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Effect of human chorionic gonadotropin on sexual maturation, sex steroids and thyroid hormone levels in Caspian lamprey (*Caspiomyzon wagneri* Kessler, 1870).

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ABSTRACT

The objective of this study was to determine the effect of human chorionic gonadotropin (hCG) on sexual maturation, plasma sex steroids [17β-estradiol, (E2) and 17α-hydroxy progesterone (17α_OHP)] and thyroid hormones (triiodothyronine, T3 and thyroxin, T4) levels in upstream - migrating Caspian lamprey. During the experiment, 36 fish (24 females and 12 males) in spring 2013 and 36 fish (24 females and 12 males) in fall 2013 were collected from the Shirud River estuary in Mazandaran Province, the Southern Caspian Sea during their upstream migration to the freshwater. All fish were injected with hCG at the doses of 1000, 1500 and 2000 IU.kg BW^{-1}. The injection was a two - step process (50% of hormone in each step) by 12 h interval. After the first injection, fish were retained in the cages in the river beds and 24 hours after the second injection, fish were checked for egg and sperm release after mild abdomen pressure. Blood samples were taken for determining sex steroid levels. Results showed that hCG hormone injections caused increase in migration of germinal vesicle in the oocyte of female and sperm release in males. Significant differences were found in the serum E2 and 17α-OHP levels in hCG - injected fish compared to the control. However, no significant differences were found in serum T3 and T4 levels in the hCG - injected fish. According to the results, the appropriate hCG dosage to induce the reproduction acceleration in Caspian lamprey is 1500-2000 IU.kg BW^{-1}.

Key words: Lamprey, Caspian Sea, Induced reproduction, Hormonal changes.

INTRODUCTION

Caspian lamprey (*Caspiomyzon wagneri* Kessler 1870) is a jawless fish (Agnatha) native to the Caspian Sea and its north, west and south basin. This species is an anadromous fish, entering the Caspian Sea south basin rivers for spawning (Coad 2012). The spawning migration occurs either in spring (mid - March through late May) or fall (mid - September through mid - December). It was told that the Caspian lamprey spawning only occurs in spring, while fall migrants spend whole winter in the river and spawn by the following spring (Jolodarzade & Abdoli 2004). But the recent studies showed that both spring and fall migrants are ready to spawn in both seasons (Ahmadi *et al.* 2011). Spawning of the Caspian lamprey usually takes place in 3 km above the estuary in the river (e.g. Shirud River, Jolodarzade & Abdoli 2004).

Nowadays, due to the dam construction, water pollution, overfishing, and lack of suitable spawning ground, Caspian lamprey's population is decreasing rapidly and according to the IUCN reports it is categorized as "near threatened" species (IUCN 1996; Kiabi *et al.*

1999). So in order to preserve and increase Caspian lamprey's stocks, achieving the reproduction technology of this fish looks necessary. Although conducted studies on some lamprey species in the world provided some information on artificial reproduction in captivity using GnRH or pituitary homogenate (Bradley *et al.* 2004), in the southern part of the Caspian Sea, only basic reproductive information about the Caspian lamprey are provided (Farrokhnejad *et al.* 2014, Vatandoust *et al.* 2015). No reliable information has been presented about artificially - induced spawning of this threatened species in the freshwater basins of the Caspian Sea using synthetic hormones and suitable spawning grounds so far. Sex steroids play a key role on the lamprey maturity (Bradley *et al.* 2004). In both Sea and Arctic lampreys (*Lampetra camtschatica*), E2 concentrations increased during spermiation (Fahien & Sower 1990), while decreased during ovulation (Bolduc & Sower 1992).

Previous studies on this species showed that the serum progesterone and testosterone concentrations were significantly higher in mature females and males compared to maturing fish, while E2 was lower in mature female compared to maturing female (Farrokhnejad *et al.* 2014). It was shown that, thyroid hormones (T3 and T4) have constructive roles on controlling growth, metabolism and osmotic adjustment of fish (Takaei 2006).

Also, these hormones seem to have prominent influence on migration behaviors and adaptive changes in migrating fish (Takaei 2006). So, study on these hormones may provide valuable biological information in order to manage the Caspian lamprey reproduction.

In this research we tried first, to induce reproduction using, hCG, (similar compound structurally and functionally to GtH) and second, elucidate responses of reproduction related sex steroid hormones (E2 and 17α-OHP). Also we attempted to place the spawning migrant fish at the natural river grounds after hormone manipulation, and monitor the spawning responses.

MATERIALS AND METHODS

Fish were caught during their spawning migration by hand under the Shirud River Bridge, Southern Caspian Sea, Northern Iran (200 m upstream from the river mouth, 34° 44′ – 34° 51′N, 50° 48′ – 50° 49′E).

The first attempt was conducted during spring spawning run on April 8, 2013, the second sampling in the same season on May 4, 2013, and the third sampling during fall migration on October 7, 2013.

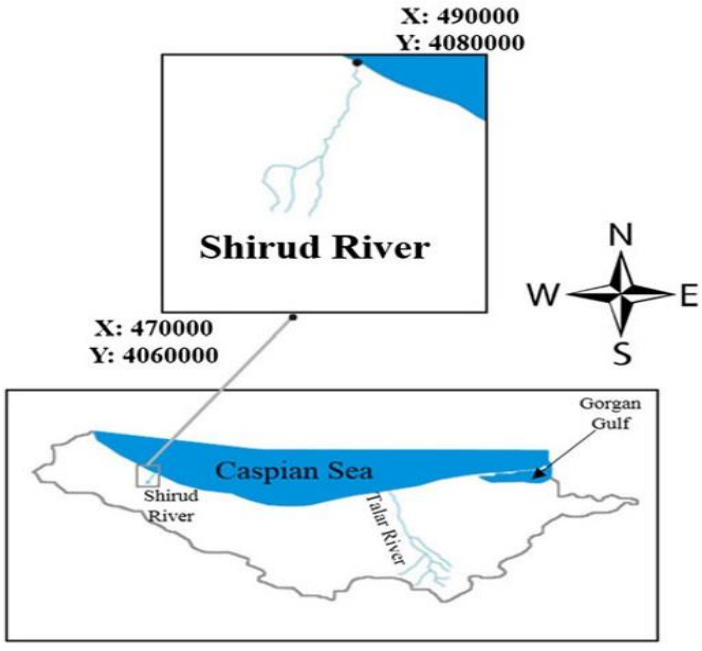


Fig. 1. Study area at the southern basin of the Caspian Sea.

After catching, the fish were anesthetized in 200 ppm clove oil solution, then after measuring their weight and length, males and females were separated based on secondary sexual characteristics including fin types and sexual papilla (Farrokhnejad *et al.* 2014). Fish were injected by three different doses of hCG hormone (manufactured by LG life sciences, Korea) as 1000 IU.kg^{-1} body weight (BW) (group 1), 1500 IU.kg^{-1} BW (group 2), 2000 IU.kg^{-1} BW (group 3) and 0.65% NaCl as control group in three replication for each treatment, containing three fish in each one (one male and two females). The desired volume of injection was 0.5 mL per 100 g Bw and the desired hormone concentration was obtained by dilution of hCG stock using a physiological saline solution (Rottmann *et al.* 1991).

HCG was injected intramuscularly with two separate dosages (50% each) by 12 h intervals as first and second injections. Males were received only one injection (50% of the doses) at the time of the second injection into females in each hCG treatment.

Treated fish were placed on the prepared nests which were installed at the lamprey's migration route in the river (Fig. 1). Each nest contained three fishes (two females and one male). Plastic nests had many holes at their ends for ease of river water flow.

To mimic the lamprey natural spawning condition, bed of each nest was covered by gravel and the nest entrance was closed by a 0.5 mm mesh net (Fig. 2). In order to prevent further manipulation which could cause negative impact on physiological activity, fish were checked once and according to Rottmann *et al.* (1991), twenty-four hours after the second injection, by removing them from the cages and checking for spawning performance by stripping. Blood samples were taken from the main artery along the spinal cord after heart using U-100, 1ml insulin syringes, then immediately transported to the lab at the refrigerator temperature (4^{o}C). Blood serum was separated using centrifuge (Labofuge 2000 centrifuge, Heraeus Spatech Company, Germany) at 2500 rpm for 10 minutes with 490 g, then stored in -20^{o}C up to hormone assay (Kubokawa *et al.* 1999).

The serum levels of 17α-OHP, E2, T3 and T4 were measured using Immunotech RIA kit (Marcel Immunotech Company, France) (Lister *et al.* 2008). In order to express the precision, or repeatability of immunoassay test results, the Coefficient of Variability (CV) were evaluated in two measures: the Inter-Assay CV variability was ≤10% and the Intra-Assay CV variability was ≤9%.

Fish were anesthetized in 200 ppm clove oil solution, then samples (small parts of the ovary or testis) were removed and fixed in Bouin's fluid and then prepared for histological studies (Rinchard & Kestemont 1996). Gonadal developmental stages were recognized according to Farrokhnejad *et al*. (2014). Water physio-chemical parameters in the river where the cages were installed, were measured and recorded during the experiment. The dissolved oxygen, water temperature, pH, electrical conductivity (EC) and total dissolved solids (TDS) of the river water where the fish were settled were 11.4 ± 0.3 mg.L^{-1}, 10.9 ± 0.2 mg.L^{-1}, 16 ± 0.5 ^{o}C, 16.4 ± 0.3 ^{o}C 7 ± 0.1-7.5 ± 0.1, 8.3 ± 0.3 µS.L^{-1}, 8.1 ± 0.2 µS.L^{-1}, 203 ± 6 and 196 ± 7 mg.L^{-1} in spring and fall, respectively.

Biometrical data of the caught fish which used for the experiment, are given in Table 1. The data were analyzed using SPSS software V. 21. Experimental treatments were compared in different levels of sex steroid parameters using One-Way ANOVA test. The data were tested to check their normality using Kolmogorov-Smirnov test. In order to compare the obtained average difference, Tukey test was used in 5% significant level.

Table 1. Biometrical data of fish used in the experiment during spring and fall.

	male Spring n = 12	**male Fall n = 12**	**female Spring n = 24**	**female Fall n = 24**
Total length (cm)	39.44 ± 3	36.8 ± 1.39	40.17 ± 2.91	38.22 ± 3.7
Total weight (g)	94.89 ± 24.45	83.57 ± 8.02	107.6 ± 24.03	94 ± 20.01

Fig. 2. hCG treatment and nest setting. 1. Plastic nest with holes. 2. Fish injection. 3. Placing injected fish in nest. 4. Placing nests in the river bed.

RESULTS

The gonad weight of males ranged 3.91–6.32g and 3-6.63g in spring and fall respectively, while in females, the gonad weight ranged 7.96 – 12.85g and 8.81–13.6g, respectively.

The mean ± SD of gonadosomatic index (GSI) in males were 5.67 ± 0.46% and 5.44 ± 0.38% in spring and fall, respectively. This factor in females were recorded as 10.01 ± 0.89% in spring and 10.36 ± 0.96% in fall, respectively. Twenty-four hours after the final injection, male breeders were removed from the baskets in order to check their sperm releasing. The results showed that all fish which were received 1500 and 2,000 IU.kg^{-1} hCG, released their sperms, while once stripping of fish, those treated with 1000 IU.kg^{-1} hCG and also control group were not successful to release. The majority of testicular lobules in all fish were filled with spermatozoa, but only the fish with higher injected hCG dose were able to release their sperms (Fig. 4).

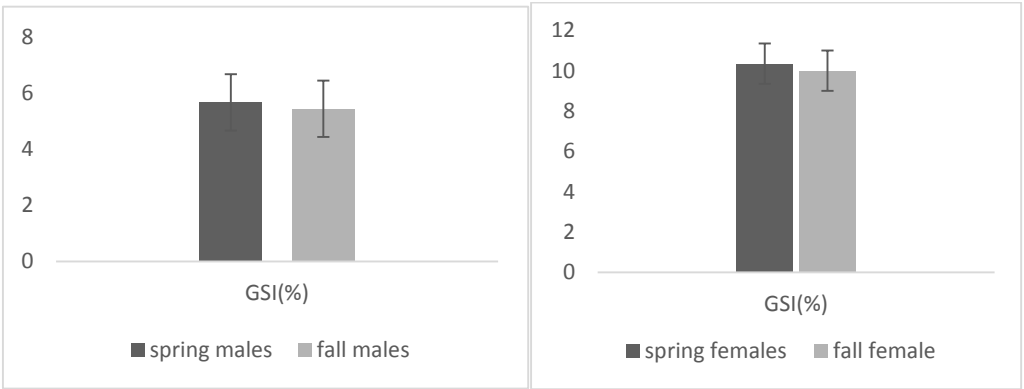


Fig. 3. The amount of GSI in Caspian lamprey in spring and fall.

Table 2. Rate of succeeded male fish in sperm releasing by stripping in various treatments (n = 3 for each).

	Control group 0 IU.kg^{-1} BW	1000 IU.kg^{-1} BW	1500 IU.kg^{-1} BW	2000 IU.kg^{-1} BW
Spring migrants	0	0	66.66%	100%
Fall migrants	0	33.33%	100%	100%

Although injection in female fish could not induce oocyte release (spawning), but promote the maturation process which was evident in histological events of oocytes. An obvious migration in the oocyte germinal vesicle (GV) was evident in treated fish. GV migration toward the animal pole and GVBD was obviously visible in fish treated with 1500 and 2000 IU (Fig. 4).

Significant increases were recognized in the levels of serum E2 after hCG injection in both seasons either in males or in females compared to control group (Fig. 5A, $P < 0.05$).

A significant hCG dose - dependent increase happened in the levels of 17α-OHP compared to control group (Fig. 5B, $P < 0.05$).

The results of validating thyroid hormones are given in Fig. 5. The results showed that the injection of hCG has no significant impact on the level of thyroid hormones. Changes in the level of T4 in blood plasma were not related to hCG injection either in spring or in fall. ($P > 0.05$). Evaluating the level of T3 in blood plasma showed the same result. The injection of hCG had no significant impact on the level of T3 neither in spring nor in fall (Fig. 5 D, $P > 0.05$).

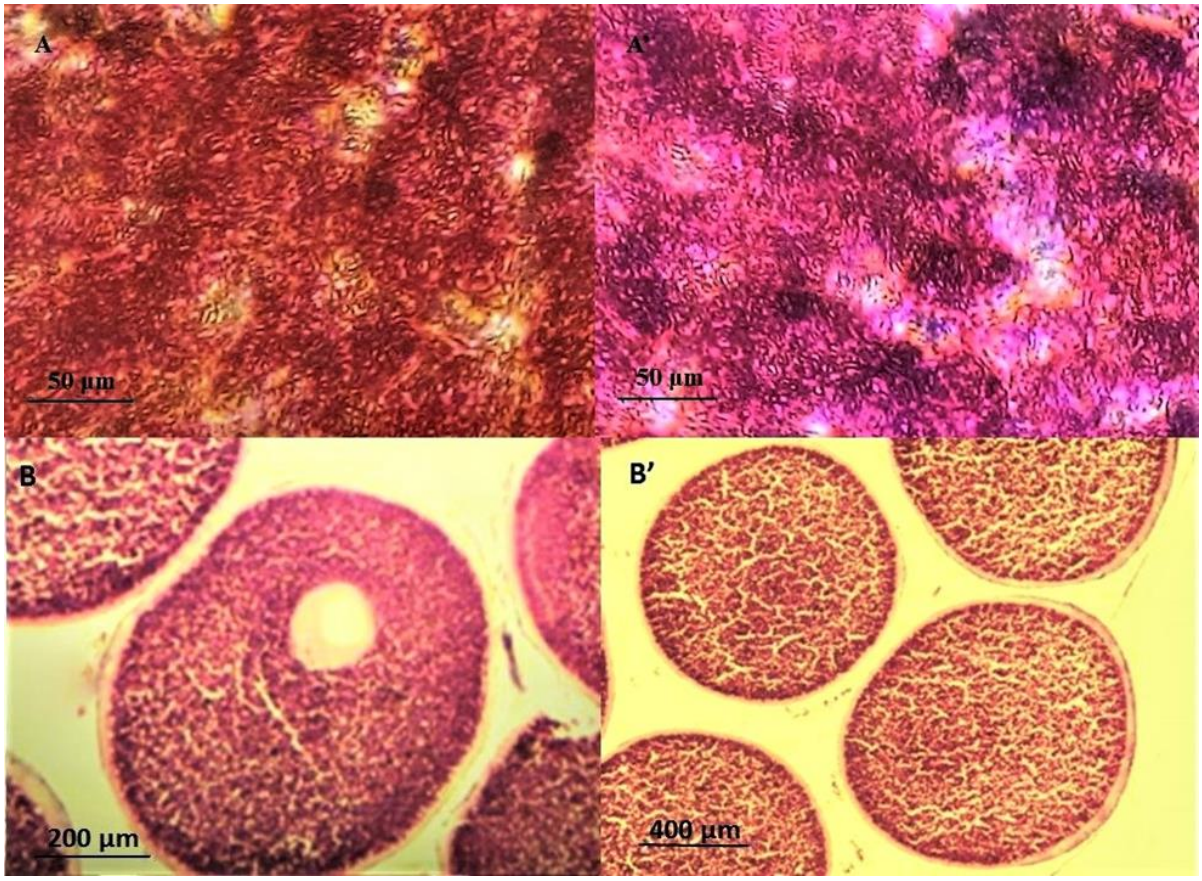
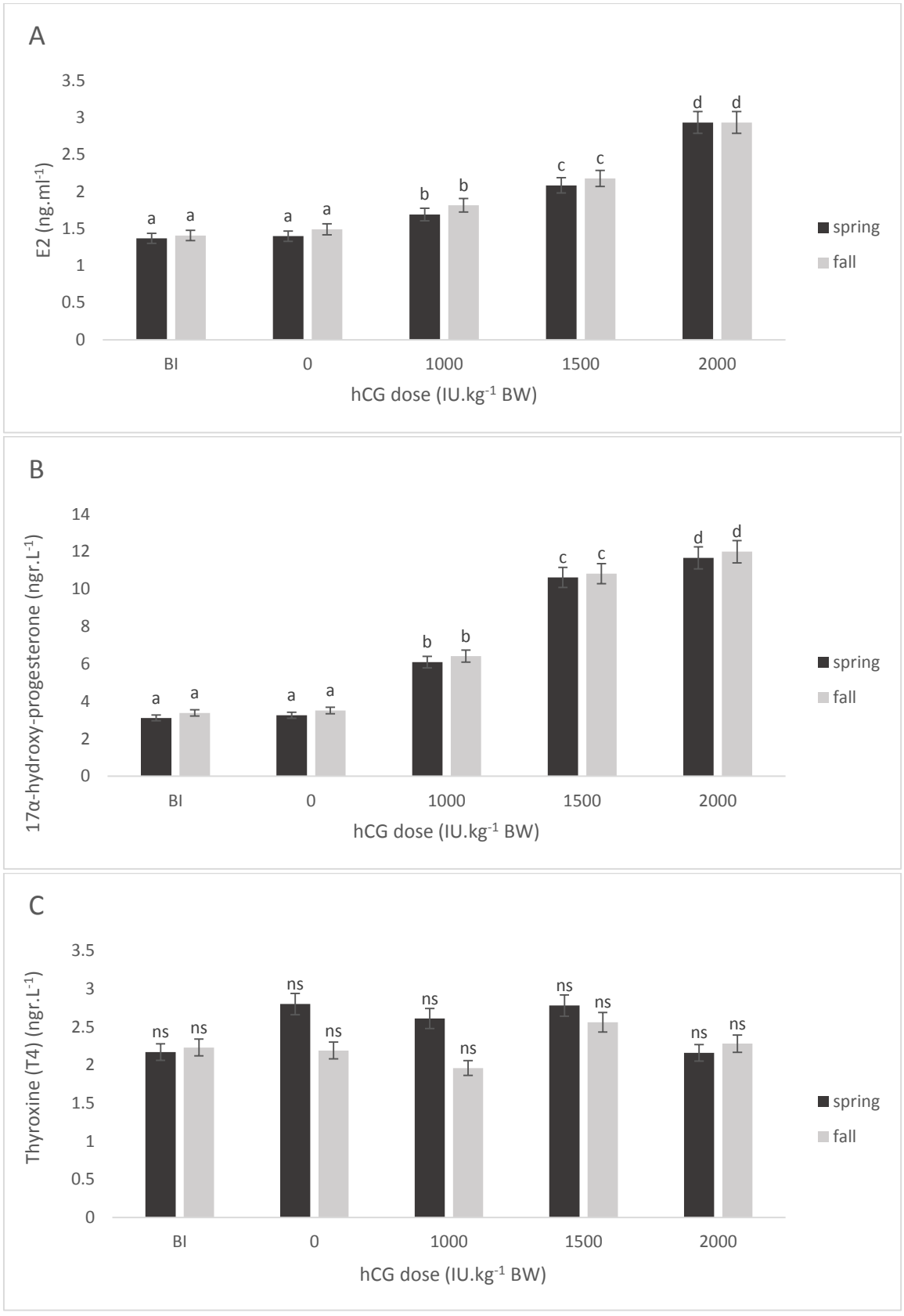


Fig. 4. The result of hCG injection on the Caspian lamprey testis and ovary (A: testis in control group filled with spermatozoa. A′: testis of fish injected by 2000 IU.kg^{-1} BW hCG hormone filled with spermatoza. B: oocyte in control group with migrating germinal vesicle (GV) B′: oocyte with broken germinal vesicle in fish injected by 2000 IU.kg^{-1} BW hCG.


A
E2 (ng.ml-1)
3.5
3
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1
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hCG dose (IU.kg-1 BW)
spring
fall
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17α-hydroxy-progesterone (ngr.L-1)
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BI 0 1000 1500 2000
hCG dose (IU.kg-1 BW)
spring
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Thyroxine (T4) (ngr.L-1)
3.5
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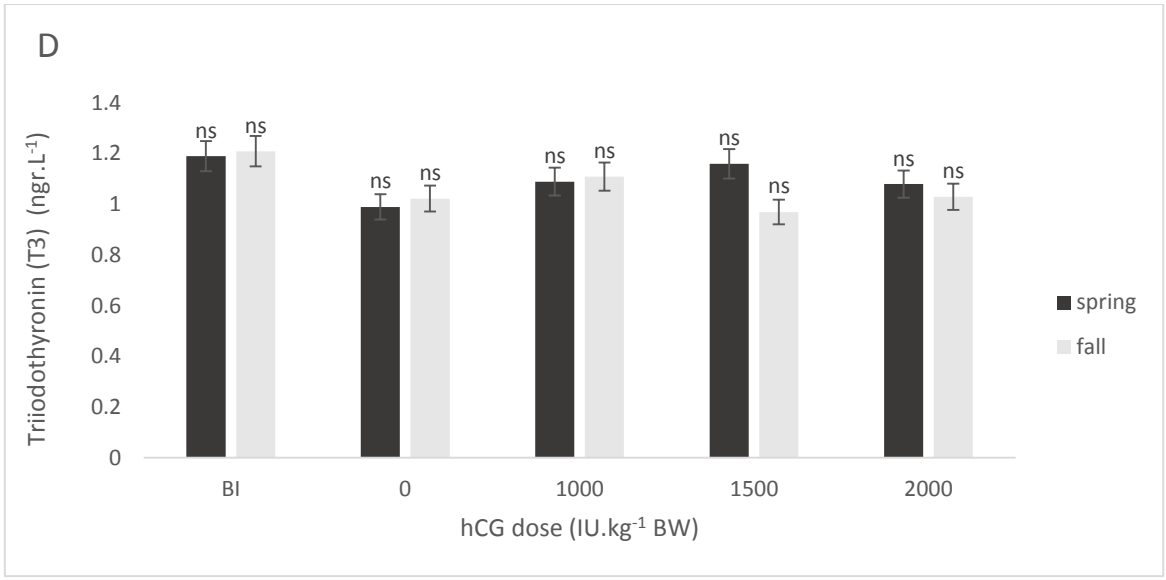

Fig. 5. Serum hormonal levels prior (left) and after (right) hCG manipulation. E2 (A,), 17α-OHP (B,), T4 (C,), T3 (D,) either in spring or fall *BI= before injection.

DISCUSSION

In the present study, the average GSI for female migrants were 10.36 ± 0.96%, and 10.01 ± 0.89% in spring and fall, respectively. The GSI of female Caspian lamprey ranged between 2.67 to 33.55% (Holcík 1986; Nazari & Abdoli 2010; Ahmadi *et al.* 2011), while it constituted up to 30% of the entire body weight in *P. marinus* (Bradley *et al.* 2004). In the present study, the average GSI of male migrants were 5.44 ± 0.38% and 5.67 ± 0.46% in spring and fall, respectively. Ahmadi *et al.* (2011) have reported that the GSI of spring males reached up to 6.45 ± 2.10%. Farrokhnejad *et al.* (2014) have reported that the average GSI of male migrants was 4.84 ± 0.39%. Totally, the values of GSI in spring were higher than in fall both in males and females. This indicates that the gonads of fall migrants were less developed rather than those of spring migrants and they might be needed more time to get developed. This information verified Jolodarzade & Abdoli (2004) who reported that the fall migrants spend the whole winter in the river and spawn by the following spring.

Generally, in the present experiment, hCG induced spermiation in male, oocyte GV migration index in female and significant elevation of sex steroid hormones in both sexes in the two seasons which may be induced through pituitary-gonadal axis. Sex steroids play a key role in lamprey maturity (Sower 2003). The results of this study showed a significant increase in serum sex steroids levels after hormonal induction which may be fully correlated to the hCG-injected dose.

Egg GVM is a great sign to evaluate the female readiness to spawn, but this index is not able to determine the quality of the eggs alone. Biological tests or egg maturing evaluation are only applicable in final maturation stage and this is the only stage that breeders respond to the hormonal injections (Iwamatsu *et al.* 1988). So, according to the promotion of polarization index in the injected fish, the results of this study confirm that hCG has a positive effect on accelerating the maturation process, but further studies are required to determine the quality of the eggs exposed to hormonal injections.

The values of sexual hormones in fishes depend on the type of the fish and the dose of the hormone. This is confirmed by one - time hCG injection with 400 IU.kg^{-1} BW which increased the sex steroid hormones, 11-ketotestosterone level in tilapia (*T. leucostica*) at early stages of sperm production (Eckstein *et al.* 1978). HCG along with $LHRH_{a2}$ and CPE hormones also caused an increase in the volume of sperm release, but not a significant sex steroid increase in pike-perch *Sander luciuperca* (Falahatkar *et al.* 2010). There was significant difference in serum concentrations of E2 and 17α-OHP in Caspian

lamprey migrant after hCG injection. This information is supported by the studies of Sower *et al.* (2011) in which the plasma E2 levels have been used as some indexes of reproductive maturation and gonadal activity in both male and female *P. marinus*. The presence of E2 has been associated with vitellogenesis in other species of lampreys (Larsen 1974; Pickering 1976; Fukayama & Takahashi 1985; Mesa *et al.* 2010), and high E2 concentration in hCG-treated Caspian lamprey as well as germinal vesicle migration, intimate that vitellogenesis has been just completed. However, high levels of E2 in mature males of other lamprey species (*P. marinus*, *L. camtschaticum* and *E.tridentatus*) are correlated to the presence of an estrogen receptor in the male testis (Fukayama & Takahashi 1985; Mesa *et al.* 2010) and also the presence of E2 has been linked to the development of secondary sexual characteristics (Mesa *et al.* 2010). While E2 is considered as a major sex steroid in male and female in lamprey, the precise function of it remains to be clarified (Sower *et al.* 2011).

HCG stimulate the production of 17α-OHP by ovarian follicles that induce oocyte maturation through activation of Phosphatidylinositol 3 (PI3) kinase pathway (Pramanick *et al.* 2014). According to Algriany *et al.* (2004) factors present in follicles at later stages of follicular growth play an important role on steroidogenesis in the oocyte maturation mechanisms and thereby enhancing developmental competence of oocytes. The present result revealed that hCG injection would significantly increase the level of 17α-OHP in blood plasma. There are conflicting evidences regarding the role of thyroid hormones in osmoregulation, and metamorphosis. Thyroid hormones play at least a supportive role in freshwater acclimation, and may interact with both the GH/IGF-I and cortisol axes. Thyroid hormones thus appear to exert their influence on salt secretory mechanisms primarily through an interaction with cortisol and the GH/IGF-I axis (Power DM *et al.* 2001). During the present study, serum thyroid hormones, T4 and T3 levels, showed no hCG dose - related change. It looks that hCG cannot influence the hypothalamus–hypophysis-thyroid axis. However studies conducted on the lampreys do not suggest an involvement of thyroid hormones, at least in the manner seen in amphibians (Fahien & Sower 1990).

From the breeder responses to the hormonal injection, sex steroids and thyroid hormone levels, it can be disclosed that, hCG is an appropriate hormone to induce the reproduction of Caspian lamprey. Furthermore, the cost and availability of this hormone makes it a good choice.

ACKNOWLEDGEMENTS

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تاثیر هورمون گنادوتروپین انسانی بر سطوح استروئیدهای جنسی و هورمون‌های تیروئیدی ماهی دهان‌گرد خزری (*Caspiomyzon wagneri*, Kessler, 1870)

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چکیده

پژوهش حاضر به منظور بررسی تاثیر هورمون گنادوتروپین انسانی (hCG) بر رسیدگی جنسی، سطح استروئیدهای جنسی پلاسما [۱۷-بتا استرادیول(E2)، ۱۷- آلفا هیدروکسی پروژسترون(17α OHP)] و هورمون‌های تیروئیدی (تری یودوتیرونین (T3) و تیروکسین (T4)) در هنگام مهاجرت ماهیان دهان گرد دریای خزر انجام شد. در طول این آزمایش ۳۶ ماهی (۲۴ ماده و ۱۲ نر) در فصل بهار و ۳۶ ماهی (۲۴ ماده و ۱۲ نر) در فصل پاییز از مصب رودخانه شیرود مازندران در حوضه جنوبی دریای خزر، هنگام مهاجرت به آب شیرین صید شدند. به تمامی ماهیان هورمون گنادوتروپین انسانی با دوزهای ۱۰۰۰، ۱۵۰۰ و ۲۰۰۰ IU.Kg-1BW تزریق شد. فرایند تزریق هورمون به صورت دو مرحله‌ای (هر مرحله ۵۰٪ از مقدار هورمون تعیین شده) و با فاصله ۱۲ ساعته انجام شد. بعد از تزریق اول ماهی‌ها به قفس‌هایی در بستر رودخانه منتقل شده و ۲۴ ساعت پس از دومین تزریق از قفس خارج شده و توسط مالش ناحیه‌ی شکمی برای امکان استحصال تخم و اسپرم بررسی شدند. به منظور ارزیابی سطح هورمون‌های جنسی و تیروئیدی از ماهی‌ها نمونه خون گرفته شد. ارزیابی نتایج نشان داد که تزریق هورمون hCG باعث افزایش سرعت مهاجرت هسته در تخمک ماهیان ماده و رهاسازی اسپرم در ماهیان نر می‌شود. تفاوت معنی‌داری در سطح هورمون‌های E2 و 17α OHP در ماهیانی که مورد تزریق گنادوتروپین انسانی واقع شده بودند، در مقایسه با ماهیان گروه کنترل مشاهده شد ($P < 0.05$). تفاوت معنی‌داری در سطح هورمون‌های تیروئیدی تریT3 و T4 پس از تزریق هورمون hCG در مقایسه با گروه کنترل مشاهده نشد ($P > 0.05$). با توجه به نتایج به‌دست آمده، دوز مناسب هورمون hCG به منظور تسریع القای تولیدمثل در ماهی دهان‌گرد خزری ۱۵۰۰ تا ۲۰۰۰ IU.kg-1BW پیشنهاد می‌شود.

*مولف مسئول