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Androgen feedback effects on LH and FSH, and photoperiodic control of reproduction in male three-spined sticklebacks, *Gasterosteus aculeatus*.

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Abstract

Sexual maturation in the stickleback is controlled by photoperiod. The aim of this study was to find out whether changes in feedback effects exerted by sex steroids could mediate the photoperiodic effect, which is regarded to be of an all-or-nothing character. To that end, males were castrated and treated with different doses of testosterone (T) and in one experiment also with the aromatase inhibitor fadrozole (AI) and kept under different photoperiods. In control fish, long day (LD 16:8) stimulated maturation, associated with more hypertrophied kidneys (a secondary sexual character) and higher levels of pituitary *lhb* and *fshb* mRNA than under short day conditions (LD 8:16). Under LD 8:16, low doses of T suppressed both *lhb* and *fshb* mRNA levels. However, with the use of high doses of T and/or longer photoperiods the inhibitory effects on *lhb* and *fshb* mRNA levels became less clear or instead positive effects were observed. Under intermediate photoperiod conditions, the negative feedback effect of a low dose of T on *fshb* was more prominent with shorter photoperiods, whereas no such shift was observed for *lhb* mRNA. The inhibitory effect of the low dose of T on *lhb* mRNA levels under LD 8:16 was abolished by AI, whereas the stimulatory effect of the high dose of T was not. The negative feedback effects were more marked under short days than under long days, whereas positive feedback effects were more marked under long days. The suppression of both *fshb* and *lhb* mRNA levels by low androgen levels, especially under short days, may inhibit maturation completely unless a rise of androgens above threshold levels would allow complete maturation.

1.Introduction

In order to reproduce at the optimal time of the year, organisms use endogenous cycles and environmental cues, the most important of which being the photoperiod [18]. In vertebrates, the circulating levels of the pituitary gonadotropic hormones (GtHs), LH and FSH, are higher under stimulatory than under non-stimulatory photoperiods, resulting in increased gonadal activity. The pituitary is under control from the brain, especially the hypothalamus, and by gonadal hormones which exert feedback effects on the brain-pituitary-gonad (BPG) axis. In mammals and birds, several studies have shown changes in steroid feedback effects on the BPG axis by photoperiod, but also gonad-independent effects, i.e. higher circulating GTH levels in castrated animals kept under a stimulatory photoperiod are known [16]. This has been studied very little in poikilothermic vertebrates.

Photoperiod is an important factor in synchronizing reproduction also in fishes, including the three-spined stickleback which is perhaps the fish where the mechanisms of photoperiodic effects have been studied most extensively [2, 3, 7, 8, 9]. At sexual maturation, the kidney of male sticklebacks hypertrophies and begins to produce a protein, spiggin, which is used to glue nest material together [15]. The proportion of sticklebacks that mature increases as the photoperiod increases and also increases as the natural breeding season approaches, but each individual either builds a nest if it is a male or ovulates if it is female within a couple of months or not at all [2, 3]. These observations suggested that the photoperiodic response in the stickleback is of an all-or-nothing type [3, 9]. Gonadal feedback effects on *fshb* mRNA levels in stickleback differed under long and short day conditions (L16:D8 and L8:D16) [14]. Under a stimulatory long day, castration reduced *fshb* mRNA levels compared to sham-operated fish, and testosterone (T) treatment increased *fshb* expression in castrated fish. In contrast, *fshb* mRNA levels in fish kept under non-stimulatory short day conditions were instead increased after castration, and, furthermore, the same dosage of T lowered *fshb* mRNA [14]. On the other hand, T increased *lhb* expression under both L8:D16 and L16:D8 [14]. However, no information is available with respect to the possibly different roles of different androgen levels in this feedback system.

Aromatase inhibitor (AI) treatments, which prevent T from being converted into estrogen, have a stimulatory effect on male stickleback maturation [11, 13], and increase the *fshb* and *lhb* mRNA levels under short day [13]. However, it is still unclear whether aromatase exerts its actions on stickleback maturation via the feedback system.

The aim of this study was to find out whether changes in feedback mechanisms could mediate photoperiodic effects on stickleback maturation, especially the all-or-nothing response. To that

end, males were castrated and treated with different doses of androgens and in one experiment also with the aromatase inhibitor fadrozole and kept under different photoperiods.

2. Material and methods

2.1. Experimental animal

Three-spined sticklebacks were caught in the Öresund, southern Sweden and transported to Stockholm University. The animals were stored in aquaria containing artificial brackish water (0.5% salinity) which was aerated and filtered. The bottom was covered with sand; ceramic pots and tubes provided hiding places. The fish were fed daily with frozen bloodworms, *Artemia* or mysids. Before operation, fish was kept under winter condition, at 4-6 °C and under a non-stimulatory short day (L8:D16 or shorter). Four sets of experiments were done between 2007 to 2011. In general, males were either castrated or sham-operated and treated with different implants. After operations fish were transferred to different aquaria under different photoperiods. The different treatments are summarized in Table 1. For more details, see supplemental material.

The experiments were carried out following permission from the Stockholm Northern Animal Experiment Ethical Committee.

2.2. Implants

All implants were made from medical grade Silastic tube (ID 0.64mm; OD 1.19mm) which was cut to 5mm in length and filled with crystalline T (Fluka), crystalline 11-ketoandrostenedione (11KA) (4-Androstene-3,11,17-trione; Sigma) or aromatase inhibitor (AI, fadrozole; CGS 16949 A, 4-(5,6,7,8-tetrahydroimidazo[1,5- α]pyridin-5-yl) benzonitrile; a gift from Novartis). Both ends were sealed with silicone glue. 11KA is a non-aromatisable androgen, whereas it can be converted to 11-ketotestosterone (11KT) in the fish. In a previous study [20] the type of 11KA implant used in present study increased the plasma 11 KT levels in castrated male sticklebacks to c. 300 ng/mL, similar to the levels found in breeding males. In another experiment (unpublished data) implants with crystalline T similar to those used in the present study in castrated males resulted in plasma levels of c. 140 ng/mL. Circulating levels of T in breeding sticklebacks vary considerably, but can reach c. 70 ng/mL [19]. Thus, the highest T treatment in the present study gives T levels somewhat higher than the natural ones. Similar types of AI (fadrozole) implants as those used in the present study drastically reduced aromatase activity in the brain of young salmon [1], even though these fish were larger than the stickleback.

Other types of T implants were made from 5 mm Silastic tubes, but filled with different concentrations of T (0.10; 0.25 or 0.50 %) dissolved in cacao butter, and not sealed at the ends. Those implants made it possible to apply lower doses than by using crystalline implants. The cacao butter-implants were stored at 4 °C before use. Empty implants or implants filled with cacao butter alone were used as controls.

2.3. Operations

Fish were either castrated or sham-operated and were given different types of implants. The fish were anaesthetized with c. 0.1% 2-phenoxyethanol (2007 and 2008) or 0.025% buffered MS-222 (Ethyl 3-aminobenzoate, methanesulfonic acid salt) solution (2010 and 2011), c. 1.5 mm long incisions were made to open into the abdominal cavity on each side and the testes were excised with fine forceps. Sham-operated fish were treated similarly, but the testes were not removed. The incisions were closed with BV-2 (0.4 Ph. Eur) suture. Categories of fish were marked with spine-clipping. After the operation, the fish were put into 700L (2007) or 1200L (2008~) aquaria and were kept under different photoperiods. The temperature was slowly increased to 20 °C and kept there over the experiments.

2.4. Dissection

All fish were euthanized with 0.1% 2-phenoxyethanol (2007 and 2008), or with 0.025% buffered MS-222 (Ethyl 3-aminobenzoate, methanesulfonic acid salt) solution (2010 and 2011) before dissection. Body and kidney weights were recorded, as were breeding color and the kidney maturity status. The kidney-somatic index (KSI) was calculated as kidney weight/body weight * 100. The pituitary was separated from the brain after decapitation and removal of the skullcap, and was then immersed in 50µl RNAlater® (Ambion) on ice for 2 hour, before storage at -70 °C until analysis. In 2011, blood was collected from the caudal artery with micro hematocrit tubes (Na-hep. Cat No. 7493 11; BRAND). After centrifugation for 2 min, the plasma samples were removed to pre-weighed Eppendorf tubes. The plasma volume was measured by weighing and plasma was stored at -70 °C. Following the standard protocol [23], the testosterone levels of those plasma samples were measured by radioimmunoassay (RIA). The plasma sample pretreatment has been carried out as described previously [17]. In brief, plasma was diluted with 2 volumes of RIA buffer, heated for 1 hr at 80C and then centrifuged. Aliquots of the supernatant were used in the RIA procedure.

2.5. Histology

Since the kidney of male sticklebacks hypertrophies and begins to produce spiggin during breeding, kidney indices, i.e. KEH and KSI, were used to indicate male maturation. The kidneys from the sham-operated and intact males in the 2007 years experiment were fixed in Bouin-Hollande solution, dehydrated in graded ethanol, cleared in xylene, embedded in paraffin and sectioned at 7µm. Kidneys were stained using the Azan method. Kidney epithelium height (KEH) was measured on ten secondary proximal tubules per kidney under a microscope with an ocular micrometer.

2.6. Quantitative real-time PCR

Total RNA was extracted from stickleback pituitaries through homogenization using TriZol reagent (Invitrogen) according to the standard protocol. The extracted RNA was treated with DNase (2U) using TURBO DNA-free kit according to the manufacturer's instruction (Ambion) to remove possible genomic DNA contamination. After reverse-transcription, the samples were diluted 1/10 for *fshb* and *lhb*, or 1/100 for *18s* measurements. The relative quantity of *fshb*, *lhb* and *18s* mRNA expression was measured by Q-PCR with the Stratagene MX3000P™ using Stratagene's MxPro™ Q-PCR Software with brilliant II Fast SYBR® Green Q-PCR Master Mix according to the manufacturer's instructions (Stratagene, Agilent Technologies) Specific primers for *fshb* (Fw: 5'-CATCGAGGTGGAGGTCTGTG-3';
Rw: 5'-GGGGCTGATGGCTGCTGT-3') and *lhb* (Fw: 5'-GGTCACTGCCTCACCAAGGA-3'; Rw: 5'-GGAGCGCGATCGTCTTGTA-3') and *18s* rRNA (Fw: 5'-CTCAACACGGGAAACCTCAC-3'; Rw: 5'-AGACAAATCGCTCCACCAAC-3') (0.05 µM) were used. The parameters for the real-time PCR were set at 95°C for 2 min followed by 40 cycles (at 95°C for 5 sec and 60°C for 20 sec). One last cycle was run to produce a melting curve to show the amplified products specificity. *fshb* and *lhb* mRNA expression data were normalized against *18s* as a reference gene.

2.7. Statistics

The data were analysed using SPSS v.14 with two tailed Student's t-test with Tukey's post-hoc test for independent samples after normality test ($p < 0.05$) for comparisons between two groups after one way ANOVA by for comparisons between multiple groups.

3. Results

Weight data are shown in Table 1. The fish kept under L11:D13 had lower body weight than the fish under L13:D11 ($p<0.05$) and L14:D10 ($p<0.05$) in the 2007 experiment. Apart from that, no significant difference in body weight was found between groups within a year.

Most control males under long day displayed breeding colours, but only few males under short day did so. Breeding colours were not recorded in castrated controls, and at most weak colours were found in T-treated males, whereas all 11KA-treated males displayed them.

3.1. Kidney epithelium height (KEH) and Kidney somatic index (KSI)

In 2007, no difference in KEH was found between sham-operated and un-operated groups in all four intermediate photoperiods. However, KEH in sham-operated and un-operated control fish increased with increased hours of light ($p<0.05$) (data not shown). The distribution of individual KEH values in all control fish showed two distinct peaks with a gap between KEH 16 μ m and 26 μ m (Figure 1A). There was no effect of photoperiod on the KEH of fish within the low and the high groups, but the proportion of males with high KEH values increased with increased hours of light (Figure 1B).

KSI in control operated group (including both unoperated and sham-operated fish) were higher than in castrated controls and castrated fish treated with 70.25 in L12:D12, L13:D11 and L14:D10 in 2007 ($p<0.005$). The average KSI for the control operation group increased with increased hours of light and was higher in L14:D10 than in L11:D13 ($p<0.001$), L12:D12 ($p<0.001$), L13:D11 ($p<0.05$) and higher in L12:D12 than in L11:D13 ($p<0.001$). There were no significant differences in KSI between the different photoperiods for castrated control males or castrated males treated with 0.25% T.

In 2008 and 2011, sham-operated males showed significantly higher KSI under L16:D8 than L8:D16 ($p<0.005$). Under L16:D8, the KSI of castrated fish were lower than sham-operated fish ($p<0.005$), but no difference was found under L8:D16. The castrated males treated with 11KA implants (11KA) showed higher KSI values than castrated males with empty implants under L16:D8 and L8:D16 in 2008, but this stimulation effect was not found in any T treatments groups (Table 1).

3.2. Plasma testosterone levels

0.5% T implants (*T 0.50*) increased plasma T level significantly compared to castrated controls both under L16:D8 and L8:D16 ($p < 0.005$ in each comparison), but no effect was found in the fish treated with 0.10% (*T 0.10*) T cacao butter implants (Figure 2). The castrated fish treated with 0.25% (*T 0.25*) had higher T levels than castrated controls under short day treatment, whereas this was not the case under long day treatment (Figure 2). The T levels of the castrated males with 0.5% T implants (*T 0.50*) were even higher than those in sham-operated mature males under L16:8 (Figure 2).

3.3. *lhb* mRNA levels

In 2007, sham-operated fish had significantly higher *lhb* mRNA levels than the castrated fish ($p < 0.05$ in each comparison) and 0.25% T (*T 0.25*) treated fish ($p < 0.001$ in each comparison) under L12:D12, L13:D11 and L14:D10, but the levels in sham-operated males were lower than in castrated males ($p < 0.05$) under L11:D13 (Figure 3A). The castrated fish had significantly higher *lhb* levels than the 0.25% T treated (*T 0.25*) fish ($p < 0.005$) in all photoperiods. However, no difference in *lhb* mRNA levels was found in castrated males or 0.25% T (*T 0.25*) treated males between different photoperiods (Figure 3A, Table 2).

In 2008 and 2011, the *lhb* mRNA levels of the sham-operated fish kept under L16:D8 (Figure 4A) were significant higher than in those kept under L8:D16 (Figure 4A; $p < 0.005$). However, the *lhb* levels were higher in the castrated control fish under L8:D16 than under L16:D8 ($p < 0.05$). Under L8:D16, the *lhb* mRNA levels of castrated males with empty implants were significantly higher than in sham-operated fish in 2008, 2010 and 2011 ($p < 0.05$ in each comparison) (Figure 4A, 5A, 6A, Table 2), but they were far lower than in sham-operated males under L16:D8 in 2008 ($p < 0.005$) and 2011 ($p < 0.05$) (Figure 4A, 6A, Table 2).

In 2008, the treatments with crystalline T (*T cryst*) or 11KA (*11KA*) increased *lhb* mRNA levels of castrated males exposed to L16:D8, but no effect was found in the castrated fish implanted with 0.25% T implant (*T 0.25*) under this photoperiod regime (Figure 4A). The castrated fish treated with 0.5% T (*T 0.50*) in 2011 showed higher *lhb* levels than the castrated control group under L16:D8. However, this stimulatory effect was not observed after treatments with lower dosages of T (0.25% or 0.10%) (Figure 6A).

Compared with castrated fish with empty implants, *lhb* mRNA levels of castrated fish treated with crystalline T implants (*T cryst*) were higher under L8:D16. However, *lhb* levels of castrated fish implanted with *T cryst* were lower than in castrated fish treated with 0.25% T (*T 0.25*) under the same photoperiod in 2008 and 2010 (Figure 4A, 5A, Table 2). There was no difference between castrated control males and the males treated with 11KA implants (*11KA*)

under L8:D16 (Figure 4A). Under L8:D16, the castrated males treated with 0.5%, 0.25% and 0.10% T implants showed lower *lhb* levels than the castrated control males in 2011 (Figure 6A).

Compared to castrated controls kept under L8:D16 in 2010, treatments with 0.25% T or AI both suppressed *lhb* mRNA levels, whereas the combined treatment with 0.25% and AI did not. However, there was no difference between the groups implanted with crystalline T alone or in combination with AI (AI) (Figure 5A).

Compared with castrated control males, there was a significant interaction between *T 0.25* and AI treatments ($p < 0.05$, two-way ANOVA). On the other hand, the crystalline T implant had a clear effect on *lhb* levels ($p < 0.05$), but no interaction was found between *T cryst* and AI treatments ($p = 0.607$, two-way ANOVA)

3.4. *fshb* mRNA levels

In 2007, the control fish (both sham-operated and un-operated) had significantly lower *fshb* levels than the castrated control fish under all photoperiods ($p < 0.005$ in all comparisons) (Figure 3B). Under L11:D13 and L12:D12 the castrated control fish had significantly higher *fshb* levels than the T treated castrated fish ($p < 0.05$), while there was no difference between these treatment groups in fish subjected to longer photoperiods (L13:D11 or L14:D10). The *fshb* mRNA level in castrated control fish was significantly lower under L14:D10 than under L11:D13 ($p < 0.05$) and L12:D12 ($p < 0.05$), i.e. there was a clear decrease as light hours increased (Figure 4B) (Table 2). Castrated males treated with 0.25% T (*T 0.25*) had higher *fshb* mRNA levels under L14:D10 than under L11:D13 and L12:D12 ($p < 0.05$ in all comparisons).

In 2008, 2009-10 and 2011, the mRNA levels of *fshb* were higher in castrated control fish than in sham-operated fish under L8:D16 (Figure 4B, 5B, 6B, Table 2). In 2011, sham-operated males had higher *fshb* mRNA levels than castrated males under long day (Figure 6B), but no significant difference was observed between sham-operated and castrated control males under L16:D8 in 2008 (Figure 4B). All types of T implants (crystalline, 0.50%, 0.25% and 0.10%) resulted in significantly lower *fshb* mRNA levels in castrated males under L8:D16 (Figure 4B, 5B, 6B, Table 2). Furthermore, 0.25% (*T 0.25*) and 0.10% (*T 0.10*) T treatments decreased *fshb* mRNA levels in castrated males under L16:D8 (Figure 4B, 6B, Table 2). However, in 2008, crystalline T treatment increased *fshb* mRNA level in castrated males under L16:D8 (Figure 4B), and 0.50% T treatment had no effect on *fshb* mRNA levels in castrated males under long day in 2011 (Figure 6B).

Treatment with 0.25% T (*T 0.25*) combined with AI resulted in higher *fshb* mRNA levels than 0.25% T (*T 0.25*) alone (Figure 5B). However, we found no difference in *fshb* mRNA levels between castrated fish treated with crystalline T alone, or treated with crystalline T in combination with AI (Figure 5B). An interaction was observed between the effects of AI and T on *fshb* mRNA levels in both 0.25% T and crystalline T experiments ($p < 0.05$ two way ANOVA, in both comparisons).

4. Discussion

Long but not short days stimulated reproduction in the stickleback as seen in kidney weights, KEH and breeding colours, which is in general agreement with previous studies [4, 5, 7, 8]. Under the intermediary photoperiods, the proportion of sham-operated males showing breeding color and elevated KSI values increased with increasing light hours. Interestingly, there was a clear gap in the distribution of the individual KEH values between the immature and mature control fish (Figure 1). This indicates that the sexual maturation response to increasing photoperiod lengths is of an all-or-nothing nature in this species. While this has been proposed before, but has not been demonstrated clearly so far. Most of the studies by Baggerman [5] used the attainment of complete maturation as parameter, which of course would not detect half-mature fish. Borg [8] and Borg and van Veen [6] analysed KEH and ovarian maturation and found that fish that did not mature fully often displayed a very low gonadal activity, but there were also many intermediates, probably since the previous experiments were run for only three weeks compared to six weeks in the present study, so that many fish were likely to still be in the process of maturation.

As expected [14], the *lhb* and *fshb* mRNA levels in sham-operated fish were higher under long day that stimulated breeding than under short day which did not (significant effects 2008, 2011). The *lhb* mRNA levels started to increase already after a few days of exposure to long day [21]. Paradoxically, the mRNA levels of *lhb* and *fshb* in castrated males were higher under short rather than under long day, though the difference was not significant for *fshb* in 2011. This effect was found previously but then for *fshb* alone by Hellqvist et al. [14]. Under intermediate photoperiods, the *fshb* mRNA levels, but not the *lhb* mRNA levels, of the castrated males decreased as the light hours increased from L11:D13 to L14:D10. Steroid-independent effects of photoperiod on GTH secretion are known from studies in mammals [16] and birds [26], i.e. circulating GTH levels are higher under stimulatory than under non-stimulatory photoperiod also in castrated animals not treated with steroids. However, the pattern in the stickleback with higher expression under non-stimulatory than under stimulatory photoperiod is opposite to the observations in mammals and birds, and its biological significance, if any, is not known.

The feedback control of both *lhb* and *fshb* mRNA levels were often influenced by the photoperiod. Negative feedback effects were more marked under short days than under long days, whereas positive feedback could be more obvious under long days (Table 2). Compared to castrated controls, sham-operated fish had significantly lower levels of *lhb* mRNA (negative feedback) under all L8:16D treatments and under the L11:D13 treatment in 2007. In contrast,

the sham-operated males had significantly higher levels of *lhb* mRNA (positive feedback) under both L16:D8 treatments and under L12:D12, L13:D11 and L14:D10. Sham-operated fish had significantly lower levels of *fshb* mRNA (negative feedback) than castrated controls under all L8:16D treatments and significantly higher levels (positive feedback) under LD 16:8 in 2011 (n.s. in 2008). *fshb* mRNA levels were also lower in sham-operated fish than in castrated fish under L11:D13, L12:D12, L13:D11 and L14:D10, but less so with increasing day-length. A similar pattern was seen in the effects of *T cryst* and *11KA*. *T cryst* had always a significantly positive effect on *lhb* mRNA, though much stronger under L16:D8 than under L8:D16. *11KA* had no significant effect on *lhb* under short day, but acted stimulatory under long day. *T cryst* and *11KA* suppressed *fshb* mRNA under LD 8:16. *T cryst* and *11KA* treatments had no significant effect on *fshb* under long day in 2008. This pattern is largely consistent with the previous study by Hellqvist et al. [14].

Treatments with comparatively low doses of T usually had negative feedbacks on *lhb* and *fshb* mRNA levels. However, these negative feedback effects were less prominent under longer photoperiods. *lhb* mRNA was suppressed by low levels of T under L8:D16 in 2008 and 2011, whereas under L16:D8 the effects were non-significant or positive (*T 0.50* in 2011). *fshb* mRNA was suppressed by low levels of T under L8:16D, and by *T 0.10* and *T 0.25* under long day in 2008 (*T 0.25* only) and in 2011. There was no photoperiodic trend for *T 0.25* treatment effects on LH in 2007, whereas *fshb* mRNA in *T 0.25* treated males increased under longer photoperiod and were significantly higher than in castrated controls under L14:D10.

Also in mammals, the photoperiod can influence the feedback effects exerted by sex steroids on the secretion GtHs in the BPG axis [22, 25, 16]. For instance, in castrated male golden hamsters, T treatment suppressed LH and FSH plasma levels under both a stimulatory long day and under a short non-stimulatory photoperiod, but a higher dosage is needed under stimulatory, long day conditions [25]. Similar effects have also been found in some studies on birds [26]. Strong negative feedbacks can suppress gonadal activity to low levels, mediating the inhibitory effects of non-stimulatory photoperiods. A difference between birds and mammals on the hand and the stickleback on the other is that in the latter there is not only a change in strength but also of polarity of the feedback effects. This is maybe not so surprising since in mammals and birds [24] positive feedbacks on the BPG axis only occur at the LH surge stimulating ovulation.

In 2008, 2009-10 and in 2011 different doses of T were used in the same experiment. The effects on gonadotropin mRNA levels were often markedly different. In 2008, *T 0.25* had a negative effect on *lhb* mRNA under L8:D16, whereas *T cryst* had a positive effect. *T 0.10* and *T 0.25* had negative effects on *fshb* mRNA under both L16:D8 and L8:D16 in 2008 and 2011,

whereas *T 0.50* and *T cryst* had no significant effects under L16:D8 (Table 2). Thus, the polarity of feedback effects was to a large extent dependent on the dosage of T. A shift from negative to positive feedback at a certain threshold (different for different photoperiods) could be part of the mechanisms that make sexual maturation in the stickleback an all or nothing response. For feedbacks exerting stabilizing effects, on the other hand, one would rather expect a stimulatory effect by low doses and inhibitory effects of high doses. One of the most studied feedback systems in fishes is the positive effect of steroids on *lhb* synthesis in salmonids where also low steroid levels are effective [12].

Aromatase activity is present in the brain of the stickleback, especially in the hypothalamus and in the pituitary [10]. A role of aromatization in mediating the inhibitory effects of short days has been shown previously [11, 13]. Male sticklebacks treated with AIs (fadrozole or ATD) had higher KEH than controls under L8:D16, whereas no difference was found under L16:D8, where also controls matured [11, 13]. Furthermore, intact males implanted with fadrozole showed higher *lhb* and *fshb* mRNA levels than controls, and also fish treated with ATD had higher *fshb* mRNA levels than controls under short day [13]. However, AI does not influence stickleback maturation [11, 13] or *lhb* and *fshb* under long photoperiod [13]. A role of aromatization in the feedback effects by low levels of T on *lhb* and *fshb* was found in the present study where AI weakened the inhibitory effect of a low dose of T. However, since no difference in *lhb* and *fshb* was found between the castrated fish treated with *T cryst* alone or combined with AI, the stimulatory effect of the high dosage T treatment was not aromatase dependent and therefore is likely to be exerted via androgen receptors. This is consistent with the observation that feedback effects of the non-aromatisable androgen 11KA are similar to *T cryst* both in the present and in a previous study [14]. However, those results were different from studies in salmonids, where aromatizable androgens and estrogens stimulated pituitary LH levels [1, 12].

Since the photoperiodic and androgen feedback effects discussed above were not always similar on *lhb* and *fshb* mRNA levels, the responses of the two types of gonadotrope cells appear to be independent and different in sensitivities. To summarize, the negative feedback effect on both *lhb* and *fshb* mRNA levels under short day conditions could be an important factor in inhibiting sexual maturation. A shift from negative to more positive feedback effects with increasing photoperiods are likely to accelerate maturation under the longer photoperiods. The suppression of both *fshb* and *lhb* by low androgen levels, especially under short day conditions, may inhibit maturation completely unless a rise of androgens above a threshold levels would trigger complete maturation. This may be a component in the control of the all-or-nothing sex maturation in stickleback.

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Figure legends

Figure 1. (A) Distribution of individual values of kidney epithelium height (KEH) in intact and sham-operated males in 2007. Results from all photoperiods (L11:D13, L12:D12, L13:D11 and L14:D10) are used. (B) Proportion of intact and sham-operated fish maturing, i.e. displaying kidney epithelium heights values $> 26 \mu\text{m}$, under different photoperiods in the 2007 experiment.

Figure 2. Plasma testosterone (T) levels in sham-operated (Sham) and castrated (Castr) males and castrated male treated with different dosage of T ($T 0.10 / T 0.25 / T 0.50$) (2011 experiment). Means $\pm$ SEM, Values with different letters differ from each other ($p < 0.05$).

Figure 3. GtHs mRNA levels under different photoperiods in sham-operated (Sham) and castrated (Castr) males, and in castrated males implanted with 0.25% T implants ($T 0.25$) (2007 experiment). (A) *lhb* (B) *fshb*. The numbers above the bars indicate sample numbers. Means $\pm$ SEM are shown. Effects of photoperiod; different letter indicate that similarly treated groups kept under different photoperiods are significantly different ($p < 0.05$). * indicates a significant difference between Castr. and Castr.T ($p < 0.05$).

Figure 4. GtHs mRNA levels in sham-operated (Sham) and castrated (Castr) males, and in castrated males implanted with different dosages of T ($T 0.25 / T \text{cryst}$) or 11-ketoandrostenedione ($11KA$) implants and kept under short day (8L:16D) (solid bar) or long day (16L:8D) (open bar) (2008 experiment). (A) *lhb* (B) *fshb*. The numbers above the bars indicate sample numbers. Means $\pm$ SEM are shown. Values with different letters differ from each other in the same photoperiod ($p < 0.05$).

Figure 5. GtHs mRNA levels under short day (LD 8:16) in sham-operated (Sham) and castrated (Castr) males, and in castrated males implanted with different dosages of T ($T 0.25 / T \text{cryst}$), aromatase inhibitor (*A*) or both ($T 0.25 + A / T \text{cryst} + A$) implants (2010 experiment). (A) *lhb* (B) *fshb*. The numbers above the bars indicate sample numbers. Means $\pm$ SEM are shown. Values with different letters differ from each other ($p < 0.05$).

Figure 6. GtHs mRNA levels in sham-operated (Sham) and castrated (Castr) males, and in castrated males implanted with different dosages of T ($T 0.10 / T 0.25 / T 0.50$) implants and kept under short day (8L:16D) (solid bar) or long day (16L:8D) (open bar) (2011 experiment). (A) *lhb* (B) *fshb*. The numbers above the bars indicate sample numbers. Means $\pm$ SEM are shown. Values with different letters differ from each other in the same photoperiod ($p < 0.05$).

Table 1. Effects of photoperiod, castration and implants on kidney weights.

Year (n)	LD	Operation	Implant(s)	Body weight (g)	KSI (%)	
2007 (35)	11:13	Control ¹	<i>Control</i>	1.83±0.03	0.81±0.12	**
(10)		Castration	<i>Control</i>	1.78±0.04	0.64±0.02	
(20)		Castration	<i>T 0.25</i>	1.80±0.03	0.71±0.06	
(35)	12:12	Control ¹	<i>Control</i>	1.88±0.03	1.21±0.15	**
(10)		Castration	<i>Control</i>	1.91±0.02	0.88±0.09	
(20)		Castration	<i>T 0.25</i>	1.86±0.04	0.76±0.05	
(35)	13:11	Control ¹	<i>Control</i>	1.96±0.04	1.72±0.22	*
(10)		Castration	<i>Control</i>	1.98±0.05	0.64±0.04	
(20)		Castration	<i>T 0.25</i>	1.95±0.03	0.65±0.08	
(35)	14:10	Control ¹	<i>Control</i>	1.99±0.03	2.65±0.16	**
(10)		Castration	<i>Control</i>	1.96±0.06	0.64±0.05	
(20)		Castration	<i>T 0.25</i>	1.91±0.07	0.62±0.03	
2008 (10)	16:8	Sham	<i>Control</i>	1.75±0.06	2.48±0.10	*
(10)		Castration	<i>Control</i>	1.65±0.05	0.65±0.05	
(10)		Castration	<i>T 0.25</i>	1.63±0.06	0.87±0.15	
(10)		Castration	<i>T cryst</i>	1.67±0.05	0.95±0.09	**
(10)		Castration	<i>11KA</i>	1.70±0.05	2.80±0.15	
(10)	8:16	Sham	<i>Control</i>	1.79±0.05	0.76±0.11	
(10)		Castration	<i>Control</i>	1.88±0.06	0.70±0.07	**
(10)		Castration	<i>T 0.25</i>	1.65±0.05	0.92±0.18	
(10)		Castration	<i>T cryst</i>	1.76±0.06	0.82±0.23	
(10)		Castration	<i>11KA</i>	1.71±0.06	2.89±0.23	
2009 (16)	8:16	Sham	<i>Control</i>	1.75±0.07	1.11±0.02	*
-10 (10)		Castration	<i>Control</i>	1.90±0.08	0.70±0.07	
(10)		Castration	<i>T 0.25</i>	1.75±0.07	0.56±0.03	
(10)		Castration	<i>T cryst</i>	1.67±0.06	0.79±0.06	
(10)		Castration	<i>AI</i>	1.92±0.09	0.60±0.05	
(10)		Castration	<i>T 0.25</i>			
			+ <i>AI</i>	1.69±0.09	0.59±0.08	
(10)		Castration	<i>T cryst</i>			
			+ <i>AI</i>	1.69±0.08	0.75±0.09	
2011 (16)	16:8	Sham	<i>Control</i>	1.62±0.04	1.81±0.16	*
(16)		Castration	<i>Control</i>	1.55±0.05	0.56±0.03	
(15)		Castration	<i>T 0.10</i>	1.53±0.04	0.60±0.04	
(15)		Castration	<i>T 0.25</i>	1.48±0.05	0.59±0.04	
(15)		Castration	<i>T 0.50</i>	1.56±0.05	0.79±0.08	
(16)	8:16	Sham	<i>Control</i>	1.55±0.05	0.59±0.02	
(16)		Castration	<i>Control</i>	1.59±0.05	0.62±0.03	
(15)		Castration	<i>T 0.10</i>	1.54±0.05	0.55±0.03	
(15)		Castration	<i>T 0.25</i>	1.53±0.05	0.67±0.04	
(15)		Castration	<i>T 0.50</i>	1.57±0.05	0.68±0.05	

Sample sizes (n), Sham-operated (Sham), KSI (kidney somatic index; Kidney weight/body weight). Mean±SEM are shown. Brackets indicate the values significantly different from each other p<0.05. Implants: *Control* (empty or cacao butter alone), *T cryst* (Crystalline testosterone), *T 0.10/0.25/0.50* (0.10%/0.25%/0.50% T in cacao butter), *11KA* (Crystalline 11-ketoandrostenedione), *AI* (aromatase inhibitor). * p<0.05; ** p<0.001.

¹Controls includes both unoperated and sham-operated fish.

Table 2. Effect of photoperiod, operation and androgen implants on pituitary mRNA levels of *fshb* and *lhb*

Year	Comparison	Photoperiod (L:D)	LH-β feedback [#]	FSH-β feedback [#]
2007	Sham vs. Cast.	11:13	0.15 *	0.04 **
		12:12	9.32 ***	0.07 **
		13:11	53.57 ***	0.32 *
		14:10	2.54 **	0.36 *
	Cast. vs. <i>T0.25</i>	11:13	0.27 *	0.15 *
		12:12	0.29 *	0.26 *
		13:11	0.22 *	0.62 ^{ns}
		14:10	0.21 *	2.06 ^{ns}
2008	Sham vs. Cast.	8:16 / 16:8	0.26 * / 19.51 *	0.05 * / 0.71 ^{ns}
	Cast. vs. <i>T0.25</i>	8:16 / 16:8	0.06 ** / 8.4 ^{ns}	0.02 ** / 0.27 *
	Cast. vs. <i>Tcryst</i>	8:16 / 16:8	5.12 * / 26.33 **	0.02 ** / 2.09 ^{ns}
	Cast. vs. <i>11KA</i>	8:16 / 16:8	1.06 ^{ns} / 20.15 *	0.11 * / 2.11 ^{ns}
2010	Sham vs. Cast.	8:16	0.33 *	0.12 *
	Cast. vs. <i>T0.25</i>	8:16	0.23 *	0.16 **
	Cast. vs. <i>Tcryst</i>	8:16	2.08 *	0.18 *
	Cast. vs. <i>T0.25</i>	8:16	0.83 ^{ns}	0.35 *
	+ <i>AI</i>			
	Cast. vs. <i>Tcryst</i>	8:16	1.86 *	0.23 *
	+ <i>AI</i>			
2011	Cast. vs. <i>AI</i>	8:16	0.32 *	0.68 *
	Sham vs. Cast.	8:16 / 16:8	0.50 * / 5.67 *	0.26 * / 2.88 **
	Cast. vs. <i>T0.10</i>	8:16 / 16:8	0.30 * / 0.39 ^{ns}	0.08 * / 0.14 *
	Cast. vs. <i>T0.25</i>	8:16 / 16:8	0.29 * / 0.34 ^{ns}	0.08 * / 0.12 *
	Cast. vs. <i>T0.50</i>	8:16 / 16:8	0.18 ** / 2.11 *	0.18 * / 0.63 ^{ns}

Castration (Cast.), Sham-operated (Sham)

Feedback[#]: The mRNA levels of sham operated fish or treated castrated fish are compared to the mRNA levels of castrated control fish in the same photoperiod and experiment. The castrated controls are set to 1 and thus ratios <1 indicate negative feedback and ratios >1 positive feedback. Implants: *Control* (empty or cacao butter alone), *Tcryst* (crystalline T), *T 0.10/ 0.25/ 0.50* (0.10%/0.25%/0.50% T in cacao butter), *11KA* (crystalline 11KA), *AI* (AI).

^{ns} not significant; * p<0.05; ** p<0.01; *** p<0.001.

Figure 1.

Figure 2.

Figure 3

Figure 4

Figure 5

Figure 6