Comparative Biochemistry and Physiology, Part A 225 (2018) 59–64

Seasonal-and dose-dependent effects of recombinant gonadotropins on sperm production and quality in the flatfish *Solea senegalensis*

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Key words: Flatfish, recombinant gonadotropins, spermatogenesis, sperm, motility.

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Abstract

Consecutive treatments with recombinant follicle-stimulating and luteinizing hormones (rFsh and rLh, respectively) stimulate spermatogenesis and potentiate sperm production in pubescent specimens of the oligospermic Senegalese sole (*Solea senegalensis*). However, sperm production in response to the hormones is highly variable, and the steroidogenic potential of the testis may be diminished due to sustained hormone supply. Here, we compared the effectiveness of low (9 μg/kg) and high (18 μg/kg) doses of rFsh and rLh to improve sperm production in adult sole during late winter-early spring (onset of the natural spawning period), and in autumn under a controlled temperature of 12ºC (period of testicular recrudescence). Treatment with rFsh over six weeks during spring, followed by a single rLh injection, did not enhance sperm production, possibly because of an advanced stage of sexual maturation of the males, as reflected by high Lh plasma levels (~17 ng/ml) before rFsh treatment. In contrast, in autumn, when the Lh circulating levels were much lower (~3 ng/ml), the low doses of rFsh and rLh generated a four-times increase in sperm production, whereas the high doses of the hormones were ineffective. However, treatment with rLh, regardless of the effect of rFsh, improved the motility of spermatozoa during both spring and autumn. These data confirm that consecutive rFsh and rLh treatments increase sperm production and quality in adult sole males, although they seem to be highly sensitive to the rFsh dose. The efficiency of recombinant gonadotropins also appears to be season-dependent despite the asynchronous nature of the sole testis.

1. Introduction

In temperate teleosts, the reproductive period is seasonal and therefore testicular development, which occurs in a cystic and synchronous manner, is highly dependent on environmental conditions such as temperature and photoperiod (Migaud et al., 2010; Schulz et al., 2010). In seasonal breeders, spermatogonial proliferation and meiosis initiation (spermatogenesis) is activated during the reproductive season, and resulting haploid spermatids embedded in the Sertoli cells transform into fully differentiated spermatozoa (spermiogenesis), which are subsequently released into the lumen of the testicular lobules and further transported to the spermatic ducts during the process of spermiation (Schulz et al., 2010; Schulz and Chauvigné, 2018). As in other vertebrates, the processes of spermatogenesis and spermiogenesis in teleosts are believed to be tightly regulated by the pituitary gonadotropins, follicle-stimulating (Fsh) and luteinizing (Lh) hormones, through activation of the Fsh receptor (Fshr) in Sertoli cells and Lh/choriogonadotropin receptor (Lhcgr) in the steroidogenic Leydig cells, respectively (Levavi-Sivan et al., 2010; Schulz et al., 2010). Thus, in this model it is established that Fsh promotes spermatogenesis by the activation of growth factor release from Sertoli cells, whereas Lh regulates spermatozoa maturation and spermiation through the activation of steroidogenesis in Leydig cells and possibly of the Lhcgr expressed by haploid germ cells (Schulz et al., 2010; Chauvigné et al., 2014b). Some unique features observed in teleosts are that while the Lhcgr is specifically activated by its ligand, the Fshr may be promiscuous in some species, and that Fsh can also exert steroidogenic actions through the activation of its cognate receptor in Leydig cells (Schulz et al., 2010; Xie et al., 2017; Schulz and Chauvigné, 2018). However, recent gene editing studies in the zebrafish (*Danio rerio*) suggest that Lh signaling and Fsh signaling are redundant and either hormone alone can support spermatogenesis in this species (Xie et al., 2017).

The use of gonadotropin-based hormone therapies is envisaged as the most potent method to counteract reproductive dysfunctions of cultured male fish. Amongst the newest biotechnological approaches to increase sperm production and quality is the use of specific recombinant gonadotropin hormones (rFsh and rLh), produced in heterologous eukaryotic systems such as the Chinese hamster ovary (CHO) cells in the form of a single-chain (Garcia-Campayo et al., 1997; Mazón et al., 2013, 2014; Yom-Din et al., 2016; Chauvigné et al., 2017). Administration of these types of rFsh and rLh triggers testicular recrudescence and promotes spermiation in juvenile European sea bass (*Dicentrarchus labrax*), which show a seasonal and cyclic pattern of testicular activity (Mazón et al., 2013, 2014). Injection of rFsh also enhances the androgen plasma concentration, and the testis volume and sperm count in other seasonal breeders (Mylonas et al., 2017).

Some flatfish species of high commercial interest, such as the Senegalese sole (*Solea senegalensis*), exhibit an asynchronous pattern of spermatogenesis, in which spermiogenesis occurs within the lumen of the seminiferous lobules (i.e. a semicystic type of spermatogenesis) (García-López et al., 2005). As a consequence, in this species spermatogenesis and spermiation occur all year-round, although these processes are enhanced during spring, coinciding with a peak in the plasma levels of Fsh, Lh and the major androgen 11-ketotestosterone (11-KT), and the seasonal occurrence of female ovulation (García-López et al, 2006a; 2006b, Cabrita et al., 2011; Chauvigné et al., 2015, 2016). The production of sperm in Senegalese sole is however very low (<130 µl), particularly in the first generation (F1) of cultured males, which complicates the

development of *in vitro* fertilization methods for selective breeding programmes at an industrial level (Morais et al., 2014). Several attempts have been made to improve sperm quality and quantity in sole by the administration of gonadotropin-releasing hormone agonist (GnRHa) or human chorionic gonadotropin (hCG) using either injections or implants (Agulleiro et al., 2006, 2007; Cabrita et al., 2011; Guzmán et al., 2011a), as well as by dietary supplementation with fatty acids and vitamins (Beirão et al., 2015), but none of these treatments have resulted in a significant increase in sperm volume, density or motility.

In a recent study, we reported for the first time that consecutive treatments of sole F1 pubescent males with Senegalese sole specific rFsh and rLh, which are able to stimulate testicular steroidogenesis and regulate genes involved in spermatogenesis both *in vitro* and *in vivo* (Chauvigné et al., 2012, 2014a), can enhance spermatogenesis and increase sperm production *in vivo* (Chauvigné et al., 2017). In this latter study, however, we found a high variability in the production of sperm by the males in response to the hormones when treatments were administered towards spring and during the spawning period. The variability may have been caused by a decreased survival and steroidogenic potential of the Leydig cells as a consequence of a weekly rFsh administration during 10 weeks (Chauvigné et al., 2017). In the present work, we conducted new experiments to identify the most effective conditions for the induction of sperm production and quality in Senegalese sole adult F1 males by evaluating the effectiveness of different doses of rFsh and rLh administered for shorter times on the same fish under different conditions of temperature and photoperiod.

2. Materials and methods

2.1. Animals

Approximately three-year old adult Senegalese sole F1 males were obtained from the commercial company Stolt Sea Farm S.A. (Spain), and the Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), Centro El Toruño. Fish were transported to the IRTA fish research facilities at Sant Carles de la Ràpita (Spain), and held in fiber glass tanks of 10 m^3 connected to a recirculation system (IRTAmar®). Fish were fed five days a week with 0.75% of wet feed (mussels and polychaetes) and 0.55% of dry feed (balance diet) of the total biomass. The procedures relating to the care and use of animals and sample collection were conducted in accordance with the protocols approved by the Ethics Committee (EC) of the Institut de Recerca i Tecnología Agroalimentàries (IRTA) following the European Union Council Guidelines (86/609/EU).

2.2. Experimental design and sample collection

The experiments carried out in this study were designed to investigate the effect of consecutive treatments with different doses of rFsh and rLh on sperm production and quality by adult Senegalese sole F1 males (Fig. 1). The homologous single-chain Senegalese sole rFsh and rLh were produced in CHO cells by Rara Avis Biotec (Valencia, Spain) as previously described (Chauvigné et al., 2017). The trials were carried out on the same groups of males (886 ± 25 g; mean ± SEM) during two periods of the reproductive cycle under natural photoperiod. The first trial was conducted slightly preceding the major

natural spawning period of sole, from late winter to early spring (from mid February to early April), when temperature naturally increased from ~13ºC to ~17ºC and photoperiod ranged from 10.5 h light (L):13.5 h dark (D) to 12 h L:12 h D. Three groups of males (*n* = 9-12) were weekly injected intramuscularly with CHO cell culture medium (control group) or 9 or 18 µg/kg rFsh during 6 successive weeks, followed by a single injection of medium or rLh at the same doses (9 or 18 µg/kg) on the sixth week (Fig. 1). Plasma samples were collected before the first rFsh injection (time zero), and 24 h after rLh injection on the sixth week, when semen quantity and quality were also evaluated. After this trial, fish were rested under a controlled temperature of ~19ºC throughout the summer until early autumn (mid October), when temperature in the holding tanks was decreased and maintained at 12.2 ± 0.03ºC for two weeks while maintaining a natural photoperiod ranging from 10 h L:14 h D to 9 h L:15 h D. Similar low temperatures have been suggested to enhance spermatogenesis and the reproductive performance of F1 Senegalese sole (Anguis and Cañavate, 2005; Agulleiro et al., 2007; Martin et al., 2014), and potentiate the effect of rFsh on germ cell development in pubescent sole (Chauvigné et al., 2017). After low temperature acclimation, fish were treated during autumn (from early November until mid December) with the same rFsh and rLh doses and time lengths as in the late winter-spring experiment. Collection of plasma and semen samples was carried out in the same manner as in spring.

For the samplings, fish were sedated with 60 mg/l tricaine methanesulfonate (MS-222; Sigma-Aldrich) and weighed. Then, a sample of 0.8 ml of blood was collected from the caudal vein using a syringe previously coated with 0.5 M EDTA pH 8. The blood was mixed with 5 µl 0.5 M EDTA into a tube and kept on ice until centrifugation (3000 x *g* for 15 min at 4 °C). The plasma was aliquoted and stored at -80 °C. The sperm was collected by applying soft pressure massage to the abdomen from the testes along the sperm ducts until the cloaca. The sperm was collected with a micro hematocrit 75 x 1.15 mm capillary (Brand GMBH) that allowed an accurate estimation of the volume of the ejaculate. The sperm was immediately transferred into a 1.5-ml tube at room temperature (~15-18ºC) and the motility of spermatozoa evaluated within 30 min.

2.3. Gonadotropin and steroid determination

Plasma levels of endogenous and recombinant gonadotropins were determined by enzyme-linked immunosorbent assays (ELISAs) as previously described (Chauvigné et al., 2015, 2016, 2017), using specific antibodies against Senegalese sole Fsh and Lh β subunits. The levels of 11-KT in plasma were determined by commercial ELISA (Cayman Chemical Company) as described previously (Chauvigné et al., 2012, 2017). Steroids were extracted from plasma (5 µl) in methanol and the resulting pellet was diluted 1:50 in ELISA buffer (0.1 M K2HPO4/KH2PO4, 1.54 mM sodium azide, 0.4 M NaCl, 1 mM EDTA, and 0.1% BSA, pH 7.4). A standard curve was run for each ELISA plate and samples were analyzed in duplicate.

2.4. Computer-assisted sperm analysis (CASA)

An aliquot of freshly collected sperm was diluted 1:10 with non-activating medium (NAM; in mM: 59.89 NaCl, 1.48 KCl, 12.92 $MgCl_2$, 3.51 $CaCl_2$, 20 $NaHCO_3$ and 10 mg/ml BSA, pH 7.7; 300 mOsm) (Fauvel et al., 1999). The spermatozoa concentration was

evaluated in three different regions of the sperm counting chamber with a CASA system (ISASv1 software, Proiser, Spain) coupled to a phase contrast microscope (Nikon Eclipse 50i, Nikon) equipped with a x20 negative phase contrast objective. The total number of spermatozoa per ejaculate was normalized by the weight of the fish. Sperm samples were subsequently diluted in NAM to 10^9 cells/ml, which for samples with low sperm concentration was unnecessary. Motility was recorded immediately (5 s) after dilution (1:6) in artificial seawater (ASW; in mM: 420.9 NaCl, 9 KCl, 22.9 $MgCl_2$, 25.5 $MgSO_4$, 9.25 $CaCl_2$, 2.1 $NaHCO_3$, pH 8.0; 1100 mOsm), and every minute until motility completely stopped (Chauvigné et al., 2017). The sperm kinetics parameters evaluated were the percentage of total motile and progressive spermatozoa, as well as the curvilinear velocity (VCL, in μm/s). Each ejaculate was assessed in triplicate.

2.5. Statistics

Data are the mean ± SEM. and were statistically analyzed by two-way ANOVA, after log or arcsine transformation of the data when needed, followed by the Duncan's multiple range test, using the Statgraphics Plus 4.1 software (Statistical Graphics Corp., USA). A *P* value < 0.05 was considered statistically significant.

3. Results and discussion

The aim of the present study was to compare the effectiveness of recombinant gonadotropin-based therapies on sperm production and quality in adult Senegalese sole F1 males at two different times of the year, when temperature increases towards spring, which corresponds to the onset of the natural spawning period, and in autumn under a controlled stable temperature of 12ºC, which mimics the time when plasma androgens start to increase and testicular recrudescence takes plaice (García-López et al., 2006b; Chauvigné et al., 2016). In both trials, treatments with low (9 μg/kg) and high (18 μg/kg) doses of rFsh during 6 weeks, followed by a single injection with equal doses of rLh, were selected based on our previous study (Chauvigné et al., 2017), which we estimated could prevent potential toxic effects of the hormones while still being effective at stimulating spermatogenesis and spermiation.

3.1. Gonadotropin-induced androgen release is season-independent

To evaluate the endocrine state of the fish before the start of the experiments in spring and autumn, as well as the steroidogenic response of the testis to the recombinant gonadotropins, we first determined the circulating levels of Fsh, Lh and 11-KT (Fig. 2). The basal plasma levels of endogenous Fsh before the start of the experiments in spring and autumn (mid February and early November, respectively) were relatively low in the three groups (2.6 ± 1.1, 2.7 ± 0.9 and 2.7 ± 1.1 ng/ml, and 2.6 ± 0.3, 2.4 ± 0.3 and 1.6 ± 0.3 ng/ml, for the control, 9 μg/kg rFsh and 18 μg/kg rFsh groups, respectively), and it did not significantly change in the non-treated males at the end of the experiments (2.8 ± 0.5 and 2.6 ± 0.6 ng/ml in spring and autumn, respectively) (Fig. 2A). The low Fsh values recorded in autumn are in general in accordance with previous results on adult sole males under the natural temperature conditions of this time of the year when Fsh is generally <10ng/ml (Chauvigné et al., 2016, 2017). However, the low Fsh plasma levels found in spring

contrast with the variations that have been previously described during the same period (from ~15 to ~20 ng/ml) (Chauvigné et al., 2016, 2017). Nevertheless, in both experiments higher plasma levels of Fsh than in the controls could still be detected in the groups treated with rFsh one week after injection at the end of the trials, which confirmed that rFsh is highly stable in Senegalese sole plasma (Chauvigné et al., 2017). However, although the plasma levels of total Fsh remained the highest in the males treated with 18 μg/kg rFsh (21.6 ± 2.6 ng/ml) at the end of the experiment in autumn, during spring the same group did not show higher Fsh concentrations than the group treated with 9 μg/kg rFsh after six weeks (10.2 ± 1.7 and 12.6 ± 3.4 ng/ml, respectively) (Fig. 2A), suggesting that the half-life of the rFsh is lower at high temperatures.

In contrast to the levels of Fsh, the basal plasma levels of total Lh in the three experimental groups before the start of the experiment in spring were about 6-times higher (18.9 ± 4.2, 15.1 ± 2.1, and 17.1 ± 3.5 ng/ml, for the control, 9 μg/kg rFsh and 18 μg/kg rFsh groups, respectively) than those in autumn (3.1 ± 0.4, 3.1 ± 0.9, and 3.2 ± 0.4 ng/ml, for the control, 9 μg/kg rFsh and 18 μg/kg rFsh groups, respectively) (Fig. 2B), suggesting a slight seasonal advancement of Lh secretion in these males with respect to previous reports (Chauvigné et al., 2016, 2017). Accordingly, the Lh plasma levels in control males significantly increased towards the end of the experiment (5.2 ± 0.6 ng/ml) in autumn, but not in spring, during which the levels remained unchanged (17.9 ± 4.7 ng/ml) (Fig. 2B). The groups injected with rLh showed a dose dependent increase in plasma Lh, both in spring and autumn (Fig. 2B), which could be a result of the determination of the hormone levels only 24 h after rLh injection, despite the fact that this recombinant gonadotropin shows a slightly lower half-life than that of rFsh (Chauvigné et al., 2017).

In both the spring and autumn trials, the plasma levels of 11-KT were relatively low in all groups before the start of the experiments (5.3 ± 1.5, 6.1 ± 1.0 and 6.7 ± 1.1 ng/ml, and 4.0 ± 0.8, 5.1 ± 1.3 and 3.4 ± 0.3 ng/ml, for the control, 9 μg/kg rFsh and 18 μg/kg rFsh groups, respectively) (Fig. 2C), which was expected based on early reports which show a clear peak of plasma 11-KT during winter (García-López et al., 2006ab; Chauvigné et al., 2016). The levels of 11-KT in control males slightly but significantly increased towards the end of the experiment in spring (10.4 ± 1.7 ng/ml), possibly as a consequence of the high plasma levels of Lh during this period, whereas at the end of the autumn experiment the androgen levels were unchanged (4.8 ± 1.1 ng/ml) (Fig. 2C). Following rLh injection, plasma 11-KT concentrations increased in a Lh dose-dependent manner with respect to those in the control fish, reaching levels up to 82.7 ± 12.0 and 70.0 ± 9.8 ng/ml during spring and autumn, respectively (Fig. 2C), which are in the same order of magnitude to those observed in previous experiments after rFsh or rLh injection (Chauvigné et al., 2017). Such active androgen synthesis was previously found to stimulate Leydig cell and germ cell proliferation and entry into meiosis of pubescent F1 sole males, although sustained accumulation of high 11-KT plasma levels as a result of repeated rFsh and rLh injections for 10 weeks in spring was found to induce apoptosis in Leydig cells and reduce their steroidogenic function (Chauvigné et al., 2017). Our data, however, revealed a good steroidogenic response to rLh, in terms of androgen release, after a 6-week treatment with rFsh both in spring and autumn, suggesting that the consecutive rFsh and rLh treatments employed in this study did not compromise the function of the testis.

3.2. Seasonal-dependent effects of rFsh and rLh on sperm production

At the end of the rFsh treatments during spring and autumn, the effect of a single injection of rLh on sperm production 24 h after the hormone treatment was assessed (Fig. 3). In both trials, all males from the three experimental groups were spermiating, and control males showed a higher production of sperm in terms of number of spermatozoa (spz) per ejaculate (877 ± 345 and 659 ± 204 x 10^6 spz, in spring and autumn, respectively) (Fig. 3A), o per kg of fish (1096 ± 424 and 802 ± 269 x 10^6 spz, in spring and autumn, respectively) (Fig, 3B), with respect to that observed in pubescent sole males at approximately the same time of the year (100-300 x 10^6 spz/kg; Chauvigné et al., 2017), or in wild and F1 adult males of similar size (10-300 x 10^6 spz/kg; Cabrita et al, 2006; Agulleiro et al., 2006, 2007; Beirao et al., 2011). The variability in sperm production between control males was rather high, ranging from 98 to 2800 x 10^6 spz/kg, and 80 to 1800 x 10^6 spz/kg, in spring and autumn, respectively, similarly to that we have previously observed in pubescent male sole (Chauvigné et al., 2017). The unexpected higher sperm counts in control fish with respect to other studies in adult sole may result from differences in the procedures employed for sperm extraction and cell density calculation, or be the consequence that in our study non-treated males were in contact in the same tanks to males injected with rFsh and rLh, which could have released androgens into the water thus potentiating the spermatogenesis of neighbour fish (Huertas et al., 2006; Sebire et al., 2007).

Interestingly, different results were obtained between spring and autumn in the response to the recombinant treatments. In the spring, although the consecutive treatments with rFsh and rLh were able to promote the 11-KT plasma levels above the controls, the hormones did not enhance sperm production with respect to the control males (Fig. 3A and 3B), suggesting that the treatment with rFsh was not effective at stimulating spermatogenesis during this time of the year. Similar results have been obtained in previous studies using GnRHa implants on adult F1 sole in spring, which despite being able to enhance testosterone and 11-KT secretion, did not increase the gonadosomatic index or the production of sperm (Agulleiro et al., 2006; Cabrita et al., 2011). The lack of response of sole males to hormone treatments during spring is likely related to an advanced stage of sexual maturation, as reflected in this study by the relatively high basal levels of circulating Lh in the males from the three groups at the beginning of the experiment. These observations thus suggest that although the Senegalese sole has an asynchronous type of spermatogenesis all year-round, when the natural spawning season approaches in spring, and the Lh plasma levels start to increase, endocrine and physiological changes occur in the testis making it refractory to Fsh stimulation. The translational and posttranslational downregulation of the Fshr in the Sertoli cells from the cortical testis at this time (Chauvigné et al., 2014a) may be an underlying mechanism, but further research is necessary to clarify the causes of the poor response to Fsh of sole males during the spawning season.

Under a constant low temperature of 12ºC in autumn, when the basal circulating levels of Lh were low, the consecutive treatment with rFsh and rLh was very effective at potentiating sperm production (Fig. 3A and 3B). Thus, males treated with 9 µg/kg of rFsh and rLh showed four-times higher sperm counts than control males, both when calculated as spz per ejaculate (2882 ± 601 and 659 ± 204 x 10^6 spz/ejaculate) or per kg of fish (3140 ± 633 and 802 ± 269 x 10^6 spz/kg, respectively). However, in the group treated with 18 µg/kg of rFsh and rLh, a strong variability in sperm production was observed, with fish producing from 190 to 5800 x 10^6 spz/kg, and consequently the mean was not significantly

different from the control group (Fig. 3A and 3B). The induction of sperm production in adult F1 sole males using low doses of rFsh and rLh in this study was similar to the increment of the spz number in the sperm duct in pubescent sole males treated with 6-15 µg/kg of rFsh during 10 weeks in autumn (Chauvigné et al., 2017), and it was higher than that reported in previous studies employing treatments based on GnRHa plus dopaminergic inhibitors (Guzmán et al., 2011b). However, extended treatments of pubescent F1 sole with higher doses of rFsh (12-24 µg/kg of rFsh) for 10 weeks during autumn did not affect spermatogenesis, but promoted a 6-times increase in the number of spz in the sperm duct (Chauvigné et al., 2017), unlike in adult fish, where we found that high rFsh doses impaired sperm production. It thus seems that adult sole males might be more sensitive than juveniles to the dose of rFsh.

3.3. Recombinant gonadotropins improve sperm quality

Evaluation of the sperm kinetics by CASA revealed that the motility, progressivity and velocity of spermatozoa were enhanced after gonadotropin injection both during spring and autumn (Fig. 4). In the two trials, the percentage of motile sperm in the control groups was of 24.6 ± 6.2% and 22.1 ± 9.1% in spring and autumn, respectively, at the time of activation, which is consistent with previous observations (Martinez-Pastor et al., 2008; Beirao et al., 2011; Cabrita et al., 2011), with the spermatozoa being motile for up to 3 min (Fig. 4A). In contrast, sperm motility was approximately doubled with 9 and 18 µg/kg rFsh/rLh with respect to that of the non-treated males (54.9 ± 4.4% and 45.1 ± 5.4%, and 42.3 ± 10.8% and 45.7 ± 7.0%, during spring and autumn, respectively); in spring the dose of 9 µg/kg rFsh/rLh being slightly more effective after 1 min of activation, whereas in autumn both doses were equally efficient, although they elicited somewhat shorter times of spermatozoa motility than in spring (Fig. 4A). Similarly, a strong increase in the percentage of progressive spermatozoa was observed in spring after rLh injection regardless of the dose applied (5.8 ± 1.8%, 15.4 ± 1.7% and 15.4 ± 2.5%, for the control, 9 µg/kg rFsh and 18 µg/kg rFsh groups, respectively), which was maintained for 2 min (Fig. 4B), while in autumn the percentage of progressivity of the sperm produced by the hormone treated males was lower than in spring, and it was significantly higher than in the controls only at the time of activation (4.2 ± 2.2%, 8.5 ± 3.3% and 10.8 ± 2.8% for the control, 9 µg/kg rFsh and 18 µg/kg rFsh groups, respectively) (Fig. 4B). A remarkable increase of the sperm VCL was also observed after rFsh and rLh treatments during both periods of the year, although the velocity of spermatozoa appeared to be slower during autumn (Fig. 4C).

The data on sperm quality suggest that, in this study, the treatment with recombinant gonadotropins enhanced spermatozoa motility regardless of the time of the year, despite observations that in spring repeated rFsh injections were apparently unable to stimulate spermatogenesis. Such an improvement of sperm motility, progressivity and velocity has not previously been observed after GnRHa administration in sole F1 males, which could only increase the time of spermatozoa motility when supplied in combination with the 11-KT precursor 11-ketoandrostenedione (Agulleiro et al., 2007). Our observations therefore suggest that the beneficial effects of recombinant gonadotropins on sperm quality were possibly mediated by rLh rather than by rFsh, which is consistent with the major role of this gonadotropin during sperm hydration and spermiation in teleosts (Schulz et al., 2010), and with its function of regulating spermiogenesis-related genes in the Senegalese sole testis *in vitro* (Chauvigné et al., 2014ab). The motility parameters during autumn were however

lower than in spring, which could be related to the low temperature at the time of spermiation, which could also be the case in our previous experiment on pubescent males where rLh treatments did not conclusively improve sperm quality (Chauvigné et al., 2017). However, in the present and previous study, sperm was collected 24 h after rLh injection, which considering that rLh-induced differentiation of spermatids to spermatozoa *in vitro* at 18ºC takes at least 20 h (Chauvigné et al., 2014b), raises the possibility that yet immature sperm stored in the sperm duct was collected. It is clear therefore that further studies are necessary to establish in Senegalese sole the best timing for sperm collection after rLh treatment.

3.4. Conclusions

The results of the present study show that consecutive treatments with rFsh and rLh can promote the steroidogenic potential of the testis of Senegalese sole F1 adult males and increase sperm production and quality. However, the effectiveness of these hormonal therapies to enhance spermatogenesis, spermatozoa differentiation, and sperm motility in adult fish appears to be highly dependent on the reproductive stage of the males, which is likely determined by the conditions of temperature and photoperiod (Morais et al., 2016), the doses of rFsh and rLh applied, and possibly by the temperature during which rLh-induced spermiogenesis and spermiation takes place. The finding that rFsh was not effective at higher temperatures may agree with the observation that warm winter temperatures may effect sperm production in Senegalese sole (Agulleiro et al., 2007), and spawning in common sole (*Solea solea*) (Devauchelle et al., 1987). Therefore, despite the fact that in the Senegalese sole spermatogenesis is asynchronous and sperm is produced all year-round, the response of the testis to recombinant gonadotropins can still change drastically depending on the environmental parameters, which probably modulate the endocrine status of the hypothalamic-pituitary-gonadal axis. The biological mechanisms underyling the poor response to Fsh of sole males during the spawning season, as well as the higher Fsh sensitivity of the adult male testis with respect to that of juvenile fish, are aspects that need to be investigated in the future in order to improve recombinant gonadotropin-based fertilization protocols for cultured Senegalese sole.

Acknowledgments

The authors thank the technical staff from IRTA for their help during the maintenance and sampling of fish. This work was funded by the Spanish Ministry of Science and Innovation (MINECO) (AGL2017-84013-R to F.C. and AGL2013-41196-R to J.C.), and the Spanish National Institute for Agronomic Research (INIA)-European Fund for Economic and Regional Development (FEDER) (RTA2014-00048 to N.D.). Participation of FC, SR and WG was funded, respectively, by a "Ramon y Cajal" contract (MINECO), a predoctoral grant from AGAUR (Government of Catalonia), and a predoctoral grant from the National Board of Science and Technology (CONACYT, Mexico). This work was also supported by the CERCA Programme (Government of Catalonia).

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Figures

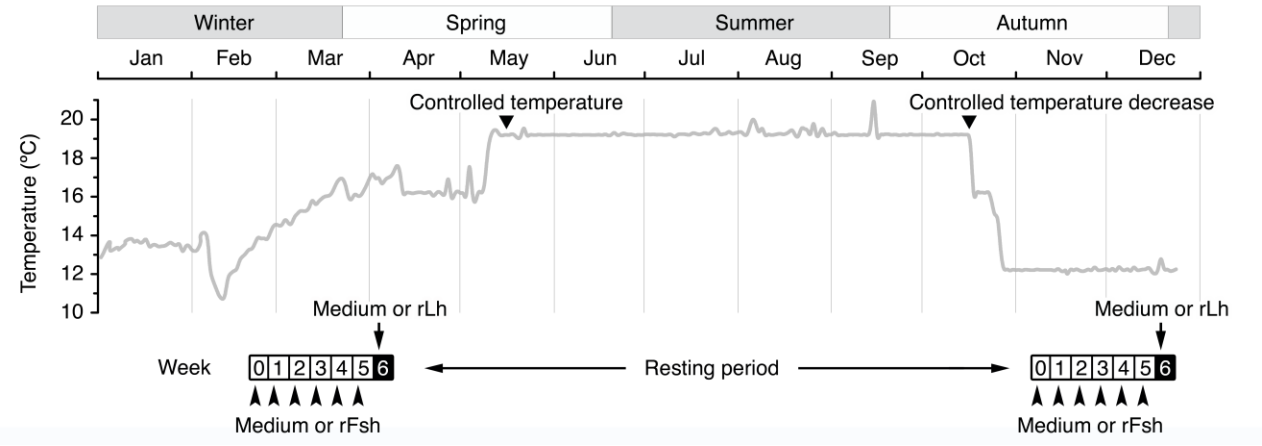

Fig. 1. Schematic representation of the experimental design. Three groups of adult Senegalese sole males (*n* = 9-12) were injected intramuscularly with rFsh (9 or 18 μg/kg) or CHO cell culture medium (control) during five consecutive weeks, followed by a single injection with rLh (9 or 18 μg/kg) or medium, under natural conditions of photoperiod and temperature during late winter-early spring. Plasma samples were collected before the first rFsh injection (time zero), and 24 h after rLh injection on the sixth week. The same groups of fish were rested during summer under a controlled temperature of ~19ºC throughout the summer until early autumn, when temperature in the holding tanks was manually decreased and maintained at 12ºC. After low temperature acclimation, fish were treated during autumn with the same rFsh and rLh doses and time lengths than in the late winter-spring experiment and sampled accordingly. In both cases, 24 h after rLh injection, semen quantity and quality were evaluated by CASA. The week of rFsh and rLh injection (black arrowheads and arrows, respectively) and the temperature of the holding tanks during each trial are indicated.

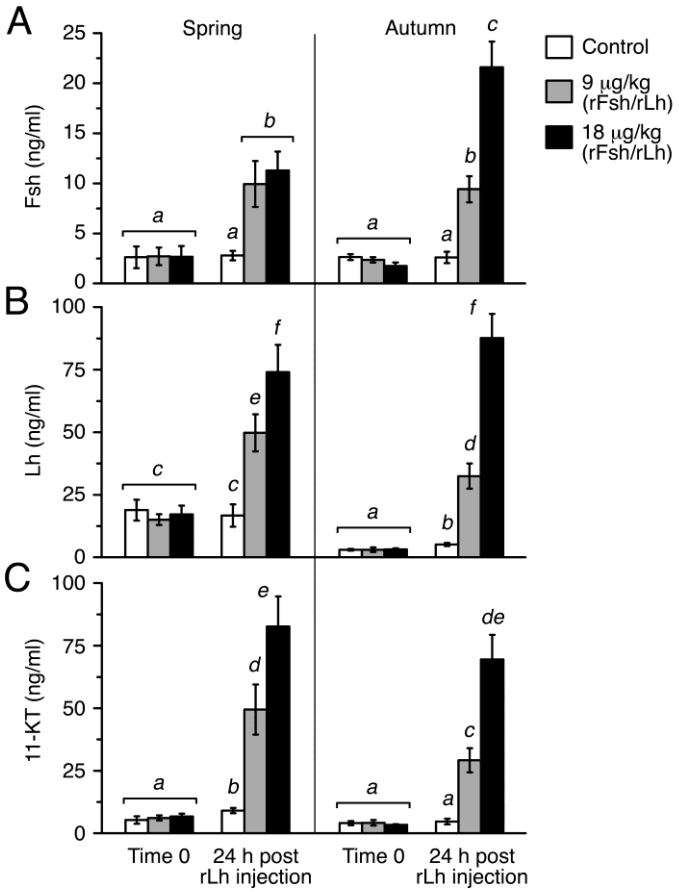

Fig. 2. Gonadotropin and androgen circulating levels in males treated with rFsh and rLh in spring and autumn. Plasma levels (mean ± SEM; *n* = 9-12 fish) of Fsh (A), Lh (B) and 11-KT (C) in each group before rFsh treatment (time 0) and after 24 h of rLh injection following the 5-weeks of rFsh treatment. In each panel, bars with different superscript are significantly different ($P < 0.05$).

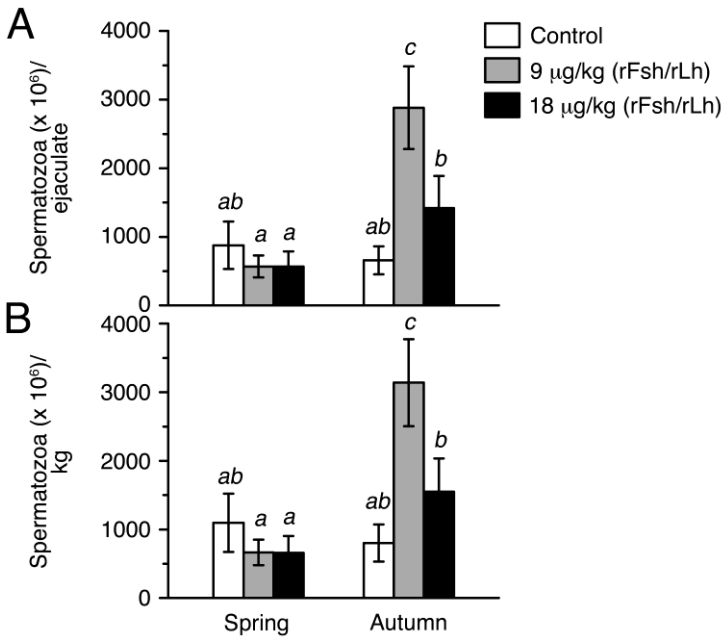

Fig. 3. Sperm production of males treated with rFsh and rLh in spring and autumn. Mean ± SEM (*n* = 9-12 fish) amount of sperm per ejaculate (A) or kg of fish (B) produced by each group 24 h after medium or rLh injection following the 5-weeks rFsh treatment. In both panels, bars with different superscript are significantly different ($P < 0.05$).

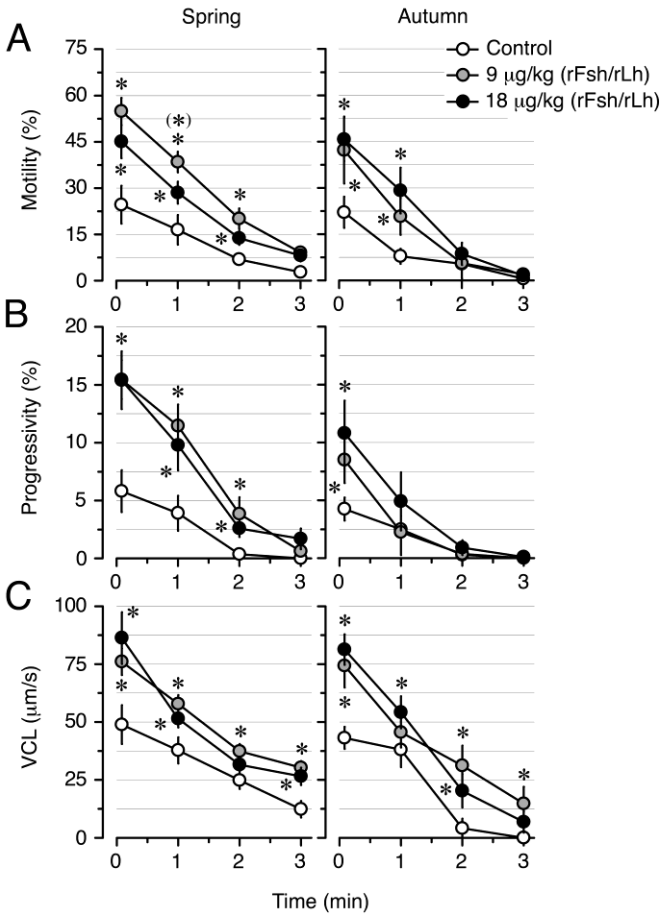

Fig. 4. Kinetic parameters of the sperm produced by males treated with rFsh and rLh in spring and autumn. Time-course of the percentage of motile (A) and progressive (B), and curvilinear velocity (VCL), of spermatozoa after activation in seawater. In all panels, the data are the mean ± SEM (n = 9-12 fish), and the asterisks denote significant differences ($P < 0.05$) with respect to the control group.