Comparative Biochemistry and Physiology, Part A 199 (2016) 38–46

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Is the kisspeptin system involved in responses to food restriction in order to preserve reproduction in pubertal male sea bass (*Dicentrarchus labrax*)?

Sebastián Escobar [1], Alicia Felip *, Silvia Zanuy, Manuel Carrillo *

Instituto de Acuicultura Torre de la Sal (IATS), Consejo Superior de Investigaciones Científicas (CSIC), 12595 Ribera de Cabanes S/N, Castellón, Spain

ARTICLE INFO

Article history:
Received 31 January 2016
Received in revised form 3 May 2016
Accepted 4 May 2016
Available online 7 May 2016

Keywords:
Kisspeptins
Food restriction
Reproductive hormones
Sexual maturation
Brain
Teleost

ABSTRACT

Previous works on European sea bass have determined that long-term exposure to restrictive feeding diets alters the rhythms of some reproductive/metabolic hormones, delaying maturation and increasing apoptosis during gametogenesis. However, exactly how these diets affect key genes and hormones on the brain–pituitary–gonad (BPG) axis to trigger puberty is still largely unknown. We may hypothesize that all these signals could be integrated, at least in part, by the kisspeptin system. In order to capture a glimpse of these regulatory mechanisms, *kiss1* and *kiss2* mRNA expression levels and those of their kiss receptors (*kiss1r*, *kiss2r*) were analyzed in different areas of the brain and in the pituitary of pubertal male sea bass during gametogenesis. Furthermore, other reproductive hormones and factors as well as the percentage of males showing full spermiation were also analyzed. Treated fish fed maintenance diets provided evidence of overexpression of the kisspeptin system in the main hypophysiotropic regions of the brain throughout the entire sexual cycle. Conversely, Gnrh1 and gonadotropin pituitary content and plasma sexual steroid levels were downregulated, except for Fsh levels, which were shown to increase during spermiation. Treated fish exhibited lower rates of spermiation as compared to control group and a delay in its accomplishment. These results demonstrate how the kisspeptin system and plasma Fsh levels are differentially affected by maintenance diets, causing a retardation, but not a full blockage of the reproductive process in the teleost fish European sea bass. This suggests that a hormonal adaptive strategy may be operating in order to preserve reproductive function in this species.

1. Introduction

Reproduction in vertebrates is coordinated by the activation of the brain–pituitary–gonad (BPG) axis (Pinilla et al., 2012). It is well-established that gonadotropin releasing hormone (GnRH or GnRH1) and gonadotropins (GTHs), which include the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), play important roles in the control of reproduction and the onset of puberty (Pinilla et al., 2012). However, puberty can be affected by certain metabolic factors and/or nutritional status (Wahab et al., 2013). A close relationship between energy balance and reproduction has been well-documented in mammals, with metabolic fuel deficiency delaying the onset of puberty in prepubertal rodents (Foster et al., 1989; Cameron et al., 1993; Wahab et al., 2013). In relation to this, suppression of the BPG axis by food restriction reduces the release of GnRH from the brains of rats (Bergendahl et al., 1992), ewes (Kile et al., 1991) and humans (Aloi et al., 1997). Nevertheless, a negative energy status has not been correlated with decreased GnRH mRNA levels in the hypothalamus of rats (Bergendahl et al., 1992), suggesting that a metabolic regulation may occur further upstream from the GnRH system.

Recent data have demonstrated that neurons expressing kisspeptins play an important role in the essential control of reproductive function (Pinilla et al., 2012). In this context, the kisspeptinergic neuron system is known to be directly exposed to metabolic factors in the nucleus arcuatus (ARC) in mammals (Chehab, 2014). Moreover, food deprivation in mammals induces a concomitant decrease in hypothalamic *kiss1* (Castellano et al., 2005; Luque et al., 2007; Wahab et al., 2008), thus transmitting metabolic status-related information to the GnRH network. Unfortunately, studies on the metabolic regulation of the BPG axis and kisspeptin system are scarce in fish. However, interactions between nutrition and reproduction have been explored in the European sea bass (*Dicentrarchus labrax*). Recently, the influence of long-term feed restriction on the reproductive performance of male European sea bass entering their first breeding season has been reported (Escobar et al., 2014a). Of note, food restriction caused a moderate delay in gonadal development stages and resulted in a lower gonadosomatic index (GSI), probably due to the increase in the number of apoptotic bodies in the germinal cells of the testes of feed-restricted European sea bass (Escobar et al., 2014a). However, no

* Corresponding authors.
E-mail addresses: afelip@iats.csic.es (A. Felip), m.carrillo@csic.es (M. Carrillo).
[1] Present address: FAVET-INBIOGEN, Faculty of Veterinary Sciences, University of Chile, Avda. Santa Rosa 11735, Santiago, Chile.

http://dx.doi.org/10.1016/j.cbpa.2016.05.005
1095-6433/

negative consequences have been observed for certain sperm parameters; on the contrary, in some cases, a food restriction regime actually increases key sperm motility measurements (Escobar et al., 2014a).

Currently, the only experimental evidence relating nutritional status to the kisspeptin system has been reported in the Senegalese sole (*Solea senegalensis*), where fasting increases hypothalamic *kiss2* expression and *lh*β and *fsh*β mRNA levels in the pituitary of male and female fish alike (Mechaly et al., 2011). In this vein, in male European sea bass, Fsh is considered to play a major role in early spermatogenesis, when active testicular growth occurs (Molés et al., 2012; Mazón et al., 2014), whereas Lh has been shown to be involved in the final stages of testicular growth and maturation (Rodríguez et al., 2000a; Rocha et al., 2009). In addition, a potential collaborative role of both gonadotropins in promoting testicular activity has also been suggested in this species (Espigares et al., 2015b). Accordingly, the aim of the present work was to study the influence of long-term (14-month) food restriction (maintenance ration regime) on kisspeptin genes and other reproductive hormones and factors of the BPG axis male European sea bass over the course of their first reproductive cycle (puberty). We analyzed the effects on the seasonal expression of the two kisspeptin genes and their receptors at the brain level, the pituitary content of Gnrh1, Fsh and Lh, plasma profiles of essential reproductive hormones and gonadal changes in terms of gonadotropin receptor genes (*fhsr*, *lhr*).

2. Material and methods

2.1. Fish and rearing conditions

The experiment was conducted at the facilities of the Instituto de Acuicultura de Torre de la Sal (IATS, Castellón, Spain, 40°N 0°E). Nine hundred juvenile male European sea bass were organized into two groups ($n = 150$ fish per tank in triplicate). In the first group, which acted as a control group (CT), the animals were fed until visual satiety, whereas in the second experimental group (EX), the animals were provided each month with an amount of food equal to 0.35% of the CT group's biomass (maintenance ratio) based on previous studies carried out in other fish species (Bermejo-Nogales., 2011). For the purposes of better comparing the results, we assumed three crucial periods throughout the annual reproductive cycle: the pre-gametogenesis period (PGP; April–August of Year 1), the gametogenesis period (GP; September of Year 1–March of Year 2) and the post-spermiation period (PSP; April–May of Year 2). In June (Year 2), male European sea bass were determined to be in their sexual resting period (SRP).

2.2. Sampling procedures

For each sampling point, 6–9 male European sea bass per treatment were randomly selected, anesthetized, and sacrificed in accordance with Spanish and European legislation concerning the protection of animals used for experimentation or other scientific purposes (Royal Decree Act 53/2013 and 2010/63EU, respectively). Tissue collection was carried out in April, September and November of Year 1, and January to March of Year 2 during PGP and GP. Thus, the brain was dissected according to the procedure described by Espigares et al. (2015a), and the hypothalamus, forebrain–midbrain (hereafter, FB-MD), and pituitary were removed. Gonad samples were also collected. All tissues were frozen on dry ice and stored at −80 °C until RNA extraction and hormone analysis was performed. Blood samples ($n = 6$–9 fish/treatment) were collected during PGP and GP and extended to PSP and SRP, as it is known that food restriction causes a moderate delay in testicular development in this species (Escobar et al., 2014a). They were taken from the caudal vein and centrifuged at 3000 rpm for 30 min at 4 °C. Plasma was obtained and stored at −20 °C until analysis. The content of gonadotropin releasing hormone 1 (Gnrh1 or sbGnrh), luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) in the pituitary was determined as described by Espigares et al. (2015a). Testicle development was staged according to the procedure described by Begtashi et al. (2004). Fish at Stage V of testicular development indicated both active spermiogenesis and sperm release (Escobar et al., 2014a), thus the percentage of males showing full spermiation ($n = 9$ fish/group) was performed from January to April.

2.3. Quantitative real-time PCR (qRT-PCR)

Changes in the expression levels of *kiss1*, *kiss2*, *kiss1r* and *kiss2r* (Alvarado et al., 2013; Espigares et al., 2015a) in the brain and pituitary and *fshr* and *lhr* (Rocha et al., 2009) in the testes were analyzed using qRT-PCR. The expression of these target genes was normalized against a reference gene. Thus, *ef1α* was used as the control gene in the brain and pituitary samples, while *18s* was used as a control gene in the testes. Both, *ef1α* and *18s* genes were appropriate reference genes as they has been previously tested for its ability to be used as control genes in these tissues in the sea bass (Rocha et al., 2009; Alvarado et al., 2013). Data were expressed as the relative value of the starting quantity of each target gene, divided by the starting quantity of each reference gene.

2.4. Hormonal analysis

Gnrh1 content was measured using a specific enzyme-linked immunosorbent assay (EIA) adapted to European sea bass (Rodríguez et al., 2004). Lh content in the pituitary and the plasmatic levels of this hormone were measured using a homologous ELISA assay developed for European sea bass (Mateos et al., 2006), while Fsh content and levels were measured according to the method used by Molés et al. (2012). Plasma 11-ketotestosterone (11-KT) (Rodríguez et al., 2001) and testosterone (T) (Rodríguez et al., 2000b) levels were analyzed using a specific EIA for this species.

2.5. Statistical analysis

The data are presented as the mean ± the standard error of the mean (SEM). Gene expression and both hormonal content and plasmatic levels were analyzed using a two-way ANOVA (SigmaStat 3.5 SYSTAT Software Inc., Richmond, CA, USA), followed by all pairwise multiple comparison procedures (Tukey's test). Before the analysis, values were appropriately transformed to meet normality and homoscedasticity requirements. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Changes in mRNA levels of kisspeptin system genes in the hypothalamus

The *kiss1* expression levels were elevated in the hypothalamus of the CT group during the early gametogenic period (September) (Fig. 1A). From the lowest values attained in November, levels gradually increased during gametogenesis (GP) and peaked in March. A similar pattern of variation was observed in the EX group, although it showed higher levels than the CT group in January. Expression levels of *kiss2* (Fig. 1B) significantly decreased in September with respect to April in the CT group and remained low and unchanged throughout the gametogenesis period, except in March when the expression levels increased sharply. As observed in the CT group, the expression of *kiss2* in the EX group was lower in September than in April, after which time levels increased in November and remained constantly high during gametogenesis, until they peaked in March. Of note, the EX group exhibited significantly higher *kiss2* expression levels than the CT group across the GP, except in September and March, when no significant differences between both groups were detected. The expression levels of *kiss1r* in the CT group (Fig. 1C) significantly decreased in September with respect to April, and remained low until November. In January, a significant