Spawning induction in Sterlet sturgeon (Acipenser ruthenus) with recombinant GnRH: Analysis of hormone profiles and spawning indices

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Running head title: Spawning induction in Sterlet sturgeon with rGnRH

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Highlights

1. rGnRH had a positive effect in final maturation in Sterlet sturgeon.
2. rGnRH at the dose of 10 µg kg body weight^{-1} was efficient in spawning induction.
3. rGnRH can stimulate the secretion of sex-steroids *in vivo*.
4. rGnRH can be used as a specific homologue to treat reproductive disorders in fish.

Abstract

In the present study, the biological function of recombinant gonadotropin-releasing hormone associate peptide (rGnRH) was tested for the induction of ovulation and spawning in Sterlet sturgeon (*Acipenser ruthenus*) broodstock. For this purpose, Sterlet sturgeon broodstock were divided into four treatments and treated with following preparing procedure: 0.9% NaCl (C); 2.5 μg kg body weight^{-1} (BW) LHRH-A2 (luteinizing hormone-releasing hormone-A2, L2.5); 2.5 μg kg BW^{-1} rGnRH (rGn2.5); 10 μg kg BW^{-1} rGnRH (rGn10). The capability of the rGnRH for eliciting biological response was studied *in vivo* by evaluating the changes of 17β estradiol (E2), progesterone (P) and testosterone (T) and by its ability for inducing final maturation. Blood samples were collected at the time of first injection, 10 h after the first injection and at ovulation time. The L2.5 or rGn10 treatments led to significantly lower E2 concentration 10 h after the first injection and at ovulation time compared to the baseline measures. E2 levels increased in the rGn2.5 treatment 10 h after the first injection and by ovulation time compared to the time of first injection. T levels were significantly increased in the L2.5 or rGn10 treatments 10 h after the first injection compared to the time of first injection and it further decreased at ovulation time. No significant differences were found in C and rGn2.5 treatments between the different sampling times. P secretion was significantly higher in the rGn10 or L2.5 treatments compared to C and rGn2.5 treatments 10 h after the first injection and at ovulation time. No significant difference was found in P secretion in the C and rGn2.5 treatments at different sampling times. The results showed that the rGnRH at the highest dose (10μg kg BW^{-1}) was more efficient than the other treatments in the induction of spawning and this dose caused P secretion and spawning *in vivo*. Our results present the possibility of using rGnRH in artificial reproduction of sturgeons and it can be introduced as a specific homologue to treat reproductive disorders in fish.

Keywords: Recombinant GnRH; *Acipenser ruthenus*; Sex steroids; Ovulation; Spawning

Introduction

Sterlet sturgeons (*Acipenser ruthenus*) are one of the 27 sturgeon species that are endangered worldwide (Muller et al., 2018). They are promising candidates for freshwater aquaculture production and they have several indisputable advantages compared with the other sturgeon species. They require smaller space in captivity due to smaller size and they display a quicker sexual development (Akhavan et al., 2015; Abdollahpour et al., 2018). In comparison with lengthy gonadal cycle in the large migratory species, they are an ideal model to study the reproductive physiology of sturgeon due to earliest maturation (Akhavan et al., 2015). However, the broodstock reproduction and the larval rearing in captivity are two major bottlenecks for its aquaculture development (Abdollahpour et al., 2018). In captivity, sturgeons do not spawn and this dysfunction may result from the combination of captivity-induced stress and absence of suitable natural spawning condition (Alavi et al., 2012). Intramuscular administrations of pituitary extracts (Linhart et al., 2003; Piros et al. 2002) and GnRHa (Linhart et al., 2000; Williot et al. 2002; Van Eenennaam et al., 2008; Nazari et al., 2009; Roosta et al., 2018) have been used to induce final maturation in both sexes. Various GnRH analogues with different half-lives exist and they are used with differential efficiency for inducing final sexual development in sturgeon broodstock.

GnRH is the main hypothalamic hormone regulating the synthesis and release of gonadotropins (Nagahama and Yamashita, 2008; Gaillard et al., 2018). At the N-terminal extremity of the precursor structure of the GnRH protein, there is a signal peptide, followed by the decapeptide, which is a bioactive site of the protein and is almost entirely preserved in all fish species. Next, there is a proteolytic site, which is conserved in all vertebrates. Ultimately, at the C-terminal extremity, there is a GnRH-associated peptide (GAP) that it is composed of 60 amino acids

117 approximately, which is less preserved in fish than other regions of the GnRH protein (Zmora et
118 al., 2002). Previous studies indicated that GAP is involved in the stabilization of GnRH structure
119 and that it is not related to the biological function of the protein (Andersen and Klungland,
120 1993).
121 In synthetic GnRH analogues, the biological active site (decapeptide region) is used for the
122 production of a hormone, because GAP region would not be involved in the biological activity of
123 GnRH. In addition, the amino acid sequences of GAP are long, so it is not economical to produce
124 by synthetic method. Apparently, a reliable, more effective, available and cheap product, useful
125 to induce the final maturation, is required (Aizen et al., 2017). Therefore, recombinant protein
126 methods are used instead of synthetic production for managing the costs (Mohammadzadeh et
127 al., 2020). The potential for the generation of recombinant hormones has been achieved in recent
128 years due to recent advances in construction cDNA encoding target protein. Different studies
129 have shown *in vivo* and *in vitro* effects of GnRH analogues or recombinant proteins to treat the
130 reproduction disorders in fish. In tench (*Tinca tinca*), treatment with GnRHa with or without a
131 dopamine antagonist increased luteinizing hormone levels and induced spawning (Podhorec et
132 al., 2016). Intramuscular administrations with different synthetic GnRH analogues and their
133 combinations with an anti-dopaminergic compound were also used to induce final maturation in
134 sterlet sturgeon (Ronyai, 2009). In the orange-spotted grouper (*Epinephelus coioides*) or
135 common carp (*Cyprinus carpio*), treatments with recombinant luteinizing hormone (LH) altered
136 the hormonal levels along the hypothalamus-pituitary-gonad axis (Cui et al. 2007; Aizen et al.
137 2017). An injection of rGnRH recently increased the plasma levels of 17α, 20β- dihydroxy-4-
138 pregnen-3-one, which led to final maturation induction in goldfish (*Carassius auratus*)
139 (Mohammadzadeh et al., 2020). Seemingly, producing rGnRH with suitable biological function

could pave the way for introducing an effective hormone therapy in aquaculture and could be applied as specific analogues to treat reproductive disorders in some related species for developing their aquaculture production. Due to the positive features regarding rGnRH for inducing final maturation in breeding female fish (Mohammadzadeh et al., 2020), this study tested rGnRH as an effective agent to stimulate sex steroids, inducing final maturation and spawning in Sterlet sturgeon.

2. Material and methods

2.1. Production of recombinant GnRH

The amino acid sequence of pre pro cGnRH Beluga (*Huso huso*) (GenBank: EF534706.2) was selected without its signal peptide. Some amino acids (1, 6 and 7) were changed in the decapeptide sequence to increase the half-life and the stability of the peptide. The GAP region was considered for this sequence in order to increase the stability of recombinant protein in production, extraction, and purification procedures. This gene sequence was synthesized and then cloned on the expression vector pET28a. Recombinant vector pET28a/GnRH was transformed into expression host *Escherichia coli* BL21 (DE3). The supernatant of cultured recombinant bacteria was applied for the purification using Batch/Gravity-Flow column purification with TALON metal affinity resin. The solubilized protein was loaded on Co^{2+}-CMA, TALON resin (Clontech) at 4°C for 1 h on a platform shaker to allow the His-tagged protein to bind the resin. Then, the column was washed with 10 bed volumes by washing buffer (50 mM Tris, 300 mM NaCl, 10 mM Imidazole and 0.05% Triton x-100 at pH 8.0). Finally, the His-tagged protein was eluted with 5 bed volumes elution buffer (50 mM Tris, 150 mM Imidazole and 1% (v/v) Triton x-100, pH 7.5) at a flow rate of 1 mL min^{-1} and before molecular

characterization (Mohammadzadeh et al., 2020). rGnRH quantity was determined by Bradford's method (1976) with bovine serum albumin as a standard. The lyophilized peptide was dissolved in saline solution (Nacl 0.9 %, pH 7) and assayed with Bradford buffer.

2.2. Broodstock and holding system

This study was conducted at Dr. Yousefpour Marine Fishes Restocking and Genetic Conservation Center (Siahkal, Guilan, Iran). Sterlet sturgeons were originally obtained from Hungary and then reproduced two generations in captivity. In this center, Sterlet sturgeon breeders were kept in round concrete tanks (185 cm diameter, 35 cm depth, and 940.7 ± 0.2 L volume). Water was supplied by river with a constant flow rate of 13 ± 0.3 L min^{-1} with a natural photoperiod and thermal regime. During the trial early in spring in April (14 h light/ 10 h dark), dissolved oxygen and water temperature was 12.5 ± 0.7 mg L^{-1} and 14.5 ± 0.5 ºC respectively. The breeders were fed with commercial sturgeon feed (Fara Daneh, Shahrekord, Iran) twice a day (9:00 and 19:00). The pelleted diet included 45% protein, 17% lipid, 4% fibre and was 8 mm in diameter.

2.3. Ovulation induction and experimental design

Female broodstocks were checked for maturity stage. To this aim, 400 ppm clove powder extract was applied for anesthetizing the fish (Ghiasi et al., 2017). A steel probe (3-4 mm diameter and 3-6 cm groove length) was used to sample a gonad part. For this purpose, it was placed between the 3rd and 4th ventral scute (Abdollahpour et al., 2019).

Germinal vesicle (GV) position was measured for selection of appropriate broodstock and injection time according Chapmman and Van Eenennaam (2007). Briefly, 20-30 oocytes were

collected that were immediately well-kept in 10% neutral buffered formalin for 1 hour and they were boiled for 5 min and then cut through the vegetal pole (VP) to the animal pole (AP). A dissecting microscope (Olympus, Tokyo, Japan) was used to measure the distances form VP to AP and from AP to nucleus under. For calculating germinal vesicle migration (GVM), GVM was examined according to this formula:

GVM = distance between nucleus and AP / distance between VP and AP (X100)

The gonad maturity of all sampled females proved to be in the pre-spawning stage IV (GV = 8.63 ± 1.74); thus, it was decided to perform the trial.

Based on this indicator, 24 seven year-old farmed females with mean weight (± SD) of 1200.9 ± 9.2 g, total length 60.3 ± 0.7 cm were selected for the spawning experiment. Female breeders were randomly distributed into eight tanks (185 cm diameter, 35 cm depth, 940.7 ± 0.2 L volume) with three fish per tank (four treatments with two replicates). Upon the optimum water temperature for spawning (15.5-16.5°C), the fish was injected with NaCl 0.9% (control); 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5, pGlu-His-Trp-Ser-His-Gly-Ttp-Arg-Pro-Gly-NH2; San Shen Ningbo, Sheng, China,) (Ghiasi et al., 2017); 2.5 µg kg body weight^{-1} rGnRH (rGn2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10). The doses were chosen according to the common practices of sturgeon propagation in Iran which use LHRH analogues in the range of 2.5-50 µg kg body weight^{-1} (Ghiasi et al., 2017; Pourhosein et al., 2018). Hormonal treatment for females was divided into 2 injections with 10 h interval (20% as priming dose and 80% as resolving dose), this procedure also stemming from the usual practices in Iran (Abdollahpour et al., 2018). Females ripping were checked based on Dettlaff curve and water temperature (Dettlaff et al., 1993). The broodstock' s abdomen was tested for ovulation 18 hours after the second injection, and this manipulation was repeated at 2 h intervals up to 48 hours (Abdollahpour et al., 2018).

The breeders spawned within 29-30 h after the first injection. Latency period was recorded as the lapse of time between the first hormone injection and ovulation. Ovulated eggs were manually stripped from the females using an incision made in the caudal section of the oviducts (Pourhosein et al., 2018). Stripping of all fish was done until the flow of oocytes had considerably reduced or stopped (Pourasadi et al., 2009; Abdollahpour et al., 2019). Spawned females were disinfected with Chloramphenicol spray (Afagh, Tehran, Iran) and the fish were injected with 1 mL of oxytetracycline 10% (Nasr, Mashhad, Iran) as an antibiotic (Falahatkar et al., 2011). Finally, the fish were moved to a recovery tank. There was no mortality monitored between the hormone injection and the stripping. Eggs were weighed, and three sub-samples were kept to count the number of eggs per gram.

2.4. Sampling and analysis

First, fish were anesthetized with 400 ppm clove powder extract (Abdollahpour et al., 2019). Blood samples (3 fish into each tank) was collected from the behind of the anal fin with 5 ml heparinized syringe at the time of first injection, 10 h after the first injection and at ovulation time. Then the samples were transferred to the tubes, centrifuged (1,600 × g for 10 min) to separate plasma and stored at -20°C for later analysis.

Sex steroid concentration (ng mL^{-1}) of testosterone (T, Cat. No: 3725–300), 17β estradiol (E2, Cat. No: 4925–300) and progesterone (P, Cat. No: 4825-300) were measured using enzyme-linked immunosorbent assay (ELISA) using commercial kits (AccuBind ELISA Microwells, Monobind, Inc. Lake Forest, CA, USA) based on manufacturer's instruction. The intra- and inter-assay coefficients of variation were 5.8%, 9.8% (n=10), 6.4%, 9.5% (n=10) and 5.1%, 7.5% (n=10) for E2, T and P, respectively. Testosterone and progesterone were selected based upon

their potential roles during the final stages of maturation in female sturgeon (Semenkova et al. 2002; Skoblina et al. 2012; Khara et al. 2014) and the very low inter-individual variability of the plasma level during the reproductive season (Barannikova et al. 2002).

2.5. Statistical analysis

Statistical analyses were performed using the SPSS software (version 16, Chicago, IL, USA). This research was done using a completely randomized design with different treatments. In order to check the data normality and homogeneity of variances Kolmogorov–Smirnov and Levene's test were applied respectively. One-way analysis of variance (ANOVA) was applied to analyze differences in reproduction performances between treatments. Sex steroid levels were analyzed by Two-way analysis of variance (ANOVA). Differences between several treatments were determined by Tukey's post-hoc tests. A Pearson correlation test was done to check the correlation between sex steroid hormones with each other (Barannikova et al., 2004). Values were reported as mean ± standard deviation (SD) in this paper.

3. Results

3.1. rGnRH quantity

After reading the absorbance of the samples at 595 nm with a spectrophotometer, the rGnRH concentration was estimated at 3.5 mg mL^{-1}.

3.2. Spawning performance

Reproductive response of Sterlet sturgeon broodstock injected with LHRH-A2 and rGnRH is shown in Table 1. 50% females injected with L2.5, 16.66% females injected with rGn2.5 and 66.66% females injected with rGn10 spawned, while none of the females of the C treatment

255 spawned. The fish injected with rGn10 treatment exhibited a significantly higher number of eggs
256 per gram (105.0 ± 3.5 eggs g^{-1}) in comparison with the other treatments (Table 1; $P < 0.001$).

257 *3.3. Hormone levels*

258 E2 levels were not significantly different in C treatment among the different sampling times (Fig.
259 1; $P = 0.983$). The L2.5 or rGn10 treatments led to significantly lower E2 concentration 10 h
260 after the first injection and at ovulation time compared to the baseline measures (Fig, 1; $P =$
261 0.002). By contrast, E2 levels increased in rGn2.5 treatment at ovulation time compared to the
262 time of first injection (Fig. 1; $P = 0.002$).

263 T levels were significantly increased in L2.5 or rGn10 treatments 10 h after the first injection
264 compared to the time of first injection (Fig. 2; $P < 0.001$). At ovulation time, a significant decline
265 compared to 10 h after the first injection in T values was observed in the L2.5 or rGn10
266 treatments (Fig. 2; $P < 0.001$). There was a significant difference between treatments with the
267 highest levels in the rGn10 treatment at both sampling times post-injection (Fig. 2; $P < 0.001$).
268 No significant differences were found in C (Fig. 2; $P = 0.173$) and rGn2.5 (Fig. 2; $P = 0.961$)
269 treatments among the different sampling times.

270 No difference in P levels was found among treatments at the time of first injection (Fig. 3; $P =$
271 0.437). Ten h after the first injection and ovulation time, a surge in P levels was observed in
272 plasma concentration in the L2.5 or rGn10 treatments, but no increases were detected in C or
273 rGn2.5 treatments (Fig, 3; $P < 0.001$).

274 Regarding L2.5 and rGn10 treatments, a Pearson linear correlation test revealed negative
275 correlation between plasma E2 and P (Fig, 4; Pearson correlation = -0.45; $P = 0.055$) and E2 was
276 negatively correlated with T, but this correlation was not significant (Fig, 5; Pearson correlation

277 = -0.45; P = 0.060). A significant positive correlation was also found between T and P levels at
278 L2.5 and rGn10 (Fig, 6; Pearson correlation = 0.48; P = 0.042). (Barannikova et al., 2004).
279
280 **Discussion**
281 In the current study, the biological function of rGnRH was confirmed *in vivo* by observing the
282 induced changes of E2, P, and T blood levels and its ability to induce final maturation and
283 spawning in the mature Sterlet sturgeon females. In the absence of a GtH (gonadotropin
284 hormone) test for Sterlet sturgeon, the indirect impacts of rGnRH have been surveyed in the
285 current study by estimating plasma levels of T, E2, and P; as peaks or drops in the circulating
286 plasma levels of these steroids can be utilized as an indirect measurement of the advancement of
287 final oocyte maturation. While it is generally admitted that E2 reaches a peak in fish undergoing
288 vitellogenesis, T is raised during vitellogenesis and also during the early stages of final oocyte
289 maturation and P is elevated during final oocyte maturation (Morehead et al., 1998; Semenkova
290 et al., 2002; Barannikova et al., 2004; Falahatkar et al., 2014).
291 In the present study, some females that received injection of recombinant GnRH and LHRH-A2
292 spawned, while none of the control fish spawned. This shows that both rGnRH and synthetic
293 LHRH-A2, were able to induce the advancement of final oocyte maturation, hence spawned
294 broodstock were most abundant in the rGn10 treatment. The induction effect of different GnRH
295 analogues has been demonstrated in different species of sturgeon (Nazari et al., 2009; Amini et
296 al., 2012; Khara et al., 2014; Roosta et al., 2018). Nazari et al. (2009) showed that using the
297 LHRH-A2 at doses of 3.5, 7, 8, and 10 μg kg^{-1} BW can induce final maturation in Persian
298 sturgeon (*Acipenser persicus*) broodstock and the doses of 3.5, 7, and 8 μg kg^{-1} yielded better
299 results than the dose of 10 μg kg^{-1}. Amini et al. (2012) reported that the implantation of LHRH-

A2 hormone at a dose of 15 µg kg^{-1} BW in premature Persian sturgeon broodstock had positive results in inducing final maturation. Also, Abdollahpour et al. (2018) showed that 4 µg kg^{-1} BW of LHRH-A2 induced the Sterlet sturgeon broodstock's final maturation. Thus, we confirm that GnRH analogues are quite effective to promote spawning in sturgeon species. Regarding the effectiveness of recombinant GnRH, no study is available, which makes it impossible to compare the present results by existing literature. Based on our observations, the number of spawned broodstock in rGn2.5 was less than those of the other hormonal treatments, which could be attributed to the inappropriate injected amount of this hormone for the induction of spawning in female Sterlet sturgeon broodstock. Similarly, low levels of injected GnRH analogues led to failed spawning in Japanese medaka (*Oryzias latipes*) and common carp broodstock (Chan, 1977; Drori et al., 1994). All together, the amount of required LHRHa seems to vary according to the species and the route of administration, and/or be due to the differences in the maturation level of female broodstock, their age, size, and sensitivity (Faridpak, 2008) and it might be also the case for recombinant GnRH.

In the current study, a positive effect of rGnRH treatment was displayed on the number of eggs per gram. It was consistent to previous research on Persian sturgeon, showing that increasing the LHRH-A2 levels from 4 to 6 µg kg^{-1} BW also increased the number of eggs per gram (Mohammadi et al., 2015). The egg quality and quantity from induced spawning appear to be partially related to the interaction between the stage of maturity or oocyte diameter and GnRHa dose (Ibarra and Duncan, 2007). Doses higher than the optimal may result in reduced egg quality (Garcia, 1989) while lower doses may result in reduced spawning frequency (Ibarra and Duncan, 2007). In the present study, egg production varies among rGnRH doses, suggesting that rGnRH 2.5 µg kg^{-1} has a lower efficiency than rGnRH 10 µg kg^{-1} to induce females' egg release.

In female broodstock, the level of E2 increases during the vitellogenesis and then decreases at the end of this stage (Mojazi Amiri et al., 1996; Barannikova et al., 1999). Majumder et al. (2015) confirmed that estrogens play an inhibitory role on the meiotic maturation of mature oocytes through activation of estrogen membrane receptors, necessitating a reduction in estrogen levels for final maturation. Significant reduction in E2 values from baseline 10 h after the first injection and at ovulation time was observed for both L2.5 and rGn10 treatments. A similar pattern for E2 level was found in common carp (Levavi-Zermonsky and Yaron, 1986) and tench injected with GnRHa (Podhorec et al., 2016) and in Persian sturgeon injected with LHRH-A2 (Khara et al., 2014). Secretion patterns for this steroid correspond to the expected natural shift in the biosynthetic pathway from the production of mainly C_{19} to C_{21} steroids (Podhorec et al., 2016). The broodstock in the L2.5 or rGn10 treatments were in final maturation stage, hence we cannot rule out the fact that a reduction in E2 levels was required in these fish to achieve the final maturation coincidently with potential increase of the maturation inducing hormone. E2 levels increased in rGn2.5 treatment 10 h after the first injection and at ovulation time compared to the time of the first injection. A similar E2 secretion pattern after GnRH or LHRH treatment was reported in previous observations on Stellate sturgeon (*Acipenser stellatus*) and Sterlet sturgeon (Bayunova et al., 2006; Yooneszadeh et al., 2010; Falahatkar et al., 2016). The slight increase in plasma E2 could occur because of a high aromatase activity in the ovary upon GnRH administration (Sharaf, 2012). Thus, in the females exposed to rGn2.5, the increase of E2 after injection could be related with the lower efficiency to trigger ovulation than the females exposed to L2.5 or rGn10, which displayed a drop in E2.

In this study, T increased significantly in L2.5 or rGn10 treatments 10 h after the first injection but it decreased following the ovulation time. A similar T secretion pattern after GnRH and

LHRH treatments was reported in Stellate and Sterlet sturgeon with more frequent blood sampling (Semenkova et al., 2002; Barannikova et al. 2005; Falahatkar et al., 2016). Increased T levels in response to GnRH injection and its decrease at ovulation time have been observed in many studies suggesting that GnRH stimulated the secretion of the pituitary GtH and, in turn, pituitary GtH stimulated the production of T by the vitellogenic follicles and mature oocytes (Morehead et al., 1993). In some teleosts, high levels of T led to accelerated maturation of oocytes, and such concentrations in the pre-ovulatory phase may affect the synchronicity of final oocyte maturation and ovulation (Bayunova et al., 2006). In the present study, the highest levels of T were observed in L2.5 or rGn10 treatments at the beginning of the experiment, which were significantly higher than those of the other treatments at all sampling stages; these treatments also contained the highest number of spawned broodstock. T levels were significantly lower in rGn2.5 treatment than the other treatments at all sampling times. Ceapa et al. (2002) found that high T concentrations might play an important role in initiating and continuing reproductive behaviors in both males and females, so that broodstock with low T levels would not reach ovulation stage. We suggest that the elevation of T post-injection after L2.5 or rGn10 treatments contributed to the better spawning success with these hormonal therapies.

In the current study, P levels showed significant increases in L2.5 or rGn10 treatments 10 h after the first injection, but no increase occurred in C or rGn2.5 treatments. At the time of ovulation, P levels were significantly higher in L2.5 or rGn10 treatments than those of the other treatments. These findings are in agreement with previous observations on sturgeon where the level of serum P was higher after LHRH-A injection (Semenkova et al., 2002; Khara et al., 2014). Elevation of P over a short period could indicate its indirect role in the final maturation of oocytes, as a precursor of the putative MIS (Maturation-Inducing-Steroid) namely 17α, 20β-progesterone and

17α, 20β, 21-thrihydroxy-4-pregnen-3-one at the time of ovulation (Semenkova et al., 2006). However, the direct role of P in the induction of final oocyte meiotic maturation in fish cannot be excluded in sturgeon (Skoblina et al., 2012). Nevertheless, the role of P in reproductive function of sturgeon remains unclear (Barannikova et al., 2002; Khara et al., 2014). P is present in measurable concentrations in sturgeon and its level increases during the final maturation, but it does not demonstrate a distinct peak (Semenkova et al., 2002). In our study, P levels increased in L2.5 or rGn10 treatments after hormone injection, coincidently with E2 decrease. A similar pattern after recombinant LH and LHRH treatment was reported in common carp (Levavi-Zermonsky et al., 1986; Aizen et al., 2017). Indeed, Aizen et al. (2017) showed that injection of recombinant LH to common carp broodstock led to increased P and decreased E2. Dropped E2 levels along with elevated P levels may confirm a shift in the steroidization pathway from estrogen formation towards P production. This process may be due to a decrease in 17-20 lyase activity and an increase in 20-β-hydroxysteroid dehydrogenase activity (Aizen et al., 2012, 2017).

To date, few *in vivo* studies have reported the usage of recombinant hormones in fish. In the Japanese eel (*Anguilla japonica*), different *in vitro* methods proved biological function of recombinant gonadotropins, which showed low activities in gonads when tested *in vivo* (Kazeto et al., 2008). In immature Japanese eel, recombinant eel Follicle-Stimulating Hormone (FSH) induced testicular development and spermatopoiesis (Kamei et al., 2006). The gonadosomatic index significantly increased after injection with recombinant FSH in Manchurian trout (*Brachymystax lenok*), (Ko et al., 2007). Injection of recombinant LH induced ovulation and altered the levels of sex steroids in common carp (Aizen et al. 2017). Based on the results of

present research, it can be speculated that recombinant GnRH is capable of binding to its receptor on pituitary surface and inducing final maturation in Sterlet broodstock.

The present study is the first report on the use of recombinant GnRH in fish that was able to stimulate P secretion and induce spawning. Complementary research is necessary concerning the effects of recombinant GnRH on expression of genes involved in the reproductive system of different fish species at different stages of sexual maturation to determine the exact mechanisms of action of this hormone. Also, further optimization of effective dose is necessary to obtain 100% of ovulation in sturgeon and higher doses than 10 μg kg^{-1} BW can be tested in further experiments. Finally, testing the efficiency of recombinant GnRH to stimulate the sperm quality and quantity in males could also open new avenues of investigation for the optimization of sturgeon reproduction in captivity. Such new findings make it possible to further use recombinant GnRH as a suitable alternative for pituitary extract and LHRH-A2 hormone in sturgeon breeders and maybe other fish species.

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Figures legends

Fig. 1. Plasma 17β-estradiol levels in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after injection of NaCl 0.9% (control); 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5); 2.5 µg kg body weight^{-1} rGnRH (rGn2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10). Mean ± SD; n = 3 for each tank. Different letters designate significant differences as determined by Tukey's post-hoc tests.

Fig. 2. Plasma Testosterone levels in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after injection of NaCl 0.9% (control); 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5); 2.5 µg kg body weight^{-1} rGnRH (rGn2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10). Mean ± SD; n = 3 for each tank. Different letters designate significant differences as determined by Tukey's post-hoc tests.

Fig. 3. Plasma Progesterone levels in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after injection of NaCl 0.9% (control); 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5); 2.5 µg kg body weight^{-1} rGnRH (rGn2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10). Mean ± SD; n = 3 for each tank. Different letters designate significant differences as determined by Tukey's post-hoc tests.

Fig. 4. Negative correlation between plasma concentrations of progesterone and 17β-estradiol in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after injection of 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10).

Fig. 5. Negative correlation between plasma concentrations of 17β-estradiol and testosterone in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after injection of 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10).

Fig. 6. Positive correlation between plasma concentrations of testosterone and progesterone in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after injection of 2.5 µg kg body weight^{-1} LHRH-A2 (L2.5) and 10 µg kg body weight^{-1} rGnRH (rGn10).

Fig. 1.

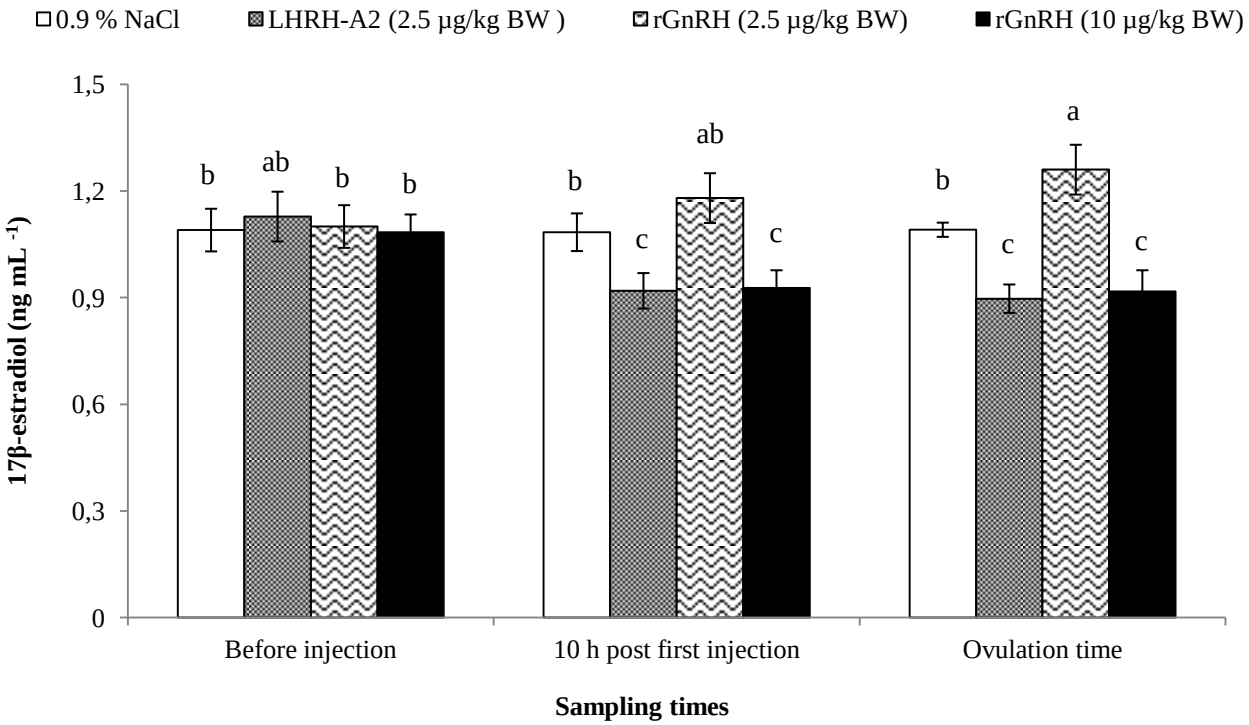

0.9 % NaCl
LHRH-A2 (2.5 µg/kg BW)
rGnRH (2.5 µg/kg BW)
rGnRH (10 µg/kg BW)
17β-estradiol (ng mL -1)
1,5
1,2
0,9
0,6
0,3
0
b
ab
b
b
b
c
ab
c
b
c
a
c
Before injection
10 h post first injection
Ovulation time
Sampling times

Fig. 2.

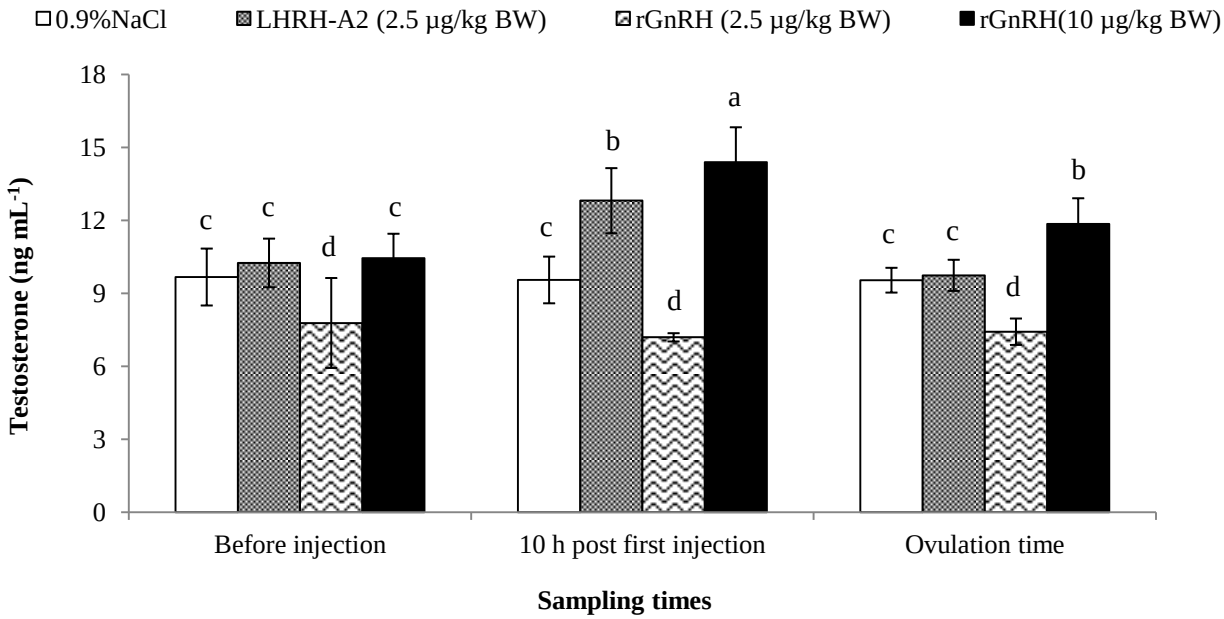

0.9%NaCl
LHRH-A2 (2.5 µg/kg BW)
rGnRH (2.5 µg/kg BW)
rGnRH(10 µg/kg BW)
Testosterone (ng mL-1)
18
15
12
9
6
3
0
c
c
d
c
c
b
d
a
c
c
d
b
Before injection
10 h post first injection
Ovulation time
Sampling times

Fig. 3.

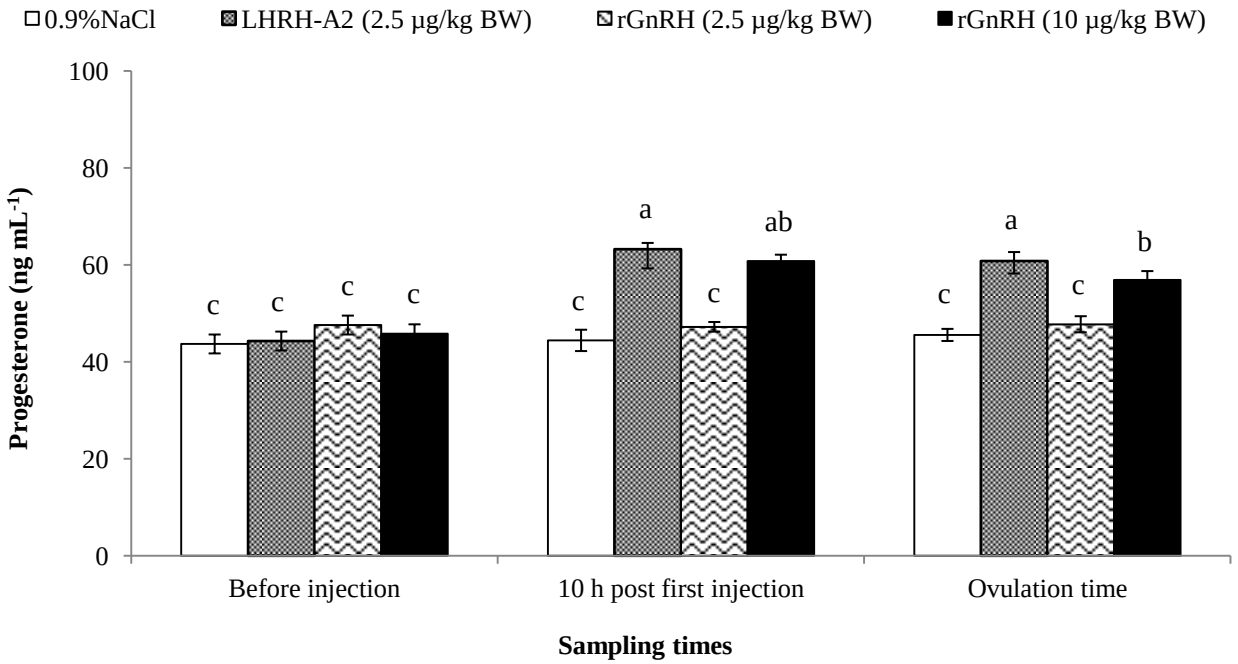

0.9%NaCl
LHRH-A2 (2.5 µg/kg BW)
rGnRH (2.5 µg/kg BW)
rGnRH (10 µg/kg BW)
Progesterone (ng mL-1)
100
80
60
40
20
0
c
c
c
c
c
a
ab
c
c
a
b
c
Before injection
10 h post first injection
Ovulation time
Sampling times

Fig. 4.

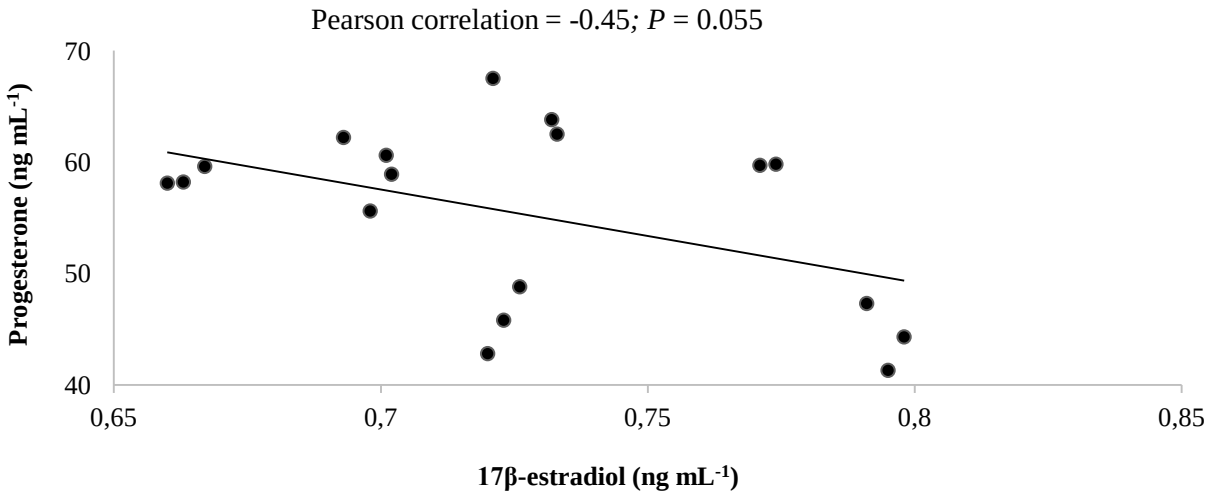

Pearson correlation = -0.45; P = 0.055
70
60
50
40
Progesterone (ng mL-1)
0,65
0,7
0,75
0,8
0,85
17β-estradiol (ng mL-1)

Fig. 5.

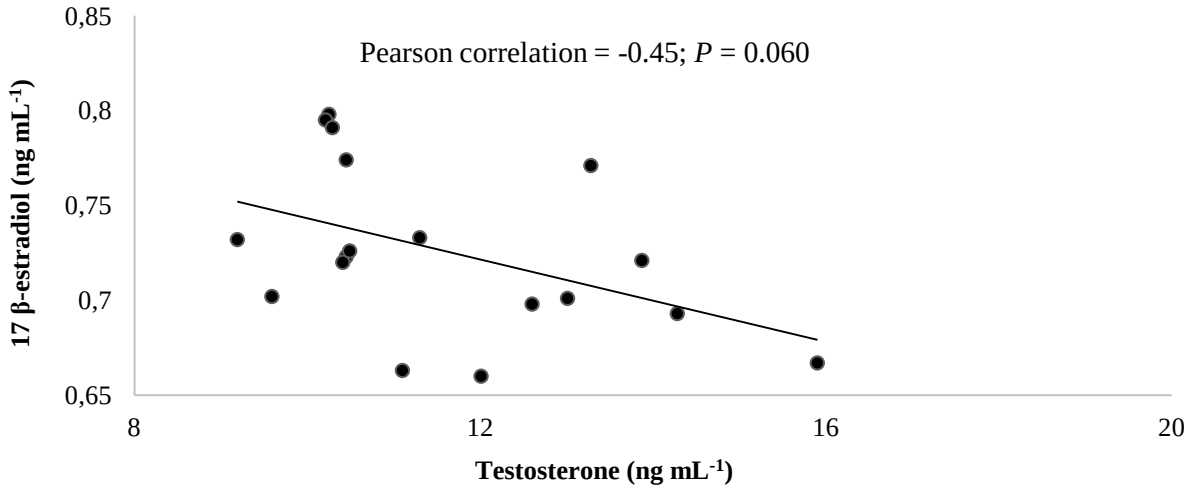
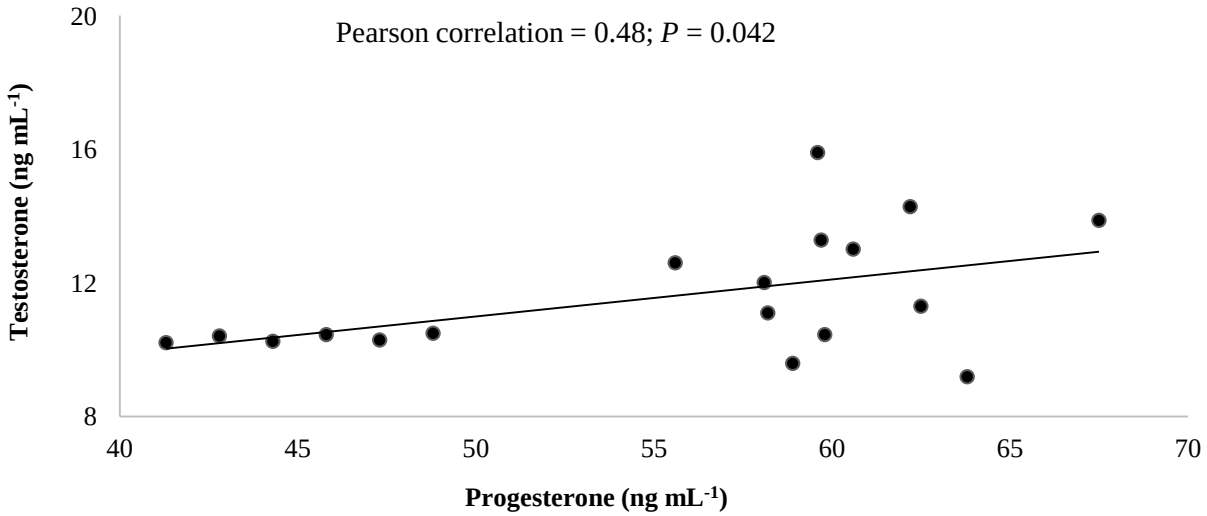

Pearson correlation = -0.45; P = 0.060
17 β-estradiol (ng mL-1)
0,85
0,8
0,75
0,7
0,65
8
12
16
20
Testosterone (ng mL-1)

Fig. 6.


Pearson correlation = 0.48; P = 0.042
Testosterone (ng mL-1)
Progesterone (ng mL-1)
20
16
12
8
40
45
50
55
60
65
70

734 **Table 1.** Reproduction performance in Sterlet sturgeon (*Acipenser ruthenus*) broodstock after
735 injection of NaCl 0.9%; 2.5 µg kg body weight^{-1} LHRH-A2; 2.5 µg kg body weight^{-1} rGnRH and
736 10 µg kg body weight^{-1} rGnRH (Mean ± SD).

Treatment	No. of injected fish	Germinal vesicle migration [1]	No. of spawned fish	Spawning success (%)[2]	Latency period (h)[3]	Number of eggs /g
0.9% NaCl	6	8.64 ± 2.23	0	-	-	-
LHRH-A (2.5 µg kg-^{1})	6	8.53 ± 2.23	3	50	29.5 ± 0.5	101.7 ± 4.7^{ab}
rGnRH (2.5 µg kg^{-1})	6	8.59 ± 0.94	1	16.66	30	96.3^{b}
rGnRH (10 µg kg^{-1})	6	8.63 ± 1.62	4	60.66	30 ± 0.5	105.0 ± 3. 5 ^{a}

737 Different letters designate significant differences as determined by Tukey's post-hoc tests.
738 [1] Germinal vesicle migration (%) = distance between nucleus and AP / distance between VP and
739 AP (X100); [2]Spawning success (%): the number of females that ovulated after injection divided
740 by the total number of injected females. [3]Latency period (h): time between the first hormone
741 injection and ovulation.
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