

## Effectiveness of carp pituitary extract and luteinizing hormone releasing hormone analogue administration (by either injection or cholesterol pellet implantation) on spawning performance in female sturgeon, *Huso huso*

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### Abstract

The effectiveness of common carp pituitary extract (CPE), luteinizing hormone releasing hormone analogue (LHRH-A<sub>2</sub>) injections and LHRH-A<sub>2</sub> implants for spawning induction in female sturgeon, *Huso huso* was examined. In the first trial, fish were injected with 7% physiological saline (control), 50 mg kg<sup>-1</sup> CPE or LHRH-A<sub>2</sub> at 3.5, 7, 8 or 10 µg kg<sup>-1</sup>. In the second trial, fish were treated with LHRH-A<sub>2</sub> cholesterol pellet implants containing 0, 3.5, 7, 8 and 10 µg kg<sup>-1</sup> LHRH-A<sub>2</sub>. Ovulated eggs were removed using a minimally invasive surgical technique and were artificially fertilized. Injection of CPE and LHRH-A<sub>2</sub> at doses of 3.5, 7, 8 and 10 µg kg<sup>-1</sup> resulted in the number of ovulated fish more than LHRH-A<sub>2</sub> implants (similar doses) or controls, although there was no significant difference at doses of 8 and 10 µg kg<sup>-1</sup> ( $P \geq 0.05$ ). The latency period of fish receiving CPE and LHRH-A<sub>2</sub> injections was approximately 20 h, which was significantly lower than in fish receiving LHRH-A<sub>2</sub> implants ( $P \leq 0.05$ ). Furthermore, there were no significant differences in rates of fertilization or hatching among the progeny produced in any of the treatment groups ( $P \geq 0.05$ ).

In conclusion, the data from this study could be useful for artificial propagation of not-fully-matured females of *H. huso* at sturgeon hatcheries.

**Keywords:** *Huso huso*, CPE, LHRH-A<sub>2</sub>, injection, implant, reproduction

### Introduction

Knowledge of the reproduction of wild populations of sturgeon is essential for ensuring the perpetuation of this ancient group of animals. Like most chondrosteans, *Huso huso* has a prolonged reproductive cycle and takes a long time to reach sexual maturity under natural conditions. Sexual maturity and first spawning occur in this species at an age of 10–16 years for males and 14–20 years for females (Holcik 1989). Under culture conditions, female sturgeon, *H. huso* usually fail to reach sexual maturity, a situation that is characterized by ovarian maturity being arrested at the previtellogenic stage (Moberg, Doroshov, Chapman, Kroll, Van Eenennaam & Watson 1991). To ameliorate this problem and to induce or accelerate spawning, a variety of approaches have been developed, most of which involve the artificial

manipulation of the endocrine system with exogenous hormones (Arabaci & Sari 2004; Kaminski, Kuszniarz, Myszkowski & Wolnicki 2004).

To induce spawning of fish in hatcheries, the broodfish are usually injected with carp pituitary extract (CPE), which is expensive, not always readily available, has unpredictable activity and has variable rates of success (Drori, Ofir, Levavi-Sivan & Yaron 1994; Dunham, Lambert, Argue, Ligeon, Yant & Liu 2000; Zohar & Mylonas 2001). Furthermore, as CPE is a crude protein product, it contains other pituitary hormones in addition to luteinizing hormone (LH) (Chatakondi, Yant, Kristanto, Umali-Maceina & Dunham 2011), which can result in adverse physiological effects on the fish. Furthermore, CPE acts at the lowest level of the hypothalamus–pituitary–gonadal (HPG) axis, giving mixed or variable results when used for induced spawning.

In recent years, synthetic analogues of luteinizing hormone releasing hormone (LHRH), referred to as LHRH-A, have been developed that are far more effective than CPE. As LHRH-A is resistance than CPE and is not rapidly metabolized by fish, it remains active for a longer period of time (Tamaru, Lee, Ha, Ginoza, Aida & Hanyu 1990; Chebanov & Billard 2001). The effect of LHRH on the gonads is indirect through the stimulation of gonadotropin(s) release from the pituitary (Swanson, Suzuki & Kawauchi 1987). In fact, this peptide hormone induces the secretion of gonadotropins or LH, which stimulates final oocyte maturation, ovulation and spermiation (Zohar & Mylonas 2001). Some of the advantages of LHRH-A use include repeatable application without a reduction in efficiency, action at a higher level in the HPG axis, and LHRH-A can be synthesized in pure form (Chatakondi *et al.* 2011) for enhanced efficiency. In addition, synthetic analogues eliminate the risk of transmission of infectious diseases and enable the administration of precise doses of LHRH-A (Kouril, Svoboda, Hamackova, Kalab, Kolarova, Lepicova, Sedova, Savina, Rendon, Svobodova, Barth & Vykusova 2007; Chen & Fernald 2008).

Generally, spawning hormones are administered at present by two general methods: injecting a water or saline solution or implanting a slow-release pellet. Drugs injected after dissolving them in saline solution enter the general circulation within minutes and are then metabolized and excreted. As an additional point, in comparison to

a single LHRH-A injection, two injection protocols can effectively shorten ovulation time in fish (Zohar & Mylonas 2001). In fact, the failure of single injection protocols to reliably induce maturation is probably the result of this short residence time of LHRH-A in circulation, which ranges from a few hours to a few days depending on LHRH-A, initial dose, fish species and water temperature (Zohar & Mylonas 2001). Thus, it is useful to make the hormone available to the animal over weeks or months, and this can be done by mixing the drug with a binding material from which it is slowly released (silicone rubber or Silastic<sup>®</sup>, normally used to deliver steroids) or which slowly breaks down allowing the hormone to escape (cholesterol or cholesterol-cellulose) (Harvey & Carolsfeld 1993; Zohar & Mylonas 2001). In addition, the methods used for administering hormone, including multiple injections and slow-release implants, have been reported to have both similar and different effects on the timing of ovulation. Implants clearly have the advantage that the fish are handled only once, thus reducing handling stress and labour (Mylonas & Zohar 2001).

The endocrine control of female reproduction using synthetic analogues has been studied in several sturgeon species (Goncharov, Igumnova, Polupan & Savieleva 1991; Williot, Gulyas & Ceapa 2002; Nazari, Modanloo, Ghomi & Ovissipor 2010; Amini, Siraj, Mojazi Amiri, Mirhashemi Rostami, Sharr & Hossienzadeh 2012). However, no published reports are currently available on hormonal induction of maturation in *H. huso*. Hence, this study was conducted to compare the effectiveness of CPE, LHRH-A<sub>2</sub> injections and LHRH-A<sub>2</sub> implants on the reproductive performance of *H. huso* from the Caspian Sea by evaluating the induction of ovulation, latency period, fertilization rate and hatching rate.

## Materials and methods

### Collection and maintenance of fish

The experiments were carried out during 2011 and 2012 with broodfish captured from the south-east coast of the Caspian Sea, Mazandaran Province, Iran (salinity of 12–14 ppt), which were transported to the Sturgeon Hatchery Centre. Fish were stocked into one of four circular concrete tanks (2.5 m diameter, 0.5 m depth, and 4 m<sup>3</sup> water volume) that were supplied with flow-

through river water at a rate of  $38.2 \pm 2.1 \text{ L min}^{-1}$  for the duration of the experiment. Water temperature remained between 14–19°C and the average dissolved oxygen content was consistently  $6.2 \pm 0.4 \text{ mg L}^{-1}$ . Fish were hand-fed three times a day with a commercial trout diet (Fara Daneh, Shahrekord, Iran; 36% crude protein, 14% crude fat, 10% ash, 11% moisture) at 1–1.5% of their biomass, depending on water temperature and appetite, and were deprived of food for 1 day prior to induction and sampling.

### Hormones

Hormones used in this study were CPE (Rajaei Fish Farm Centre, Sari, Mazanzdaran, Iran) and LHRH-A<sub>2</sub> (D-Ala<sup>6</sup> GnRH Pro<sup>9</sup>-Net; Ningbo Renjian Pharmaceutical Group Co. Ltd., Ningbo, China).

### Experimental procedures

A total of 100 female sturgeon [with polarization index (PI) more than 10, described by Chapman & Van Eenennaam 2007] were selected from among a total of 200 fish and were randomly allocated into 11 groups: nine experimental groups consisting of 10 fish and two control groups consisting of five fish. The control groups contained fewer individuals as a limited number of females at the appropriate stage of oocyte maturation were available. Fish in each group were marked by placing a soft plastic tag on the dorsal fin. All fish manipulations were conducted in accordance with the guidelines on the care and use of animals for scientific purposes (National Health and Medical Research Council, Canberra, Australia). Details of the experimental groups, treatments, dosages and the number and weight of fish in each experimental group are presented in Table 1.

### CPE and LHRH-A<sub>2</sub> injections

The spawning induction treatments were as follows: (i) 0.7% physiological saline (control group); (ii) 50 mg kg<sup>-1</sup> CPE; and (iii) LHRH-A<sub>2</sub> injections at dosages of 3.5, 7, 8 and 10 µg kg<sup>-1</sup> (Nazari *et al.* 2010; see Table 1). Hormones were injected into each female with an initial dose that equalled 10% of the total dose and the remaining dose was administered 12–14 h later. All hormone injections were given intramuscularly.

### LHRH-A<sub>2</sub> – cholesterol implants

Five treatments, consisting of cholesterol pellets containing LHRH-A<sub>2</sub> at doses of 0 (control), 3.5, 7, 8 and 10 µg kg<sup>-1</sup>, were implanted intramuscularly into female sturgeon (see Table 1). The LHRH-A<sub>2</sub> was incorporated into the cholesterol pellets as described by Lee, Tamaru and Kelley (1986). Each pellet weighed approximately 20 mg and had an average length and diameter of 5.5 and 2.4 mm respectively. Fish were removed individually from the tanks and anaesthetized with 30 mg L<sup>-1</sup> clove oil (Aramli, Golshahi & Nazari 2014). A small incision (8–10 mm) was made on the ventral surface of the fish with a sterile scalpel and the LHRH-A<sub>2</sub> cholesterol pellet was implanted into the peritoneal cavity. The abdominal incision was closed with one suture and the sturgeon was returned to the rearing tank.

### Reproductive performance parameters

For the removal of ovulated eggs from fish, a minimally invasive surgical technique was used. A finger was inserted into the gonopore to stretch the opening slightly. A scalpel (with a straight blade narrower than the gonopore) was inserted carefully into the gonopore opening, and a 1.5–3 cm incision was made through the ventral area of the oviductal (Mullerian duct) wall. The scalpel was withdrawn and the incision probed with one finger to ensure that the opening was not obstructed. The fish was inverted and slight pressure applied to the abdominal region by two individuals: the ova flowed through the incised opening in the oviduct and out of the gonopore (Aramli *et al.* 2014). Fertilization was performed using a pool of semen obtained from three males at a rate of 10 mL semen per kg<sup>-1</sup> of ovulated eggs. Fertilized eggs were washed with clay solution to prevent adhesion and placed in upwelling incubation units (Youshchenko). To measure fertilization rate, 2 g of eggs (approx. 150 eggs) were examined under a light microscope at 40 × magnification 3 h after fertilization. Fertilization rate (%) was calculated as the number of fertilized eggs divided by the total number of eggs (Dettlaff, Ginsburg & Schmalhausen 1993). Larvae hatched 5–8 days post fertilization and hatching rate (%) was calculated as the number of hatched larvae divided by the number of fertilized eggs. Spawning success (number of ovulating fish / total number of treated fish) and

**Table 1** Details of experimental groups, treatments [Carp pituitary extract (CPE), luteinizing hormone releasing hormone analogue (LHRH-A<sub>2</sub>) injections, and LHRH-A<sub>2</sub> implants], dosages and number and weight of fish in each group

Experiment	Group	Treatment	Dose (kg <sup>-1</sup> b.w.)	No. of fish	Weight (g)
1	Negative control	Physiological saline (0.7%)	–	5	6743 ± 395.2
		CPE	50 mg	10	7523 ± 421.2
	Injections	LHRH-A <sub>2</sub>	3.5 µg	10	6554 ± 239.2
			7 µg	10	7442 ± 425
			8 µg	10	6854 ± 290.6
2	Implants		10 µg	10	5897 ± 325.9
		LHRH-A <sub>2</sub> cholesterol pellets	0 (control)	5	5698 ± 214.3
			3.5 µg	10	6912 ± 378.1
			7 µg	10	7482 ± 298.5
			8 µg	10	6845 ± 426.1
		10 µg	10	7892 ± 410.9	

the latency period (time elapsed between treatment and ovulation) were calculated according to Drori *et al.* (1994) and Szabo, Szabó, Urbanyi and Horvath (2000).

#### Statistical analyses

Data were statistically analyzed using GraphPad Prism software v. 6.0 (GraphPad Software Inc., San Diego, CA, USA). The normality of data was checked by the Shapiro–Wilk test. The significance of differences in ovulation between the groups was calculated using the  $\chi^2$  test (Mikolajczyk, Chyb, Szczerbik, Sokolowska-Mikolajczyk, Epler, Enright, Filipiak & Breton 2004). Data on latency period and fertilization success were analyzed using Kruskal–Wallis one-way analysis of variance (ANOVA) with an alpha value of 0.05. Data are presented as mean ± SD.

## Results

#### Effects of treatments on ovulation induction

Ovulation response of females to hormone and saline (control) treatments are summarized in Table 2. None of the control fish that received the saline injection ovulated during the experiment. Ovulation success in group that received injection of CPE was 60%. The rate of ovulation was 70% for the fish injected with 7, 8 and 10 µg kg<sup>-1</sup> LHRH-A<sub>2</sub>. The rate of spawning in fish that received LHRH-A<sub>2</sub> implants at doses of 8 and 10 µg kg<sup>-1</sup> was 50%, and was only 20% and 30% in fish given implants containing 3.5 and 7 µg kg<sup>-1</sup> LHRH-A<sub>2</sub> respectively.

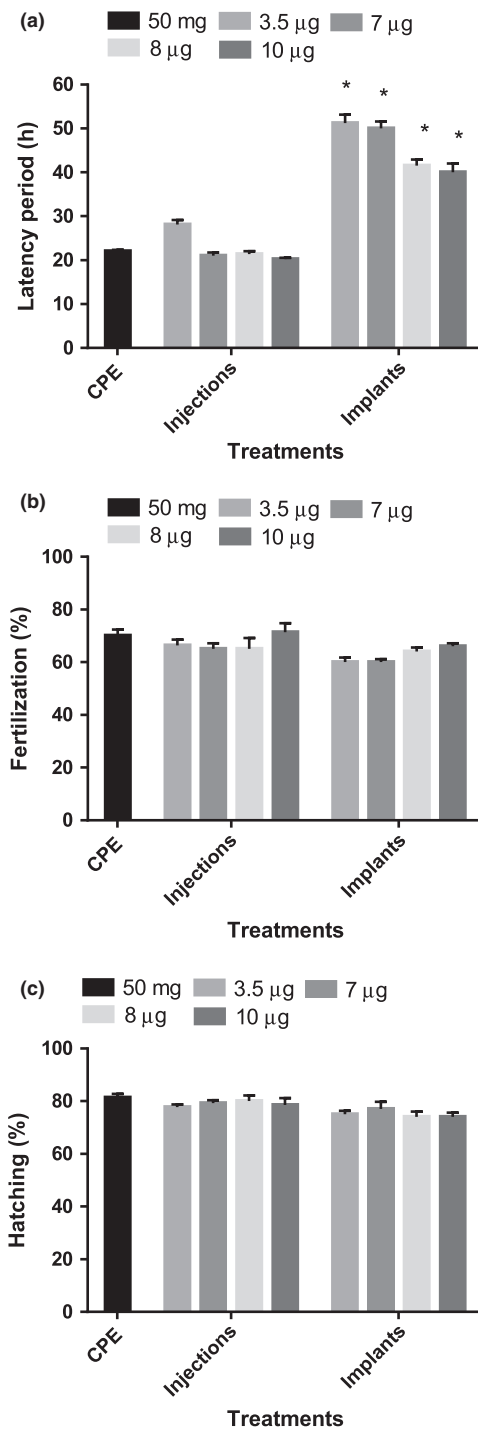
**Table 2** Ovulation response of *H. huso* to physiological saline (control), Carp pituitary extract (CPE), luteinizing hormone releasing hormone analogue (LHRH-A<sub>2</sub>) injections and LHRH-A<sub>2</sub> implants

Experiments	Groups	Dose (kg <sup>-1</sup> b.w.)	No. of ovulated / treated fish
1	Negative control	–	0/5 <sup>a</sup>
		CPE	50 mg
	Injections	3.5 µg	4/10 <sup>b</sup>
		7 µg	7/10 <sup>c</sup>
		8 µg	7/10 <sup>c</sup>
2	Implants	10 µg	7/10 <sup>c</sup>
		0 µg	0/5 <sup>a</sup>
		3.5 µg	2/10 <sup>b</sup>
		7 µg	3/10 <sup>b</sup>
		8 µg	5/10 <sup>c</sup>
		10 µg	5/10 <sup>c</sup>

Data are presented as mean ± SD. Means with different superscript letters are significantly different ( $P \leq 0.05$ ).

#### Effects of treatments on latency period in treated fish

Ovulation occurred at approximately 20 h post treatment in the LHRH-A<sub>2</sub> injected fish and at 45 h in the LHRH-A<sub>2</sub> implanted fish (Fig. 1a). A single dose of CPE and injections of LHRH-A<sub>2</sub> at doses of 7, 8 and 10 µg kg<sup>-1</sup> significantly shortened the latency period when compared to fish treated with implants ( $P \leq 0.05$ ). Furthermore, among the doses tested in implants, 8 and 10 µg kg<sup>-1</sup> LHRH-A<sub>2</sub> significantly shortened the latency period when compared to the lower-dose implants and the controls ( $P \leq 0.05$ ).



**Figure 1** Latency period (a); fertilization (b) and hatching success (c) of *Huso huso* in relation to hormonal different treatments [Carp pituitary extract (CPE); luteinizing hormone releasing hormone analogue (LHRH-A<sub>2</sub>) injection and LHRH-A<sub>2</sub> implant]. Data are presented as mean ± SD. Asterisks indicate significant differences among treatments ( $P < 0.05$ ).

**Effects of treatments on fertilization and hatching rate in treated fish**

No significant differences were observed in rates of fertilization or hatching of larvae between any of the treatment groups ( $P > 0.05$ ). Hormonal treatments did not affect egg quality or result in any side effects in fish or progeny. The mean (±SD) fertilization and hatching rates were  $66 \pm 2.9\%$  and  $78 \pm 1.72\%$  for the LHRH-A<sub>2</sub> injected fish, and  $62 \pm 1.4\%$  and  $75 \pm 2.1\%$  for LHRH-A<sub>2</sub> implanted fish respectively (Fig. 1b and c).

**Discussion**

Both liquid injections and controlled-release delivery systems (implants) that release LHRH-A for a prolonged period of time have been shown to be effective in inducing maturation in female fish (Mylonas & Zohar 2001). Furthermore, the two methods may have applicability in different commercial aquaculture situations, depending upon the egg production requirements (number and frequency) and broodfish management procedures (tank and breeder size, available expertise in fish handling and hormone administration) (Mylonas, Fatira, Karkut, Papadaki, Sigelaki & Duncan 2015).

This study indicates that LHRH-A<sub>2</sub> injections induce spawning in captive beluga sturgeon by increasing the number of follicles completing final oocyte maturation (FOM) and stimulating ovulation. The percentage of fish that ovulated increased from 0% in the saline-injected control group to 70% in the LHRH-A<sub>2</sub> injection groups. Results from our study are consistent with those of Goncharov *et al.* (1991), who used LHRH-A to induce reproduction in starry sturgeon (*Acipenser stellatus*), Russian sturgeon (*Acipenser gueldenstaedti*) and sterlet (*Acipenser ruthenus*). Nazari *et al.* (2010) injected similar doses of LHRH-A<sub>2</sub> into Persian sturgeon (*Acipenser persicus*) and observed the same effectiveness as reported in this study. In another study, Chebanov and Savelyeva (1999) found that LHRH-A was more effective than pituitary gland extract for the induction of breeding in *A. stellatus*. Williot *et al.* (2002) also demonstrated that the GnRH<sub>a</sub> D-Phe-6 NH<sub>2</sub> was as potent as CPE for inducing gamete release in Siberian sturgeon (*Acipenser baerii*).

The most widely used system for the sustained delivery of LHRH-A in the induction of fish

spawning is the cholesterol pellet (Lee, Tamaru, Banno, Kelley, Bocek & Wyban 1986; Crim, Sherwood & Wilson 1988; Mylonas & Zohar 2001). To date, cholesterol pellets have been used in the control of reproduction in many wild-caught and captive fish including Atlantic salmon (*Salmo salar*) (Crim, Evans & Vickery 1983), milk fish (*Chanos chanos*) (Lee, Tamaru *et al.* 1986), winter flounder (*Pseudopleuronectes americanus*) (Harmin & Crim 1992), striped bass (*Morone saxatilis*) (Hodson & Sullivan 1993), yellowtail flounder (*Pleuronectes ferrugineus*) (Larsson, Mylonas, Zohar & Crim 1997), black sea bass (*Centropristis striata*) (Watanabe, Smith, Berlinsky, Woolridge, Stuart, Copeland & Denson 2003) and *A. persicus* (Amini *et al.* 2012). In contrast to the most previous studies demonstrating a high efficiency of spawning induction using LHRH-A-loaded cholesterol pellets in diverse species of fish (Mylonas & Zohar 2001), in this study, cholesterol pellets containing LHRH-A<sub>2</sub> were resulted in the number of ovulated fish less than LHRH-A<sub>2</sub> injections (with no significant difference at higher doses). One explanation for this difference may be that the rate of LHRH-A release from the cholesterol pellets was too slow to supply adequate hormone required to induce FOM and ovulation. A slower release of LHRH-A<sub>2</sub> from the cholesterol pellets is supported by the observed increase in the latency period in fish receiving the implants, which was nearly twice as long as that of the fish injected with LHRH-A<sub>2</sub>. Alternatively, the constant but relatively lower levels of LH release achieved in fish receiving the LHRH-A<sub>2</sub> pellet implants may not have been adequate to trigger FOM and ovulation in beluga sturgeon. In other words, the concentration of LH may be more important in beluga sturgeon than the timing or duration of LH release.

In this study, fertilization success and rates of hatching were not significantly different between any groups. Similarly, the rates of fertilization and hatching in the Persian sturgeon were not significantly different between female broodstock receiving LHRH-A<sub>2</sub> injections at different doses (Nazari *et al.* 2010). Williot *et al.* (2002) also demonstrated that an analogue of GnRH did not influence egg quality in Siberian sturgeon.

In addition, the results revealed that the effectiveness of LHRH-A<sub>2</sub> treatment was dependent upon the method of administration. Injection of *H. huso* with LHRH-A<sub>2</sub> increased the number of ovulated fish compared to other treatments,

although there was no significant difference at higher doses. The latency period of fish receiving CPE and LHRH-A<sub>2</sub> injections was significantly lower than in fish receiving LHRH-A<sub>2</sub> implants. Furthermore, the rates of fertilization and hatching in this study were not significantly different among the treatment groups. Data from this study is recommended for the induction of ovulation in female *H. huso* under conditions similar to those reported here.

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