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New insights into the factors mediating the onset of puberty in sea bass

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ABSTRACT

In populations of 1-year-old male European sea bass (*Dicentrarchus labrax*), only large males are able to acquire for the first time a functional competence of their reproductive axis; in other words, to attain puberty. To examine the causes and mechanisms involved in the onset of puberty in this species, a size sorting sampling was carried out to obtain two experimental groups of small and large male fish exhibiting different growth rates. As expected, only large fish reached full spermiogenesis (stage V of testicular development) by the end of the experiment. Our study suggests that fish size is a permissive condition to ensure full effectiveness of the hormonal (Gnrh1, gonadotropins and sexual steroids) actions. Thus, though small fish had endocrine profiles similar to those of large fish, their amplitude was much lower, and was most likely the reason why functional competence of the reproductive axis was not achieved. Moreover, this work provides evidence of the involvement of kisspeptin and Gnrh1 systems in the onset of puberty in a marine teleost fish. It also indicates that very likely kisspeptin and Gnrh1 may regulate gonadotropins and sex steroids at specific stages of testicular development.

1. Introduction

In fish, puberty is defined as the developmental period during which immature juveniles acquire the capacity to sexually reproduce for the first time. It involves functional activation of the brain–pituitary–gonad (BPG) axis (Schulz and Goos, 1999; Zanuy et al., 2001; Okuzawa, 2002; Schulz and Miura, 2002; Jalabert, 2005; Carrillo et al., 2009; Taranger et al., 2010). In males, the end of puberty can be recognized by the first spermiation and milt hydration (Okuzawa, 2002). However, the numerous developmental and neuroendocrine events involved in this process make it difficult to ascertain when puberty begins. Based on several studies, the transition to the first wave of rapid spermatogonial proliferation has been proposed as the starting point of puberty in male fish (Schulz et al., 2010; Taranger et al., 2010). Farming conditions increase the growth of cultured fish as compared to wild fish of the same stock (Svasand et al., 1996), and the age at puberty is frequently reduced. Thus, early puberty or precocity commonly observed in farmed fish may occur as a phenotypic response to enhanced growth conditions and feed availability, and the likely associated higher energy stores. On the contrary, in order to maintain fitness, slower growth may result in delayed puberty (Taranger et al., 2010). On the other hand, previous studies have suggested that gonadal growth is an energetically demanding process, and the fish very likely have to reach a certain threshold of body size and/or energy stores for this to occur (Rowe et al., 1991; Chen and Ge, 2013).

The European sea bass, *Dicentrarchus labrax*, henceforth sea bass, is a gonochoristic species in which growth is clearly related to sex, with the majority of large females reaching first maturity at three years of age, while males do so at only two. However, under intensive culture conditions, the presence of a considerable proportion (around 20–30%) of mature males during their first year of life has been repeatedly observed (Carrillo et al., 1995). Interestingly, precocious males are significantly larger than non-precocious ones during their first year, with reported average values of 107.80 g and 19.35 cm for the maximum precocity rate (21.9%) (Begtashi et al., 2004). Thus, it seems that to be precocious male fish must exceed a critical size threshold, while individuals below this limit remain immature until the next reproductive season.

However, the causes and mechanisms that lead to fish precocity are still unknown. It is well known that full activation of the reproductive axis is mediated by a series of neuroendocrine events. Gonadotropin-releasing hormones (Gnrh) play an essential role in the control of the reproductive function. It has been established that specific populations of Gnrh neurons modulate a wide range of

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reproductive function regulatory signals in a variety of fish species (Millar, 2005; Zohar et al., 2010). Thus, the onset of puberty in fish is characterized by the secretion of Gnrh, which in turn stimulates the synthesis and secretion of pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Finally, plasma gonadotropins stimulate the testes, triggering pubertal development of the gonads. However, studies in mammals suggest that the kisspeptin (KISS)/kisspeptin receptor (KISSR) system is essential for accurate GnRH action on gonadotropin secretion (deRoux et al., 2003; Funes et al., 2003) and provide convincing evidence that kisspeptins are important players in the central control of reproduction in vertebrates.

Unlike placental mammals, in which only one *KISS1* and *KISSR* gene are present, several teleosts species have a duplicate kisspeptin system. Thus, sea bass express two distinct genes encoding kisspeptins (*kiss1* and *kiss2*) and two cognate receptors (*kiss1r* and *kiss2r*) (Felip et al., 2009; Tena-Sempere et al., 2012). The essential role of kisspeptins in regulating reproductive function in fish has been studied following systemic administration of synthetic peptides. Injection of Kiss1–10 in early-mid pubertal fathead minnows elicited an increase of *gnrh3* (hypophysiotropic form) expression in the brain, but not of *gnrh2* (Filby et al., 2008). Furthermore, an increase of hypothalamic *gnrh1* (probably the hypophysiotropic form), but not of *gnrh3*, was detected in orange spotted grouper after the injection of Kiss2–10 (Shi et al., 2010). Meanwhile, at the pituitary level, Kiss2–10 and Kiss1–10 administration increased the release of Lh and Fsh in pre-pubertal sea bass (Felip et al., 2009), while only Kiss1–10 injections increased Lh levels in goldfish (Li et al., 2009). The proven ability of kisspeptins to activate the gonadotropic axis suggests a potentially important role for this system in the timing of fish puberty. In fact, recent studies have suggested that kisspeptin pathways are involved in timing the onset of puberty in diverse groups of teleosts (Mohamed et al., 2007; Nocillado et al., 2007; Biran et al., 2008; Martinez-Chavez et al., 2008; Filby et al., 2008).

However, the factors and mechanisms involved in the onset of puberty, as well as the physiologic regulation of key aspects of the first reproductive maturation in fish, still remain poorly understood and deserves attention. The aim of the present work was to study the effects of body size on the onset of puberty and its relation with relevant molecular neuroendocrine and hormonal events inherent to the first gametogenesis of male sea bass.

2. Materials and methods

2.1. Fish and sampling

Fish of the same origin (Gravelines, France), born the same day in September 2008, were maintained in triplicate groups under natural temperature and simulated natural photoperiod (40°LN) conditions at the Instituto de Acuicultura Torre la Sal (IATS) facilities, from March 2009 to February 2010. For the experiment, fish were sized and weighed at each sampling point and only the smallest 15% and the largest 25% of the fish were selected for analysis. A larger percentage of large fish were selected in order to offset the female-biased sex ratio in this group. Samples were first collected on August 20, 2009 (during the warmest month of the year) and was repeated every two weeks, until the middle of November. Based on preliminary histological data, at least two additional samplings were scheduled, one in mid-December and another at the end of February. At each sampling point, a sub-sample of the small fish population (15 fish) and another of the large fish population (25 fish) were sacrificed, and the liver and peri-visceral fat were quickly removed and weighed (±0.001 g) in order to estimate the hepatosomatic index (HSI) and mesentery fat index (MFI). HSI and MFI were calculated as $100 \times$ Lw/Bw and $100 \times$ VFw/Bw, respectively, where Bw is the body weight (g), Lw is the liver weight (g), and VFw is the peri-visceral fat weight (g). In addition, the forebrain–midbrain (FB–MB), the hypothalamus and the pituitary were collected for gene expression (Espigares et al., 2015), and the gonads were harvested for molecular identification of the sex. All tissues were frozen in liquid nitrogen and stored at −80 °C until use. Blood samples were collected from the caudal vein and plasma was extracted and stored at −20 °C until use for hormone analysis. The onset of testicular development was analyzed by histology. At the first sampling the fish gonads were too small to be used for both molecular and histological analysis. Consequently, the gonads from another eight fish (from both the small and large populations) were dissected in parallel and fixed by immersion in 4% formaldehyde: 1% glutaraldehyde (McDowell and Trump, 1976), embedded in 2-hydroxyethyl methacrylate polymer resin (Technovit 7100, Heraeus Kultzer, Germany), sectioned (2 μm) and stained as in Bennett et al. (1976). The different stages of testicular development were classified by light microscopy following the previously established criteria by Begtashi et al. (2004): stage I, immature testes; stage II–IV, testicular growth (early, mid and late recrudescence); stage V, fully spermiating testes and stage VI, post-spawning. For better interpretation and greater clarity of results and discussion, Table 1 shows the relationship between these testicle stages and the successive developmental phases of testicular gametogenesis in large fish throughout the experimental period. It also indicates the most characteristic germ cell type of each testicle stage, and its abundance.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the frozen gonads. Samples were loaded in FastPrep Lysing Matrix Tubes (1.4-mm ceramic spheres) and lysed in presence of the TRIzol reagent (Invitrogen) using a FastPrep system (Qbiogene Inc., Irvine, CA). When the amount of gonadal tissue was too small (<10 mg) to be processed using the FastPrep system, the tissue was lysed using disposable insulin syringes with needles. Purity and concentration of the total RNA was assessed spectrophotometrically with a NanoDrop instrument (Thermo Scientific NanoDrop 2000). RNA integrity (sharp 28S and

Table 1
Succession of the different developmental phases of testicular gametogenesis shown by large fish during their first reproductive cycle (upper part) and summary of the most characteristic germ cell type(s) of each testicular stage[a] (lower part). Bold roman numerals indicate the predominant testicle developmental stage at each sampling point.

Date	Testicular stages	Testicular phase
Aug 20	I	Immature
Sep 04	I	Immature
Sep 16	**I**, II	Early proliferative
Sep 30	I	Early proliferative
Oct 14	I,II	Late proliferative
Oct 29	**II**, III	Early meiotic
Nov 11	II, **III**	Mid-meiotic
Dec 14	II, **III**, **IV**, **V**	Late meiotic/Early spermiogenic
Feb 24	II, III, **V**	Spermiogenic

Stage/cell type[b]	SgA	SgB	Sc1	Sc2	Spt	Sz
Stage I	+++[c]					
Stage II	++	++	(+)			
Stage III	+	+	++	++	+	
Stage IV	(+)	(+)	++	++	++	++
Stage V	+			+	+	+++

[a] Adapted from Begtashi et al. (2004).
[b] SgA, A spermatogonia; SgB, B spermatogonia; Sc1, primary spermatocytes; Sc2, secondary spermatocytes; Spt, spermatids; Sz, spermatozoa.
[c] The abundance of each cell type is indicated with + signs; (+) indicates residual presence.