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Interaction Between Photoperiod and Gonadal Feedback on *cck* Expressions in Three-spined Stickleback, *Gasterosteus aculeatus*

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Cholecystokinin (CCK) is a peptide hormone that plays crucial roles not only in the digestive system but also in the central nervous system as a neurotransmitter. Recent research indicate that it is involved in the reproductive endocrine system, inducing the release of follicle-stimulating hormone (FSH) in medaka, *Oryzias latipes*. The present study tested whether *cck* expression is involved in the control of maturation in three-spined stickleback (*Gasterosteus aculeatus*). To that end, castrated and sham-operated males were exposed to short-day (8L:16D) or long-day (16L:8D) photoperiods for 3 or 30 days. Pituitary *fsh*β was strongly elevated in castrated fish, particularly under short-day conditions, whereas *lh*β increased only in sham-operated fish after 30 days under long-day conditions. Castration increased hypothalamic *cck*a and *cck*b expression under short days, but decreased *cck*a after long-day exposure for 30 days. Photoperiod influenced whole-brain and hypothalamic *cck*s expression differently. Whole-brain *cck*s levels were generally higher under short days and lower under stimulatory long days, whereas these photoperiodic effects were largely absent in the hypothalamus. These findings suggest that hypothalamic *cck* genes may participate in feedback regulation on the BPG axis of the stickleback; however, FSH secretion and photoperiodic reproductive control likely involve additional factors.

Keywords: Cholecystokinin (CCK), Brain pituitary gonadal axis (BPG), Stickleback, Photoperiod, Feedback, Life below water (SDG14)

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BACKGROUND

Animals synchronize reproduction by integrating environmental cues with internal endocrine

rhythms. Teleosts, or bony fishes, represent the most diverse group of vertebrates. Due to their varied evolutionary histories and ecological adaptations, teleosts utilize a wide range of environmental factors (*e.g.*, photoperiod, salinity, lunar phase, pH, food availability, and social hierarchy) as external signals to regulate reproductive cycles and sexual maturation (Stacey 1984). However, by which mechanisms these external factors specifically regulate reproductive maturation and spawning seasons remains unclear.

In nature, three-spined sticklebacks (*Gasterosteus aculeatus*) usually reproduces in late spring to early summer. Mature males display distinct nuptial coloration, including a bright red throat and iridescent blue eye rings. Sticklebacks can be reliably and rapidly induced to mature under controlled photoperiods: short-day conditions (8 h) suppress sexual maturation, whereas exposure to long-day conditions (16 h) triggers reproductive readiness in immature, winter-conditioned fish within 3-4 weeks (*e.g.*, Borg 1982). The onset of photoperiodic control maturation in stickleback is related to feedback effects on the brain-pituitary-gonadal (BPG) axis. Under short-day conditions, negative feedback on gonadotropins suppresses maturation. A shift toward positive feedback with longer photoperiods, coupled with rising androgens above a threshold, likely drives the all-or-nothing onset of sexual maturation in stickleback (Hellqvist et al. 2008; Shao et al. 2013).

In vertebrates, the reproductive system is mainly regulated by the BPG axis, where gonadotropin-releasing hormones (GnRHs) from hypothalamus trigger pituitary release of FSH and LH, which act on the gonads to produce sex steroids that, in turn, feedback to control the cycle (Goos 1991; Peter et al. 1991). For more than five decades following the discovery of GnRH (Paulson and Gordon 2023), it was universally accepted that this hypothalamic neuropeptide was the common pathway for the regulation of both FSH and LH in pituitary. In vertebrate, there are three main phylogenetic groups of GnRH, which are GnRH1, GnRH2, and GnRH3 (Millar 2005; Okubo and Nagahama 2008). Although GnRH1 is absent in stickleback (O'Brien et al. 2012; Shao et al. 2015), both of GnRH2 (formerly chicken GnRH-II) and GnRH3 (formerly salmon GnRH) have been found in this species (Shao et al. 2015) and at least the latter appears to be involved in the

control of stickleback maturation (Andersson et al. 1995; Shao et al. 2015 2019).

While other substances, such as dopamine, are known to exert inhibitory control on gonadotropin secretion, GnRH was traditionally viewed as the main stimulatory releasing hormone. However, this classical model, with GnRH as the principal hypothalamic factor stimulating the pituitary's release of gonadotropins, has been challenged for the teleosts. In mammals, FSH and LH are secreted from the same gonadotroph cells, whereas in teleosts, they are produced by separate, distinct cell populations (*e.g.*, Hollander-Cohen et al. 2021). This anatomical separation facilitates different regulatory systems, and recent research in medaka and zebrafish now supports a "dual GnRH model." In this model, cholecystokinin (CCK) acts as the primary FSH-releasing hormone driving early gonadal development, while GnRH primarily functions as the LH-releasing hormone, inducing final maturation and ovulation (Kayo et al. 2025).

The present study aimed to investigate whether *cck*s are associated with the photoperiod-induced maturation in three-spined stickleback, and if they participate in BPG-axis feedback regulation. To this end, castrated and sham-operated males were subjected to either short-day (8L:16D) or long-day (16L:8D) photoperiods for 3 or 30 days, after which *cck*s mRNA levels were quantified. Our study suggests that hypothalamic *cck* genes contribute to both positive and negative feedback regulation within the BPG axis, but do not appear to be the primary photoperiodic trigger for sexual maturation in male three-spined sticklebacks.

MATERIALS AND METHODS

Experimental animals and treatments

Adult three-spined stickleback males were caught in brackish water in the Öresund, Southern Sweden, 9-10 December 2018 and transported to Stockholm. They were initially kept under short

day (8L:16D) and c. 17°C in 1200 L aquaria containing 0.5% salinity water which was aerated and filtered. The aquaria were provided with sand and ceramic hiding places and the fish were fed daily with blood worms or mysids. Groups of fish were taken out and castrated or sham operated under 0.025% buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate) anesthesia. On both sides of the abdomen c. 1.5 mm long incisions were made and testis were removed using fine forceps (or not for sham operated fish) and each incision was closed with a suture. When the fish had recovered, they were placed in 200 L aquaria under short day (8L:20D) or long day (16L:8D) under 20°C, where they were kept as above. After the operations 10 castrated and 10 sham operated fish were kept in each 200 L aquarium for 3 days and similar groups for 30 days (Table1). The experiments were carried out in late January-early March 2019.

Table 1. The samples size of each group

Photoperiod	Operation	Period (days)	Sanple size (N)
8L16D	Cstrated	3	10 (5WB+5HP)
	Sam-operated		9 (5WB+4HP*)
	Cstrated	30	9 (5WB+4HP*)
	Sam-operated		9 (5WB+4HP*)
16L8D	Cstrated	3	10 (5WB+5HP)
	Sam-operated		10 (5WB+5HP)
	Cstrated	30	10 (5WB+5HP)
	Sam-operated		10 (5WB+5HP)

WB: Whole brain samples were collected from these specimens for qPCR measurements. HP: hypothalamus and pituitary samples were collected from these specimens for qPCR measurements.
* The tissue sample was damaged during dissection and therefore excluded from the experiment.

Dissection

The fish were euthanized using a 0.025% buffered MS-222. Body and kidney weights were recorded, and the presence of breeding coloration was assessed visually. The completeness of the castrations was inspected and the kidneys were examined under a stereomicroscope to evaluate

maturity, as kidney hypertrophy is a well-known androgen dependent male sexual characteristic in the stickleback (*e.g.*, Borg 1994). Kidney somatic index (KSI) was calculated as (kidney weight/body weight) × 100. Following decapitation and removal of the skullcap, the pituitary gland was carefully separated from the brain. The entire brain and pituitary were then immersed in 500 μL and 100 μL of RNAlater® (Ambion), respectively. Samples were kept at 4°C overnight and subsequently stored at −70°C until analysis. The experiment was conducted with permission from the Stockholm Northern Animal Experiment Ethical Board (N44/14).

Real-time quantitative PCR (q-PCR)

The *cck* gene is found across multiple regions of the fish brain, including the olfactory bulbs, telencephalon, preoptic area, hypothalamus, optic tectum–thalamus, and posterior brain regions (Peyon et al. 1999). In medaka, the cholecystokinin genes (*cck*a and *cck*b) are expressed in both preoptic area (POA) and nucleus ventralis tuberis (NVT) within the hypothalamus that are known to contain hypophysiotropic (pituitary-projecting) neurons (Uehara et al. 2024). Therefore, the present study quantified *cck*a and *cck*b expression in both entire brain (with hypothalamus, but without pituitary) or in dissected hypothalamus alone (Fig. 1). To isolate the hypothalamus, frozen brains were immersed in RNAlater at 4°C, and the diencephalon lobe containing the hypothalamus and thalamus was carefully excised with iris scissors from the whole brain sample under a stereomicroscope. Pituitaries from these fish from which the hypothalamus were sampled were collected to measure mRNA levels of *fsh*β and *lh*β.

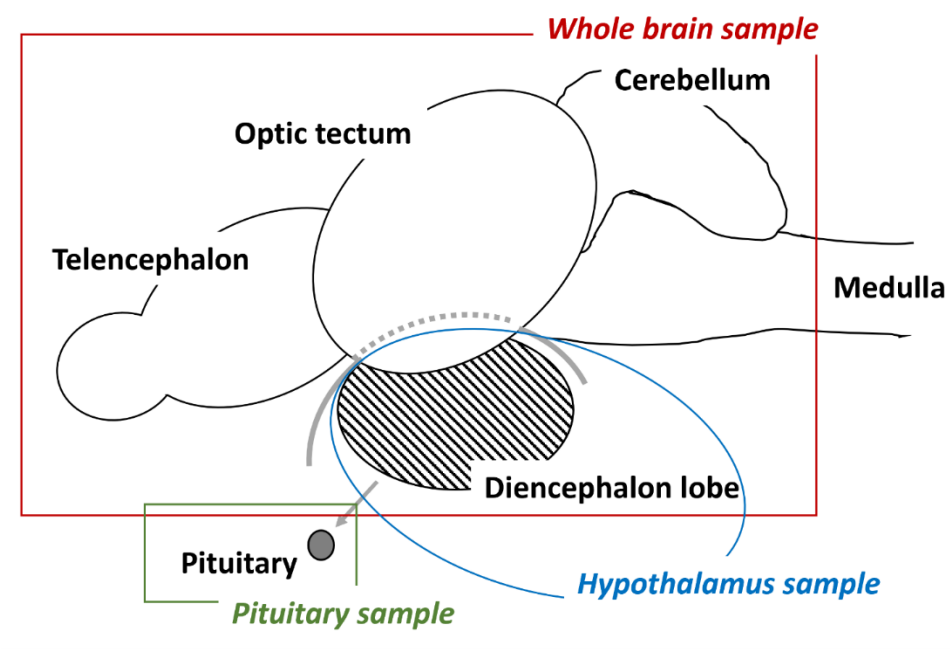

Fig. 1. Schematic diagram showing dissected areas used for expression studies.

Total RNA was extracted from brain or hypothalamus tissue using the RNeasy® Plus Universal Kit (Qiagen, Cat. No. 73404), and from pituitary tissue using the RNeasy® Micro Kit (Qiagen, Cat. No. 74004), following the manufacturers' protocols. Two micrograms of total RNA from each sample were reverse-transcribed into cDNA using the SuperScript IV Reverse Transcriptase (RT) Kit (Invitrogen, Cat. No. 18090200) with RNaseOUT (Invitrogen, Cat. No. 10777019) according to the recommended procedure.

Two *cck*-like gene copies were retrieved from the three-spined stickleback (*G. aculeatus*) genome by Ensembl ortholog/paralog phylogenetic tree tool (Assembly: GAculeatus_UGA_version5, Ensembl Release 115; data version 115.5): ENSGACG00000033105 and ENSGACM00000023919. The gene annotations were based on the Ensembl genebuild, Build 4 released in August 2023, and these *cck* genes were mapped as orthologs by use of Ensembl's comparative genomics pipeline. In order to validate our phylogenetic inferences, all transcript variants were retrieved: ENSGACT00000070369, ENSGACT00000038702 for ENSGACG00000023919; ENSGACT00000060618, ENSGACT00000037713 for ENSGACG00000033105. To verify their classification within the core CCK family, the transcript

variants of these two *cck*-like genes were downloaded along with the other 23 fish *cck* genes (listed in Table S1) for conducting the phylogenetic analysis. The protein-coding regions of these 25 fish *cck* gene sequences were aligned in MACSE v2 software based on amino acid translation of the protein-coding nucleotide sequences (Ranwez et al. 2018). A Neighbor-Joining (NJ) analysis using the Kimura 2-parameter (K2P) distance metric was conducted to reconstruct a genetic distance tree with 10,000 bootstrap replicates in MEGA 11.0.10 (Tamura et al. 2021).

qPCR reactions were carried out in a final volume of 10 μL, containing 40 ng of cDNA, 50 nM of each primer (Table 2), and LightCycler® 480 SYBR Green I Master Mix (Roche). Thermal cycling consisted of an initial step at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Specificity of amplification was confirmed by melting-curve analysis, and representative products were electrophoresed to verify single bands. No-template controls using RNA-free water were included to assess background signal. The geometric mean expression of two reference genes, β-actin (*act*β, DQ018719.1) and ribosomal protein L8 (*rpl8*, ENSGACG00000002035), was used to normalize target gene expression. Relative expression levels were quantified using an efficiency-corrected method (Roche Applied Science, 2001; Weltzien et al. 2005).

Table 2. The primer sequences used in q-PCR (the reference genes were *rpl8* and *act*-β)

Gene name	Nucleotide sequence (5' → 3')		Product size (bp)
ccka	F	ACGACTCATTGACAGGTGTGT	106
E_i = 100%	R	GTGGCTTCAAATGTCCCCAAC	
cckb	F	AACATCACACTCTGCTCACGA	119
E_i = 105%	R	ACGTAGCCGACTTTCAGTGC	
fsh-β (Hellqvist et al., 2004)	F	CATCGAGGTGGAGGTCTGTG	101
E_i = 103%	R	GGGGCTGATGGCTGCTGT	
lh-β (Hellqvist et al., 2004)	F	GGTCACTGCCTCACCAAGGA	103
E_i = 99%	R	GGAGCGCGATCGTCTTGTA	
rpl8 (Shao et al., 2013)	F	CGACCCGTACCGCTTCAAGAA	143
E_i = 101 %	R	GGACATTGCCAATGTTCAGCTGA	
act-β (Shao et al., 2015)	F	ATGGGCCAGAAGGACAGCTA	91
E_i = 96 %	R	TCACAATACCGTGCTCAATGG	

Data Analysis

Since some of the datasets were not normally distributed, the numeric analyses were conducted by nonparametric Kruskal-Wallis test followed by post-hoc analysis with a Dunn test (Olsvik et al. 2005). Pearson correlation analyses were used to determine the possible correlation between different groups (Pascal et al. 2008). Those tests were done using SPSS 26.0.

RESULTS

By day 30, most sham-operated males exposed to long-day (16L8D) conditions displayed breeding coloration and all of them had hypertrophic kidney, whereas no such traits were observed in any other treatment. There was no difference in the body weights among the groups, whereas KSI of the long photoperiod sham-operated fish at 30 days were higher than in all other treatments ($p <$ 0.001 in each of the seven comparisons). No significant weight or KSI differences were observed between treatments in other comparisons (Fig. 2). Additionally, the phylogenetic analysis clearly indicated that the two stickleback *cck*-like genes correspond to the two teleost *cck* gene groups respectively. The average K2P distance within the teleost ccka group is 0.3914 and that within the teleost cckb group is 0.3340; the average K2P distance between these 2 groups is 0.6096 (Fig. 3).

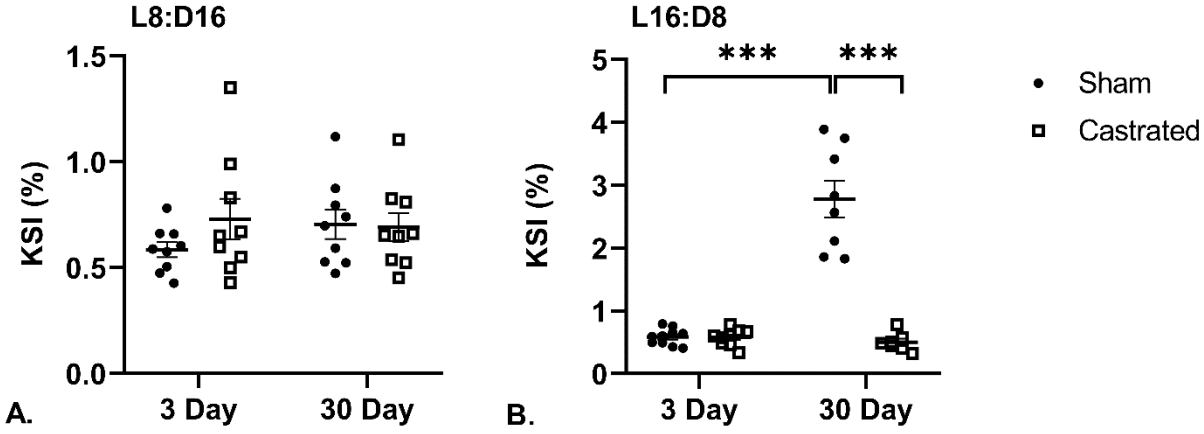

Fig. 2. Kidney somatic index (KSI) of sham-operated and castrated fish under short day (L8:D16, A.) or long day (L16:D8, B.) photoperiod (N = 10 in each group, means ± SE shown, ***$p <$ 0.001).

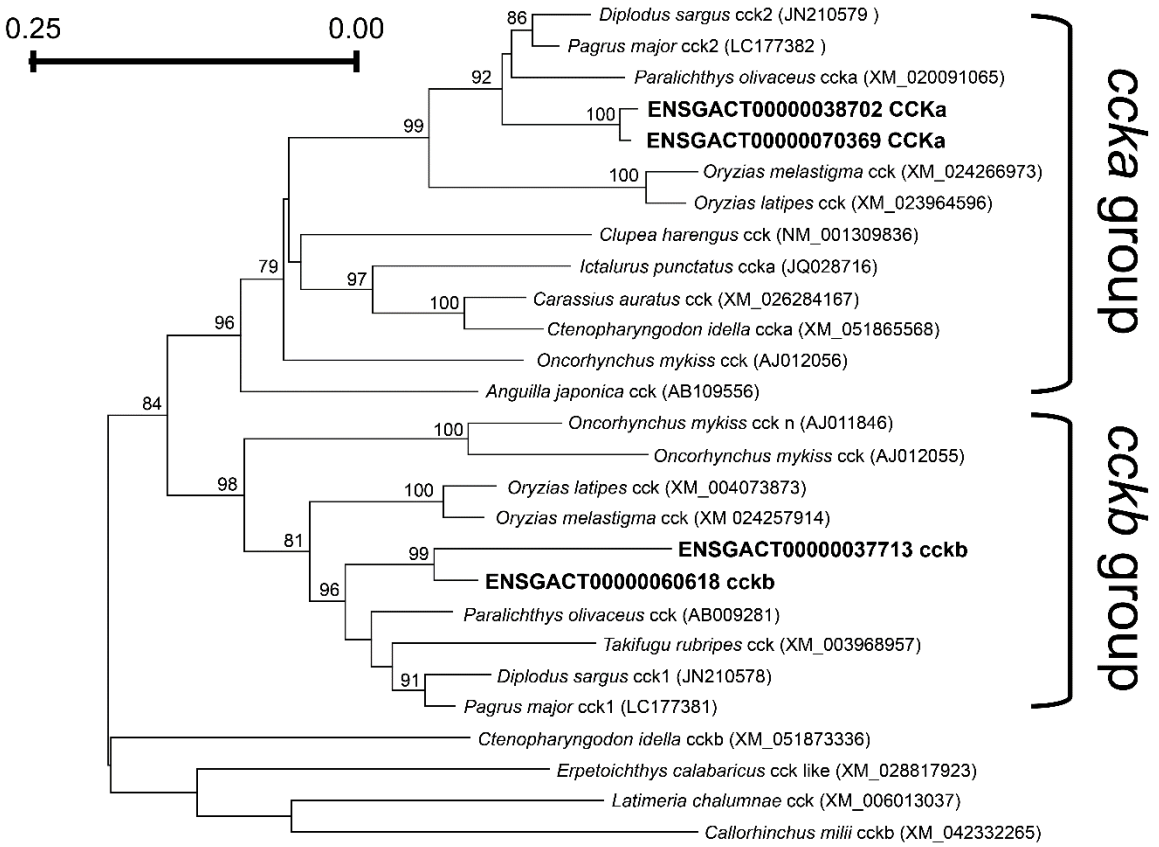

Fig. 3. Neighbor-joining (NJ) tree of fish *cck* gene inferred from dataset comprising 858 aligned base pairs with 10,000 bootstrap replicates. Each terminal node is marked with the scientific name, followed by the gene definition, and the GenBank accession. The four three-spined stickleback *cck* transcript variants are shown in bold. Only nodes with bootstrap values of 70 or higher are labeled.

Brain *cck*s mRNA levels

In the whole brain, *cck*a expression was significantly higher under long-day than short-day conditions (one-way Kruskal-Wallis test, $df = 1$, $p = 0.016$) at 3 days, but no difference was observed between sham-operated and castrated fish within the same photoperiod at this time point (Fig. 4A). In contrast, *cck*b expression was higher under short-day conditions than long-day conditions in both sham-operated and castrated fish (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.009$ in both comparisons) (Fig. 4B). By 30 days, both sham and castrated fish showed significantly higher levels under short-day than under long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.028$ and 0.009, respectively) (Fig. 4C). Similarly, *cck*b expression at 30 days was higher under short-day conditions compared to long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.009$), with no significant difference between sham and castrated fish (Fig. 4D).

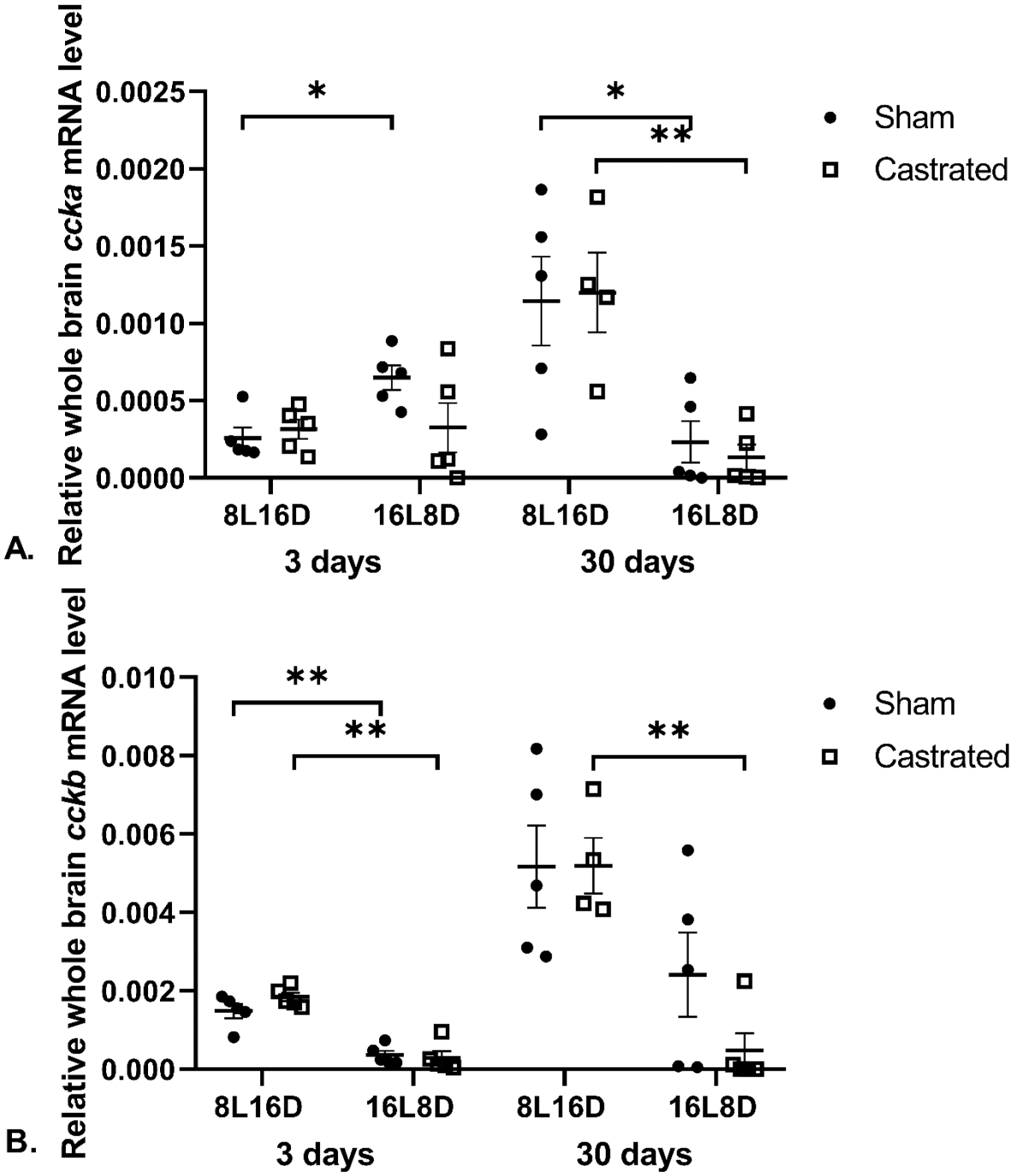

Fig. 4. Whole brain *cck*a (A) and *cck*b (B) mRNA levels in the stickleback males under short day (L8:D16) or long day (L16:D8) photoperiod for 3 days or 30 days (N = 5 in each group, means ± SE shown, *$p < 0.05$; **$p < 0.01$).

Hypothalamic *cck*s mRNA levels

At 3 days, no significant differences were detected in *cck*a mRNA levels between sham-operated and castrated fish under long-day conditions; however, under short-day conditions, castrated fish showed significantly higher *cck*a expression than sham-operated fish (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.05$) (Fig. 5A). At 30 days, castrated fish exhibited reduced *cck*a expression compared to sham fish under long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.047$), whereas no significant differences were observed under short-day conditions (Fig. 5C). For *cck*b, expression remained low at 3 days and did not differ significantly between groups under either photoperiod (Fig. 5B). By 30 days, however, castrated fish showed significantly higher *cck*b expression under short-day than under long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.014$). Under short-day conditions, castrated fish exhibited a marked increase in expression compared to the sham-operated fish (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.021$) (Fig. 5D). Regardless of the photoperiods, the hypothalamic *cck*a and *cck*b mRNA levels of both sham-operated and castrated fish remained consistent throughout the treatment period, as no difference was found between the time points.

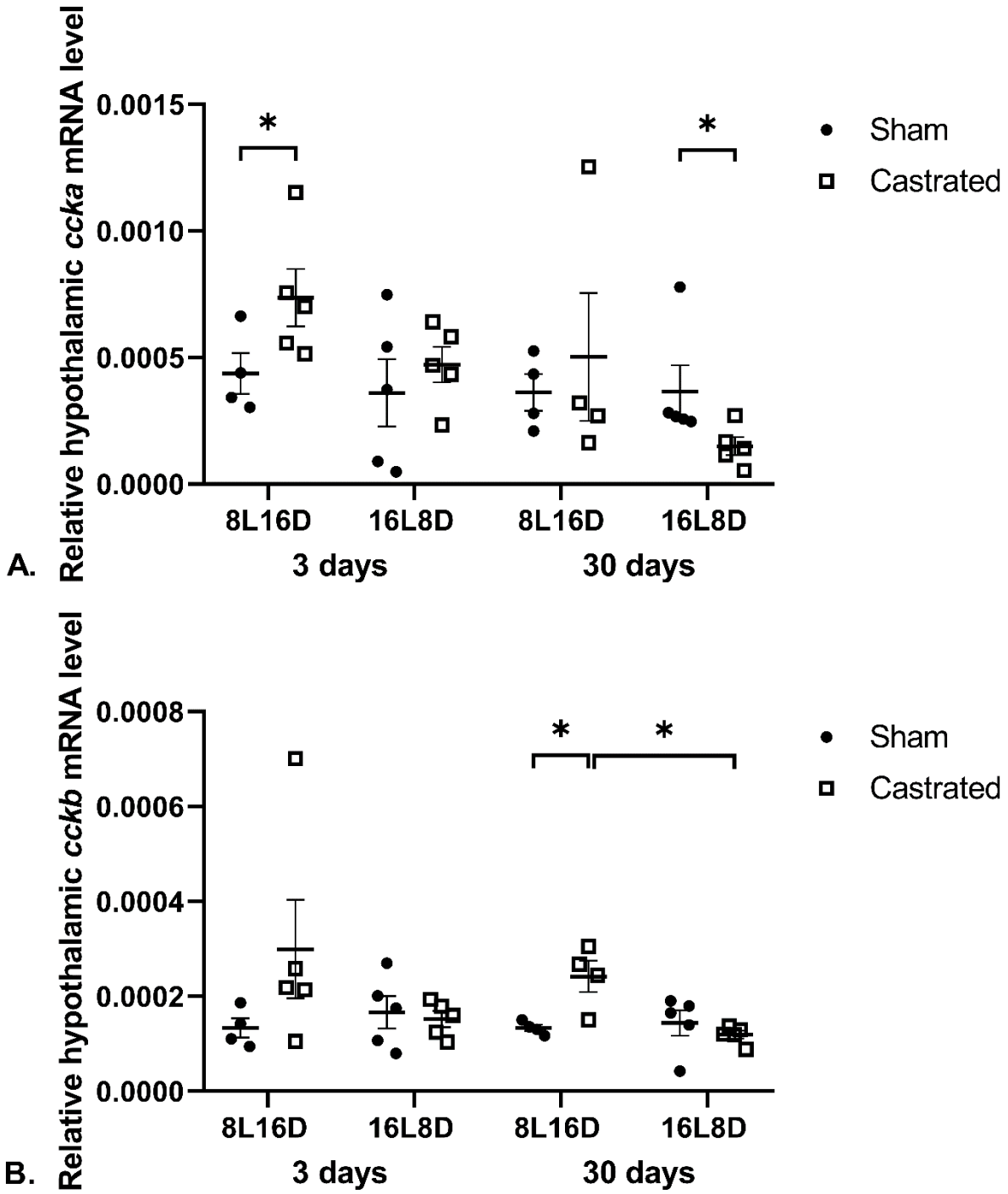

Fig. 5. Hypothalamic *cck*a (A) and *cck*b (B) mRNA levels in the stickleback males under short day (L8:D16) or long day (L16:D8) photoperiod for 3 days (A and B) or 30 days (C and D) (N = 4 - 5 in each group, means ± SE shown, * $p < 0.05$).

Pituitary *fsh*β and *lh*β mRNA levels

In the pituitary, *fsh*β expression was strongly influenced both by gonads and photoperiod. At 3 days, castrated fish exhibited markedly higher *fsh*β expression than sham-operated fish under both long-day (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.009$) and short-day (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.014$) conditions (Fig. 6A). By 30 days, *fsh*β expression in sham-operated fish remained low across both photoperiods, whereas castrated fish showed significantly elevated levels under short-day conditions compared to both sham fish (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.05$) and castrated fish maintained under long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.021$) (Fig. 6C). In contrast, *lh*β expression was low at 3 days, with only a modest increase in castrated fish compared to sham fish under short-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.014$) (Fig. 6B). At 30 days, however, sham-operated fish displayed significantly higher *lh*β expression than at 3 days under long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.009$) (Fig. 6D). Notably, in sham-operated fish, both *fsh*β and *lh*β expression levels were significantly higher at 30 days compared to 3 days under long-day conditions (one-way Kruskal-Wallis test, $d.f. = 1$, $p = 0.009$), whereas no such temporal difference was observed in castrated fish or under short-day conditions. Importantly, Pearson's correlation analysis revealed no significant associations between individual hypothalamic *cck*a or *cck*b expression and pituitary *fsh*β or *lh*β expression in either sham-operated or castrated fish at either point of time (Fig. S1).

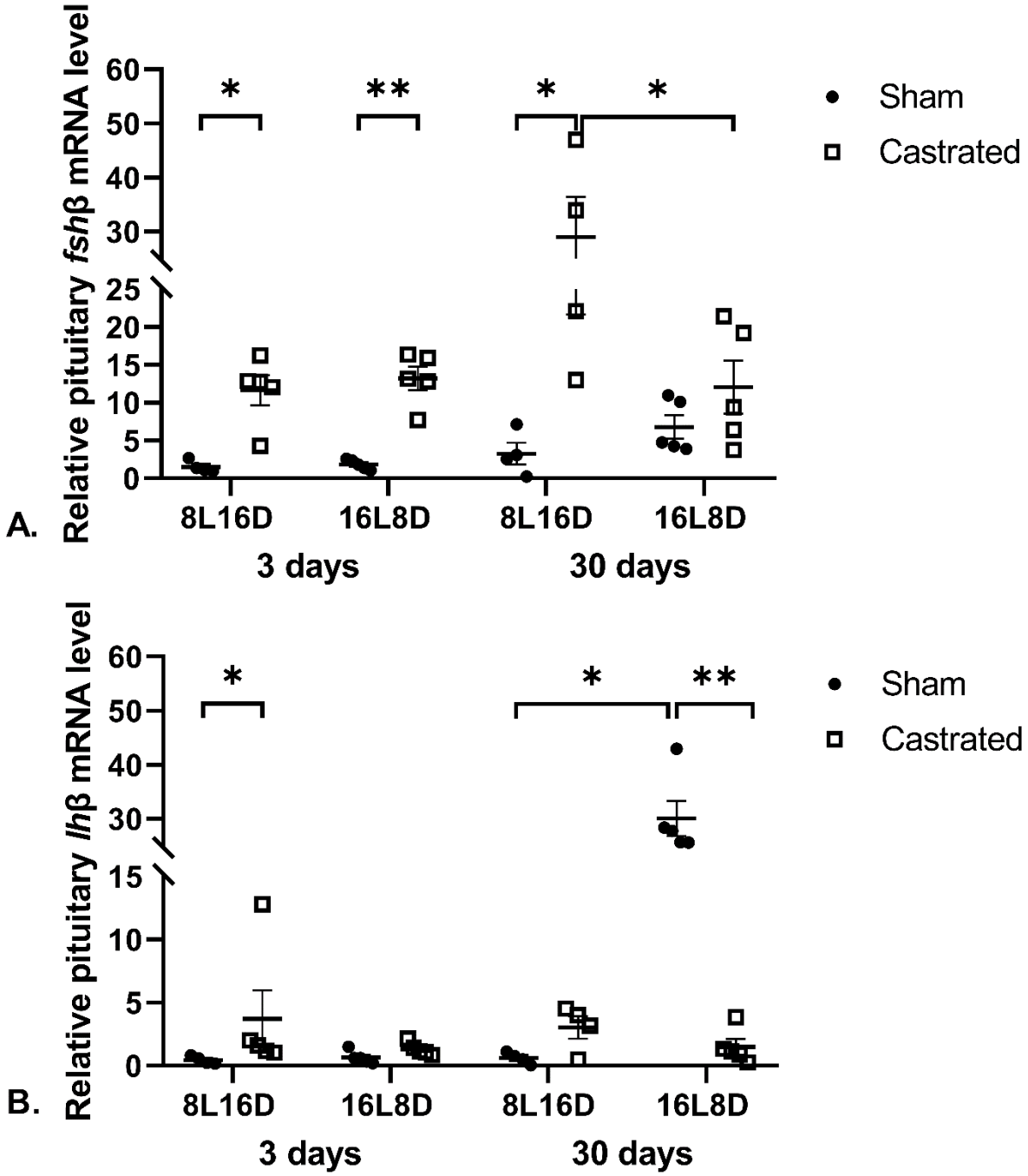

Fig. 6. Pituitary *fsh*β (A) and *lh*β (B) mRNA levels in stickleback males under short day (L8:D16) or long day (L16:D8) photoperiod for 3 days (A and B) or 30 days (C and D) (N = 4 - 5 in each group, means ± SE shown, * $p < 0.05$; ** $p < 0.01$).

DISCUSSION

Recent studies have showed that hypothalamic CCK acts as an FSH-releasing hormone in teleosts (Hollander-Cohen et al. 2023; Uehara et al. 2024; Li et al. 2025). Phylogenetic analyses confirmed that the two identified *cck*-like genes in the stickleback genome correspond to the teleost paralogs *cck*a and *cck*b, and that both are expressed in the hypothalamus, consistent with observations in other teleosts such as medaka. This study examined whether those homologous CCK contributes to the photoperiodic regulation of sexual maturation in the three spined stickleback, a species with a well-characterized reproductive cycle governed by photoperiod and steroid feedback.

Sham operated males kept under long photoperiod for 30 days underwent sexual maturation with all fish displaying hypertrophied kidneys and most also breeding colours, traits absent in other groups. These photoperiod effects and the suppressive effect of castration on secondary sexual characters agrees with earlier studies (*e.g.*, Hellqvist et al. 2008; Shao et al. 2013).

Photoperiod had markedly different effects on the expression of *cck*s in hypothalamus and in whole brains. There was never any significant effect of photoperiod on hypothalamic *cck*a and *cck*b mRNA levels in sham operated fish. *cck*b mRNA levels were, however, higher in castrated fish kept under short than under long photoperiod after 30 days, when the levels in the former were higher than in the corresponding sham operated fish. In whole brains, on the other hand, mRNA levels are higher under short than under long photoperiod in both sham operated and castrated males at 30 days for both peptides and for *cck*b also after 3 days, though not all comparisons are significant. As photoperiodic effects were largely absent from the hypothalamus, the clear effects on the whole brains have been due to changes in other brain areas. The generally higher whole brain *cck* expression under short photoperiod, which inhibits spawning in the stickleback, and the lower expression under the stimulatory long photoperiod, does not support that the regulation of *cck* genes play a part in photoperiodic control of stickleback sexual maturation. Instead, *cck* genes may participate in other photoperiod-dependent neural processes, as reported in mammals (Reuss et al. 1991; Hannibal et al. 2010; Hastings et al. 2018).

After 3 days of treatment, there were no effects of photoperiod on pituitary mRNA levels of *fsh*β or *lh*β, which agrees with similar lack of effects on intact stickleback males found by Shao et al. (2019). The mRNA levels of *lh*β after 30 days were considerably higher in sham operated males kept under long than under short photoperiod, which is consistent with the higher sexual maturation under the former and in agreement with earlier studies on expression of GTHs (Hellqvist et al. 2008; Shao et al. 2013 2019). In the present study, there was no significant effect of photoperiod on *fsh*β in sham operated males, whereas also *fsh*β mRNA levels were found to be higher under long than under short photoperiod by these previous studies.

Hypothalamic *cck* expression demonstrated clear sensitivity to gonadal hormones, though the polarity of this feedback appeared to depend on photoperiods. Under the inhibitory short-day photoperiod, gonadectomy resulted a significant upregulation of hypothalamic *cck*s mRNA levels, paralleling the rise in pituitary *fsh*β expression. This pattern indicates that the gonads exert negative feedback on hypothalamic *cck* gene regulation under non-breeding conditions. Conversely, under stimulatory long-day conditions, castration after 30 days resulted in decreased hypothalamic *cck*a mRNA levels, suggesting a possible shift in feedback polarity from negative to positive during the breeding season, when gonadal steroids are required to maintain reproductive status. This decrease in *cck*a, together with the reduction in *lh*β, is broadly consistent with findings in zebrafish in which CCK receptor knockout suppressed both FSH and LH secretion (Hollander-Cohen et al. 2023), suggesting that CCK may have a broader regulatory role extending beyond FSH alone. Nevertheless, CCK dynamics cannot explain all fluctuations in gonadotropins; for instance, *fsh*β increased shortly after castration (3 days) under long-day conditions without a corresponding change in *cck* expression. This indicates that while *cck* is an integral component of the feedback loop, the photoperiodic control of the BPG axis involves additional factors

CONCLUSIONS

Our data suggest that hypothalamic *cck* genes do participate in photoperiod-dependent feedback regulation on the BPG axis. The parallel rise of *cck* and *fsh*β mRNA after castration under short days implies a potential role in FSH stimulation, though this relationship was inconsistent under long days. Thus, while *cck* is likely involved in the feedback of BPG axis, photoperiodic reproductive control in this species is also influenced by other factors.

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Consent for publication: Not applicable.

Ethics approval consent to participate: All animal care and experiments were done in Stockholm, Sweden, and were followed protocols approved by Stockholm Northern Animal Experiment Ethical

Board.

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Supplementary materials

Table S1. List of 23 fish *cck* genes used for phylogenetic analysis. (download)

Fig. S1. Pearson's correlation analysis between hypothalamic *ccka* or *cckb* and pituitary *fshβ* or *lhβ* gene expressions