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Changes in kisspeptin, GnRH, and gonadotropin mRNA levels in male threespine stickleback (*Gasterosteus aculeatus*) during photoperiod-induced sexual maturation

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ABSTRACT

Background: Both gonadotropin-releasing hormone (GnRH) and kisspeptin have a critical role in the photoperiodic control of sexual maturation in mammals.

Question: What are the roles of GnRH and kisspeptin in the photoperiodic control of sexual maturation in the threespine stickleback, *Gasterosteus aculeatus*?

Method: If kisspeptin and GnRH trigger the photoperiodic induction of maturation in fishes, they would be expected to be activated prior to, or at least no later than, the gonadotropins. The mRNA levels of brain *gnrh2*, *gnrh3*, *kiss2* and its receptor, *gpr54*, as well as of pituitary *lh-β* and *fsh-β*, were measured in males under standard laboratory conditions [16 hours light/8 hours dark (L16/D8) or 8 hours light/16 hours dark (L8/D16)] after 3, 10, 19, and 29 days at 20°C.

Results: From day 19, males in the long-day photoperiod (L16/D8) condition were visibly mature with bright breeding coloration. Compared with short-day photoperiod (L8/D16) fish, L16/D8 fish had higher *lh-β* mRNA levels at days 10, 19, and 29, and higher *fsh-β* mRNA levels at day 10 only. Furthermore, L16/D8 fish had higher *gnrh3* mRNA levels at days 10 and 29, and higher *gnrh2* mRNA levels at day 29, than L8/D16 fish. Finally, *kiss2* and *gpr54* mRNA levels of L16/D8 fish were significantly higher than those of L8/D16 fish at days 19 and 29, respectively.

Conclusions: At the onset of sexual maturation, the levels of *lh-β*, fsh-*β*, and *gnrh3* rose earlier than those of *kiss2* and *gpr54*. These results are consistent with GnRH3 having a role in the early phases of maturation, but do not support a role for kisspeptin in photoperiodic initiation of maturation in male stickleback.

Keywords: *fsh-β, lh-β, gnrh2, gnrh3, gpr54, kiss2,* reproduction, stickleback, teleosts.

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INTRODUCTION

Most temperate animals have a predictable and restricted breeding period. This is critical for individuals to mature in the right season to maximize reproductive success. For this reason, temperate species use both endogenous cycles and environmental cues for the timing of growth and maturation (Bradshaw and Holzapfel, 2007). The role of photoperiod in synchronizing the timing of seasonal reproduction in fishes has been widely studied. It is the major cue used in many temperate species, such as salmon, seabass, and stickleback (Randall *et al.*, 1998; Howell *et al.*, 2003; Begtashi *et al.*, 2004; Borg, 2010). In the threespine stickleback (*Gasterosteus aculeatus*), sexual maturation is stimulated by a long-day photoperiod but inhibited by a short-day photoperiod (e.g. Baggerman, 1957; Borg, 1982). A short-day photoperiod of 8 hours light/16 hours dark (L8/D16) can keep stickleback in an immature state for long periods, but the fish mature quickly under a long-day photoperiod, such as 16 hours light/8 hours dark (L16/D8). The mature males show bright breeding coloration (i.e. red belly and blue eyes), as well as nesting and courtship behaviour. Breeding male stickleback build a nest of algae glued together with threads of the protein spiggin produced by the kidney, which hypertrophies under androgen stimulation. Hence, the kidney somatic index (KSI = kidney weight/body weight) can be used as an indicator of maturation of males (Páll *et al.*, 2005).

As in other vertebrates, gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH) play central roles in fish reproduction. The stimulatory effects of a long-day photoperiod on gonadotropic cells, as shown in their ultrastructure (Borg *et al.*, 1987) and in gonadotropin mRNA levels, have been reported previously (Hellqvist *et al.*, 2004; O'Brien *et al.*, 2012; Shao *et al.*, 2013). The expression of both *lh-β* and *fsh-β* in stickleback changes markedly with the season of the year (Hellqvist *et al.*, 2006). Under a manipulated long-day photoperiod (L16/D8), male stickleback mature rapidly within weeks (Borg, 1982), and have higher mRNA levels of both *lh-β* and *fsh-β* than L8/D16 fish (Hellqvist *et al.*, 2008; Shao *et al.*, 2013).

The development of gonads and other sexual characters is regulated by the endocrine system, including the brain–pituitary–gonadal (BPG) axis, in which the secretion of gonadotropins from the pituitary gland is stimulated by gonadotropin-releasing hormone (GnRH) from the brain. Two types of gonadotropin-releasing hormone – GnRH2 (previously called chicken GnRH II, or cGnRHII) and GnRH3 (previously called salmon GnRH, or sGnRH) – have been found in the stickleback genome, while GnRH1 is absent (O'Brien *et al.*, 2012; Shao *et al.*, 2015). Both *gnrh2* and *gnrh3* appear to be involved in the control of reproduction in stickleback (Shao *et al.*, 2015), but the role that they play in photoperiod-induced onset of sexual maturation is largely unknown (O'Brien *et al.*, 2012; Shao *et al.*, 2015). Changes in gonadotropins and GnRH can be paradoxical in the early phases of long-day exposure. O'Brien *et al.* (2012) observed that whereas *lh-β* mRNA levels increased in adult males exposed to a photoperiod of L17/D7 for 5 days, *gnrh3* mRNA levels decreased.

The kisspeptin system is another possible link mediating environmental cues on maturation via the BPG axis (Tena-Sempere, 2006). Kisspeptin, a protein that is encoded by the *kiss1* gene in humans and which was originally identified as a human metastasis suppressor (Lee *et al.*, 1996; Gottsch *et al.*, 2006), is also essential for reproductive function and gonad development (Kuohung and Kaiser, 2006). For example, signals from kisspeptin and the kisspeptin ligand G-coupled protein receptor 54 (GPR54) can stimulate GnRH and/or gonadotropin secretion in mammals (for a review, see Roa and Tena-Sempere, 2007). Kisspeptin signals are also important in the photoperiodic control of mammalian seasonal reproduction (Mason *et al.*, 2007). Recently, the effects of kisspeptin on GnRH and gonadotropins in fish have been

reported (Shi *et al.*, 2010; Pasquier *et al.*, 2011, Ohga *et al.*, 2018). It is less clear, however, whether kisspeptin controls seasonal reproduction in fish as it does in mammals (Felip *et al.*, 2009; Cowan *et al.*, 2012). Two paralogous genes of kisspeptin (*kiss1* and *kiss2*) have been reported in a number of teleost species, including zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), goldfish (*Carassius auratus*), and European seabass (*Dicentrarchus labrax*) (for a review, see Akazome *et al.*, 2010). However, it appears that *kiss1* is absent in threespine stickleback (Felip *et al.*, 2009; Tena-Sempere *et al.*, 2012), which means *kiss2* is the only kisspeptin that could be involved in the control of reproduction in stickleback.

Kisspeptin and GnRH have critical roles in the photoperiodic control of reproduction in mammals. The aim of the present study was to determine whether the kisspeptin and GnRH systems regulate the onset of maturation in stickleback. If kisspeptin and GnRH do control the onset of maturation in fishes, then under photoperiod stimulation they ought to be activated prior to, or at least no later than, the secretion of gonadotropins. To test this hypothesis, the gene expression of brain *kiss2*, *gpr54*, *gnrh2*, and *gnrh3*, as well as pituitary *lh-β* and *fsh-β*, was measured in male stickleback fishes at different time points during the rapid maturation process under both long-day (L16/D8) and short-day (L8/D16) photoperiod conditions.

MATERIALS AND METHODS

Experimental animals and treatments

Stickleback were caught in the Baltic Sea at Skåre (55°22′35″N, 13°3′8″E), southern Sweden on 16 December 2012 and transported to Stockholm University. The fish were kept in aquaria containing artificial brackish water (0.5% salinity) that was aerated and filtered. The bottoms of the aquaria were covered in sand, and ceramic pots and tubes were provided as refuges for the fish. The fish were fed daily with frozen bloodworms or mysids and kept under standard laboratory conditions (L8/D16 photoperiod and temperature maintained at 20°C). For the photoperiod experiment, we used six 200-litre aquaria similar to the above: three under short-day photoperiod (L8/D16) conditions and three under long-day photoperiod (L16/D8) conditions. Controls (day 0) were dissected on 25 January 2013. Then, on 26 January, 15 males and 15 females (note: females were not used in this study) were transferred to each aquarium. Ten fish of each sex (6–7 fish per aquarium) were sampled during the light phase after 3 days, another 10 fish of each sex after 10 days, and a third group of 10 fish of each sex after 19 days; all remaining males (11 under L16/D8 conditions and 14 under L8/D16 condition) were sampled after 29 days.

Dissection

The fish were euthanized with 0.025% buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate salt) solution before dissection. Body and kidney weights were recorded, and the presence of breeding colour was noted after visual inspection. The kidney was studied under a stereo microscope for hypertrophy of tubules. The kidney somatic index (KSI) was calculated as kidney weight/body weight × 100. The pituitary gland was separated from the brain after decapitation and removal of the skullcap, and the whole brain and pituitary were immersed in 500 μL and 100 μL of RNAlater® (Ambion), respectively. The samples were kept at 4°C overnight, before storage at −70°C until analysis.

The experiment was conducted with permission from the Stockholm Northern Animal Experiment Ethical Committee (N174/11).

Real-time quantitative PCR (q-PCR)

Total RNA was extracted from the brain or pituitary gland using RNeasy Plus Universal kits (Qiagen, Cat. No. 73404) following the manufacturer's instructions. The samples extracted from each brain provided enough RNA for analysis, whereas samples from two pituitary glands from the same treatment group were pooled to ensure the same. Two micrograms of total RNA from each RNA sample were reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystem, LN: 00285957) and NxGen® RNase inhibitor (Lucigen, LN: 11549) according to the recommended protocol.

mRNA expression of target genes was measured by q-PCR with the Roche LightCycler® 480 System (Roche Applied Science, Mannheim, Germany). PCR reactions contained 40 ng of cDNA, 50 nM of each primer (Table 1), and the LightCycler® 480 SYBR Green I Mastermix (Roche) in a final volume of 10 μL. All q-PCR reactions were performed as follows: 1 cycle at 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute using 0.05 μM of specific primers. The PCR products were subjected to a melting-curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with RNA free water to determine levels of the background. The mRNA levels of *gnrh2* (GenBank: JQ673490.1), *gnrh3* (GenBank: JQ673491.1), *kiss2* (GenBank: KT202354.1), and *gpr54* (ENSGACG00000017220) were determined using brain tissue; those of *fsh-β*

Table 1. The primer sequences used in q-PCR (the reference genes were *rpl8* and *act-β*)

Gene name	Primer sequence	Product size (bp)
gnrh2 (Shao *et al.*, 2015) E_i = 91%	F = 5′-CCGTCGGAGATTTCAGAGGAGATT-3′ R = 5′-TCTGAAGCTCTCTGGCTAAGGCAT-3′	121
gnrh3 (Shao *et al.*, 2015) E_i = 107%	F = 5′-AGCGATGGTGCAGGTGTTGATGTT-3′ R = 5′-TTAAGTCTCTCTTGGGTCTGGGCA-3′	189
kiss2 (Roufidou *et al.*, 2016) E_i = 93%	F = 5′-CAACGACCGCAGGAATCAAT-3′ R = 5′-GGCTCTTCTGTAAATGTAGCGTTTC-3′	76
gpr54 (Roufidou *et al.*, 2016) E_i = 104%	F = 5′-GTCCACCTTCTGTCCGAGAGAA-3′ R = 5′-CCAGCAAACGGCGAAAAG-3′	90
fsh-β (Hellqvist *et al.*, 2008) E_i = 103%	F = 5′-CATCGAGGTGGAGGTCTGTG-3′ R = 5′-GGGGCTGATGGCTGCTGT-3′	101
lh-β (Hellqvist *et al.*, 2008) E_i = 99%	F = 5′-GGTCACTGCCTCACCAAGGA-3′ R = 5′-GGAGCGCGATCGTCTTGTA-3′	103
rpl8 (Shao *et al.*, 2013) E_i = 101%	F = 5′-CGACCCGTACCGCTTCAAGAA-3′ R = 5′-GGACATTGCCAATGTTCAGCTGA-3′	143
act-β (Shao *et al.*, 2015) E_i = 96%	F = 5′-ATGGGCCAGAAGGACAGCTA-3′ R = 5′-TCACAATACCGTGCTCAATGG-3′	91

Note: E_i represents the efficiency of each q-PCR primer.

(NM_001267631.1) and *lh-β* (NM_001267650.1) were measured from the pooled pituitary samples. The geometric means of the expression of two reference genes, *β*-actin (*act-β*) (DQ018719.1) and ribosomal protein (*rpl8*) (ENSGACG00000002035), were used to normalize the target gene expression levels. The efficiency-corrected relative expression method was used to quantify the relative expression levels (Roche Applied Science, 2001; Weltzien *et al.*, 2005).

Statistical analyses

As some of the data were not normally distributed after a Shapiro-Wilk normality test ($P < 0.05$), non-parametric statistics were used in the analyses. Data from two groups were compared using non-parametric Mann-Whitney *U*-tests (SPSS v. 19). Multiple group comparisons were performed using the one-way Kruskal-Wallis test, followed by *post-hoc* analysis with a Dunn test (Olsvik *et al.*, 2005).

RESULTS

The body weights of the control group (day 0) were lower (mean ± SEM = 1.51 ± 0.07 g) than those of the other groups of fish (means 1.72–2.02 g; $P < 0.05$). Fish maintained under different photoperiods showed no significant difference in weight at any time point except for day 19, when short-day photoperiod (L8/D16) fish had lower body weights (1.76 ± 0.10 g) than long-day photoperiod (L16/D8) fish (2.02 ± 0.06 g; $P < 0.05$).

Most males under L16/D8 conditions displayed breeding colour and a hypertrophied kidney at days 19 and 29, whereas no L8/D16 fish did. On these days, L16/D8 fish had a significantly higher kidney somatic index than the fish dissected at earlier time points (day 0, day 3, and day 10), as well as L8/D16 fish at all times (Fig. 1).

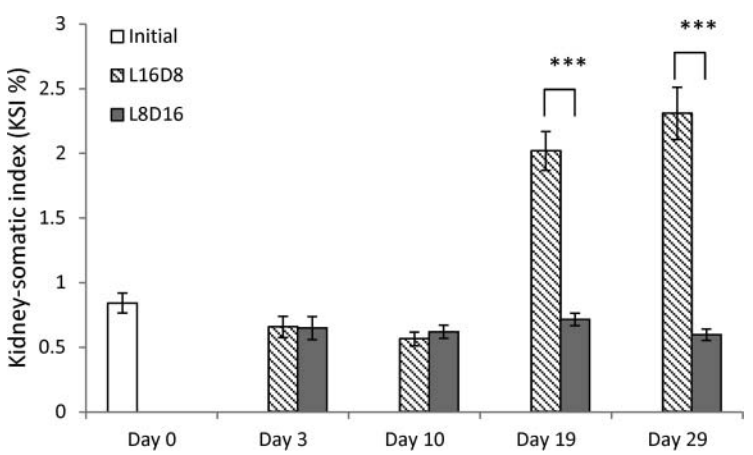

Fig. 1. Kidney somatic index (KSI) of male stickleback in the L16/D8 and L8/D16 conditions. Means ± standard error are shown ($n \geq 10$; ***$P < 0.001$).

Brain *gnrh2*, *gnrh3*, *kiss2*, and *gpr54* mRNA

Under L8/D16 conditions, the mRNA levels of *gnrh2*, *gnrh3*, *kiss2*, and *gpr54* did not change significantly (Figs. 2 and 3). Under L16/D8 conditions, however, *gnrh2* mRNA levels were slightly higher than under L8/D16 conditions at day 3, but not significantly so ($P = 0.071$) (Fig. 2). Also under L16/D8 conditions, *gnrh2* mRNA levels remained unchanged until day 29, when they were higher than in controls on day 0 as well as in L8/D16 fish (Fig. 2). On the other hand, although the *gnrh*3 mRNA levels of L16/D8 fish tended to be lower than those of L8/D16 fish at day 3 ($P = 0.058$), they were higher at days 10, 19, and 29 compared with L8/D16 fish (Fig. 2). Under the L16/D8 photoperiod, *kiss2* and *gpr54* mRNA levels were significantly higher than in controls on day 0 and in the L8/D16 fish at days 19 and 29 respectively (Fig. 3), whereas no difference was observed in either *kiss2* or *gpr54* mRNA levels between the L8/D16 fish at any time and the controls at day 0.

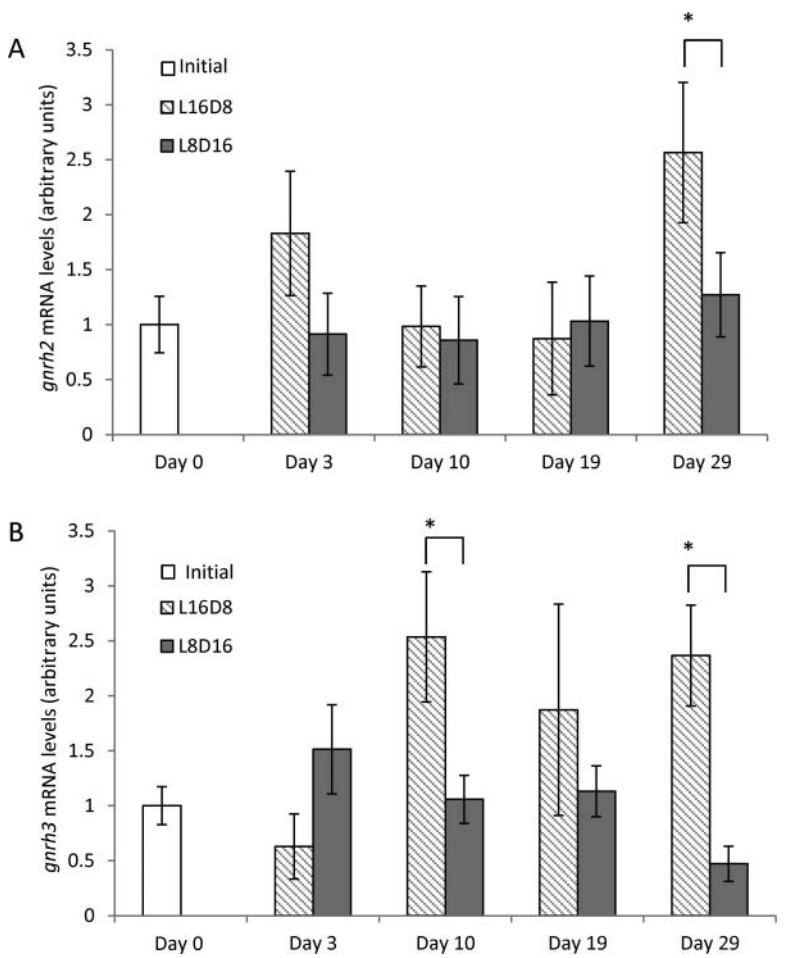

Fig. 2. Brain *gnrh2* (A) and *gnrh3* (B) mRNA levels. Means ± standard error are shown ($n = 5$–7; $^*P < 0.05$; $^{**}P < 0.01$).

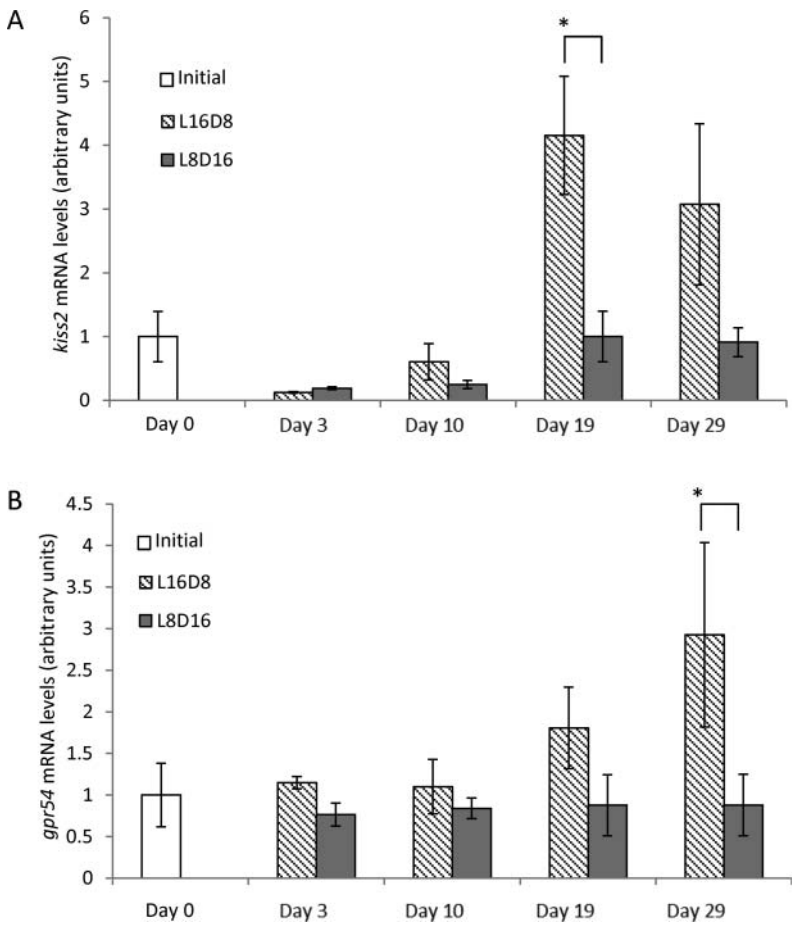

Fig. 3. Brain *kiss2* (A) and *gpr54* (B) mRNA levels. Means ± standard error are shown ($n = 5–7$; $^*P < 0.05$).

Pituitary *fsh-β* and *lh-β* mRNA

Long-day photoperiod (L16/D8) fish had higher pituitary *lh-β* mRNA levels than short-day photoperiod (L8/D16) fish from day 10 and the levels continued to increase gradually at the following time points (Fig. 4). Pituitary *fsh-β* mRNA levels of L16/D8 fish reached their peak at day 10, when they were higher than in L8/D16 fish. However, *fsh-β* expression returned to baseline values at day 19. At day 29, the mRNA levels of *fsh-β* of L16/D8 fish were again higher than those of L8/D16 fish (Fig. 4).

DISCUSSION

Kisspeptin can mediate the effects of environmental and metabolic signals and may be involved in the control of the onset of puberty and/or seasonal maturation in teleosts (for reviews, see Tena-Sempere *et al.*, 2012; Ogha *et al.*, 2018) as in mammals (Revel *et al.*, 2007). Ohga *et al.* (2015) studied the development of *kiss1*, *kissr1*, *kiss2*, *kiss2r*, as well as *gnrh1* in the brains of

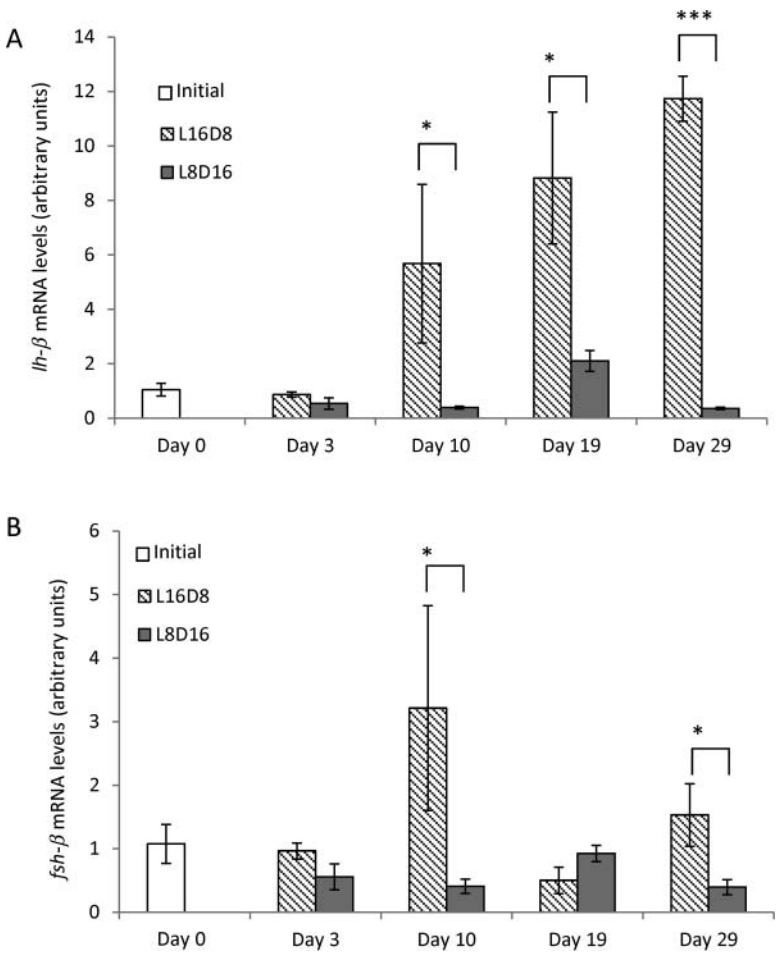

Fig. 4. Pituitary *lh-β* (A) and *fsh-β* (B) mRNA levels. Means ± standard error are shown ($n = 5$; $^{*}P < 0.05$; $^{***}P < 0.001$).

male and female chub mackerel (*Scomber japonicus*) in the first year of life. All of these parameters displayed a significant rise, or at least a non-significant tendency to rise, during – or a little earlier than – the early stages of puberty, which occurred earlier in males than in females. This suggested a positive effect of kisspeptin in puberty in this species. At least in females, *kiss1* levels rose earlier than those of *gnrh1* (Ohga *et al.*, 2015), and it is possible that gonadotropin-releasing hormone (GnRH) is activated by kisspeptin, especially since *gnrh1* and *kiss1* are located in the same brain areas and even co-expressed in the same cells (Ohga *et al.*, 2017). Using *in situ* hybridization, medaka (*Oryzias latipes*) were observed to have a higher number of *kiss1* expressing neurons in the nucleus ventral tuberis under L14/D10 compared with inhibitory L10/D14 conditions (Kanda *et al.*, 2008). Likewise, continuous illumination, which inhibits puberty in Nile tilapia (*Oreochromis niloticus*), also suppressed *gpr54* mRNA levels (Martinez-Chavez *et al.*, 2008). Nevertheless, the photoperiodic effects of kisspeptin expression are not consistent. Goldfish exposed to a photoperiod of L14/D10 for months had

higher *kiss1* and *gpr54* mRNA levels, as well as higher gonadotropin gene expression and plasma gonadotropin levels, than goldfish kept under L12/D12 or L10/D14 conditions (Shin *et al.*, 2014). For European seabass, long-term exposure to continuous light, which suppresses reproduction, also suppressed *kiss2* and *gnrh1* mRNA levels. Furthermore, *gnrh1*, *gnrh2*, *kiss2*, and *kiss1rb* mRNA levels were up-regulated when the photoperiod shifted from L15/D9 to a stimulatory L9/D15 (Martins *et al.*, 2015). On the other hand, there was no significant temporal pattern in the mRNA levels of *kiss2* or *kissr4* in Atlantic cod (*Gadus morhua*) maturing under an inhibitory photoperiod or maturing for the first time under a stimulatory photoperiod (Cowan *et al.*, 2012). In the present study, a stimulatory long-day (L16/D8) photoperiod resulted in higher *kiss2* and *gpr54* mRNA expression. However, this occurred after the elevation in *gnrh3* and *lh-β*/*fsh-β* levels and the development of secondary sexual characters, which does not lend support to kisspeptin mediating photoperiodic effects on reproduction in stickleback. This does not negate that changes in kisspeptin expression in some brain areas, which might not change mRNA levels in the whole brain very much, and/or in the release of kisspeptin not coupled to changes in expression, could influence reproduction. Perhaps future *kiss2* knockout studies could shed more light on this roll of kisspeptin.

Some previous studies showed that the administration of kisspeptin induces sexual maturation in fishes (for reviews, see Zmora *et al.*, 2014; Ogha *et al.*, 2018). Nevertheless, the kisspeptin signal is not always required for maturation. A gene knockout study indicated that mutant lines of zebrafish (*Danio rerio*) that do not express *kiss1*, *kiss2*, *kiss1r*, and *kiss2r* still mature and breed normally (Tang *et al.*, 2015). Ablations of kisspeptin neurons or the neurons that express GPR54 do not affect the maturation and fertility of female mice, although the gonads are smaller than normal (Mayer and Boehm, 2011). As for teleosts, a recent review suggests that kisspeptin may play a role in the regulation of homeostasis or behaviour in accordance with reproductive states; however, unlike in mammals, kisspeptin may not be involved in the steroid feedback regulation of GnRH neurons (Kanda, 2019).

It is generally believed that gonadotropin-releasing hormones of the forebrain lineage, especially GnRH1, are involved in the regulation of reproduction (for a review, see Okubo and Nagahama, 2008). Hypothalamic or brain *gnrh1* mRNA levels reach their apex in the natural breeding season and decline rapidly after that in red seabream (*Pagrus major*) and Brazilian flounder (*Paralichthys orbignyan*us) (Okuzawa *et al.*, 2003; Campos *et al.*, 2011). GnRH1 is the most important isoform in the regulation of reproduction in most vertebrates. However, in some species that do not have GnRH1, such as salmon, zebrafish, and goldfish, GnRH3 stimulates gonadotropin release (for reviews, see Okubo and Nagahama, 2008; Zohar *et al.*, 2010). In Atlantic cod (*Gadus morhua*), which also lacks GnRH1, brain *gnrh3* mRNA increased significantly during the spawning stage, whereas no such change was observed in *gnrh2* mRNA (Hildahl *et al.*, 2011). A previous study suggested that both GnRH2 and GnRH3 may be involved in reproduction in the stickleback, as the brain mRNA levels of both decline after the breeding season (Shao *et al.*, 2015). Under a long-day (L16/D8) photoperiod, elevated *gnrh2* mRNA levels were not found prior to 29 days, when the fish were already fully mature. Even if GnRH2 does not have a role in the control of gonadotropin release, both GnRH2 and GnRH3 may act as neuromodulators (for a review, see Okubo and Nagahama, 2008). For instance, GnRH2 has been implicated in the spawning behaviour of goldfish (Volkoff and Peter, 1999) and the nest-building behaviour of dwarf gouramis (*Colisa lalia*) (Yamamoto *et al.*, 1997). Under L16/D8 conditions, significantly elevated *gnrh2* mRNA levels were not found prior to 29 days, when the fish were already fully mature, suggesting that GnRH2 is not involved in the onset of the photoperiodic response.

The role of GnRH in photoperiodic control in mammals is well established (Kriegsfeld and Bittman, 2010). However, it is unclear whether it also plays a similar role in fishes. For example, a stimulatory photoperiod does not necessarily increase the pituitary GnRH content. Davies *et al.* (1999) showed that the adjustment of photoperiod from an inhibitory L18/D6 to a stimulatory L6/D18 reduced pituitary GnRH3 (sGnRH) contents in rainbow trout. However, it cannot be stated clearly whether the L6/D18 photoperiod increased the release of GnRH from the nerve endings or reduced the overall production of GnRH. In stickleback, males maintained under a long-day (L16/D8) photoperiod for 3 weeks had higher *gnrh2* mRNA levels in the thalamus and higher *gnrh3* mRNA levels in both the telencephalon and hypothalamus than fish under a short-day (L8/D16) photoperiod (Shao *et al.*, 2015). However, the stimulatory effects of L16/D8 on GnRHs were not detected in castrated males, which suggests that the *gnrh* genes are up-regulated by gonadal hormones secondarily to an increase in gonadotropins stimulating the gonads (Shao *et al.*, 2015). Furthermore, a short period (2–5 days) of long-day exposure suppresses overall brain (including the pituitary) *gnrh3* mRNA levels in male stickleback (O'Brien *et al.*, 2012). Although the reason why *gnrh3* mRNA expression is down-regulated under the stimulatory photoperiod is unknown, its occurrence is clear (O'Brien *et al.*, 2012). Likewise, the present study showed lower *gnrh3* mRNA levels under L16/D8 than L8/D16 conditions at day 3, whereas they rose at day 10 and day 29.

The *lh-β* mRNA levels of male stickleback in the L16/D8 condition were higher than in the L8/D16 condition from day 10, which is line with the findings of O'Brien *et al.* (2012), who also observed stimulation as early as day 5 in one of the studied populations. The levels continued to increase at the following time points, paralleling the development of the kidney. In contrast, although *fsh-β* mRNA levels in L16/D8 fish were higher than in L8/D16 fish at day 10, no such difference was observed at the following time points. In earlier studies on male stickleback, a long-day photoperiod (>12 hours) increased mRNA levels of *lh-β* and *fsh-β*, although the effects on the latter were not as consistent (Hellqvist *et al.*, 2004; Shao *et al.*, 2013). Our results indicate that a photoperiod of L16/D8 may up-regulate both *lh-β* and *fsh-β* within a short period of time. Nevertheless, the change in *lh-β* mRNA levels persisted for longer than the change in *fsh-β*, and *lh-β* expression was more strongly correlated with secondary sex characters, which are stimulated by gonadal hormones (i.e. androgens).

In summary, *lh-β*, fsh-*β*, and *gnrh3* levels rose earlier than those of *kiss2* and *gpr54* at the onset of sexual maturation in response to a long-day photoperiod. This suggests a role for GnRH3 in the early phases of maturation, but not one of kisspeptin in the photoperiodic initiation of maturation in male stickleback.

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