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

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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

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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 *lhβ* and *fshβ* 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

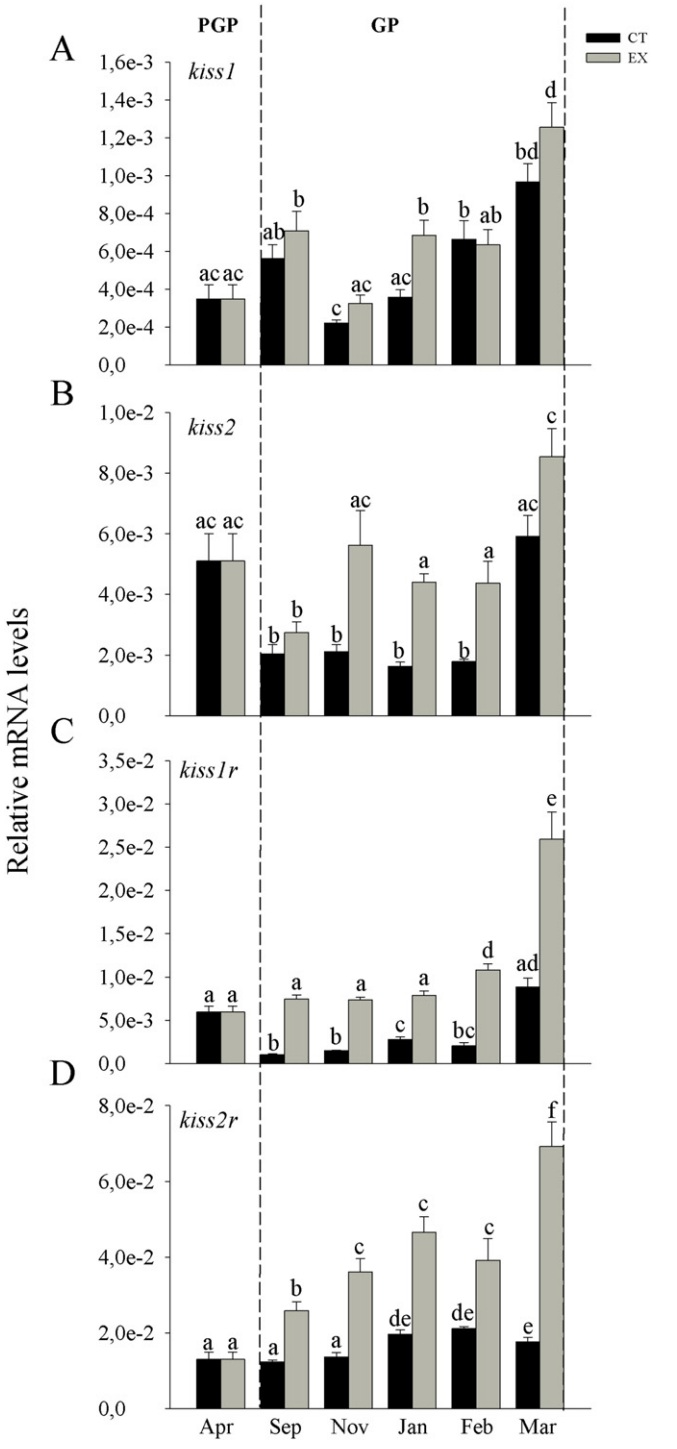

Fig. 1. Relative changes in *kiss1* (A), *kiss2* (B), *kiss1r* (C) and *kiss2r* (D) mRNA levels in the hypothalamus male European sea bass kept under maintenance feed protocols over 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 6 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between the CT and EX groups or differences in the same group throughout the experimental period. Expression values were normalized to the *ef1α* housekeeping gene. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period) and GP (gametogenesis period).

($P < 0.05$) increase was observed; levels remained high until February and then significantly increased again in March. In treated fish, constantly high levels of *kiss1r* expression were observed from April to January, although a steady increase was observed in the following months, peaking in March. Of note, treated fish showed higher expression levels than the controls during the GP. Finally, hypothalamic *kiss2r* expression in the CT group was low and remained unchanged from April to November (Fig. 1D). In January, a significant increase was observed, after which constantly high levels were maintained until March. In contrast, *kiss2r* expression in the EX group steadily increased from September onwards, peaking in March. Treated fish showed significantly higher expression levels of *kiss2r* than the control animals throughout the entire GP.

3.2. Changes in mRNA levels of kisspeptin system genes in the forebrain–midbrain

The expression of *kiss1* in the FB-MD of controls was higher in September than in April, and then remained unchanged until January. A significant increase in the expression of *kiss1* was observed in February, subsequently returning to basal levels in March (Fig. 2A). Treated fish also showed significantly higher levels of *kiss1* expression in

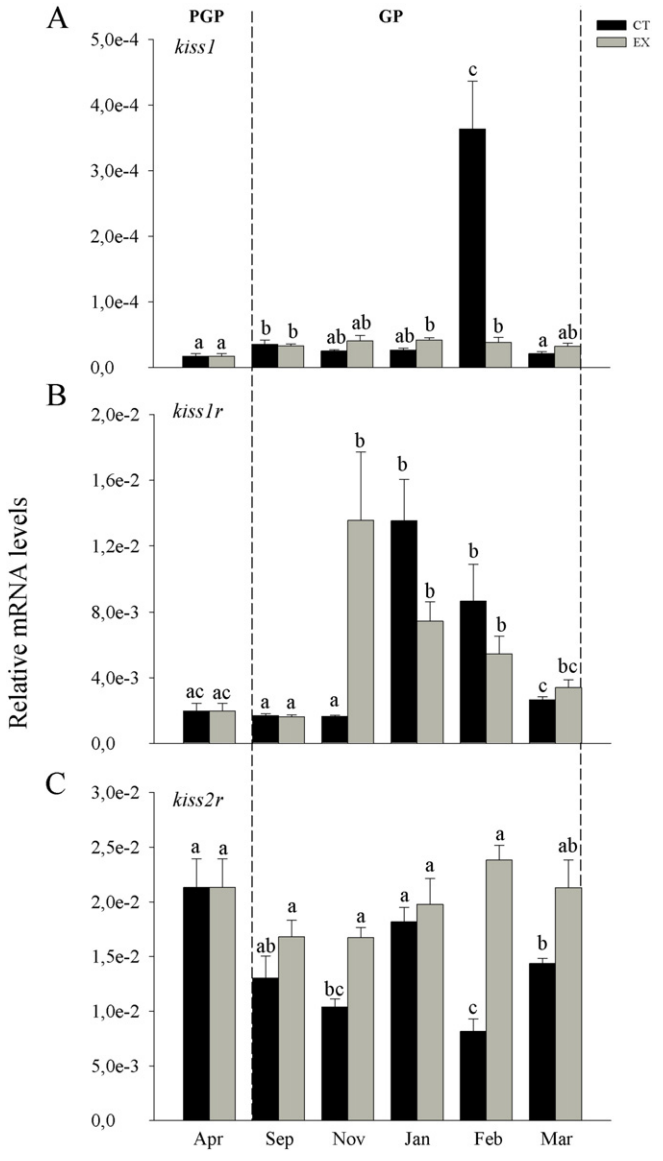

Fig. 2. Relative changes in *kiss1* (A), *kiss1r* (B) and *kiss2r* (C) mRNA levels in the forebrain–midbrain (FB-MD) regions of male European sea bass kept under maintenance feed protocols for 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 6 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between CT and EX groups or differences in the same group throughout the experimental period. Expression values were normalized to the *ef1α* housekeeping gene. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period) and GP (gametogenesis period).

September as compared to the month of April. Afterwards, however, the levels of the EX group remained unchanged until the end of the experiment, while the CT group showed significantly higher levels than treated fish (9.5 fold) in February. The expression of *kiss1r* was low in the CT group from April to November, reached significantly higher levels between January and February, and then decreased in March (Fig. 2B). The expression pattern of *kiss1r* in the EX group revealed a first increase in mRNA levels in November (8.5-fold, as compared to the previous sampling point). During the following months, a progressive decrease in *kiss1r* RNA levels was observed. Interestingly, treated fish showed higher levels ($P < 0.05$) than those of the CT group in November. The levels of *kiss2* could not be determined in the FB-MD, due to their low expression in this study. Finally, a steady decrease in the expression of *kiss2r* in the CT group was observed from April to November (Fig. 2C). Expression levels were significantly increased in January, and then decreased until they reached basal levels in February. A second increase of *kiss2r* mRNA levels was observed in March. The expression of *kiss2r* showed constantly high levels throughout the experimental period in the EX group. Of note, *kiss2r* mRNA levels in the EX group were significantly higher than those of the CT group in November and February ($P < 0.05$).

3.3. Changes in kiss2 and kiss1r mRNA levels in the pituitary

The pituitary expression of *kiss2* showed higher levels in September than in April in the CT group (Fig. 3A). The highest values were observed in November (GP), and then they remained low on the following sampling dates. Although expression levels in the EX group were higher in September than in April, *kiss2* mRNA levels in September were lower than those of the CT group. Furthermore, the EX group exhibited a significant increase in November and February, when *kiss2* mRNA levels were higher than those of the CT group. Changes in *kiss1r* expression were similar between the two groups. *kiss1r* mRNA levels peaked in November, but otherwise remained low throughout the experiment, with no differences being observed between the groups (Fig. 3B). *kiss1* expression was not considered in this study, due to the undetectable levels found in the pituitary. For similar reasons, *kiss2r* expression was not included in the study.

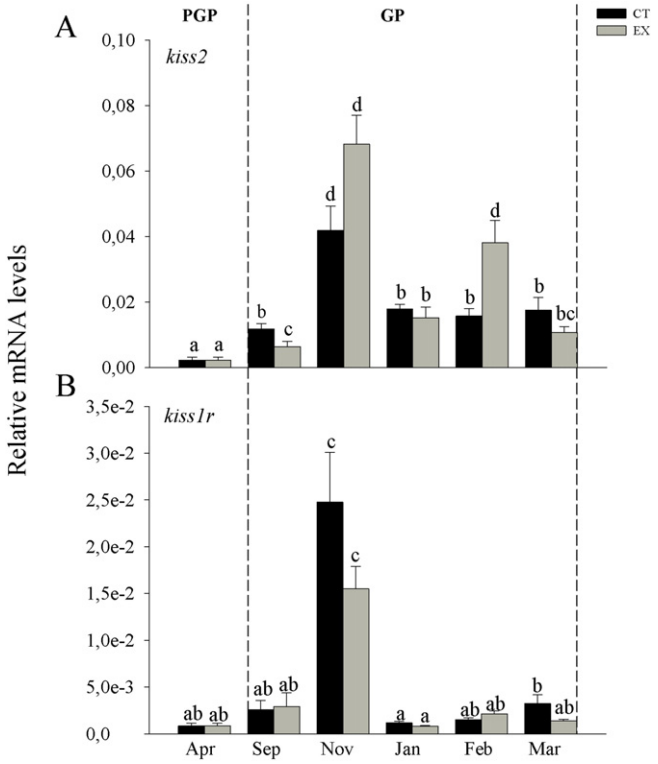

Fig. 3. Relative changes in *kiss2* (A) and *kiss1r* (B) mRNA levels in the pituitary of male European sea bass kept under maintenance feed protocols for 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 6 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between the CT and EX groups or differences in the same group throughout the experimental period. Expression values were normalized to the *ef1α* housekeeping gene. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period) and GP (gametogenesis period).

3.4. Changes in pituitary Gnrh1, Fsh and Lh content and plasma gonadotropin levels

No significant differences were found in the content of Gnrh1 in the CT group throughout the sexual cycle, except during March, when a significant increase in Gnrh1 content was observed with respect to the previous month. The EX group presented the lowest Gnrh1 pituitary content in January and March. These low values were statistically different from those of the CT group (Fig. 4A). In September, Fsh content in the CT group was higher than in April, and remained constant until November, only to be followed by a significant increase in January, which was maintained until March. Fsh content then significantly decreased from April onwards (Fig. 4B). The EX group exhibited a similar pituitary Fsh content profile, except that the Fsh content in September was similar to that in April, and the first significant increase took place in November. Overall, values in the CT group were higher than those observed in the EX group throughout the experiment, except for the month of November, when the situation was reversed. On the other hand, the Lh pituitary content of the CT group showed higher levels in September than in April, and remained constantly low until November. A steady increase was observed from January onwards, peaking in March, and followed by a sharp decrease in the following months (Fig. 4C). The EX group also showed higher levels in September than in April, but these values were lower than those of the CT group. In November, a significant increase in Lh content was observed in the EX group, which remained constantly high until February. In March, a dramatic elevation was observed, and similar to the C group, a sharply decrease in their values was observed in the following months. The EX group presented a lower Lh pituitary content as compared to the CT group throughout their sexual cycle, except for the month of November, in which the opposite was true.

Plasma Fsh levels in the CT group were higher in September than in April, and they remained high and constant until November. A significant increase was observed in January, and these levels remained high until February. By the end of the GP (March), plasma levels decreased, although they increased once again in April. Then these levels decreased steadily in the following months (Fig. 5A). Plasma levels in the EX group followed a somewhat similar pattern of evolution to those of the C group, except that Fsh plasma levels in the EX group were lower than those of the CT group during early GP, between September and November, and during the entire PSP. However, an up-regulation of plasma Fsh was observed in the treated fish between January and March. Plasma Lh levels in males were not detected in April (Fig. 5B), although consistently low Lh levels were observed in the CT group from September to January. A steady increase in Lh plasma levels was observed in February, which later peaked in March. In April, a decrease was observed, and Lh levels remained constant until the end of the experiment. The EX group exhibited lower Lh levels than the CT group throughout most of the GP and early PSP.

3.5. Changes in fshr and lhr mRNA levels in the testes

The *fshr* expression profile in the CT group showed a progressive and significant decline from April onwards, reaching minimum values in January. A moderate, yet significant increase was observed in February, but it returned to low levels in March (Fig. 6A). A similar pattern of variation was observed in the EX group, thus attaining minimum values

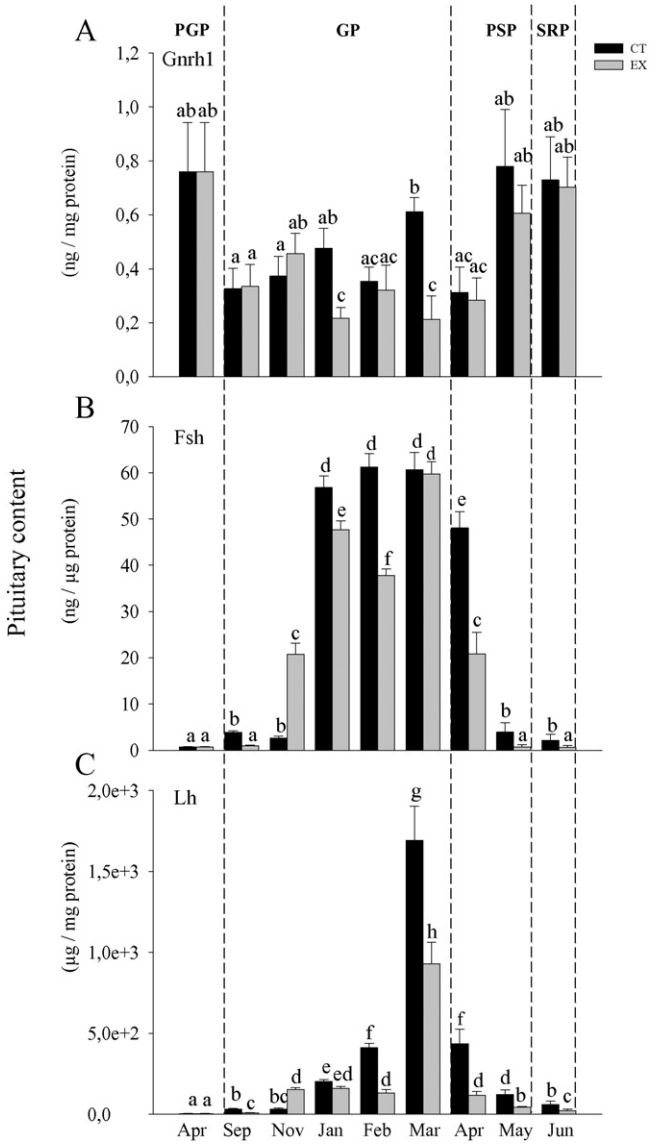

Fig. 4. Profile of Gnrh1 (A), Fsh (B) and Lh (C) content in the pituitary of male European sea bass kept under maintenance feed protocols for 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 9 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between CT and EX groups or differences in the same group throughout the experimental period. Lh content in PGP is depicted as zero, as fish showed undetectable values. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period), GP (gametogenesis period), PSP (post-gametogenesis) and SRP (sexual resting period).

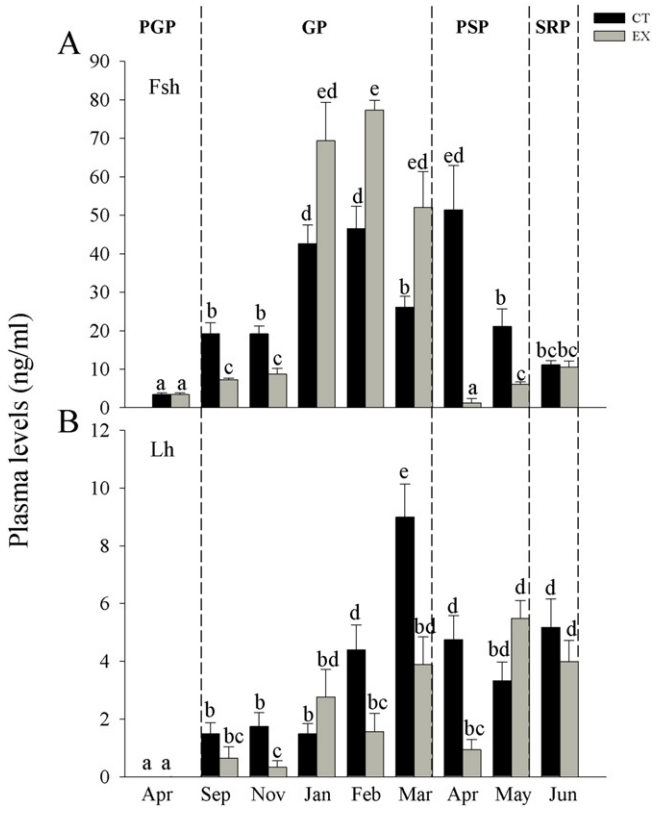

Fig. 5. Profile of Fsh (A) and Lh (B) plasma levels of male European sea bass kept under maintenance feed protocols for 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 9 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between the CT and EX groups or differences in the same group throughout the experimental period. Lh content in PGP is depicted as zero, as fish showed undetectable values. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period), GP (gametogenesis period), PSP (post-gametogenesis) and SRP (sexual resting period).

from January onwards, although November values were higher than those of the CT group. Expression levels of *lhr* remained low and unchanged in the CT group during the PGP and part of the GP (November). The expression of *lhr* sharply increased in January, remained high in February and then significantly decreased in March. In the EX group, expression levels of *lhr* showed a first increase in September and a second elevation in January, after which they gradually decreased (Fig. 6B). Interestingly, *lhr* mRNA levels in the EX group were higher in September, although lower in January and February, as compared to those of the CT group.

3.6. Changes in plasma sex steroid levels

Plasma 11-KT levels in the CT group started to increase in September and peaked in January and February. These high levels significantly dropped from March onwards (Fig. 7A). A similar hormonal profile was observed in the EX group. However, this group presented lower levels than those of the CT group throughout the GP and PSP periods. On the other hand, T levels in the CT group peaked in January and February and then progressively decreased, reaching minimum values in the PSP (April–May). Similarly, the EX group showed lower T levels than the CT group, with significant differences in November and April (Fig. 7B). In January, 20% of mature testes was observed in group C (Table 1). The proportion of mature testes increased in February (50%) and March (55%), whereas in the experimental group 70% of fish at Stage V was observed in March and subsequently it declined in April (40%) (Table 1).

4. Discussion

In this study, we investigated the involvement of the kisspeptin system and other key components of the BPG axis in the response to long-term feeding of a maintenance ration in pubertal male European sea bass. In this teleost fish, as occurs in other non-mammalian vertebrates, the onset of puberty is controlled by the activation of the BPG axis, with the kisspeptin system playing an important role in the control of this process (Taranger et al., 2010; Tena-Sempere et al., 2012; Carrillo et al., 2015). In fact, it is known that in European sea bass (Migaud et al., 2012; Alvarado et al., 2013; Bogevik et al., 2014) and other teleost fish (Shanjahan et al., 2010; Mechaly et al., 2013), the expression levels of both *kiss1* and *kiss2* genes in the brain are low during the early stages of spermatogenesis, although high levels of kisspeptin expression are

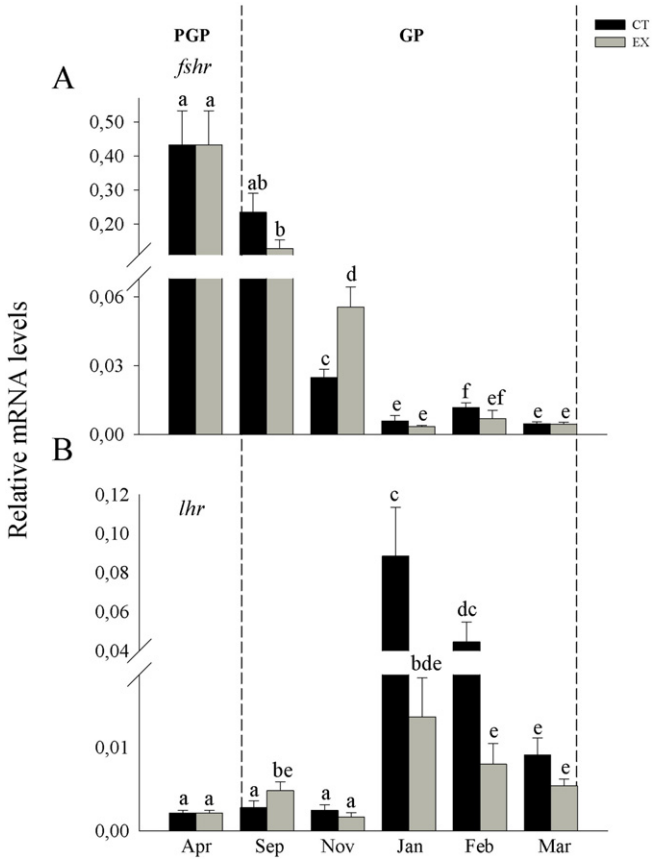

Fig. 6. Relative changes in *fshr* (A) and *lhr* (B) mRNA levels in the testes of male European sea bass kept under maintenance feed protocols for 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 6 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between the CT and EX groups or differences in the same group throughout the experimental period. Expression values were normalized to the *18s* housekeeping gene. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period) and GP (gametogenesis period).

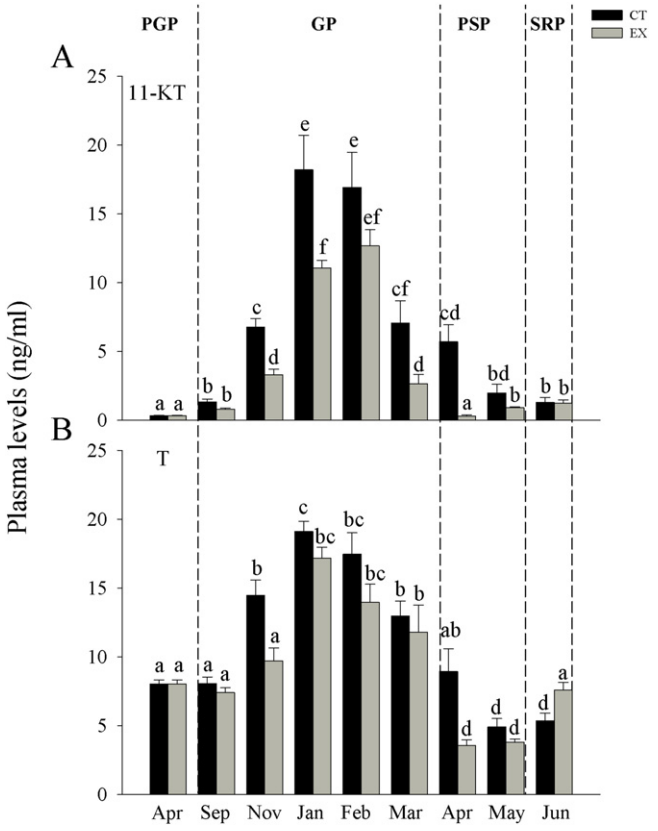

Fig. 7. Profile of 11-KT (A) and T (B) plasma levels of male European sea bass kept under maintenance feed protocols for 14 months. Values shown as the mean ± SEM for the control (CT) and experimental (EX) groups (n = 9 males/group). Different lowercase letters indicate either significant differences ($P < 0.05$) between the CT and EX groups or differences in the same group throughout the experimental period. The different periods of the annual reproductive cycle, separated by vertical dashed lines, were PGP (pre-gametogenesis period), GP (gametogenesis period), PSP (post-gametogenesis) and SRP (sexual resting period).

observed at spermiation (Migaud et al., 2012; Bogevik et al., 2014; this study). It is worth noting that male sea bass under restricted feed protocols consisting of either 1/4 or 1/8 of the ration received by the control animals, exhibit lower concentration of sperm compared to controls (Escobar et al., 2014a). Related to this, the present study demonstrates that control fish are able to reach full spermiation from January to March, whereas treated fish shows lower rates of spermiation and a delay in its accomplishment (from March to April). Furthermore, treated fish exhibited the lower gonadosomatic index (data not shown) likely related to the smaller number of proliferating spermatogonial cells and larger proportion of apoptotic bodies in these fish compared to controls (Escobar et al., 2014b). Interestingly, the reproductive process is not fully impaired in treated fish. Thus, results reported in this study report that a sustained food restriction regime enhances the hypothalamic expression of kisspeptin system genes, including *kiss1* and *kiss2* and their receptors (*kiss1r*, *kiss2r*), which is accompanied by a significant elevation in circulating Fsh levels during gametogenesis. This situation apparently also evokes an increase in *kiss1*, *kiss1r* and *kiss2r* mRNA levels in the forebrain–midbrain. In this line, these findings demonstrate that kisspeptins and Fsh are differentially affected by maintenance diets, causing a delay, but not a full blockage, of the reproductive process in this species (Escobar et al., 2014b). This situation suggests that the kisspeptin system and Fsh are crucial elements able to modulate the onset of puberty in the sea bass.

In this context, it is worth noting that although certain components involved in the metabolic regulation of puberty and adult reproductive function are relatively well-conserved among vertebrate groups, some differences can be found between mammals and non-mammalian species (Volkoff et al., 2009). Fish occupy different habitats, with distinct environmental characteristics, which lead to specific adaptive feeding and reproductive strategies (Taranger et al., 2010). Thus, many fish species alternate between periods of normal feeding and sustained negative energy balance (fasting) during their annual reproductive cycles (Trombley and Schmitz, 2013). In European sea bass, a seasonal spawning species, maximum food intake occurs from late spring to early autumn. Food intake then gradually declines during the GP period, coinciding with gonadal development (October–December), and reaches minimal values during the spawning period (December–March) (Zanuy and Carrillo, 1985). Of note, in male European sea bass (this study), the mRNA levels of both kisspeptins and their receptors have been observed to increase in the hypothalamus after a prolonged period of food restriction. A similar situation has been observed in the Senegalese sole (*Solea senegalensis*), where fasting (21 days) enhanced the expression of *kiss2* and *kiss2r*, which also evoked an increase of the expression of gonadotropin genes (Mechaly et al., 2011). In contrast, fasting in mice decreased the expression of *kiss1* and *kiss1r* (Luque et al., 2007), producing a decrease in LH levels (Roa et al., 2009). Moreover,

Table 1
Percentage of fish at Stage V during the spermiation period of male sea bass kept under maintenance feed protocols over 14 months (n = 9 males/group). CT = control; EX = experimental.

Months	January		February		March		April	
Group	CT	EX	CT	EX	CT	EX	CT	EX
Stage V (%)	20	0	50	0	55	70	0	40

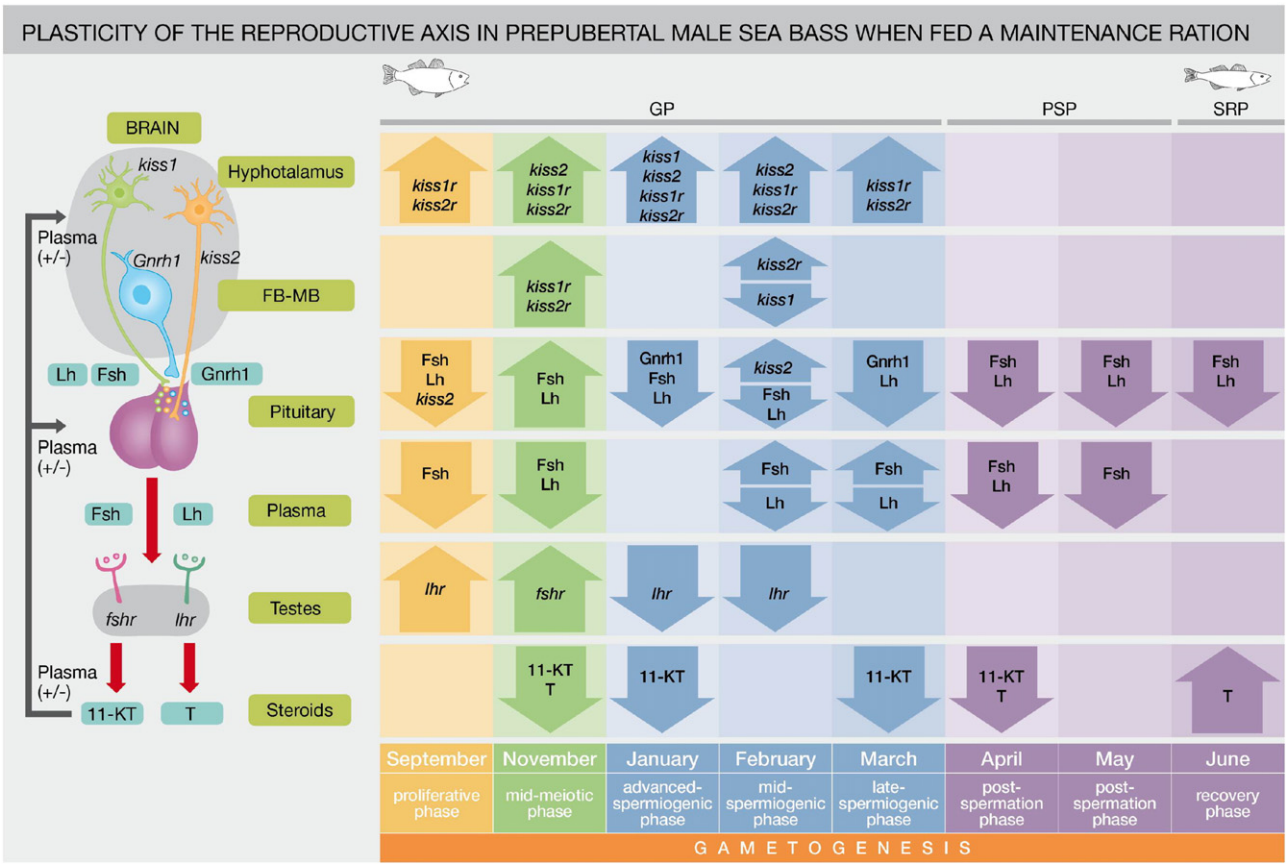

Fig. 8. Summary of physiological and molecular changes on the reproductive axis of male European sea bass subjected to maintenance feed restriction (experimental group, EX) for 14 months. Arrows indicate the timing when the first significant increase (up arrows) or decrease (down arrows) occurred in the EX group as compared to the control group (CT). See the details in the text.

fasting induced the suppression of the BPG axis in adult rhesus macaques, due to a reduction in the sensitivity of GnRH neurons (Wahab et al., 2008). It is noteworthy that while mRNA levels of *kiss1* decrease in the hypothalamus of mice (Castellano et al., 2005) and monkeys (Wahab et al., 2008), its receptor is overexpressed (Castellano et al., 2005), suggesting that an increase in *kissr* sensibility may exist in animals after fasting. Overall our results provide additional experimental evidence of the existence of a potential link between nutritional status and reproduction in European sea bass.

The present work thus provides a framework for a better understanding of neuroendocrine mechanisms and the metabolic regulation of fish reproduction, where much still remains uncertain. Interestingly, neuroanatomical studies in fish indicate that *kiss1* neurons in the medial basal hypothalamus (NLTm) and Kiss2 fibers from the dorsal nucleus recessus lateralis (NRLd) region may emerge toward the pituitary gland (Zmora et al., 2012, 2014; Escobar et al., 2013a, 2013b), thus suggesting a role of kisspeptins in the control of gonadotroph function. The distribution and nature of *kiss1*- and *kiss2*-expressing cells in the pituitary have been investigated in the European sea bass. In this line, a putative co-expression between *kiss1* and *kiss1r* genes and *fsh* expressing cells was shown to exist in the pituitary (Escobar et al., 2013a), while *kiss2* cells were found in the proximal pars distalis and colocalized with gonadotropin-immunoreactive cells (Espigares et al., 2015c). Furthermore, *in vivo* studies in European sea bass have revealed that both intraperitoneal (Felip et al., 2009) and intracerebroventricular (ICV) administration of kisspeptins (Espigares et al., 2015a), and in particular of Kiss2 peptide, elicited potent increases in circulating gonadotropin hormone levels. In this sense, the Kiss2/Gnrh1 system in the FB-MD of European sea bass is considered to be involved in the regulation of gonadotroph activity and, presumably, favors gamete maturation in this teleost fish (Espigares et al., 2015a). Increased expression of pituitary *fshβ* and lhβ 12 h post-injection with Kiss2 into the ICV region has been also recently described in the chub mackerel (*Scomber japonicus*), a scombroid fish (Ohga et al., 2014). Of note, we consider that the high expression levels of *kiss2* in pituitary of the animals in our study may reflect the existence of a profuse innervation of *kiss2* fibers that penetrate into the neurohypophysis in these animals, although we do not rule out that these expression levels might also correspond to the presence of certain *kiss2* cell populations in this tissue (Escobar et al., 2013b; Espigares et al., 2015a, 2015c). On the other hand, it is known that the expression of *kiss1* is extremely low in the pituitary as it has been reported by Alvarado et al. (2013). So, when the expression is so low (only few cells are expressing the gene), although it can be observed by *in situ* hybridization, the detection by qRT-PCR can be diluted (undetectable) as the pituitary is analyzed as a whole tissue in this quantitative approach (this study). Unfortunately, we do not have a specific antibody against Kiss1, since it would be useful to determine the degree of innervation of these fibers in this region. Thus, this situation is suggesting that, presumably, the expression of these genes might be due to a summed expression of cells and fiber innervations which may be playing a key role in the control of the onset of puberty and reproduction in this species. In addition, the present study demonstrates that a sustained food restriction regime influences the pituitary content of Gnrh1, Fsh and Lh, and the expression of gonadotropin receptors in the testes of prepubertal male European sea bass. These findings strongly indicate that the synthesis and release of gonadotropins depend on body size, as previously reported in this species (Carrillo et al., 2015; Espigares et al., 2015b). In fact, it has been demonstrated that body size has a significant influence on the number and size of GnRH cells in other species, such as the plainfin midshipman,

(*Porichthys notatus*) (Grober et al., 1994) and ballan wrasse (*Labrus bergylta*) (Elofsson et al., 1999). Our findings show that although the plasmatic Fsh levels were low in European sea bass fed a restricted diet during the early GP, fish preserved their reproductive competence as Fsh levels increased during spermatogenesis, between January and March, suggesting that Fsh might play an important role in the control of spermatogenesis as part of a reproductive strategy in response to different nutritional statuses of the fish. In addition, although 11-KT and T plasma levels were lower in prepubertal male European sea bass fed a maintenance ration than in the control group, their levels were high enough to guarantee that the reproductive function was merely delayed in this species, which was ultimately reflected by a lower concentration of sperm (Escobar et al., 2014b). Of note, in populations of 1-year-old male European sea bass, only large fish are able to attain puberty, as the amplitude of the endocrine profiles (Gnrh1, gonadotropins and sexual steroids) in small fish is much lower, thus affecting the functional competence of their reproductive axis (Espigares et al., 2015b). Although it is necessary to gain new insights into how food restriction influences reproductive axis function in European sea bass, our findings provide experimental evidence for the enhancement of Fsh levels during advanced gametogenesis, and the overexpression of *lhr* and *fshr* genes in testes during early gametogenesis in food restricted fish. This could be, in part, due to the plasticity and up-regulation of kisspeptin systems at the brain level that characterize this species throughout its sexual cycle when subjected to a food restriction protocol. Finally, it is demonstrated that fish are able to maintain spermatogenesis, thus suggesting that a hormonal adaptive strategy may be operating, in order to preserve reproductive function in this species.

In conclusion, the present study reports some important points, which are depicted in Fig. 8. Prepubertal and pubertal male European sea bass subjected to maintenance feed restriction for 14 months are able to up-regulate the expression levels of all four kisspeptin system genes in the hypothalamus, which in turn modulates the onset of reproduction in this teleost species. In this line, although the distinctive projection patterns of Kiss1 neuronal populations are still unknown in European sea bass, it is possible to assume that axonal projection from Kiss1 cell bodies in the hypothalamus may reach the pituitary gland in this species, as has been recently found in the striped bass (Zmora et al., 2014). On the other hand, there is evidence supporting Kiss2 neuronal innervation in the hypothalamus and the neurohypophisis (Escobar et al., 2013b), which colocalizes with gonadotropin immunoreactive cells in the pituitary gland (Espigares et al., 2015c). The observed enhancement of kisspeptin expression during European sea bass spermatogenesis could thus be connected to an increase in Fsh and Lh pituitary content and circulating gonadotropin levels. Altogether, these data highlight the fact that a negative energy status and/or food restriction evoke regulatory mechanisms which modulate the BPG axis and thus control the onset of puberty in male European sea bass.

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