Molecular and Cellular Endocrinology 519 (2021) 111056

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Photoperiodic regulation of pituitary thyroid-stimulating hormone and brain deiodinase in Atlantic salmon

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ARTICLE INFO

Keywords:
Photoperiod
Thyroid hormone
Thyroid stimulating hormone
Deiodinase
Growth hormone
Na+/K+-ATPase
Osmoregulation
Smolt
Salmon

ABSTRACT

Seasonal timing is important for many critical life history events of vertebrates, and photoperiod is often used as a reliable seasonal cue. In mammals and birds, it has been established that a photoperiod-driven seasonal clock resides in the brain and pituitary, and is driven by increased levels of pituitary thyroid stimulating hormone (TSH) and brain type 2 iodothyronine deiodinase (DIO2), which leads to local increases in triiodothyronine (T_3). In order to determine if a similar mechanism occurs in fish, we conducted photoperiod manipulations in anadromous (migratory) Atlantic salmon (*Salmo salar*) that use photoperiod to time the preparatory development of salinity tolerance which accompanies downstream migration in spring. Changing daylength from short days (light:dark (LD) 10:14) to long days (LD 16:8) for 20 days increased gill Na^+/K^+-ATPase (NKA) activity, gill NKAα1b abundance and plasma growth hormone (GH) levels that normally accompany increased salinity tolerance of salmon in spring. Long-day exposure resulted in five-fold increases in pituitary *tshβb* mRNA levels after 10 days and were sustained for at least 20 days. *tshβb* mRNA levels in the saccus vasculosus were low and not influenced by photoperiod. Increased daylength resulted in significant increases in *dio2b* mRNA levels in the hypothalamus and midbrain/optic tectum regions of the brain. The results are consistent with the presence of a photoperiod-driven seasonal clock in fish which involves pituitary TSH, brain DIO2 and the subsequent production of T_3, supporting the hypothesis that this is a common feature of photoperiodic regulation of seasonality in vertebrates.

1. Introduction

For temperate animals, environmental seasonal changes in daylength and temperature function as important *zeitgebers* for the seasonal timing of critical life history transitions such as metamorphosis, foraging/hibernation cycles, migration, puberty and reproduction. Not surprisingly, photoperiod, is most often the main signaling pathway for controlling seasonality, having absolute annual consistency. Thus, for anadromous salmonid fish species such as the Atlantic salmon, photoperiod has been found to be the key signal for the timing of the juvenile parr-smolt transformation (Hoar, 1988) and the associated seawards migratory behavior, as well as the onset of puberty, spawning migration and reproduction later in the life cycle (Duston and Bromage, 1988).

However, surprisingly little is known about the mechanisms that control photoperiodism in fishes. In birds and mammals, the local production of triiodothyronine (T_3) in the brain has been identified as a key component of signaling changes in daylength and subsequent hormonal and physiological responses (Dardente et al., 2014). In this mechanism, exposure to long days results in the production of thyroid-stimulating hormone (TSH) in the pars tuberalis of the pituitary gland (Wittkowski et al., 1988), which stimulates increased levels of type 2 iodothyronine deiodinase (DIO2) in the hypothalamus, converting thyroxine (T_4) to the more biologically active triiodothyronine (T_3) (Yoshimura et al., 2003). Lower levels of DIO3, which converts T_3 to less active

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https://doi.org/10.1016/j.mce.2020.111056
Received 15 July 2020; Received in revised form 11 September 2020; Accepted 6 October 2020
Available online 15 October 2020
0303-7207/Published by Elsevier B.V.

compounds, may also promote higher brain levels of T_3 (Yoshimura et al., 2003; Yasuo et al., 2005). T_3 then stimulates hypothalamic signaling, such as increased gonadotropin-releasing hormone (GnRH), that stimulates onset of puberty and reproduction (Viguié et al., 1999). There is evidence that the saccus vasculosus (SV) may play a role in seasonality of fish, as it possesses both TSH and DIO2 and is responsive to changes in daylength (Nakane et al., 2013). Atlantic salmon (*Salmo salar*) have recently been found to have two paralogs of the beta subunit of TSH, *tshβa* and *tshβb* (Fleming et al., 2019). These are present in distinct cells of the pituitary, and transcription of *tshβb* is greatly stimulated in spring, during the downstream migratory phase of juvenile salmon (Fleming et al., 2019). However, the photoperiodic entrainment of pituitary *tshβb* in salmon and other fishes has yet to be investigated. Two paralogs of *dio2* (*dio2a* and *dio2b*) have been found in Atlantic salmon; *dio2b* is present in the circumventricular regions of the hypothalamus, thalamus and optic tectum, and increases in transcription after exposure to increased daylength (Lorgen et al., 2015). This indicates that a pituitary TSH – brain DIO2 axis may be involved in photoperiodic signaling of fish, similar to what occurs in birds and mammals.

As part of their anadromous life history, juvenile salmon undergo preparatory adaptations for downstream migration and seawater entry, known as the parr-smolt transformation or smolting. This transformation occurs in spring and is primarily controlled by changes in daylength (Hoar, 1988). A key part of smolting is the development of salinity tolerance, which is critical for the transition from freshwater to seawater. Increased levels of gill Na^+/K^+-ATPase (NKA, the sodium pump), and more specifically, the seawater isoform (NKAα1b), is of critical importance for increased salinity tolerance (McCormick et al., 2013). The advent of salinity tolerance and other physiological changes during smolting are regulated by several hormones, including growth hormone (GH), insulin like growth factor I (IGF-I) and cortisol (McCormick, 2013). These hormones increase in spring and are regulated by photoperiod, although GH appears to be the most responsive to changes in daylength and may be the keystone hormone for initiation of smolting (Björnsson, 1997).

In the present study we used the well-established photoperiodic control of the parr-smolt transformation as a model system to determine whether a pituitary TSH – brain DIO2 system is involved in the control of seasonality in fish. To do this, we exposed Atlantic salmon to increased daylength for 10 and 20 days and examined critical physiological and endocrine changes that normally occur in smolting. To test our hypothesis, we further measured the transcription of *tshβa, tshβb, dio2b* and *dio3a* in the pituitary and several regions of the brain, as well as in the SV, to determine their potential role in photoperiodic signaling.

2. Materials and methods

2.1. Animals and experimental protocols

On September 16, 2017, juvenile Atlantic salmon (*Salmo salar*) were transferred from the Kensington State Fish Hatchery (Kensington, CT, USA) to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA). Fish were reared in 1.7 m diameter tanks supplied with ambient Connecticut River water at a flow rate of 4 L min^{-1} and provided with supplemental aeration. They were maintained under natural photoperiod and temperature conditions until early February and fed to satiation (Bio-Oregon, Westbrook, ME, USA) using automatic feeders. All experiments were carried out in accordance with U.S.

Geological Survey institutional guidelines and an approved IACUC protocol (LSC-9070).

On February 7, 2018, 64 smolts (13.2–17.2 cm total length) were transferred to four 1 m diameter, light-proofed tanks (16 fish per tank). Tanks were supplied with heated Connecticut River water at a rate of 2 L min^{-1} to maintain a temperature of 10.7–12.1 °C and provided continuous aeration. All fish were exposed to short daylength (SD) (10 L:14D) by 3000 K LED illumination (GE, Boston, MA, USA) connected to an automatic timer to regulate photoperiod. The time of lights on was 07:00 eastern standard time (EST) and lights off was 17:00. Fish were hand-fed twice daily (9:00–10:00 a.m. and 3:00–4:00 p.m.) with a ration equaling 0.5% of their body mass throughout the study.

On February 23, 2018, the daylength in 2 tanks was increased to LD (16 L:8D) while the remaining 2 tanks remained on SD. For the LD group the time of lights on was 04:00 and lights off was 20:00 EST. Eight fish were sampled from each tank 10 and 20 days after changing the photoperiod. Food was withheld for 24 h prior to sampling of fish, which was carried out between 13:00 and 16:00 EST. Fish were anesthetized with MS-222 (200 mg L^{-1}, pH 7.0) and blood was drawn from the caudal vessels into a 1 ml ammonium heparinized syringe. Blood was centrifuged at 3000 g for 5 min at 4 °C and resulting plasma was separated and stored at −80 °C. Gill arches were removed, gill filaments were trimmed from the ceratobranchial cartilage, placed in 1.5 ml microcentrifuge tubes and frozen immediately at −80 °C for Western blot analysis. Four to six gill filaments were placed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C for measurement of NKA activity. The brain was removed and dissected according to neuroanatomical landmarks of the salmonid brain (Bernier et al., 2008) into the following regions: pituitary (Pit), *saccus vasculosus* (SV), hypothalamus (Hypo), preoptic area (POA), and midbrain/optic tectum (MB/OT), placed in microcentrifuge tubes and frozen immediately at −80 °C for mRNA extraction.

2.2. Physiological parameters

Gill NKA activity was determined using a temperature-regulated NADH-linked microplate method (McCormick, 1993). Gill samples were homogenized in 150 μL of SEID (0.1% sodium deoxycholate in SEI buffer, pH 7.3) and centrifuged at 3000 g for 30 s. Ten μl of this homogenate was run in duplicate in the presence and absence of 0.5 mM ouabain. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Both assays were run on a BioTek Synergy 2 spectrophotometer using Gen 5 software (BioTek, Winooski, VT, USA). Plasma GH levels were measured by a radioimmunoassay validated for Atlantic salmon (Björnsson et al., 1994).

2.3. Real-time quantitative PCR assay

Total RNA was extracted from dissected brainregions regions using Tri Regent according to the manufacturer's instructions (Molecular Research Center Inc, Cincinnati, OH, USA). The concentration and purity of RNA was assessed using a Take 3 microvolume plate (BioTek, Winooski, VT, USA) and integrity of total RNA was assessed via gel electrophoresis. After extraction total RNA was reverse transcribed to cDNA using high capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Relative mRNA levels of thyroid stimulating hormone subunit beta (*tshβ*) *a* and *b*, type-II deiodinase b (*dio2b*), and type-III deiodinase a (*dio3a*) were measured in each brain region along with growth hormone (*gh*) in pituitary using quantitative real-time PCR (qPCR) in 96-well reaction plates with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the primers listed in electronic supplementary material. Ten μL reactions contained 10 ng cDNA, 200 nM forward and reverse primers (Table 1), and 5 μL of PowerUp SYBR master mix (Thermo Fisher Scientific, Walltham, MA, USA). Elongation factor 1α (*ef1α*) was used as the reference gene for this study and did not show significant change in relation to treatment. For each assay, amplification efficiencies were verified through a tenfold dilution series and specificity was confirmed through melt curve analysis.

Table 1
Nucleotide sequences of Atlantic salmon primers used for qRT-PCR.

Gene	Sequence (5′-3′)	Reference	Accession no.
tshβa	F: CTCCTTTGCCTGCTCTTCAG R: GGCCAGCTCCTTCATGTTAC	Fleming et al. (2019)	AF060566
tshβb	F: TTGCCGTCAACACCACCAT R: GGGATGATAGACCAGGGAGTG	Fleming et al. (2019)	MG948546
dio2b	F: TCAATGACAACGACCCTGAC R: ACCTGGTTCCATGCTTGTTC	This paper	KP851705
dio3a	F: GGACCCAGAGAGCTCACTTT R: CTCCAGAGGCCCATACGTAG	This paper	DY699231
gh	F: TGTTTCTGCTGATGCCAGTC R: GATGTTGAAGAGCCGTTGGT	This paper	AB462418
ef1α	F: CCTGTGGAAGTTTGAGACTGG R: GAGTCTGCCCGTTCTTTGAG	Johnstone et al., 2011	NM001141909

Table 2
dio2b mRNA levels in Atlantic salmon juveniles exposed to short day (SD) and long day (LD) photoperiods for 10 and 20 days. mRNA levels are expressed relative to *ef1a* and expressed as mean ± standard error (n = 10–12 fish per group). There was no significant effect of photoperiod, day of sampling or interaction in forebrain or saccus vasculosus ($p > 0.43$).

	SD day 10	LD day 10	SD day 20	LD day 20
POA	1.46 ± 0.14	1.82 ± 0.44	1.71 ± 0.38	1.57 ± 0.37
Saccus vasculosus	0.019 ± 0.012	0.012 ± 0.005	0.009 ± 0.002	0.011 ± 0.002

2.4. Immunoblotting

Gill NKAα1b protein abundance was quantified by western immunoblotting analysis as outlined previously (McCormick et al., 2013). Gill tissue was homogenized in 10 vol of SEID (0.1% sodium deoxycholate in SEI buffer, pH 7.3) with Complete Mini protease inhibitor tablets (Roche, Indianapolis, IN, USA). The tissue homogenate was centrifuged at 2000g for 7 min at 4 °C. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Ten μg of supernatant protein was run on a 7.5% SDS-PAGE gel for electrophoretic separation. Two lanes were reserved on each gel for 5 μg of Precision Plus relative molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) and a standard consistent tissue preparation reference to control for blot-to-blot variation. Blotted proteins were exposed to primary antibody rabbit anti-NKAα1b. Resulting blots were digitally imaged and quantified (Syngene PXi, GeneTools, Frederick, MD, USA).

2.5. Statistics

Two-way analysis of variance (2-way ANOVA) was used to examine the impact of photoperiod and time on all parameters. Assumptions of normality and equal variance were met by all parameters. If photoperiod or an interaction of photoperiod and time was significant ($p < 0.05$), then a Tukey's test was used to determine significant differences between short day and long day treatments at each time point.

3. Results

Photoperiod manipulation was conducted on juvenile Atlantic salmon that were large enough to undergo the parr-smolt transformation (>13 cm) and that had previously been reared on normal photoperiod. On March 24, fish were exposed to either short days (light: dark (LD) 10:14) or long days (LD 16:8) and sampled after 10 and 20 days (Fig. 1). There were no mortalities during the study and fish were observed to feed similarly in each group. Body mass increased in both groups from day 10–20 and was not significantly affected by photoperiod treatment (photoperiod effect $p = 0.32$, sampling date $p = 0.001$, two-way ANOVA). Condition factor (mass relative to length), which normally decreases during smolt development, was similar in SD and LD fish after 10 d, but at 20 d, it was significantly lower in LD fish (1.01 ± 0.02) compared to SD fish (1.06 ± 0.01) (photoperiod effect $p = 0.017$; sampling date $p = 0.009$, two-way ANOVA).

There was no difference in gill NKA activity or gill NKAα1b abundance in SD and LD fish after 10 d of photoperiod treatment (Fig. 2a and b), but after 20 d gill NKA activity was 30% higher and gill NKAα1b abundance was 2.0-fold higher in LD fish compared to SD fish (Fig. 2a and b). Pituitary *gh* mRNA levels were not affected by changes in day-length (Fig. 2c). Plasma GH levels were slightly but not significantly higher in the LD group at day 10, but at 20 d were 2.1-fold higher in LD fish compared to the SD group.

tshβa and *tshβb* mRNA levels were more than two orders of magnitude higher in the pituitary than in the MB/OT, preoptic area, hypothalamus and SV (Fig. 3a and b). In the pituitary, *tshβb* mRNA levels of LD fish were 4.3-fold higher than SD fish after 10 d and remained elevated at 20 d (Fig. 4a), while *tshβa* mRNA levels were not significantly affected by photoperiod treatment (Fig. 4b). *tshβa* and *tshβb* mRNA levels in the SV were low compared to the pituitary and were not significantly impacted by photoperiod treatment (Fig. 4c and d).

dio2b mRNA levels were high in the preoptic area and MB/OT, moderate in the hypothalamus, and low in the pituitary and SV (Fig. 3c). *dio2b* mRNA levels in the MB/OT were 80% higher in LD fish compared to SD fish after 10 d and were further elevated (2.8-fold) after 20 d (Fig. 5a). Hypothalamic *dio2b* mRNA levels were also 75% higher in

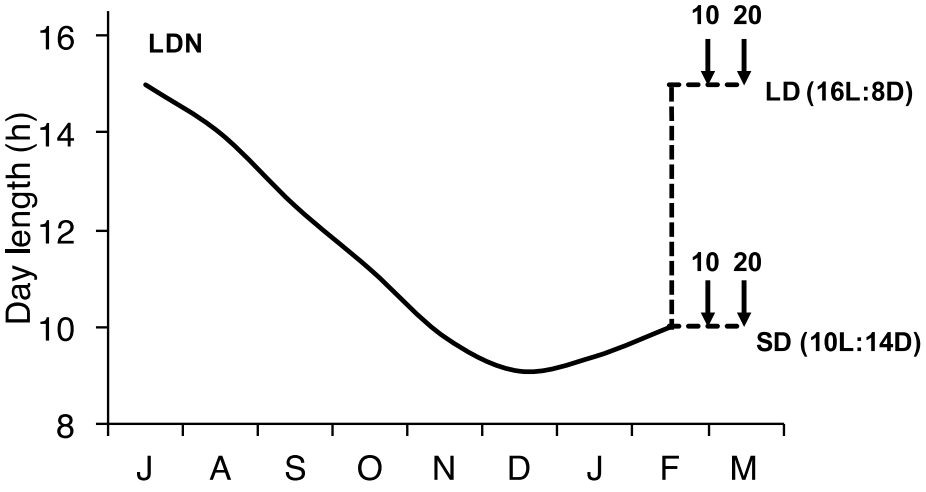

Fig. 1. Photoperiod history and photoperiod treatments used in the present study. Fish were maintained under normal photoperiod conditions (LDN) until Feb. 7 when they were maintained at on short days (10 L:14D). On Feb. 23 half the fish were moved to long days (16 L:8D) in replicate tanks and fish from both groups were sampled after 10 and 20 days. Fish were maintained on seasonally changing water temperature until Feb. 7 when they were kept at 10.7–12.1 °C for the remainder of the experiment.

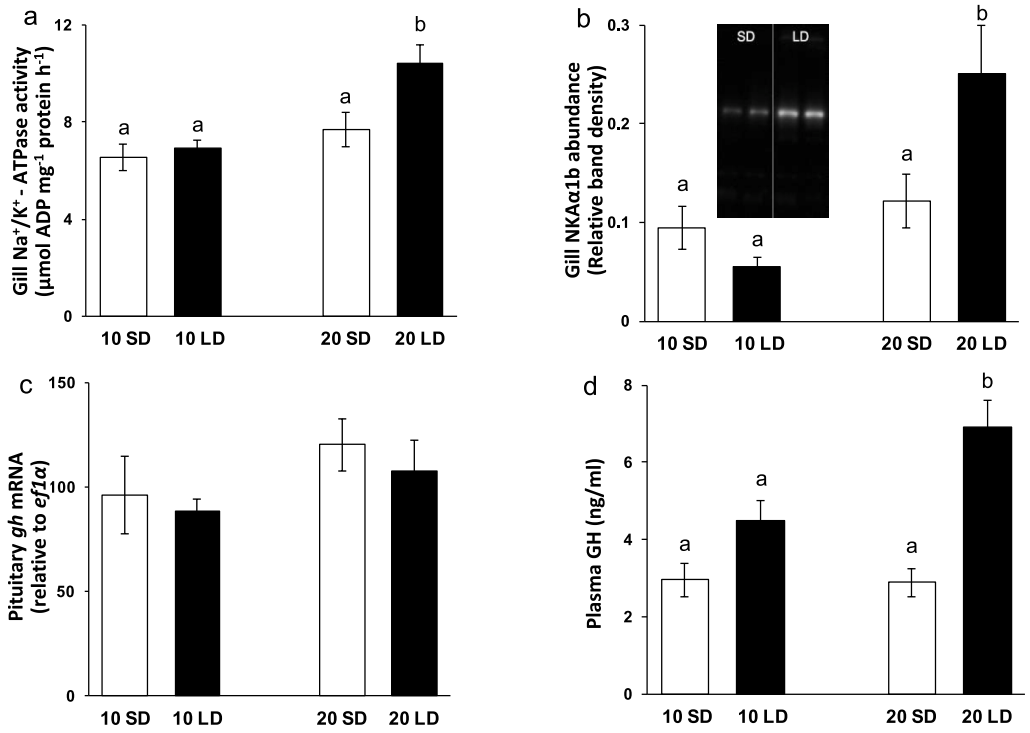

Fig. 2. Gill Na^+/K^+-ATPase (NKA) activity (a), gill NKAα1b abundance (b), pituitary *gh* mRNA levels (c) and plasma growth hormone (GH) levels (d) in juvenile Atlantic salmon exposed to short days (SD, open histograms) and long days (LD, filled histograms) for 10 or 20 days. mRNA values were normalized to housekeeping gene (*ef1a*). Values are mean ± s.e.m. of 12–16 fish. Inset: Representative Western blots of NKAα1b abundance in SD (two left lanes) and LD (two right lanes) sampled on day 20. For gill NKA activity there was a significant effect of day of sampling ($p = 0.00024$), photoperiod ($p = 0.010$) and no significant interaction ($p = 0.052$). For gill NKAα1b abundance there was a significant effect of day of sampling ($p = 0.00034$), no effect of photoperiod ($p = 0.12$) and a significant interaction ($p = 0.0049$). For pituitary *gh* mRNA levels there was no significant day of sampling, photoperiod or interaction ($p > 0.15$). For plasma GH levels there was a significant effect of day of sampling ($p = 0.029$), photoperiod ($p = 0.000002$) and a significant interaction ($p = 0.021$). Values with different letters are significantly different from one another (Tukey's test, $p < 0.05$).

LD fish as day 20 compared to SD fish (Fig. 5b), though these differences were not statistically significant. *dio2b* mRNA levels were not affected by photoperiod treatments in POA or SV (Table 2).

The levels of *dio3a* mRNA were high in the POA and MB/OT and hypothalamus and low in the pituitary and SV (Fig. 3d). Hypothalamic *dio3a* mRNA levels were lower in LD fish relatively to SD fish at both 10 and 20 d, but this effect was not statistically significant (Table 3). There was no significant effect of photoperiod treatment on *dio3a* mRNA levels in the preoptic area, MB/OT, hypothalamus or SV (Table 3).

4. Discussion

In the present study we found a large and sustained increase in pituitary *tshβb* mRNA levels in response to increased daylength. This increase was followed by elevated *dio2b* mRNA levels in the midbrain/optic tectum and hypothalamus in fish exposed to long days for 20 days. Increases in plasma GH, gill NKA activity and gill NKAα1b abundance, all of which are well established indicators of smolt development (Hoar, 1988; McCormick, 2013), also increased with increased daylength. These observations are consistent with a TSH/deiodinase/thyroid hormone system in the pituitary and brain acting as a photoperiod signaling system in fish (Fig. 6), similar to what has been observed in birds and mammals (Nishiwaki-Ohkawa and Yoshimura, 2016).

Two paralogs of TSH, *tshβa* and *tshβb,* appear to be present in most teleosts, a likely outcome of the teleost-specific genome duplication event (Fleming et al., 2019). Previous work in Atlantic salmon indicates that pituitary *tshβa* does not change seasonally, whereas *tshβb* increases in spring coincident with increasing daylength, smolt development and downstream migration (Fleming et al., 2019). We have extended these findings to demonstrate that pituitary *tshβb* is directly responsive to increased daylength. Studies on birds and mammals indicate that increases in pituitary *tshβ* in response to increased daylength can be sustained for several weeks (Hanon et al., 2008; Nakao et al., 2008), similar to what we have observed in Atlantic salmon. In birds and mammals, TSH is found both in the pars distalis as well as the pars tuberalis which is adjacent to the median eminence and third ventricle stalk of the hypothalamus, but only TSH in the pars tuberalis region appears to be responsive to changes in daylength (Hanon et al., 2008). The anatomical position of TSH in the pars tuberalis which is adjacent to the median eminence and the third ventricle is thought to facilitate the 'retrograde' diffusion of TSH to the median eminence. Although fish lack a pars tuberalis, *tshβb* cells are located in the dorsal region of the proximal pars distalis which is immediately adjacent to the pars nervosa of the pituitary stalk (Fleming et al., 2019), a location that may facilitate diffusion of the TSH protein to the hypothalamus where it can act to stimulate production of DIO2.

Increased levels of pituitary *tshβb* occurred within 10 days of increased daylength and were followed by increased *dio2b* mRNA in the MB/OT and hypothalamus at 20 days. These brain regions contain the dorsal and ventral portions of the third ventricle, respectively, where *dio2b* has previously been localized in Atlantic salmon (Lorgen et al., 2015). In birds and mammals, photoperiodic-responsive *dio2* is primarily located in ependymal cells, which are restricted to the ventral region of the third ventricle (Dardente et al., 2014). The presence of elevated levels of *dio2b* in both the MB/OT and hypothalamus suggests that the area of photoperiodic signaling may be more widespread in fish than in birds and mammals. This is consistent with a relatively wide distribution of *dio2b* in the brain of Atlantic salmon, which has been found throughout the inner areas of the third ventricle as well as in the inner regions of the optic tectum (Lorgen et al., 2015). It should also be

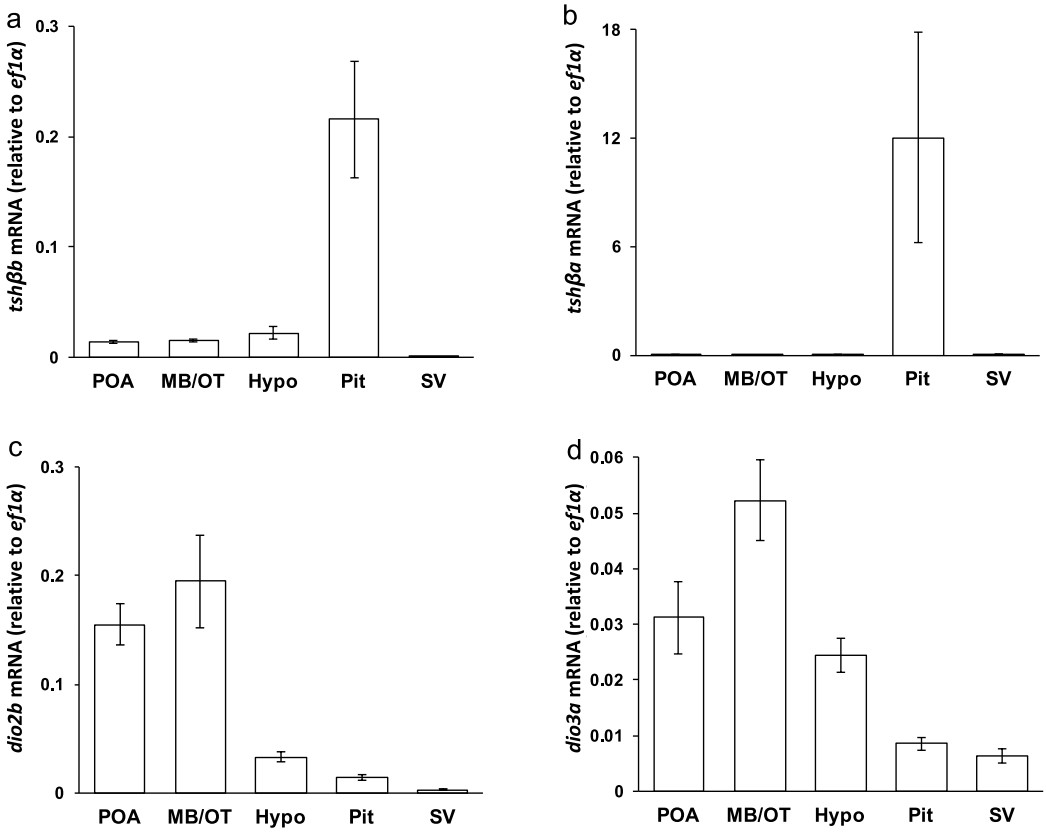

Fig. 3. Tissue distribution of *tshβb* (a), *tshβa* (b), *dio2b* (c) and *dio3a* (d) mRNA levels in brains regions of juvenile Atlantic salmon. mRNA levels were normalized to the reference gene *ef1a*. Values are mean ± s.e.m of 6 fish from the SD group sampled on day 10. Abbreviations: POA, preoptic area; MB/OT, midbrain/optic tectum; Hypo, hypothalamus; Pit, pituitary; SV, saccus vasculosus.

noted that individual cells may originate in the POA and have their terminal endings in the hypothalamus.

Increased brain levels of *dio2b* following increased daylength have been shown to correspond with elevated levels of T_3 (Yasuo et al., 2005; Yoshimura et al., 2003). In some species, increased daylength has also been shown to result in lower levels of *dio3*, which would also promote elevated levels of T_3 by reducing its conversion to reverse T_3 or diiodo-L-thyronine (T_2) (Dardente et al., 2014). Although a trend for lower levels of *dio3a* mRNA were seen in the hypothalamus of long day-treated Atlantic salmon (Table 3), significant differences were not found for this or any other part of the brain, indicating that *dio2b* may be the main pathway for photoperiodic signaling in Atlantic salmon. It should be noted, however, that post-translational control (e.g. ubiquination) may be involved in regulating both DIO2 and DIO3, and it will be of interest to determine if such pathways exist in the photoperiodic clock of fishes.

Previous work has suggested that the saccus vasculosus (SV) may act as a photoperiodic sensor in fish (Nakane et al., 2013). Using an *in situ* hybridization approach, it was found that the abundance of both *tshβ* and *dio2* mRNA (paralogs were not distinguished, although *tshβa* is the likely *tsh* paralog measured based on the primers they used) in the SV were higher under long days than short days (Nakane et al., 2013). In contrast, the present study found high *tshβb* mRNA levels in the pituitary and a robust response to increased daylength, whereas *tshβb* and *dio2b* mRNA levels in the saccus vasculosus were low and did not show a detectable change in response to changes in daylength (Fig. 4c and d). It is possible that pituitary and SV *tshβa* influences *dio2a* mRNA levels in the SV, and that this structure is more important for the control of autumnal (short day) reproduction in salmon. In any event, the high levels and strong photoperiodic signal of *tshβb* in the pituitary and *dio2b* mRNA in the brain found in the present study provides evidence that the pituitary *tshβb* is an important contributor to photoperiodic signaling of fish as it is in birds and mammals. In addition to the transcriptional regulation of pituitary *tshβb* demonstrated here, post-translational and secretory control of TSH may also play a role in regulating the photoperiodic response in fish.

Our finding that plasma GH increases in response to increased daylength is consistent with a number of previous studies on Atlantic salmon (McCormick et al., 1995; McCormick et al., 2000; McCormick et al., 2007). Although the current study was limited to 20 days, long day treatments have been shown to elevate plasma GH for as long as 60 days (McCormick et al., 2000). As in the present study, circulating levels of GH have been shown to increase in the absence of changes in *gh* mRNA (Ágústsson et al., 2001), indicating that secretory control is more important than changes in gene expression. The timing of increased plasma GH following exposure to 20 days of long days is consistent with prior elevation of TSH (10 days or earlier), followed by elevated DIO2 which would result in higher T_3 levels. A large number of hypothalamic factors that upregulate GH have been identified in teleosts in general (Canosa et al., 2007), but knowledge of their relative importance in regulating GH in Atlantic salmon is limited. We hypothesize that elevated levels of T_3 in the brain stimulates one or more types of hypothalamic neurons that control pituitary GH secretion, such as through production of growth hormone releasing hormone. Such a scenario would be analogous to the photoperiodic stimulation of seasonal reproduction in mammals, in which brain T_3 stimulated kisspeptin neurons, which then stimulate GnRH production with subsequent stimulation of the pituitary to produce gonadotropic hormones (Simonneaux, 2020). Similar manipulative studies on Atlantic salmon and other fishes will help establish this possible connection between photoperiod signaling, the TSH/DIO pathway and physiological endpoints such as smolting and reproduction.

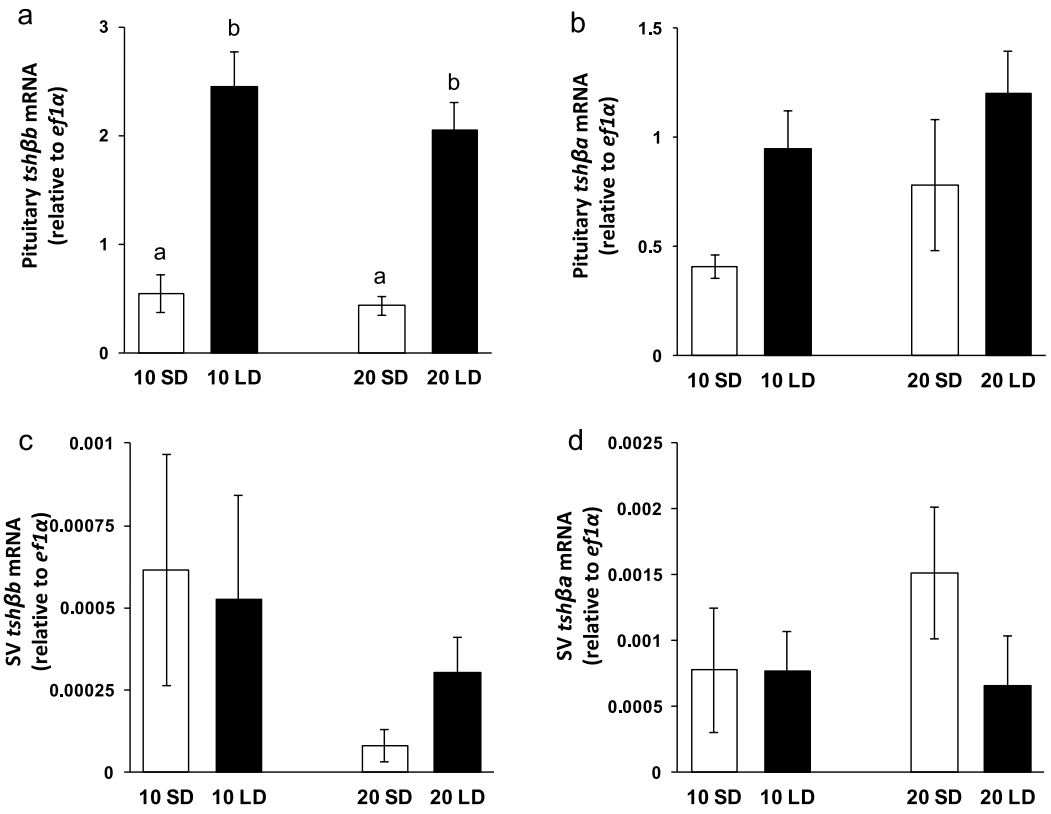

Fig. 4. Pituitary *tshβb* (a), *tshβa* (b) and saccus vasculosus *tshβb* (c), *tshβa* (d) mRNA levels in juvenile Atlantic salmon exposed to shortdays (SD, open histograms) and longdays (LD, filled histograms) for 10 or 20 days. mRNA values were normalized to the reference gene *ef1a* and are mean ± s.e.m of 10–12 fish. For pituitary *tshβb* mRNA levels there was a significant effect of photoperiod ($p < 0.000001$) and no effect of day of sampling or interaction ($p > 0.32$). For pituitary *tshβa* mRNA levels there was a significant effect of photoperiod ($p < 0.043$) and no effect of day of sampling or interaction ($p > 0.18$). For saccus vasculosus *tshβb* and *tshβa* mRNA levels there was no significant effect of photoperiod, day of sampling or interaction ($p > 0.16$). Values with different letters are significantly different from one another (Tukey's test, $p < 0.05$).

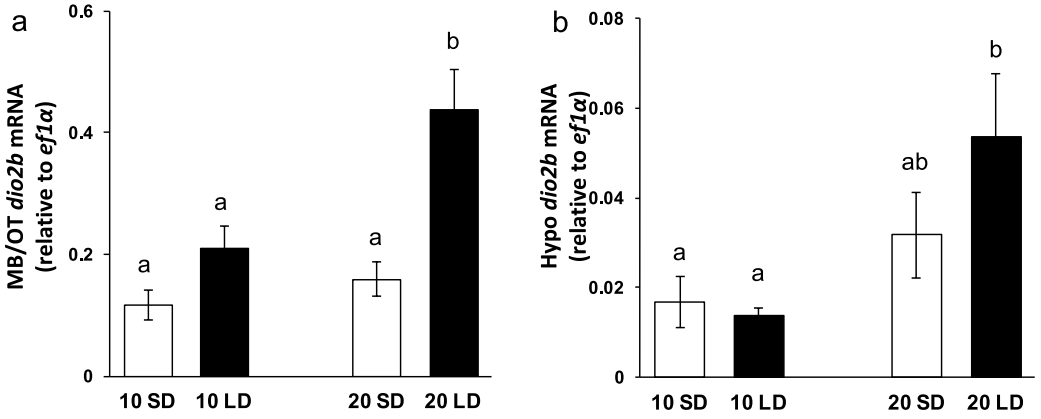

Fig. 5. *dio2b* mRNA levels in preoptic area (POA) (a) and hypothalmus (b) and of juvenile Atlantic salmon exposed to shortdays (SD, open histograms) and longdays (LD, filled histograms) for 10 or 20 days. mRNA values were normalized to housekeeping gene (*ef1a*) and are mean ± s.e.m of 8–10 fish. For POA *dio2b* mRNA levels there was a significant effect of photoperiod ($p = 0.0048$), day of sampling ($p = 0.00018$) and interaction ($p = 0.027$). For hypothalamic *dio2b* mRNA levels there was a significant effect of photoperiod ($p = 0.0049$), and no effect of day of sampling or interaction ($p > 0.17$). Values with different letters are significantly different from one another (Tukey's test, $p < 0.05$).

Table 3
d*io3a* mRNA levels in Atlantic salmon juveniles exposed to short day (SD) and long day (LD) photoperiods for 10 and 20 days. mRNA levels are expressed relative to *ef1a* and expressed as mean ± standard error (n = 10–12 fish per group). There was a significant effect of day of sampling on hypothalamic d*io3a* mRNA levels ($p = 0.034$) and no significant effect of daylength or interaction ($p > 0.35$). There was no significant effect of photoperiod, day of sampling or interaction in preoptic area, forebrain or saccus vasculosus ($p > 0.21$).

	SD day 10	LD day 10	SD day 20	LD day 20_
Hypothalamus	0.232 ± 0.085	0.150 ± 0.022	0.494 ± 0.198	0.373 ± 0.117
MB/OT	0.106 ± 0.029	0.115 ± 0.021	0.101 ± 0.014	0.119 ± 0.017
Preoptic Area	0.024 ± 0.003	0.023 ± 0.003	0.024 ± 0.003	0.026 ± 0.003
Saccus vasculosus	0.033 ± 0.019	0.025 ± 0.012	0.014 ± 0.004	0.012 ± 0.001

We observed that gill NKA activity increased after 20 days of increased daylength, concurrent with elevated plasma GH. This ion transport enzyme is critical to development of salinity tolerance, which is a key component of smolt development, having an obvious adaptive value for an animal migrating from freshwater to seawater. Increased gill NKA activity in smolts has been shown to result primarily from increased abundance of NKAα1b (McCormick et al., 2013), known as the 'SW isoform' of NKA, which also increased following long day treatment in the current study. Prior research has shown that this isoform is under control of both GH and cortisol (McCormick, Regish, O'Dea et al., 2008) (Tipsmark and Madsen, 2009), which have an important interaction to control osmoregulation. GH can 'sensitize' the interrenal to produce more cortisol in the presence of ACTH (Young, 1988), as well as increasing cortisol receptors in the gill (Shrimpton et al., 1995). Although both GH and cortisol have been shown to increase following exposure to increased daylength, plasma GH levels generally increase earlier and more robustly, indicating that GH may be the critical first endocrine signaling pathway for initiating osmoregulatory changes during smolt development (McCormick et al., 2000). GH may also have a variety of other effects on metabolism, growth and behavior during smolt development (Björnsson, 1997).

This results presented here are consistent with the hypothesis that pituitary TSH/brain DIO2 is involved in photoperiodic signaling in fish. In mammals, pineal-derived melatonin is important in transmitting photoperiodic information to TSH cells of the pars tuberalis (Dardente et al., 2010), and though the role of melatonin in seasonality of fish is uncertain (Falcon et al., 2007), it would be of valuable to determine if melatonin has a prominent role in pituitary TSH production. There is strong evidence for the presence of deep brain photoreceptors in birds that signal photoperiodic information to the pituitary (Kang et al., 2010), and it will be of interest to examine whether the brains of fish also contains photoreceptors and is directly responsive to changes in day-length. Many other aspects of the TSH/DIO2 photoperiod signaling of fish also remains to be uncovered, such as the localization and control of TSH and thyroid hormone receptors and their downstream signaling pathways. Manipulation of the system with TSH, thyroid hormones and receptor inhibitors will provide more direct evidence of the involvement of TSH, DIO and thyroid hormones in photoperiodic control of

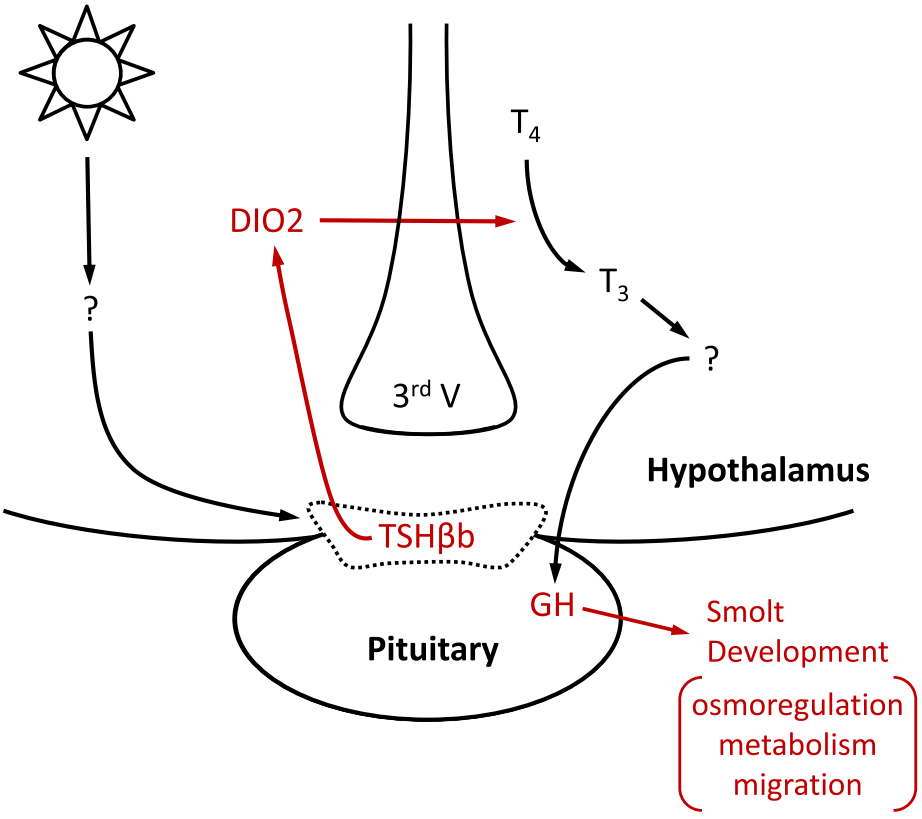

Fig. 6. Proposed scenario for the photoperiodic signaling in Atlantic salmon and other fishes. Items in red are those stimulated by increased daylength as revealed by the present study. The light-sensing pathway for photoperiodic control of TSHBb in fishes has yet to be established. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

seasonality in fish. Our work on smolt development presents a model system for examining photoperiodism in fish, as well as opening up other avenues for studying photoperiod control of reproduction and other critical life history stages of fish.

Credit authorship statement

Shotaro Irachi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – orginal draft, Writing – review & editing, Visualization, Supervision, Project Administration, Funding acquisition. **Daniel J. Hall:** Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Mitchell S. Fleming:** Methodology, Writing – review & editing. **Gersende Maugars:** Methodology, Writing – review & editing. **Björn Thrandur Björnsson:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Sylvie Dufour:** Methodology, Writing – review & editing. **Katsuhisa Uchida:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Stephen D. McCormick:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project Administration, Funding acquisition.

Funding

SI received financial support from the Tobitate! (Leap for Tomorrow) Young Ambassador Program Fellowship, sponsored by the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Declaration of competing interests

The authors declare there are no competing interests that could be, perceived as prejudicing the impartiality of this article.

Data accessibility

All data collected as part of this work are directly presented in the text, tables and figures of the manuscriptl. All real-time PCR primers are made available in Table 1 alongside the relevant NCBI GenBank accession numbers.

Acknowledgements

We thank Amy Regish, Ciaran Shaughnessy, Jessica Norstog, Diogo Ferreira-Martins and Lian Guo for their help in sampling fish. Amy Regish also trained SI in preparation of western blots. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

References

Ágústsson, T., Sundell, K., Sakamoto, T., Johansson, V., Ando, M., Björnsson, B.T., 2001. Growth hormone endocrinology of Atlantic salmon (*Salmo salar*): pituitary gene expression, hormone storage, secretion and plasma levels during parr-smolt transformation. J. Endocrinol. 170, 227–234.

Bernier, N.J., Alderman, S.L., Bristow, E.N., 2008. Heads or tails? Stressor-specific expression of corticotropin-releasing factor and urotensin I in the preoptic area and caudal neurosecretory system of rainbow trout. J. Endocrinol. 196, 637–648.

Björnsson, B.T., Taranger, G.L., Hansen, T., Stefansson, S.O., Haux, C., 1994. The interrelation between photoperiod, growth hormone, and sexual maturation of adult atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 93, 70–81.

Björnsson, B.T., 1997. The biology of salmon growth hormone: from daylight to dominance. Fish Physiol. Biochem. 17, 9–24.

Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. Gen. Comp. Endocrinol. 151, 1–26.

Dardente, H., Wyse, C.A., Birnie, M.J., Dupre, S.M., Loudon, A.S., Lincoln, G.A., Hazlerigg, D.G., 2010. A molecular switch for photoperiod responsiveness in mammals. Curr. Biol. 20, 2193–2198.

Dardente, H., Hazlerigg, D.G., Ebling, F.J., 2014. Thyroid hormone and seasonal rhythmicity. Front. Endocrinol. 5, 19.

Duston, J., Bromage, N., 1988. The entrainment and gating of the endogenous circannual rhythm of reproduction in the female rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. 164A, 259–268.

Falcon, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol. Metabol. 18, 81–88.

Fleming, M.S., Maugars, G., Lafont, A.G., Rancon, J., Fontaine, R., Nourizadeh-Lillabadi, R., Weltzien, F.A., Yebra-Pimentel, E.S., Dirks, R., McCormick, S.D., Rousseau, K., Martin, P., Dufour, S., 2019. Functional divergence of thyrotropin beta-subunit paralogs gives new insights into salmon smoltification metamorphosis. Sci. Rep. 9, 4561.

Hanon, E.A., Lincoln, G.A., Fustin, J.M., Dardente, H., Masson-Pevet, M., Morgan, P.J., Hazlerigg, D.G., 2008. Ancestral TSH mechanism signals summer in a photoperiodic mammal. Curr. Biol. 18, 1147–1152.

Hoar, W.S., 1988. The physiology of smolting salmonids. In: Hoar, W.S., Randall, D. (Eds.), Fish Physiology, Vol. XIB. Academic Press, New York, pp. 275–343.

Johnstone, K.A., Lubieniecki, K.P., Koop, B.F., Davidson, W.S., 2011. Expression of olfactory receptors in different life stages and life histories of wild Atlantic salmon (Salmo salar). Molecular Ecology 2011 (20), 4059–4069. https://doi.org/10.1111/j.1365-294X.2011.05251.x.

Kang, S.W., Leclerc, B., Kosonsiriluk, S., Mauro, L.J., Iwasawa, A., El Halawani, M.E., 2010. Melanopsin expression in dopamine-melatonin neurons of the premammillary nucleus of the hypothalamus and seasonal reproduction in birds. Neuroscience 170, 200–213.

Lorgen, M., Casadei, E., Krol, E., Douglas, A., Birnie, M.J., Ebbesson, L.O.E., Nilsen, T.O., Jordan, W.C., Jorgensen, E.H., Dardente, H., Hazlerigg, D.G., Martin, S.A.M., 2015. Functional divergence of type 2 deiodinase paralogs in the atlantic salmon. Curr. Biol. 25, 936–941.

McCormick, S.D., 1993. Methods for non-lethal gill biopsy and measurement of Na+,K+-ATPase activity. Can. J. Fish. Aquat. Sci. 50, 656–658.

McCormick, S.D., Björnsson, B.T., Sheridan, M.A., Eilertson, C., Carey, J.B., O'Dea, M., 1995. Increased daylength stimulates plasma growth hormone and gill Na+,K+-ATPase in Atlantic salmon (*Salmo salar*). J. Comp. Physiol. 165, 245–254.

McCormick, S.D., Moriyama, S., Björnsson, B.T., 2000. Low temperature limits photoperiod control of smolting in Atlantic salmon through endocrine mechanisms. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278, R1352–R1361.

McCormick, S.D., Shrimpton, J.M., Moriyama, S., Björnsson, B.T., 2007. Differential hormonal responses of Atlantic salmon parr and smolt to increased daylength: a possible developmental basis for smolting. Aquaculture 273, 337–344.

McCormick, S.D., Regish, A., O'Dea, M.F., Shrimpton, J.M., 2008. Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na+,K+-ATPase activity and isoform mRNA levels in Atlantic salmon. Gen. Comp. Endocrinol. 157, 35–40.

McCormick, S.D., 2013. Smolt physiology and endocrinology. In: McCormick, S.D., et al. (Eds.), Euryhaline Fishes. Academic Press, Amsterdam, pp. 199–251.

McCormick, S.D., Regish, A.M., Christensen, A.K., Björnsson, B.T., 2013. Differential regulation of sodium-potassium pump isoforms during smolt development and seawater exposure of Atlantic salmon. J. Exp. Biol. 216, 1142–1151.

Nakane, Y., Igegami, K., Iigo, M., Ono, H., 2013. The saccus vasculosus of fish as a sensor of seasonal changes in day length. Nat. Commun. 4, 2108. https://doi.org/10.1038/ncomms3108.

Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y., Kageyama, S., Uno, Y., Kasukawa, T., Iigo, M., Sharp, P.J., Iwasawa, A., Suzuki, Y., Sugano, S., Niimi, T., Mizutani, M., Namikawa, T., Ebihara, S., Ueda, H. R., Yoshimura, T., 2008. Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 452, 317–322.

Nishiwaki-Ohkawa, T., Yoshimura, T., 2016. Molecular basis for regulating seasonal reproduction in vertebrates. J. Endocrinol. 229, R117–R127.

Shrimpton, J.M., Devlin, R.H., Mclean, E., Byatt, J.C., Donaldson, E.M., Randall, D.J., 1995. Increases in gill corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon (*Oncorhynchus kisutch*) treated with growth hormone and placental lactogen. Gen. Comp. Endocrinol. 98, 1–15.

Simonneaux, V., 2020. A Kiss to drive rhythms in reproduction. Eur. J. Neurosci. 51, 509–530.

Tipsmark, C.K., Madsen, S.S., 2009. Distinct hormonal regulation of Na+,K+-ATPase genes in the gill of Atlantic salmon (*Salmo salar* L.). J. Endocrinol. 203, 301–310.

Viguié, C., Battaglia, D.F., Krasa, H.B., Thrun, L.A., Karsch, F.J., 1999. Thyroid hormones act primarily within the brain to promote the seasonal inhibition of luteinizing hormone secretion in the Ewe. Endocrinology 140, 1111–1117.

Wittkowski, W., Bergmann, M., Hoffmann, K., Pera, F., 1988. Photoperiod-dependent changes in TSH-like immunoreactivity of cells in the hypophyseal pars tuberalis of the Djungarian hamster, *Phodopus sungorus*. Cell Tissue Res. 251, 183–187.

Yasuo, S., Watanabe, M., Nakao, N., Takagi, T., Follett, B.K., Ebihara, S., Yoshimura, T., 2005. The reciprocal switching of two thyroid hormone-activating and -inactivating enzyme genes is involved in the photoperiodic gonadal response of Japanese quail. Endocrinology 146, 2551–2554.

Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., Hirunagi, K., Ebihara, S., 2003. Light-induced hormone conversion of T_4 to T_3 regulates photoperiodic response of gonads in birds. Nature 426, 178–181.

Young, G., 1988. Enhanced response of the interrenal of coho salmon (*Oncorhynchus kisutch*) to ACTH after growth hormone treatment in vivo and in vitro. Gen. Comp. Endocrinol. 71, 85–92.