero in all hormonal treated groups except for cholesterol groups ($P \leq 0.05$).

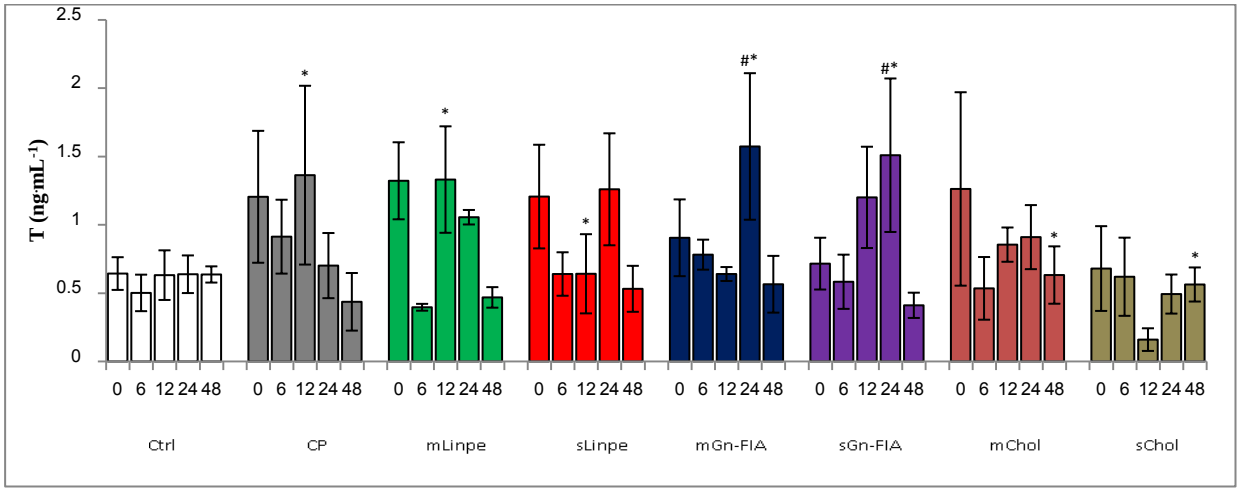

Fig. 1. Changes in plasma T concentrations (mean±S.D.) after induction of ovulation in wild carp with acute and sustained delivery methods of mammalian and salmon GnRHas (containing 20 µg·kg $^{-1}$ GnRHa plus 40 mg·kg^{-1} metoclopramide) measured by RIA at 0, 6, 12, 24 and 48 h after final injection. The level of T increased at ovulation time (indicated by *) in all hormonal treated groups except for sLinpe and cholesterol groups, but were significantly different from control group and time of final injection (time 0) only in GnHa-FIA receiving groups (indicated by #). Data were analysed by two-way ANOVA looking for interaction of treatments and times of bleeding; ($p \leq 0.05$). Except for control group, data are from ovulated individuals.

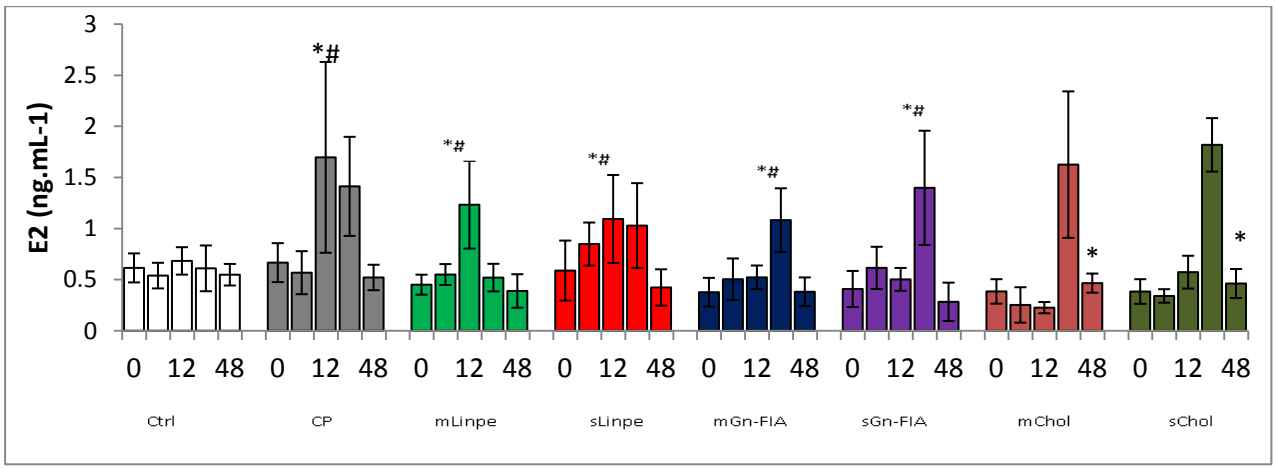

Fig. 2. The effects of acute versus sustained delivery methods of mammalian and salmon GnRHas (containing 20 µg·kg $^{-1}$ GnRHa plus 40 mg·kg^{-1} metoclopramide) on plasma level of E2 (mean±S.D.) during ovulation induction in wild carp measured by RIA at 0, 6, 12, 24 and 48 h after final injection. Levels of E2 increased in all treatments at ovulation time (indicated by *) and were significantly higher than those in control group and time of final injection (time 0) (indicated by #) except for two cholesterol groups in which E2 levels decreased at ovulation time. Other details are same as for **Fig.1**.

Effects of treatments on plasma DHP levels

Plasma levels of DHP in different groups over different time intervals are shown in Fig. 3. DHP levels showed an increment at spawning time before a sharp decrease in all treated groups, which were significantly higher than zero time. Interestingly the levels of hormone in acute treatments were higher than those in sustained delivery groups except for fish receiving sGnRHa-FIA (T6). Unlike the hormone levels, the duration of being at peak was longer in sustained delivery treatments than acute and CP groups. As in T7 and T8 (cholesterol pellets) the

DHP level increased gradually at 6 h post-treatment and remained constantly at peak until 48 h after injection. The same patterns were also observed in T5 and T6 (GnRHa-FIAs) in which DHP increased gradually from 6 h post-injection, peaked at 24 h and decreased thereafter. The levels of DHP at ovulation time in GnRHa-FIA groups were significantly higher than those in cholesterol groups, and were similar to Linpe and CP groups. The variations of DHP between individuals were high, as for instance, it varied from 2-90 ng·mL^{-1} in two ovulated fish.

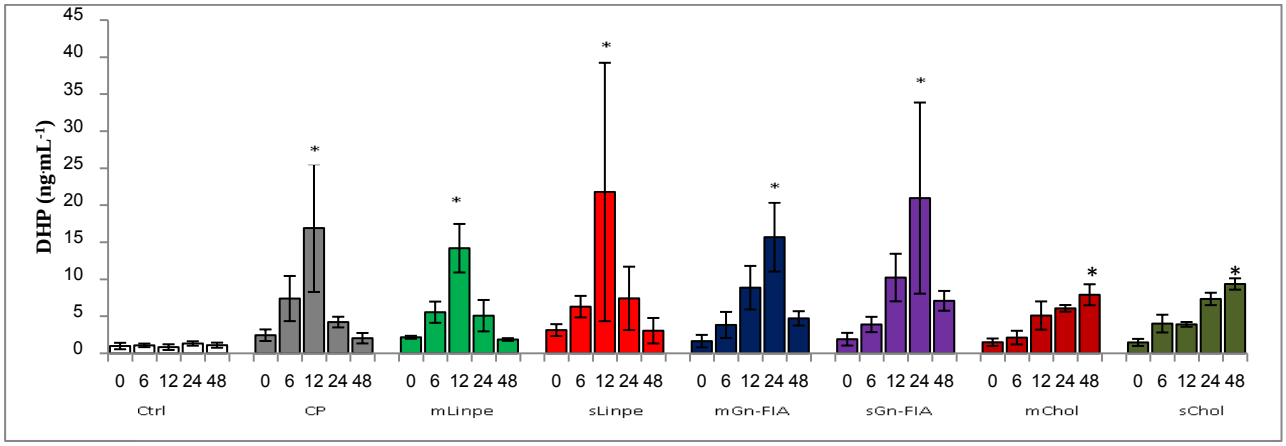

Fig. 3. Plasma levels of DHP (mean±S.D.) during ovulation induction in wild carp by acute versus sustained delivery methods of mammalian and salmon GnRHas (containing 20 µg·kg $^{-1}$ GnRHa plus 40 mg·kg^{-1} metoclopramide) measured by RIA at 0, 6, 12, 24 and 48 h after final injection. The levels of DHP in all hormonal treated groups peaked at ovulation time which were significantly higher than those in control group and all other bleeding times (indicated by *). The levels of DHP rapidly decreased after ovulation except for cholesterol groups in which the levels of DHP remained high even after 48 h. High individual variations were observed in level of DHP except for cholesterol groups. Other details are same as for **Fig.1**.

Discussion

Most of the studies on endocrine control of carp reproduction have been focused on sex steroid changes during final oocyte maturation and ovulation in domestic strains after being induced by natural cues or acute hormonal treatments (Weil et al. 1980; Santos et al. 1986; Drori et al. 1994; Yaron 1995; Diniz et al. 2005) and there is no study on the effects of sustained delivery methods of GnRHa in multiple spawner carps. Thus, this study aimed to investigate the circulating changes of sex steroids in wild caught carp after spawning induction by sustained versus acute delivery methods of GnRHa.

The increase of T plasma levels was reported in common carp after induction of ovulation by acute hormonal treatments in previous studies (Santos et al. 1986; Aida 1988). It has been suggested that T acts as precursor of E2 and it may have vitellogenic action in liver as well as positive feedback on the GtHs secretion from pituitary (Fostier et al. 1983). Recently the role of T and other androgens in primary growth of oocyte during endogenous vitellogenic stage was suggested in some fish (Lokman et al. 2007; Kortner et al. 2009a; Kortner et al. 2009b). In asynchronous fish whose ovary contains oocytes at different stage of maturity, T may have a role during final maturation and ovulation in promoting the growth of previtellogenic oocytes and maintaining GtH secretions (Nagler and Idler 1992). Qin et al. (2009) also reported the increase

of T during the ovulation induction of loach *Misgurnus anguillicaudatus* (Cantor 1842), (Cobitidae) by GnRHa. Increase of sex steroid concentrations during the spawning season in the juvenile and adult male common carp was also reported (Chang and Chen 1990).

A probable explanation to the decrease of T at ovulation time in T4 and cholesterol groups may be due to individual variations. However, the decrease of T prior to ovulation has also been reported in some asynchronous species. The decrease of testosterone arises prior to the increase of E2, which can be expected as T is the precursor of E2 and this event reflects an active conversion of T to E2 at the time of ovulation. Rinchard et al. (1993) and Sun and Pankhurst (2004) reported the decrease of T before ovulation of gudgeon *Gobio gobio* (Linnaeus 1758), (Cyprinidae) and greenback flounder *Rhombosolea tapirinia* Gunther 1862, (Pleuronectidae), respectively. Recently, a time-dependent decrease in plasma T levels was reported in Waigieu seaperch *Psammoperca waigiensis* (Cuvier 1828), (Latidae), a tropical asynchronous marine fish (Pham et al. 2010) and catfish *Heteropneustes fossilis* (Bloch 1794), (Silluridae) (Chaube et al. 2014). Therefore, the conclusion is that T showed various circadian patterns in different fish and even in a given fish after treatment with different stimulating agent.

The increase of plasma level of E2 at ovulation time has also been demonstrated in domestic carp in previous study (Weil et al. 1980). Another study on female carp injected with pituitary extract or sGnRHa along with metoclopramide has shown an increase in E2 up to 10 $ng{\cdot}mL^{-1}$ at ovulation time, then a subsequent decrease (Drori et al. 1994). Whereas in natural GnRH injected carp, E2 level showed a short increase (Weil et al. 1980). Also in carp when ovulation was induced with water temperature the E2 level showed an increase but much lower than that of the above mentioned studies (Santos et al. 1986). However in another study the level of E2 rose after priming pituitary extract injection, decreased after second injection and again increased at the spawning time (Levavi-Zermonsky and Yaron 1986). The increase of T and E2 in goldfish during spawning time has also been reported (Kobayashi et al. 1987). Vazirzadeh et al. (2014) also reported the increase of T and E2 during natural spawning of wild carp caught from the south-eastern Caspian Sea. It has been mentioned that T and E2 increase had positive feedback on GtH release and efficacy (Kobayashi et al. 1987; Yaron 1995; Peter and Yu 1997; Yaron et al. 2009). The researchers have attributed the E2 increase during ovulation to vitellogenic oocyte presence in the ovary (Weil et al. 1980; Santos et al. 1986; Drori et al. 1994), as the case in wild carp with asynchronous ovarian development pattern.

Similar to T levels, E2 decreased at ovulation time in cholesterol implanted groups. These fish had the longest latency period and 40% and 50% of implanted fish ovulated in mChol and sChol groups, respectively. They also had highest practical fecundity among all treated groups which were stripped manually several times (Vazirzadeh et al. 2011). The responsive rates of cholesterol implanted fish were also lower than groups receiving sLinpe and GnRHa-FIAs probably due to lower rate of GnRHa release from pellets as discussed in Vazirzadeh et al. (2011). So, the different pattern of T and E2 changes in cholesterol groups may be attributed to the low releasing rate of GnRHa from pellets as well as coincident maturation of oocytes with more variance in maturity stages compared to other groups, which finally resulted in lower ovulating rate but higher fecundity in ovulated fish.

Plasma levels of DHP peaked at ovulation time in all treated groups which were significantly higher than those in control and time zero, but the pattern of DHP increase in acute Linpe groups were different from sustained releasing delivery treatments. While in 12 h post-injection DHP peaked in T2, T3 and T4, no significant changes were observed in sustained releasing delivery groups. Also the absolute levels of DHP were significantly different between sustained releasing delivery groups. In cholesterol groups DHP level rose to approximately 8 $ng{\cdot}mL^{-1}$ at the time of ovulation, a level much lower than that in GnRHa-FIA receiving groups (25-35 $ng{\cdot}mL^{-1}$). These variations are probably due to different releasing rate of GnRHas from vehicles to blood of treated fish. Studies have shown that hypothalamic hormonal therapies lead to an increase in DHP level during final oocyte maturation and ovulation in carps (Levavi-Zermonsky and Yaron 1986; Santos et al. 1986; Drori et al. 1994; Yaron 1995; Yaron et al. 2009); consequently this hormone has been described as final oocyte maturation hormone in carps (Nagahama 1997). DHP is also important for previtollogenic oocyte growth (Zapater et al. 2012) particularly in species with an asynchronous ovarian development pattern. There are some growing evidences showing the positive effects of DHP in whole reproductive event in fish species (Zapater et al. 2012). Comparison of the trends of E2 and DHP changes (Figs. 2 and 3) shows that both hormones rose at ovulation time indicating a probable synergistic action between these two steroids during the ovulation of this species (Fitzpatrick et al. 1986; Kazeto et al. 2011).

The results showed that both hormone types and administration routes had significantly different time-dependent effects in the plasma levels of DHP. Injecting fish with mGnRH by Linpe method (mLinpe) or implanting by cholesterol pellets of both GnRHa types (mChol and sChol) led to lower DHP levels compared to other treatment groups. Intrinsic lower ability of mGnRHa in comparison with sGnRHa to stimulate the GtH secretion (Peter et al. 1988; Zohar and Mylonas 2001; Vazirzadeh et al. 2011) and eventually DHP release as a consequence as well as slow release of substance from cholesterol pellets (Mylonas and Zohar 2001) could be the possible causes for the observed differences.

Conclusion

The results of this study showed that both hormone types and administration methods had significantly different time-dependent effects in the plasma levels of sex steroids. sGnRHa was more potent than mGnRHa in raising the plasma steroids, but both analogues had similar effects when applied in sustained delivery methods. While acute methods of GnRHa increased higher levels of plasma steroids, sustained delivery systems resulted in lower but significantly longer duration of sex steroids increase in induced wild carp.

Acknowledgement

This work was partially supported by ICRP, Ministry of Science, Research and Technology and Science & Technology Deputy of President, Iran. The financial support of Shiraz University Research Council is also acknowledged. The kind assistance of Dr. B. Heidari (Department of Crop Breeding, Shiraz University) for statistical analysis is acknowledged. The author would like to thank the manager and staff of Sijowal Center for Fish Propagation and Culture for providing the fish and their assistance during fish breeding. The kind help and support of people in SCRIBE-INRA Lab during the stay of the author in France are greatly appreciated. The author would like also to thank the anonymous reviewers of this manuscript.

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Received: 08/02/2015; Accepted: 26/02/2015 (MS15-9)