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

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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 12 °C (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

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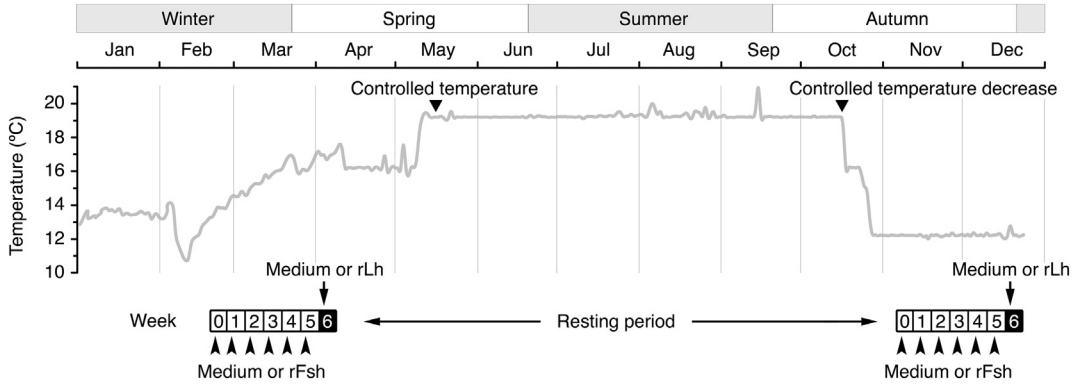

Fig. 1. Schematic representation of the experimental design. Three groups of adult Senegalese sole males (n = 9–12) were injected intramuscularly with rFsh (9 or 18 μg/kg) or CHO cell culture medium (control) during five consecutive weeks, followed by a single injection with rLh (9 or 18 μg/kg) or medium, 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 ~19 °C throughout the summer until early autumn, when temperature in the holding tanks was manually decreased and maintained at 12 °C. 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.

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., 2016). 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 ~13 °C to ~17 °C 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