Animal Reproduction Science 217 (2020) 106470

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

GnRHa treatments of Atlantic Salmon broodstock suppresses effects of endocrine disruptors, benefitting offspring quality

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ARTICLE INFO

Keywords:
Endocrine disruptor
GnRHa
Malformations
Salmo salar

ABSTRACT

The use of synthetic hormones to regulate sexual maturity in captive fish is a common practice. With aquaculture practices, fish production is desired throughout the year, necessitating the maintenance of quality standards, mainly regarding the characteristics of the fish produced. Embryonic development may be affected by toxins in the environment and by a variety of pathologies. The aim of this study was to determine the effects of treatment with gonadotropin-releasing hormone analog (GnRHa) on captive male and female Atlantic Salmon (*Salmo salar*) broodstock, observing the effects on the hormonal milieu and impacts on breeding outcomes. Sexually mature fish were fertilized with and without imposing a GnRHa treatment to evaluate the development of offspring up to the fry stage. The concentrations of 17β-estradiol (E2) and testosterone (T) were determined using commercially available ELISA kits. The results indicate the administration of GnRHa had marked effects on reductions of morphological deformities in the offspring and promoted development during the larval stage by inducing sexual maturity in both treated parents. The E2/T ratio results indicate the presence of endocrine disruptors. It is concluded that the use of GnRHa at a dose of 10 ug/kg in captive male and female Atlantic salmon broodstock has an inhibitory effect on the impacts of endocrine disruptors, does not affect fertilization rate, and has positive effects on development of offspring by reducing the number of morphological deformities during the larval stage of development.

1. Introduction

The main goal of aquaculture is to obtain maximum yields (growth and survival rates) by imposing optimal growth conditions during larval developmental cycles and fish maturation (Argüello-Guevara et al., 2014). These practices in commercial aquaculture enterprises in Chile as well as worldwide require technological developments and extensive knowledge of the species being cultivated so that there can be adequate control of the reproductive processes of fish. Gonadotropin-releasing hormone (GnRH) has been used for about 5 decades to synchronize timing of and induce ovulation by stimulating the secretion of endogenous luteinizing hormone (Lh) in a wide range of species (Zohar and Mylonas, 2001). The use of hormones for the control of reproduction in fish has focused on the induction of final oocyte maturation, ovulation, spermiation, and spawning in fish species that do not have a complete

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https://doi.org/10.1016/j.anireprosci.2020.106470
Received 18 October 2019; Received in revised form 14 April 2020; Accepted 15 April 2020
Available online 21 April 2020
0378-4320/

reproductive cycle in fresh water, or as a way to optimize the aquaculture management (performance) practices by advancing the process of sexual maturation, ovulation, and spawning (Donaldson, 1997). The effects of using these hormonal analogues on offspring, however, have not yet been investigated.

Chile is the world's second-largest producer of salmon species. The Atlantic salmon (*Salmo salar*) is a species of great commercial interest, but the farming conditions for embryos and young salmon have serious effects on the reproduction of adult salmon and, certainly, on its production (Figueroa et al., 2015). With development of new aquaculture practices, there has been a reduction in Atlantic Salmon populations due to a large number of anomalies or morphological deformities rarely observed in salmon in their natural habitat, with negative consequences on the development, behavior, and well-being of the farmed animals, leading to economic losses due to the reduction of the value of the product and negative marketing effects as a result of production of individuals with morphological deformities (Barahona-Fernandes,1982).

Results of several retrospective epidemiological studies indicate that abnormal environmental conditions during the first year after hatching has long term consequences on reproductive functions (Ravelli et al., 1998). Similarly, results from studies performed *in vivo* and *in vitro* have supported the conclusion that endocrine-altering chemicals affect the hormone-dependent pathways responsible for male gonadal development, either through direct interaction with hormone receptors or through modulation of the epigenetic regulatory or cell cycle (Di Nisio and Foresta, 2019). The chemicals that induce these changes in the hormonal milieu are called endocrine disruptors, which are natural or artificial molecules present in environment in which the salmon are located and are associated with hormone receptors that stimulate or inhibit the hormonal regulation of reproductive and morphological maturation. Endocrine disruptors can stimulate or prevent the functions of specific cells as well as the synthesis and transport of hormones or receptors and can disrupt hormonal imprinting during critical developmental periods, with subsequent consequences on development and morphological characteristics, such as alterations of cell functions that are regulated by hormones or susceptibility to disease (Gore et al., 2015; Csaba, 2017a). For example, liposoluble vitamins (A, D, E, and K), which are exohormones, can cause defective perinatal hormonal imprinting, with consequences similar to those of synthetic endohormones, which are members of the same family of hormones. Industrial, communal or medical endocrine disruptors can also have similar effects on reproduction and morphological characteristics of fish during development. In addition, because hormonal imprinting is an epigenetic process, this effect is inherited by offspring in subsequent generations (Csaba, 2017b). The use of a GnRHa, in Atlantic Salmon aquaculture production could be an endocrine disruptive practice affecting reproductive development and/or the prevalence of morphological deformities. The aim of the present study, therefore, was to determine the effects of treatment with GnRHa on captive male and female Atlantic Salmon (*Salmo salar*) broodstock, evaluating the effects of the hormone on offspring.

2. Materials and methods

2.1. Animal ethics

All methods and experimental procedures were conducted according to the approved (Approval ID: 160727012) guidelines and regulations of the institutional animal care and use committee (IACUC) of Pontificia Universidad Católica de Chile, Chile.

2.2. Broodstock

Atlantic salmon (*Salmo salar*), 3 years of age (sexually mature), with an average body weight of 8 kg and an approximate length of 86 cm were evaluated in this study. The specimens were provided by and grown in the facilities of Hendrix Genetics, a fish farm in Curarrehue, Region of La Araucanía, Chile. During the experiment, the broodstock were maintained in 3,000 L fiberglass tanks with an open water flow (500 L/h) at 10 to 17 °C with there being natural photoperiodic conditions.

2.3. Experimental design

For this study, groups of male (n = 10) and female (n = 20) sexually mature Atlantic Salmon were administered or not administered with various combinations of GnRHa. Spawning was induced, and the resulting gametes were used to produce the experimental groups and to induce fertilization to assess the development of embryos to the alevin (newly hatched) developmental stage. The experimental groups of fertilized broodstock were: 1) Males not treated with the hormone (UM) and females treated with the hormone (TF), 2) males treated with the hormone treatment (TM) and females not treated with the hormone (UF), 3) males not treated with the hormone (UM) and females not treated with the hormone (UF), and 4) males treated with the hormone (TM) and females treated with the hormone (TF). The GnRHa was injected intraperitoneally for the hormonal treatment at a dose of 10 μg/kg with administrations occurring a week before the spawning and collection of semen.

2.4. Gamete collection

Semen was collected using the procedures previously published by Ciereszko et al. (2014) with some modifications. Briefly, males were anesthetized in a 50 L tank with 125 mg/L MS-222 for 10 minutes (n = 10 for each group). The urogenital pore was dried, and semen was collected using the smooth abdominal massage procedure directly into a sterile and dry disposable plastic container maintained at 4 °C, taking care not to contaminate the sample with feces, mucus or urine. Oocytes from 20 females from each group was used to evaluate fertility using the procedures previously described by Figueroa et al. (2015). All the fertilization evaluations

were replicated 20 times using 1,000 oocytes. The number of sperm used in all treatments was 1.5×10^7 sperm/oocyte. The eggs were incubated in open flow conditions at 8 °C.

2.5. Embryo, larvae and offspring production

Fertilization rate (%) was estimated one day after fertilization after 20 h of incubation at 8 °C. In each group of fertilized eggs when there were the respective GnRHa treatment regimens, 20 embryos were fixed in Stockard 20 solution, and the fertilization rate of each group was determined. An egg was considered fertilized when a segmented blastodisc was detected, while numbers of embryos with normal eye development were determined at 8 °C at 40 days. Larvae and fry were maintained at 12 ± 0.3 °C (pH 6.5 ± 0.2, 108.2 ± 3.3 % O_2, 10.5 ± 4.6 mg/L CO_2). Embryonic mortality was considered to have occurred when there was no discernible heartbeat. From birth to sac resorption (115 days at 8 °C), dead larvae and fry were collected to estimate relative survival (%) and prevalence of morphological deformities of the fry (%).

2.6. Evaluation of morphological deformities

In the sac resorption stage, prevalence of morphological deformities in the fry were quantified and recorded as the percentage (%). Morphological deformities were classified as abnormalities in development of the spine (scoliosis, lordosis, kyphosis, kypholordosis), deformities of the yolk sac (sac strangulation, edema), deformities of the head (cyclopia, open wounds, shortened opercula, microstomia, prognathism, or loss of an eye), and deformities of the caudal complex (coiling) (Midtlyng et al., 1996; Aunsmo et al., 2008). This classification was performed until the first feeding of the fry. Hemorrhaging in the head of the larvae was evaluated by observation of a dense red region, and jaw defects were determined when the angle of the lower jaw was obviously deformed (Newman et al., 2015).

2.7. Quantification of hormones

In males ($n = 10$) and females ($n = 20$), the concentrations of 17β-estradiol (E2) (Estradiol ELISA Kit, N° 582251, Cayman Chemical, Ann Arbor, Michigan, United States) and testosterone (T) (Testosterone ELISA Kit, N° 582701, Cayman Chemical, Ann Arbor, Michigan, United States) were quantified using enzyme-linked immunosorbent assay (ELISA) kits linked to commercially available enzymes (Cayman Chemical, Ann Arbor, Michigan, United States) and were performed according to the manufacturer's instructions. The concentrations of E2 and T were standardized to the volume (mL) of plasma used for each sample and are expressed as pg-E2 or T per mL of plasma. The T assay had a range of detection from 3.9 to 500 pg/mL and a sensitivity of approximately 6 pg/mL, a cross-reactivity that was equal to 23% for androgens of similar structure (5a-dihydrotestosterone and 5b-dihydrotestosterone), a 17.2% of recovery rate of added T, a 60.3% of binding percentage, an intra-assay variation of 5.4% and an inter-assay variation of 3.8%. The E2 assay had a range of detection from 6.6 to 4,000 pg/mL and a sensitivity of approximately 15 pg/mL, with a cross-reactivity equal to 14.5% for estrogens of similar structure (estradiol-3-sulfate, estradiol-3-glucuronide, estrone and estradiol-17-glucuronide), a 45.2% recovery of added E2, a 71.7% binding percentage, an intra-assay variation of 12.2% and an inter-assay variation of 15.3%. In addition, the ratio of E2 to T was calculated (E2/T).

2.8. Statistical analysis

Normality and homogeneity of variance (F-test) were evaluated for all the data and are provided as percentages. For $P < 0.05$ according to the F-test, the Kruskal-Wallis test was used. In addition, Dunn's *post hoc* test was utilized to determine differences between the groups if there was a $P < 0.05$ utilizing the Kruskal-Wallis test (nonparametric data) or the unpaired t-test. The data were analyzed using GraphPad Prism v.4.0 software (GraphPad Software, San Diego, CA). The software was used for the preparation of the corresponding graphs and statistical analysis. The results are presented as the mean ± SD of at least three independent experiments.

3. Results

3.1. Effect of GnRHa treatment on breeding of broodstock

Even though there were differences in the numbers of fertilized ova among the groups, the average fertilization rate (%) was of 92%, with no difference among groups ($P > 0.05$; Table 1). The percentage of viable embryos compared to nonviable or asymmetric embryos was determined based on the total fertilized ova (Fig. 1). Thus, there was an average of 49% viable embryos in the group consisting of males without hormonal treatment (UM) and females treated with GnRHa (TF) with the females of this group as compared with those of the other groups, producing the largest percentage of viable embryos. This result was different from those of the groups composed of broodstock in which there were the other treatments (UM + TF compared with TM + UF, $P < 0.05$; Table 1).

Both relative survival (%) and mortality (%) were determined for each group at the end of the study (Table 1). There were other causes of death, such as the presence of fungi or deaths occurring without any apparent cause, however, the total mortality was estimated at no more than 5% (data not shown). The percentage of dead larvae in the group consisting of females and males not

Table 1
Prehatching, hatching and posthatching rates of *Salmo salar* offspring

Groups of Fry	Number of Eggs	Fertilization Rate %	Viable Embryos %	Number of Hatches	Relative Survival %	Deformities Mortality %
UM + UF	10,000	94.6 ± 4.3 [a]	46.5 ± 8.9 [a,b]	6,623	84.3 ± 10.5 [a]	10.8 ± 2.9[a]
UM + TF	19,000	90.7 ± 9.2 [a]	54.0 ± 7.7 [a]	12,061	93.4 ± 12.5 [a]	0.5 ± 0.1[b]
TM + UF	19,000	87.6 ± 7.4 [a]	41.0 ± 9.9 [b]	11,648	94.7 ± 5.6 [a]	2.7 ± 1.0[b]
TM + TF	19,000	92.0 ± 8.6 [a]	51.8 ± 7.0 [a,b]	12,236	93.3 ± 13.7 [a]	0.5 ± 0.1[b]

Data are shown as the mean ± SD; Different letters indicate differences ($P < 0.05$ – Kruskal-Wallis test – Dunn's *post hoc*); Experimental groups: Untreated male (UM), Untreated Female (UF), Treated Male (TM) and Treated Female (TF)

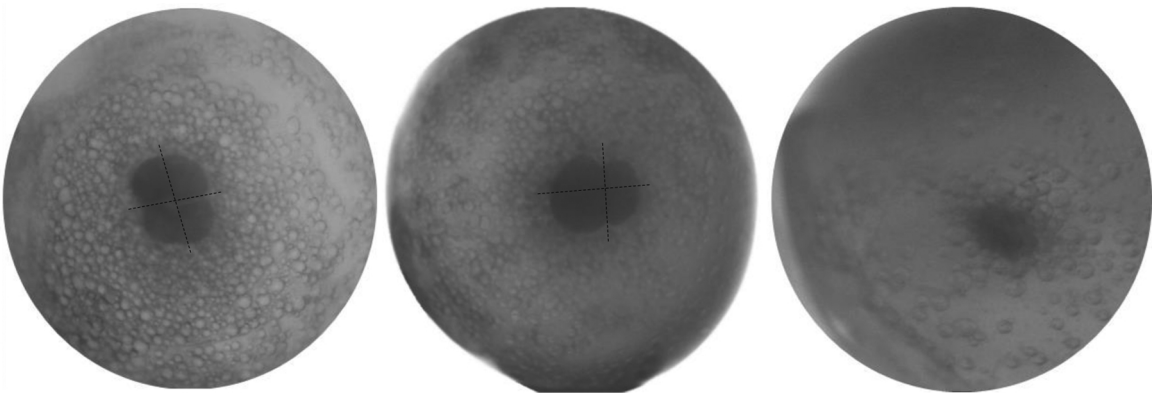

Fig. 1. Formation of an embryo at 20 hours postfertilization ($n = 40$).
A) Formation of a viable embryo with symmetric cells developing on the radial axis on the animal pole; B) formation of an unviable embryo, observing the development of asymmetric cells; C) unfertilized ovum

treated with GnRHa was greater when compared to those of the other three groups in which there were other GnRHa treatment regimens imposed (UM + UF compared with other groups, $P < 0.05$). The average relative larval survival (%), however, was 91% for all groups, with no difference between any of the groups.

The larvae from the group consisting of females and males not treated with GnRHa had a percentage of developmental abnormalities (Table 1) when compared to those of the other three groups in which there were other GnRHa treatment regimens (UM + UF compared with other groups, $P < 0.05$). The percentages of the different types of morphological deformaties are not summative because most of the larvae had more than one deformity. In all the groups, the most marked morphological deformity was associated with the spine (Fig. 2A and B).

3.2. 17β-estradiol and testosterone concentrations in broodstock treated with GnRHa

Males not treated with GnRHa (UM) had a greater concentration of testosterone when compared to the concentration in males with a prior hormone treatment (TM; Fig. 3A), indicating that in untreated males during spawning, there was ongoing spermatogenesis, while in treated males there were lesser testosterone concentrations. By contrast, there was an inverse effect on testosterone concentrations in female fish (Fig. 3B). In males, the E2 concentrations were completely inverse when compared to those of testosterone (Fig. 3C). In female fish, there was no difference in those treated with and those not treated with GnRHa ($P > 0.05$, Fig. 3D).

The results of this study indicate that both males and females not treated with GnRHa have normal E2/T ratios (male ratio < 1 and female ratio > 1), and that there are differences in these ratios when there are comparisons of the untreated group compared with the groups treated with GnRHa ($P < 0.05$, Fig. 3E-F).

4. Discussion

There are actions of GnRH and its potentiated analogs at the pituitary-gonadal axis in inducing a reproductive hormone cascade of changes in hormonal secretions that result in spawning and spermiation, and inducing final maturation of the oocyte (Zohar & Mylonas, 2001; Marino et al., 2003; Mylonas et al., 2004). In female *S. salar*, *in vivo* GnRHa implantation can result in a premature increase in plasma E2 and/or T (Celotti et al., 1991; Hess, 2003; Roselli, 2007; King and Pankhurst, 2007). In both female and male fish, the enzyme aromatase has actions in conversion of T into E2 (Gillies and McArthur, 2010). In relation to results from the present study, it is suggested that untreated female fishes might metabolize T more rapidly than females treated with GnRHa, but the latter

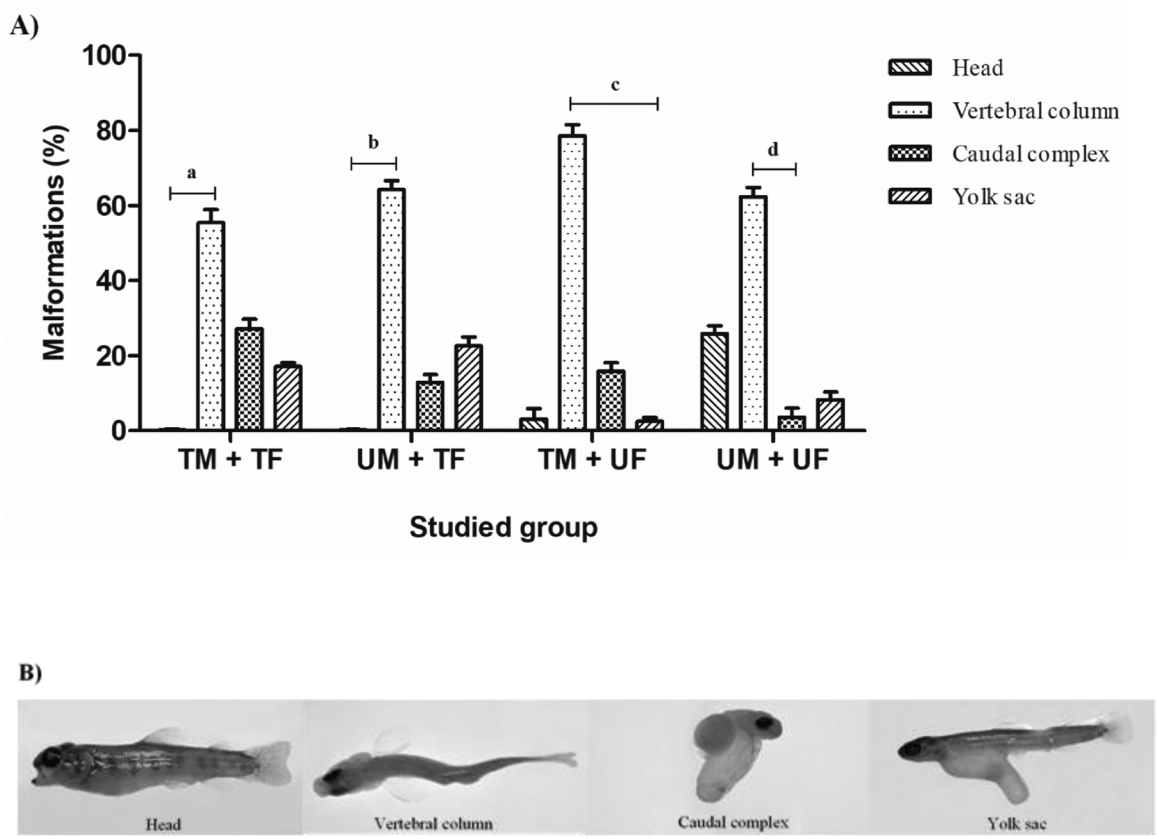

Fig. 2. Distribution of the specific types of *Salmo salar* morphological deformities, recorded from hatching to first feeding in each experimental group (550 °C-day).
A) Types of morphological deformities (vertebral column, caudal complex, yolk sac, and head) that can be observed, expressed as a percentage of the offspring number, with the morphological deformities present in each group (mean ± SD) obtained by the fertilization of broodstock with and without GnRHa treatment; B) This image represents the types of morphological deformities identified; Experimental groups: Untreated male (UM), Untreated Female (UF), Treated Male (TM) and Treated Female (TF); Differences were determined using the Kruskal-Wallis test with the *post hoc* Dunn test for each group: $^{a,b}P < 0.05$, head compared with vertebral column; $^{c}P < 0.05$, vertebral column compared with yolk sac; and $^{d}P < 0.05$, head compared with caudal complex.

are able to regulate and control the conversion of T to E2 to a greater extent than untreated females. Results from previous studies indicate there would be a resulting stimulation of the hepatic synthesis of vitellogenin, which is transported to the ovary to be incorporated into developing oocytes (Nagahama, 1994; Schultz et al., 2010). A relatively lesser concentration of E2 is observed in untreated, as compared with treated males indicating the suprarenal glands and testicles produce small quantities of estrogen (Celotti et al., 1991; Hess, 2003; Roselli, 2007). On the other hand, when they were treated with GnRHa, concentrations completely different than those observed in normal males were observed, which can be explained because GnRHa induces increased activity of the enzyme aromatase in males; regarding this, different investigations have shown that when the aromatase enzyme is activated, T levels are reduced, which allows stimulating spermiation (Nagahama, 1994; Valdebenito, 2008; Schultz et al., 2010).By assessing the E2/T ratio, there can be determination of the profiles of endocrine disruptor components, which are associated with alterations in hormone production, metabolism, and/or hormone action as a result of direct or indirect actions. The estrogen to androgen indices are often used to evaluate endocrine disruption. Males, therefore, generally have E2/T ratios of less than 1 and gametogenic females have ratios greater than 1 with deviations from these being indicative of environmental stress, including effects of pollutants on the reproductive endocrine system (Goodbred et al., 1997). For males and females treated with GnRHa, the E2/T ratio was altered, indicating this hormone functions as an endocrine disruptor or that it can alter the normal concentrations of T and E2 of fish relative to untreated specimens, but without generating negative effects on offspring because the number of morphological deformities was less in the groups where one or both parents were treated with this hormone, similar to the effects of endocrine disruptors. This finding may perhaps be mainly because the structure of the synthetic hormone used in the present study is similar to the natural hormone regulating gametic maturity, which is essential to the regulation of the different hormones that control the fertilization process. This beneficial effect of endocrine disruptors was described by Csaba (2018), where it was determined in humans, the consumption of enormously large amounts of phytoestrogens, which are considered to be endocrine disruptors, decreased the negative effects of industrial ED to which people were also exposed, a likelihood according to Mills and Chichester (2005) since each

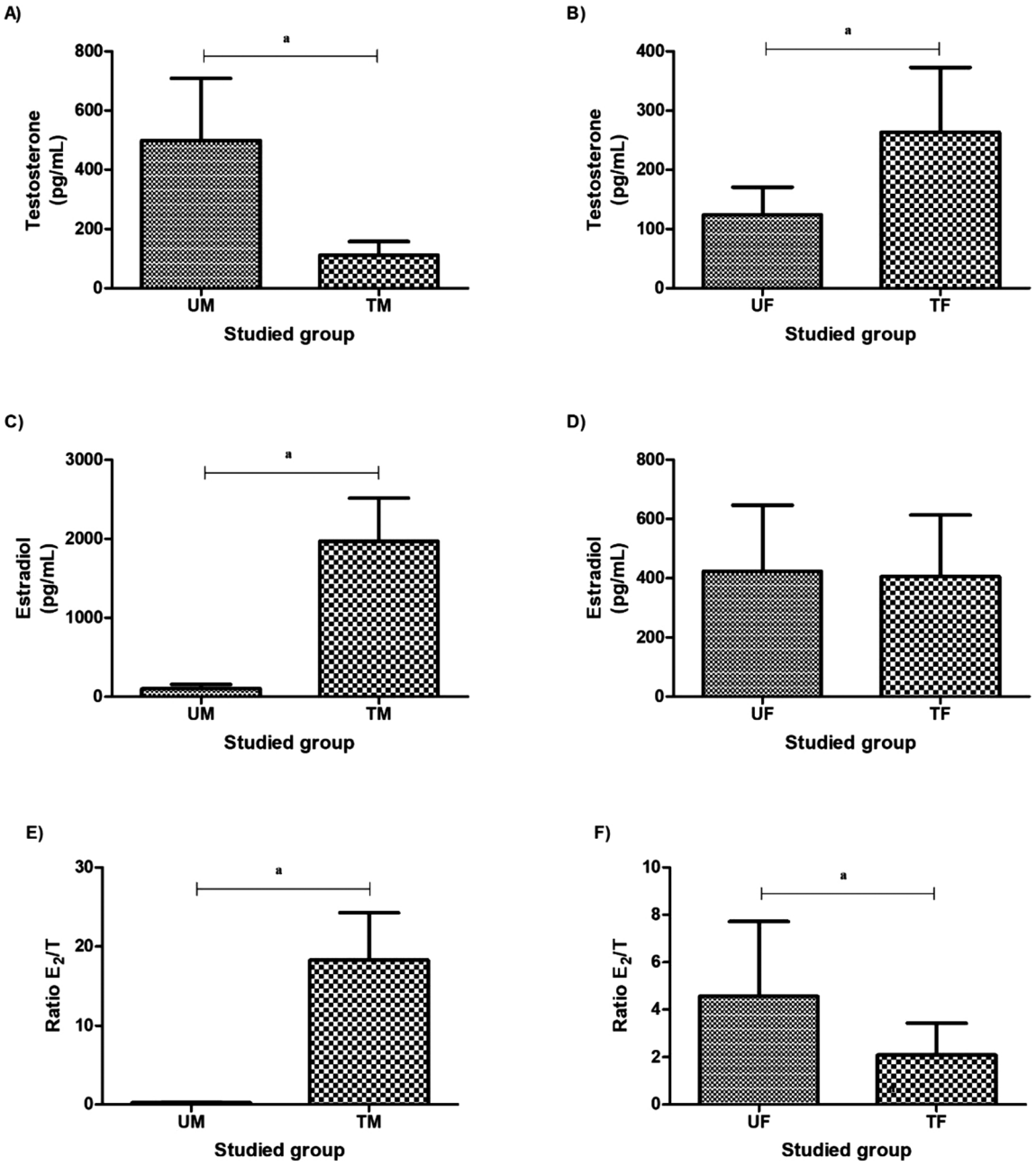

Fig. 3. Hormonal concentrations in plasma samples from *Salmo salar* broodstock ($n = 20$ for each treatment).
Testosterone and estradiol concentrations are expressed as the pg of testosterone per mL of plasma sample analyzed for each study group. E2/T ratio is determined using the concentrations of both hormones; A) T concentration comparing Untreated male (UM) compared with Treated Male (TM); B) T concentration comparing Untreated Female (UF) compared with Treated Female (TF); C) E2 concentration comparing U) vs. TM; D) E2 concentration comparing UF compared with TF; E) E2/T ratio comparing UM compared with TM; F) E2/T ratio comparing UF compared with TF
Data are presented as the mean ± SD. Differences were determined using the unpaired t-test ($^{a}P < 0.05$, untreated compared with treated)

organism might be in the presence of various synthetic chemical substances classified as ED, and the effects would be diverse due to the many possible interactions generated between the different EDs, which might enhance the negative effects of some substances, as well as on the contrary might have beneficial effects by inhibiting these negative effects; in this case, GnRHa would act by inhibiting the effect of the other disruptors. Among the possible endocrine disruptors are pesticides and contaminants in the water. The physiological, behavioral and population endpoints affected by pesticides include lesser or delayed hatching and growth suppression, as well as embryonic or larval mortality (Pašková et al., 2011). In relation to water contamination, the first stages of fish development are particularly sensitive, because the presence of heavy metals can affect several developmental processes during the embryonic

period, resulting in reductions in the number and quality of offspring. Metals transported by water can accumulate in the gonads of broodstock and negatively affect the production and viability of gametes or exert a direct toxic effect on developing embryos (Jezierska et al., 2009). The secretion of GnRH normally changes in response to environmental signals or endocrine disruptors (Mills and Chichester, 2005), suggesting that these effects might have been observed in the group in which none of the parents were treated with GnRHa in the present study.

In relation to the observed effects on offspring, it is important to note that in the present study the fertilization percentage was high. This finding indicates the batch of ova evaluated in the present study was of good quality (Bobe and Labbé, 2010). Because relative survival was similar among the groups in the present study, it is possible that there was a variable inducing death and morphological deformities in the offspring different from handling and farming conditions. There have been reports that when Atlantic Salmon are in cold water and air during the embryonic stage, and post-hatching stage, or both, there are alterations in the methylome and the transcriptome and effects on growth performance in later life (Moghadam et al., 2017; Robinson et al., 2019). Wagner et al. (2002) described the stress of captivity on fish as possibly affecting the immune system, growth, and reproduction. The stress of captivity is thought to lead to alterations in secretions from several organs of the endocrine system in ways that larval quality as well as survival and development of eggs and larvae are compromised. The latter might explain why groups without hormone treatment had the greatest number of morphological deformities in the offspring, because the use of GnRHa “repairs” the endocrine alterations produced by captivity and results in induction of sexual maturation. This response would only occur when there is use of a dose of 10 µg/kg, of GnRHa because when Bonnet et al. (2007) evaluated the use of a GnRHa at doses of 60 µg/kg in females there was an increase in the defects in yolk sac resorption and other deformities when there was treatment with GnRHa at this dose.

With regard to the morphological effects of breeders treated with GnRHa on the offspring, there were marked deformities of the spine due to the interruption of the genesis of the paraxial mesoderm, somites or axial bones (Chen et al., 2016). In the embryogenesis of vertebrates, the paraxial mesoderm is adjacent to the neural tube and develops from the anterior portion of the embryo to the somites during specific processes of somitogenesis. Each somite subsequently subdivides into a ventromedial sclerotome (from which derives the vertebral body) and a dorsolateral dermomyotome (from which the skeletal muscles of the body and the dorsal dermis form). Any defect in the paraxial mesoderm or the formation of somites can contribute to the congenital vertebral morphological deformities (CVM) (Hubaud and Pourquie, 2014). The CVM includes a group of serious congenital defects that can present as congenital scoliosis, kyphosis, or Klippel Feil syndrome (Tawk et al., 2002), which can appear together with other birth defects or as part of an underlying genetic syndrome (Giampietro et al., 2009). It should be emphasized that CVM represents a complex condition with multiple causes that have not yet been clarified (Chen et al., 2016); therefore, it can be hypothesized that the group produced from broodstock without hormonal treatment (UM + UF) in the present study had CVM because these larvae had the greatest percentage of morphological deformaties, suggesting that GnRHa treatments of the broodstock resulted in a decrease in these morphological deformities in the offspring.

5. Conclusion

The results of the GnRHa treatment in Atlantic salmon broodstock indicate that although treatments with this hormone induce gametogenesis, there is no effect on the fertilization rate or the viability of embryos. There, however are beneficial effects of treatment with the GnRHa on embryonic development and the larval stage of offspring in ways that the quality standards for larvae of aquaculture companies are met as a result of the GnRHa treatments. In groups with one or two parents treated with the GnRHa, the number of morphological deformities detected was considerably reduced. Although this is not common, in Atlantic Salmon, the concentration of GnRHa used in the present leads to endocrine disruption with favorable effects on the development of offspring.

Funding

This work was supported by Post-doctorate FONDECYT/CONICYT (AZP) [N°3170193] and REGULARFONDECYT (JGF) [N°1180387].

CRediT authorship contribution statement

Andrea B. Zepeda: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft. **Ignacia B. Miranda:** Methodology, Validation, Data curation. **Iván Valdebenito:** Methodology, Resources. **Ricardo D. Moreno:** Conceptualization, Investigation, Writing - review & editing. **Jorge G. Farías:** Resources, Supervision.

Declaration of Competing Interest

The authors have no conflict of interests regarding the publication of this manuscript.

Acknowledgements

We would like to thank Hendrix Genetics for providing animal resources.

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