



Effects of a temperature rise on melatonin and thyroid hormones during smoltification of Atlantic salmon, *Salmo salar*

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Abstract

Smoltification prepares juvenile Atlantic salmon (*Salmo salar*) for downstream migration. Dramatic changes characterize this crucial event in the salmon's life cycle, including increased gill Na⁺/K⁺-ATPase activity (NKA) and plasma hormone levels. The triggering of smoltification relies on photoperiod and is modulated by temperature. Both provide reliable information, to which fish have adapted for thousands of years, that allows deciphering daily and calendar time. Here we studied the impact of different photoperiod (natural, sustained winter solstice) and temperature (natural, ~ +4° C) combinations, on gill NKA, plasma free triiodothyronine (T3) and thyroxine (T4), and melatonin (MEL; the time-keeping hormone), throughout smoltification. We also studied the impact of temperature history on pineal gland MEL production in vitro. The spring increase in gill NKA was less pronounced in smolts kept under sustained winter photoperiod and/or elevated temperature. Plasma thyroid hormone levels displayed day–night variations, which were affected by elevated temperature, either independently from photoperiod (decrease in T3 levels) or under natural photoperiod exclusively (increase in T4 nocturnal levels). Nocturnal MEL secretion was potentiated by the elevated temperature, which also altered the MEL profile under sustained winter photoperiod. Temperature also affected pineal MEL production in vitro, a response that depended on previous environmental acclimation of the organ. The results support the view that the salmon pineal is a photoperiod and temperature sensor, highlight the complexity of the interaction of these environmental factors on the endocrine system of *S. salar*, and indicate that climate change might compromise salmon's time “deciphering” during smoltification, downstream migration and seawater residence.

Keywords Atlantic salmon · Smoltification · Temperature · Thyroid hormones · Melatonin

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Abbreviations

NKA	Na ⁺ /K ⁺ -ATPase activity
T4	Thyroxine
T3	Triiodothyronine
NPT	Natural photoperiod and temperature
WPT	Winter photoperiod and natural temperature
NPT+	Natural photoperiod and increased temperature
WPT+	Winter photoperiod and increased temperature
MEL	Melatonin
LD	Light:Dark
GH	Growth hormone
PRL	Prolactin

Introduction

Fish have developed light and temperature detection systems that allow orientation in space and time and regulation of metabolism, physiology and behavior. Light detection is performed by the retina, pineal organ, deep brain photoreceptors and skin (Falcón 1999; Falcón and Zohar 2018). Temperature detection is performed by neurons located in the pineal organ, brain, lateral line and spinal cord (Nisembaum et al. 2015 and refs). The concomitant action of light and temperature in the photoreceptor cells of the pineal organ regulates the rhythmic secretion of the time-keeper hormone melatonin (MEL) (Falcón 1999, 2007; Falcón and Zohar 2018). The alternation of light [L] and dark [D] phases of the 24 h cycle [LD cycle] dictates the duration of the melatonin surge, while the amplitude of the melatonin surge reflects the ambient temperature, a response that depends on the thermal preference of the species (Cazaméa-Catalan et al. 2013, 2014; Falcón 1999). Thus, the daily and seasonal variations of light and temperature shape the melatonin oscillation, providing a reliable indication of daily and calendar time. In turn melatonin regulates the daily and annual timing of a number of important physiological parameters, including the control of plasma glucose and electrolytes levels, cell division cycle, growth, hormonal regulations, feeding, and behavior (locomotor activity, vertical and horizontal migration, schooling, skin pigmentation, reproduction) (Falcón et al. 2007; Falcón et al. 2010; Falcón and Zohar 2018). For these reasons, the possible involvement of melatonin in the control of salmonid smoltification has been suggested.

Smoltification is a crucial and precisely timed event in the salmon's life cycle; it consists of dramatic changes at all levels of organization that conditions juveniles for downstream migration, seawater entry and marine residence (Björnsson et al. 2011; Hoar 1976, 1988; McCormick et al. 1998; Stefansson et al. 2008). One main event during salmon smoltification is the increase in the activity of Na^+/K^+ ATPase (NKA) in the gills, which is strongly correlated to increased salinity tolerance (Boeuf et al. 1989; McCormick 1996; McCormick et al. 1987). While photoperiod is considered the primary environmental factor controlling smoltification (Björnsson 1997; Duston and Saunders 1990; Hoar 1988), temperature plays an important role in the development and loss of seawater tolerance (Handeland et al. 2004; McCormick et al. 1999). In addition, smolt development has proven to be strongly related to the cumulative temperature experienced (degrees day; Sigholt et al. 1998) as well as the initiation and termination of downstream migratory behavior (Zydlewski et al. 2005). The role of melatonin in controlling smoltification is unclear; experiments using photoperiod manipulation,

pinealectomy and/or melatonin administration have led to contrasting results (Handeland et al. 2013; Iigo et al. 2005; Kulczykowska et al. 2004; López-Patiño et al. 2011; Mardones et al. 2018; Porter et al. 1998; Rourke 1994; Sangiao-Alvarellos et al. 2007). However, as highlighted elsewhere, melatonin effects depend on many factors, including time of day and of year, age, sex, and the daily and seasonal regulation of the receptor subtypes expressed (Falcón et al. 2007, 2010; Falcón and Zohar 2018). Accumulated evidence suggests that melatonin interacts with several regulators of smoltification: (i) Entry into seawater is accompanied by a transitory increase in plasma melatonin titers in Coho Salmon (*Oncorhynchus kisutch*; Gern et al. 1984), in agreement with the demonstration that increased salinity promotes melatonin synthesis in the rainbow trout (*Oncorhynchus mykiss*) pineal organ (López-Patiño et al. 2011). (ii) Melatonin modulates, in vitro, the production of growth hormone (GH) and prolactin (PRL) in *O. mykiss* (Falcón et al. 2003), and of cortisol in goldfish (*Carassius auratus*; Azpeleta et al. 2010); these hormones play a crucial role during smoltification in controlling gill NKA activity and salinity tolerance (Björnsson et al. 2011; McCormick 2013). (iii) Melatonin receptors have been identified in *O. mykiss* gills (Kulczykowska et al. 2006). (iv) Melatonin interacts with NKA (Sangiao-Alvarellos et al. 2007), and with thyroid hormones (Kulczykowska et al. 2004; Premabati et al. 2018). Although the impact of thyroid hormones (T3, T4) on the development of salinity tolerance is thought to be limited (Madsen and Kosgaard 1989; McCormick 2001), their actions on other aspects of smolting include control of growth, metabolism, pigmentation (body silvering), olfactory imprinting and locomotor activity (McCormick 2013).

Global changes, particularly those related to increased temperature, are likely to affect all timed processes in salmon species in particular, and in all fish species in general. Indeed, the rise in temperature observed these last decades may seriously compromise the photoperiod/temperature interaction established after thousands of years of adaptation, and thus disrupt the appropriate synchronization of the fish rhythmic processes as well as affecting the general metabolism. The mismatch between the timing of downstream migration and appropriate ocean conditions is predicted to have substantial impacts on the sustainability of salmon populations (McCormick et al. 1998; Marschall et al. 2011). This constitutes a serious threat, with dramatic consequences particularly in species like the Atlantic salmon, *Salmo salar*, from the Loire-Allier basin (France), which are at the southern edge of their distribution and perform an extremely long migration distance from the spawning area to the sea (~900 km; Imbert et al. 2013).

Predicting and mitigating the impact of temperature change on the timing of salmon migration will require an

increased understanding of the effect of temperature on mechanisms controlling smolt development and downstream migratory behavior. A temperature rise, up to nearly 4 °C, is predicted for the end of this century in the Loire basin, especially in summer and autumn periods (Moatar et al. 2010). As part of a larger project that aims at better understanding the impact of the ongoing rise in temperature on *S. salar* from the Loire/Allier basin (Gosse et al. 2008; Martin et al. 2012), we report here the impact of a ~4 °C rise in water temperature on physiological markers of smoltification, thyroid hormones and melatonin in fish maintained under natural or winter photoperiod. We also report on the in vitro effects of temperature challenges on melatonin response from pineal glands with different temperature history.

Materials and methods

Animal housing and sampling

S. salar (Linnaeus) were raised indoors at the *Conservatoire National du Saumon Sauvage* (CNSS, France, 45°N; <http://www.saumon-sauvage.org/>). The hatchery produces juvenile salmon which are released into the river at different developmental stages as part of a restoration program to enhance the population of the Loire-Allier basin, currently under threat of extinction. Wild male and female adult salmon, caught in the Allier River at the Vichy Dam (620 km from the Loire Estuary) were used to produce 1st generation hatchery-reared progeny. Early rearing occurred under natural photoperiod and standard hatchery conditions as described elsewhere (Martin et al. 2012).

In vitro experiments

Adult fish (600 ± 50 g b.w.), raised at the hatchery in fresh water, under natural temperature and simulated natural photoperiod, were sacrificed by anesthetic overdose (Eugenol) followed by decapitation. The pineal organs were sampled, dipped and kept in ice cold culture medium until arrival at the laboratory (less than 7 h).

In vivo experiments

In December, pre-smolt fish (23.7 ± 0.6 g b.w.) in the “upper mode”, i.e., large enough to become smolts in spring (Elson 1957; Thorpe 1977; Thorpe and Morgan 1980), were distributed in four 5.5 m³ cylindrical tanks (100 fish/tank), supplied with running water from the River Allier, at a flow of 3 l/s until April, and then progressively increased to 7 l/s when water temperature reached values above 13 °C. This ensured a concentration of dissolved oxygen higher than 7 mg/l (Martin et al. 2012). Fish were kept under a simulated

natural photoperiod using an outside light sensor controlling the light above each tank. The light source was a 125 W high pressure mercury vapor light (Hpl N-E27-125 W—4200 K; Philips, Amsterdam, Netherlands) placed in a central position above the tank and providing 50 lx at the level of the water surface. Fish were fed using automatic feeders.

At the time of winter solstice (December 21) four tanks were kept under a natural photoperiod (NP) regime, while the other four were kept under the winter photoperiod (WP) regime for the whole duration of the experiment (Fig. 1). Two tanks of each photoperiod condition were maintained in river water at the natural temperature (NPT [= control fish] and WPT); in the other two tanks, temperature was increased as indicated in Fig. 1 (NPT+ and WPT+, respectively). For each experimental condition, one tank was intended for samplings and another tank for filming the swimming behavior.

Maintaining a steady state temperature increase throughout the investigation was made difficult because of technical and economic constraints. As indicated in Fig. 1, an increase of 3 ± 0.5 °C was reached during the first 25 days and of 5 ± 1 °C between days 25 and 100. From April, the difference diminished progressively as the water temperature from the river increased with season, until no more difference was seen anymore.

Fish were sampled at noon at the times of the year indicated on the x-axis of Fig. 1. They were anesthetized as mentioned above, weighed, measured and then decapitated for

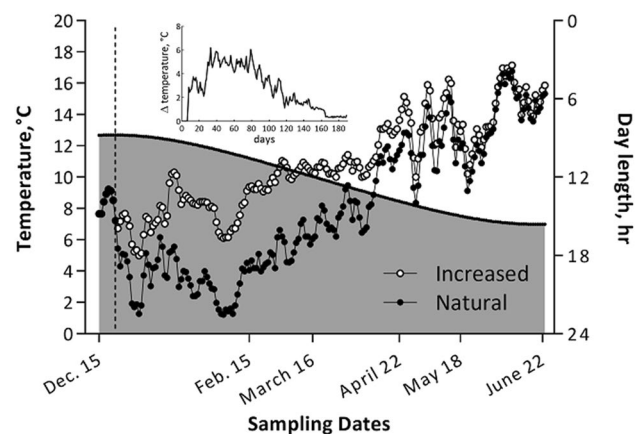


Fig. 1 Experimental design. The y axis on the right indicates day length (0 h on top and 24 h on the bottom), which is also reflected by the white and dark backgrounds in the figure. The temperature conditions are indicated by the black (natural) and white (experimental) dots (y left axis). The fish were selected and placed in their respective tanks in December. The first sampling (December 15th) was performed before initiating the experimental conditions. The interrupted vertical line indicates the time (December 21st) at which the different experimental conditions were initiated (natural or sustained winter solstice photoperiod, combined to the temperature's regimes); the dates of the sampling are indicated in the x-axis. The inset indicates the measured difference in temperature between the normal and the elevated condition. Error bars are removed for clarity

tissue samplings. Gill filaments were placed into SEI buffer (150 mM sucrose, 10 mM ethylene diamine tetra-acetic acid (EDTA) and 50 mM imidazole) immediately frozen on dry ice, and stored at $-80\text{ }^{\circ}\text{C}$ until use. Every 2 months 8 fish were sampled every 4 h of a daily cycle and plasma was sampled from the caudal blood vessels using heparinized syringes. Blood was kept at $4\text{ }^{\circ}\text{C}$ for 24 h, and then centrifuged 4 min at 5000 rpm; the plasma was stored at $-80\text{ }^{\circ}\text{C}$ until melatonin and thyroid hormones were quantified.

The study was carried out indoors at the Conservatoire National du Saumon Sauvage (CNSS), Chanteuges, France (Agreement N° B43 056 005; according to the “ARRETE N° DDCSPP/CS/2016/40”). The research project and experiments were performed in accordance with the guidelines and regulations approved by the “Ethics Committee for Animal Experiment of Languedoc-Roussillon (C2EA-LR/C2EA-36)” N° A6601601, and following the European Union regulations (European directive 2010/63/EU).

Culture of pineal glands

Each pineal gland was cultured in a well of a 24-well culture plate (Nunclon™ Surface; VWR International, Fontenay-sous-Bois, France), containing 500 μL of medium (RPMI 1640 without phenol red, complemented with penicillin [100 U/ml], streptomycin [100 $\mu\text{g}/\text{ml}$], glutamine [2 mM], and fungizone [2.5 $\mu\text{g}/\text{ml}$]). The culture plates were placed in MIR-154 incubators (Sanyo; Osaka, Japan) at the natural photoperiod and temperature the fish were acclimated to, unless otherwise specified. The media were renewed every 24 h. The response to temperature challenges was investigated in two series of experiments.

Series 1

The glands collected at different times of the year were cultured for 48 h before the temperature challenge, performed in the dark from 12:00 to 18:00. At the end of the incubation at different temperatures, the culture media were collected and frozen at $-20\text{ }^{\circ}\text{C}$.

Series 2

Three groups of pineal glands were cultured in three different incubators running in parallel, at a temperature of $10\text{ }^{\circ}\text{C}$, $15\text{ }^{\circ}\text{C}$ or $20\text{ }^{\circ}\text{C}$, respectively; photoperiod was as the natural one at the moment of sampling (14L/10D). After a 3 week acclimation period, the organs were placed 6 h in the dark, starting at 09:00. The culture medium was then collected and frozen at $-20\text{ }^{\circ}\text{C}$. The 14L/10D photoperiod was restored for the next 2 days, and then the organs were challenged with different temperatures as in the *series 1*.

Melatonin quantification

Plasma

Melatonin was extracted by solid phase extraction using C18 cartridges (Waters; Saint-Quentin-En-Yvelines, France) as detailed elsewhere (Enzyme-Linked Immuno-Sorbent Assay, IBL International^{GmbH}, Hamburg, Germany). A 100% methanol eluate was kept and vacuum dried. Melatonin in the dry samples was recovered in 200 μL of water containing 1 nM of an internal standard (5-Methoxy-*N*-cyclopropanoyl-tryptamine, MCPT). Its concentrations were determined by High Performance Liquid Chromatography (HPLC) using a $125 \times 4.6\text{ mm}$ C18(2) reversed phase analytic column (Luna, Phenomenex; Le Pecq, France), with particles length of 5 μm and a Dionex™ ULTIMATE™ 3100 fluorescence detector (Thermo Scientifique™; Villebon-sur-Yvette, France). Due to the low melatonin levels in the samples, a derivatization method was performed to enhance melatonin's natural fluorescence. The derivatization protocol was based on a previously described method (Inuma et al. 1999) with slight modifications. Briefly, Na_2CO_3 and H_2O_2 were added at a final concentration of 200 mM and 5 mM, respectively, followed by a 30 min incubation at $100\text{ }^{\circ}\text{C}$. The melatonin indole ring undergoes oxidation, and transforms into a highly fluorescent compound, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA) (Tomita et al. 2003). The reactions were carried out in sealed glass ampoules. Controls included a blank (containing all reagents and ultrapure water instead of samples), duplicates of the internal standard (0.8 nM final concentration) and different concentrations (6.24 to 0.025 nM) of commercial melatonin standards. A 100 μL volume of each sample was then injected. The calibration curve was linear with a correlation coefficient of 0.999. Under these conditions, the detection limit of 6-MOQMA corresponded to an initial melatonin concentration of 5.8 pg/ml. Melatonin recovery was $97.7 \pm 9.81\%$, and repeatability of MCPT was $94.69 \pm 1.26\%$.

The column temperature was $40\text{ }^{\circ}\text{C}$ and the excitation and emission wavelengths were of 245 nm and 380 nm, respectively. The mobile phase consisted of 50 mM of sodium acetate at pH 6.5 adjusted with orthophosphoric acid, and a crescent gradient of acetonitrile (from 10 to 20% in 7 min) with a plateau of 3 min, followed by an increase from 20 to 100% in 3 min, and return to the initial conditions in 1 min, ending on 4 min stabilization. The mobile phase flow was of 1.5 ml/min and the peaks were detected at 4.6 and 7.2 min for melatonin and MCPT derivatives, respectively.

Culture medium

Because of the high concentrations of melatonin released by the pineal in the culture medium, no derivatization was

needed. A 10 μl volume of each sample was directly injected in the HPLC system. The detection method was as described above with the following modifications: the excitation and emission wavelengths were 280 nm and 340 nm, respectively; the column temperature was maintained at 30 °C; the mobile phase consisted of 0.1 M of sodium acetate, with 20% acetonitrile; the pH was adjusted to 6.5 using orthophosphoric acid; the mobile phase flow was of 1.5 ml/min. Under these conditions, the retention time of melatonin in the samples and standards was ~ 7 min.

Thyroid hormones quantification

Because of technical limitations (number of fish available, levels of blood collected in young fish), it was not possible to measure the daily variations in thyroid hormone levels at all months of the experiment. For this reason we report the data obtained in April only.

The quantification of plasma free T3 and T4 was performed by “Unilians, Lyon” (<http://www.unilians.fr/laboratoire-de-gerland-lyon>) by means of the Roche electrochemiluminescence assay (ECLIA, Roche Diagnostics; Mannheim, Germany) and a Cobas® analyzer, according to manufacturer’s protocol.

Na⁺/K⁺-ATPase activity

Gill Na⁺/K⁺-ATPase activity (NKA) measurements were carried out at the Conte Anadromous Fish Research Laboratory (U.S. Geological Survey, Turners Falls, MA, USA) using a kinetic assay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min as described previously (McCormick 1993). Gill tissue was homogenized in 150 μL of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000 $\times g$ for 30 s. Two sets of duplicate 10 μL samples were run, one set containing assay mixture and the other, assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity is expressed as $\mu\text{moles ADP mg protein}^{-1} \text{ h}^{-1}$. Protein concentrations are determined using BCA (bicinchoninic acid) Protein Assay (Pierce, www.piercenet.com, Rockford, IL, USA). Both assays were run on a THERMOMax microplate reader using SOFTmax software (Molecular Devices, www.moleculardevices.com, Menlo Park, CA, USA).

Statistics and graphics

The analysis included one- or two- way ANOVA followed by the Holm-Sidak or Sidak post-hoc tests depending on the dataset. Individual means were compared using the Two-tailed Students’ *t* test. Drawings and statistics were performed using the Prism.v6 (GraphPad™ Software Inc., San

Diego, CA) or the Sigma Plot version 11 (Systat Software, Inc., San Jose, CA; statistics only).

Compounds and chemicals

EDTA, eugenol, fungizone (Amphotericin B), imidazole, L-Glutamine-Penicillin–Streptomycin solution, sucrose and RPMI culture medium were from Sigma-Aldrich (Saint-Quentin Fallavier, France). 5-Methoxy-N-cyclopropanoyltryptamine (MCPT) was from Santa Cruz Inc. (CliniSciences, France). Melatonin standard was from Acros Organics™ (Fisher Scientifics, Villebon-sur-Yvette, France). Acetonitrile and hydrogen peroxide solution-HPLC grade were from Fisher Scientifics (Villebon-sur-Yvette, France).

Results

Fish weight and length

All fish used in this experiment were in the upper mode in terms of mass and fork length. In contrast to photoperiod, temperature had a significant impact on both parameters (Fig. 2a, b). Both mass and length increased during winter to a greater degree in the groups experiencing an increase in temperature, and then, in spring, both temperature groups followed the same increasing rate as observed under natural (NPT) or winter (WPT) photoperiod. However, at the end of the experiment (May to June) the weight and fork length of the WPT + fish was the same as that observed for fish maintained under natural temperature throughout the whole experiment. This impacted the condition factor of WPT + fish that was lower compared to those from fish of all other groups (Fig. 2c).

Na⁺/K⁺-ATPase activity (NKA) throughout smoltification

Under the NPT condition, NKA displayed a gradual increase from winter (December 15th) to spring (April 22nd), followed by a decrease in late-spring (May 18th) (Fig. 3). Increasing temperature above natural values (NPT+) did not change the shape of the curve but the activities measured at the peak and at the end of the experiment were significantly reduced (by ~ 25 –30%) (Two-way ANOVA reports in Table 1). A similar observation applied for the WPT and WPT + conditions (Fig. 3).

Daily levels of circulating thyroid hormones

Under the NPT condition in April both the free T3 and T4 plasma levels displayed daily variations. They were

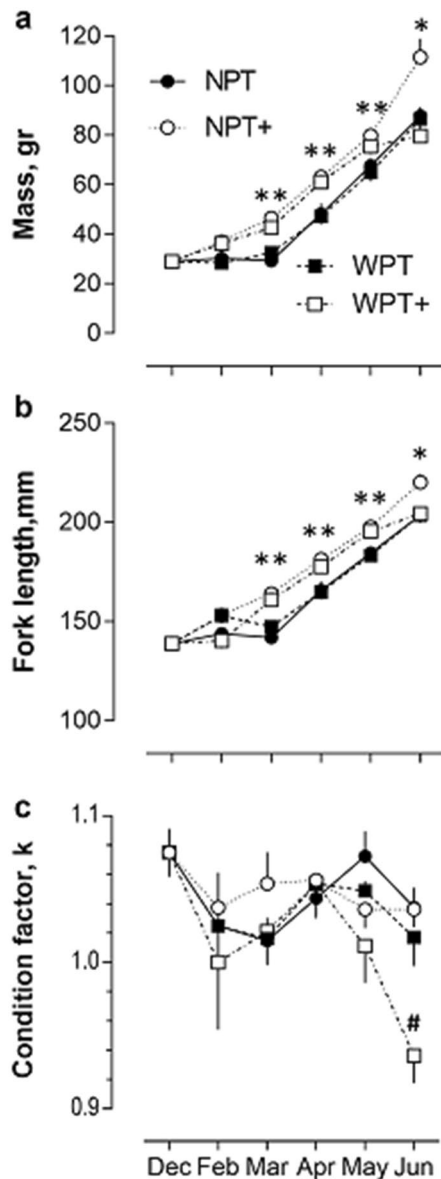


Fig. 2 Mass, fork length, and condition factor of juvenile salmon throughout smoltification under the experimental conditions described in Fig. 1. Natural temperatures under natural (NPT) and winter (WPT) photoperiods; elevated temperatures under natural (NPT+) and winter (WPT+) photoperiods. **a**: mass; **b**: fork length; **c**: condition factor K (weight [g]/fork length [cm^3] $\times 100$). Data are presented as the mean \pm S.E.M. ($n = 8$ /condition/sampling date). Two-way ANOVA indicated (i) a significant effect of photoperiod only in **c** ($P < 0.02$), (ii) a significant effect of temperature in **a**, **b** and **c** ($P < 0.001$), (iii) there was an interaction between the effects of temperature and the month investigated in **a** ($P < 0.001$) **b** ($P < 0.001$) and **c** ($P < 0.01$). *Post-hoc* Holm-Sidak's multiple comparison test indicated significant differences between means (* or # $P < 0.005$ and ** $P < 0.001$); *natural vs. corresponding increased temperature, and #NPT vs. WPT

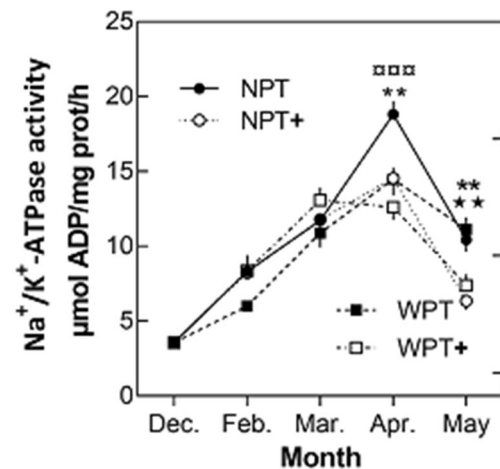


Fig. 3 Na^+/K^+ ATPase activity in the course of smoltification. Variations in activity were similar at all three conditions except that the peak was reached ~a month earlier in the WPT+ vs. all the other groups, and the amplitude was lower for all the experimental groups (NPT+, WPT, WPT+) compared to the group of fish maintained under natural conditions (NPT). Mean \pm SEM, $n = 8$ –10. Two-way ANOVA indicated significant differences amongst groups (Table 1). *Post-hoc* Sidak's test of comparison of individual means indicated significant differences (** or ** $P < 0.005$, and *** $P < 0.0005$); *NPT vs. NPT+, \star WPT vs. WPT+, and $\#$ NPT vs. WPT

Table 1 Na^+/K^+ , ATPase activity: Two-way ANOVA comparison of responses

Experimental condition	Two-way ANOVA	
	F	P
NPT vs. NPT+	Interaction	4.31 0.003***
	Temperature Month	11.59 < 0.0011***
		83.13 < 0.0001****
WPT vs. WPT+	Interaction	5.96 < 0.0003****
	Temperature Month	0.21 0.65 ^{ns}
		60.23 < 0.0001****
NPT vs. WPT	Interaction	3.63 0.009**
	Photoperiod Month	8.44 < 0.005***
		93.12 < 0.0001****
NPT+ vs. WPT+	Interaction	1.27 0.29 ^{ns}
	Photoperiod Month	0.05 0.83 ^{ns}
		58.51 < 0.0001****

** $P < 0.01$, *** $P < 0.005$ and **** $P < 0.001$

ns not significant

high at the end of the day, decreased during the night and increased again after lights on (Fig. 4; Supplementary material Fig. 8). The plasma free T4 increase preceded the T3 increase in the morning. As a result, the plasma free T3/T4 ratio appeared higher at night than during day (Fig. 4; Supplementary material Fig. 9). The shape of the plasma free T3 daily variations was not significantly modified either by temperature or photoperiod (Fig. 4);

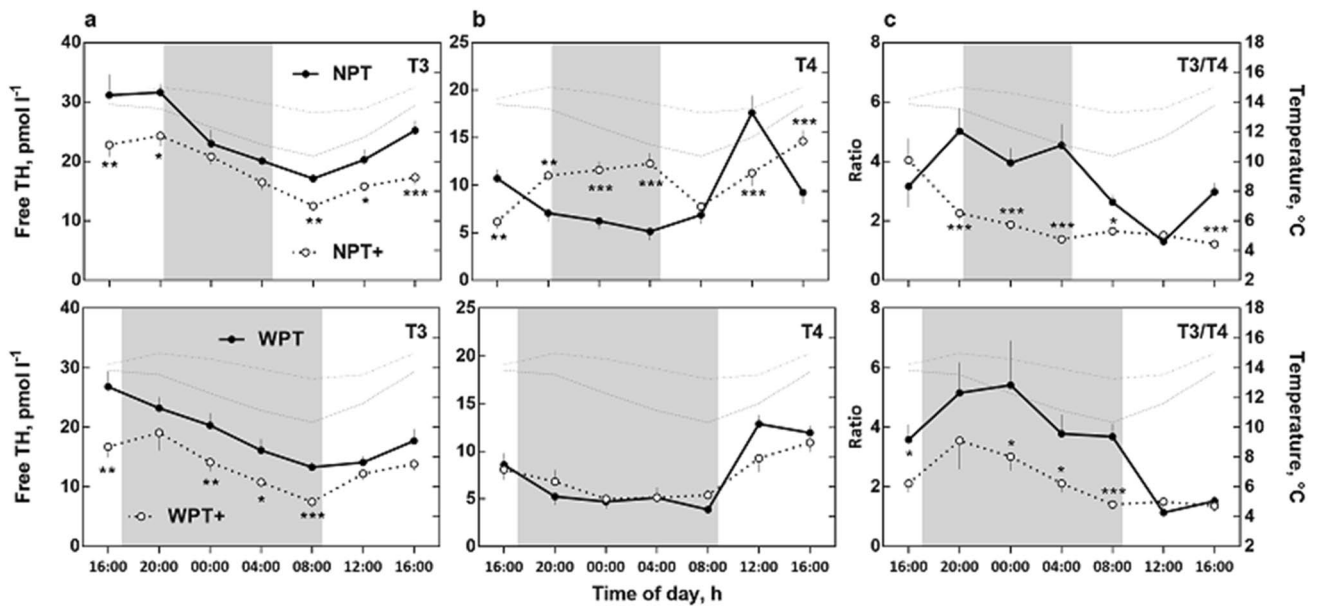


Fig. 4 Daily variations of plasma thyroid hormones (TH) levels. Free T3 (column **a**) and T4 (column **b**) levels were measured in April. Column **c** reports the T3/T4 ratio. The grey boxes correspond to the duration of the night and the thin interrupted lines indicate the daily variations in temperature under the natural (NPT, WPT; dash

lines) and increased (NPT+, WPT+; dash-dot lines) conditions. Mean \pm SEM, $n=6-8$. Two-way ANOVA values are given in Table 2. *Post-hoc* Sidak’s test of comparison of means at the same sampling time indicated significant differences (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$)

Table 2 Plasma thyroid hormone levels in April: Two-way ANOVA comparison of responses

Experimental condition		Two-way ANOVA		
			F	P
NPT vs. NPT+	Free T3	Interaction	1.07	0.389 ^{ns}
		Temperature	39.41	< 0.0001 ^{****}
		Time of day	16.67	< 0.0001 ^{****}
	Free T4	Interaction	12.82	< 0.0001 ^{****}
		Temperature	9.5	0.0027 ^{***}
		Time of day	11.34	< 0.0001 ^{****}
T3/T4	Interaction	6.13	< 0.0001 ^{****}	
	Temperature	38.15	< 0.0001 ^{****}	
	Time of day	7.59	< 0.0001 ^{****}	
WPT vs. WPT+	Free T3	Interaction	1.11	0.36 ^{ns}
		Temperature	32.92	< 0.0001 ^{****}
		Time of day	11.48	< 0.0001 ^{****}
	Free T4	Interaction	1.92	0.086 ^{ns}
		Temperature	0.25	0.61 ^{ns}
		Time of day	21.14	< 0.0001 ^{****}
T3/T4	Interaction	1.53	0.179 ^{ns}	
	Temperature	16.06	< 0.0001 ^{****}	
	Time of day	7.9	< 0.0001 ^{****}	

*** $P < 0.005$ and **** $P < 0.001$

ns not significant

however, the levels were significantly lower at elevated temperature than in the corresponding controls (Table 2). The plasma free T4 profile was dramatically affected in the NPT+ group compared to its NPT counterpart, while temperature had no clear effect under winter photoperiod

(compare WPT vs. WPT+) (Fig. 4; Table 2). The variations in the plasma free T3/T4 ratio were not affected by photoperiod, i.e., it was high at night and low during day; increasing temperature abolished the night increase in the natural photoperiod group only, while reducing the

amplitude of the daily variations under the winter photoperiod (Fig. 4; Supplementary material Fig. 9) (Table 2).

In vivo circulating melatonin levels during smoltification

In December, plasma melatonin levels of pre-smolt fish were high at night and low during the day, displaying a plateau that matched the duration of the night (Fig. 5). These daily variations were also observed in February and April, whatever the experimental condition (see Table 3 for statistics).

Natural photoperiod and natural temperature (NPT)

The profiles of the plasma melatonin rhythms did not change greatly from December to February. A slightly different profile was observed between February and April regarding the time at which the nocturnal peak was reached (Fig. 5). Two-way ANOVA indicated a possible combined effect of time of day and month (Table 3).

Natural photoperiod and increased temperature (NPT+)

In both, February and April, the increase in temperature resulted in a significant increase in plasma melatonin

content, compared to the natural (NPT) condition, particularly in February (Fig. 5; Table 3). Also, the nocturnal NPT+ profiles observed in February were significantly different from the NPT+ profiles observed in April ($P < 0.0001$; Table 3).

Sustained winter photoperiod and natural temperature (WPT)

Plasma melatonin content reached similar levels in December, February and April (Fig. 5). The major difference was in the shape of the oscillations: in February and April the WPT groups displayed a decrease from dawn to dusk, not observed in the December fish, but this difference was not statistically significant (Fig. 5; Table 3). Both in February and April, the WPT groups differed from their corresponding NPT groups as a result of an interaction between photoperiod and time of day (Table 3).

Sustained winter photoperiod and increased temperature (WPT+)

Both in February and April the increased temperature resulted in a change in the melatonin oscillation pattern. Compared to their corresponding natural temperature

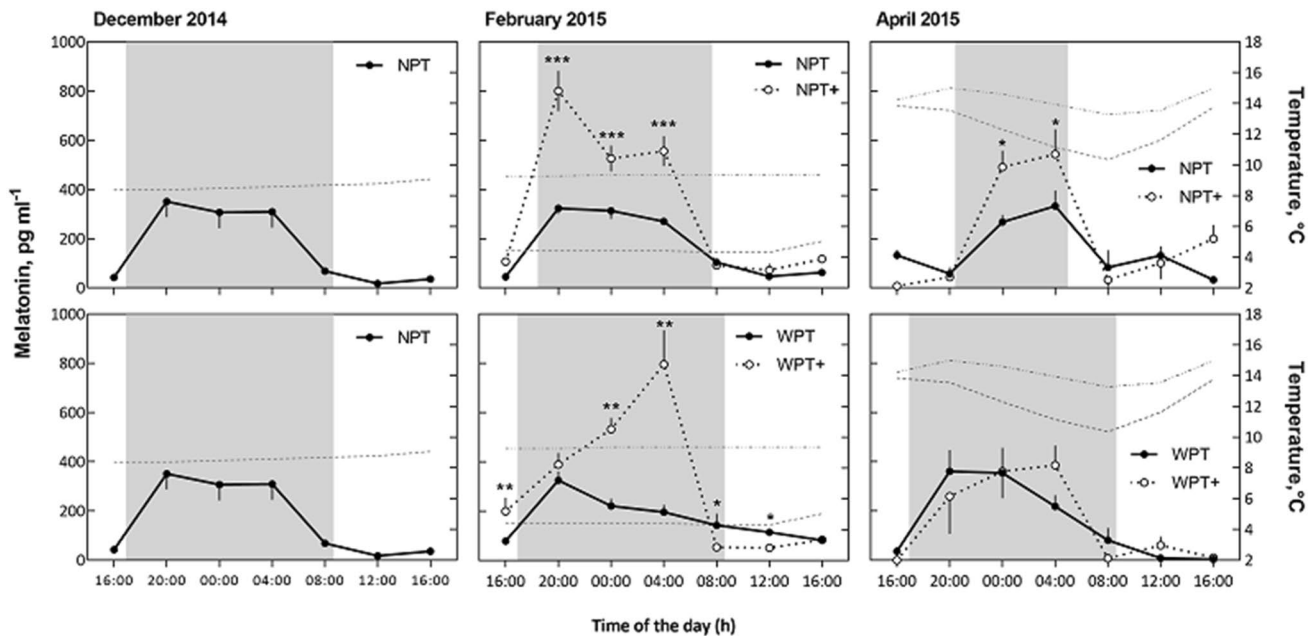


Fig. 5 Daily variations of plasma melatonin levels. Melatonin was measured in December (similar conditions for all groups), February and April. The grey boxes correspond to the duration of the night and the thin interrupted lines indicate the daily variations in temperature under both the natural (NPT, WPT; dash lines) and increased (NPT+, WPT+; dash-dot lines) conditions. The December values (corresponding to time-zero of the experiment) are plotted twice

to facilitate the comparisons with the corresponding groups. Values correspond to melatonin measurement after derivatization into 6-MOQMA (see text for details); mean \pm SEM, $n = 6-8$. Two-way ANOVA values are given in Table 3. *Post-hoc* Sidak's test of comparison of means at each sampling time indicated significant differences ($*P < 0.05$, $**P < 0.005$, $***P < 0.0005$)

Table 3 Plasma melatonin content: Two-way ANOVA data

Experimental condition	Month	Two-way ANOVA		
			F	P
<i>Month effect</i>				
NPT	Dec. vs Feb.	Interaction	0.35	0.91 ^{ns}
		Month	0.07	0.79 ^{ns}
		Time of day	30.87	<0.0001 ^{****}
	Feb. vs. Apr.	Interaction	5.80	<0.0001 ^{****}
		Month	0.8	0.37 ^{ns}
		Time of day	16.17	<0.0001 ^{****}
NPT+	Feb. vs. Apr.	Interaction	14.46	<0.0001 ^{****}
		Month	17.41	<0.0001 ^{****}
		Time of day	33.10	<0.0001 ^{****}
WPT	Dec. vs Feb.	Interaction	2.43	0.032 [*]
		Month	0.04	0.8 ^{ns}
		Time of day	19.99	<0.0001 ^{****}
	Feb. vs. Apr.	Interaction	1.69	0.13 ^{ns}
		Month	0.33	0.56 ^{ns}
		Time of day	14.16	<0.0001 ^{****}
WPT+	Feb. vs. Apr.	Interaction	1.94	0.08 ^{ns}
		Month	14.57	<0.0003 ^{****}
		Time of day	21.07	<0.0001 ^{****}
<i>Temperature effect</i>				
NPT vs. NPT+	Feb.	Interaction	11.46	<0.0001 ^{****}
		Temperature	59.42	<0.0001 ^{****}
		Time of day	63.50	<0.0001 ^{****}
NPT vs. NPT+	Apr.	Interaction	3.27	<0.006 ^{**}
		Temperature	3.37	0.07 ^{ns}
		Time of day	18.36	<0.0001 ^{****}
WPT vs. WPT+	Feb.	Interaction	11.02	<0.0001 ^{****}
		Temperature	22.84	<0.0001 ^{****}
		Time of day	21.83	<0.0001 ^{****}
WPT vs. WPT+	Apr.	Interaction	0.97	0.45 ^{ns}
		Temperature	0.01	0.92 ^{ns}
		Time of day	12.7	<0.0001 ^{****}
<i>Photoperiod effect</i>				
NPT vs. WPT	Feb.	Interaction	2.64	0.02 [*]
		Photoperiod	0	0.95 ^{ns}
		Time of day	33.28	<0.0001 ^{****}
NPT vs. WPT	Apr.	Interaction	4.11	0.0011 ^{***}
		Photoperiod	0.01	0.9 ^{ns}
		Time of day	9.2	<0.0001 ^{****}
NPT+ vs. WPT+	Feb.	Interaction	6.05	<0.0001 ^{****}
		Photoperiod	0.53	0.47 ^{ns}
		Time of day	42.28	<0.0001 ^{****}
NPT+ vs. WPT+	Apr.	Interaction	2.07	0.064 ^{ns}
		Photoperiod	1.87	0.174 ^{ns}
		Time of day	17.14	<0.0001 ^{****}

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ and **** $P < 0.001$.

ns not significant

groups (WPT), in which the plasma melatonin peak was found early at night, in the February and April groups the higher amounts were found in the second half of the night (Fig. 5), but the differences were significant only in February (Table 3). Also, the pattern changed significantly from February to April.

Differences were also observed between the NPT + and WPT + groups in February as well as in April, although two-way ANOVA indicated a strong interaction between the time of day and photoperiod only in February (Fig. 5; Table 3).

Table 4 Impact of temperature acclimation on the in vitro pineal melatonin response to temperature challenges: two-way ANOVA data

Acclimation conditions	Two-way ANOVA		
		<i>P</i>	
10° vs. 15 °C	Interaction	4.58	0.0002****
	Challenge	9.18	<0.0001****
	Acclimation	57.81	<0.0001****
10° vs. 20 °C	Interaction	2.58	0.0176*
	Challenge	15.66	<0.0001****
	Acclimation	25.53	<0.0001****
15° vs. 20 °C	Interaction	3.03	0.0065**
	Challenge	12.48	0.0296*
	Acclimation	4.88	<0.0001****

P* < 0.05, *P* < 0.01, *****P* < 0.001

Acclimation conditions affect in vitro melatonin production in response to temperature

In vitro, melatonin secretion by *S. salar* pineal glands was high in the dark and low under illumination. The nocturnal secretion was modulated by temperature in the dark. However, temperature exposures of pineal glands maintained in the dark resulted in different profiles depending on the time of the year the experiments were done (Fig. 6). In the experimental set of 2014, all data curves displayed a more or less pronounced biphasic response with two peaks, one in the cold range (between 10 and 15 °C) and one in the warm range (between 15 and 25 °C). Different profiles were also observed from one year to another, although the external water temperature was the same (compare Oct. 2013 with Oct. 2014 in Fig. 6). To determine whether the previous temperature history of the gland could explain the differences observed, we conducted an experiment in which glands from

the same pool of individuals were first cultured at 3 different temperatures (10, 15, 20 °C). After 3 weeks of acclimation, each group was exposed to a temperature challenge and melatonin was measured in the medium. The profile obtained changed with the prior acclimation temperature (Fig. 7). The most noticeable features were a peak response (1) at 12 °C in the 10 °C-acclimated glands only and (2) at 20/25 °C in the 10 °C- and 20 °C-acclimated glands. The 15 °C-acclimated glands were those that displayed the flattest response to temperature changes, and the 14/16 °C values appeared as a turning point in all groups (Fig. 7).

Discussion

Markers of smoltification

Morphological and behavioral characteristics of the parr-smolt transformation consist mainly in the loss of parr marks, body silvering, darkening of fins extremities, adoption of schooling rather than a territorial behavior, and a switch from positive to negative rheotaxis. All these criteria were observed in *S. salar* of the present study, which were exclusively in the upper mode. The observed changes in swimming behavior (data not shown) were the same as shown in the duplication of this experimental design in the following year (reported by Fleming 2018). The beginning of the negative rheotaxis, indicating readiness for downstream migration, was observed in all groups at the beginning of April and this behavior was maintained until the end of the experimental period, at the end of June. Gill NKA, used as a marker of smoltification, was low in parr (December), increased from March to reach a peak in April, and

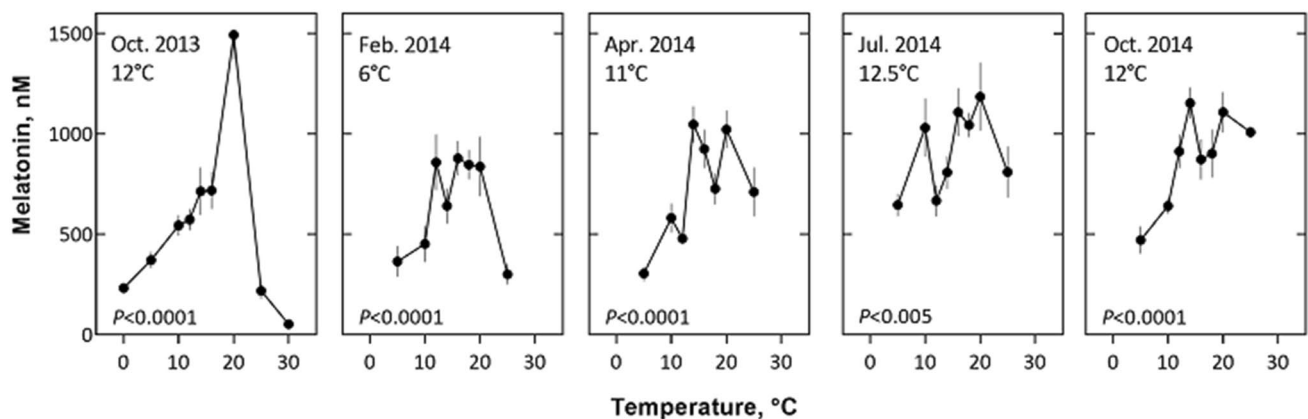


Fig. 6 In vitro pineal melatonin response to temperature challenges. Pineal organs were cultured as indicated in the “Materials and methods”. Melatonin was quantified after 6 h of culture in the dark and at the temperatures indicated in the abscissae. The months at which the

experiments were performed and the corresponding water temperature are indicated at the upper left of the graphics. Mean \pm SEM, *n* = 6–8. ANOVA indicated that all the variations were statistically significant

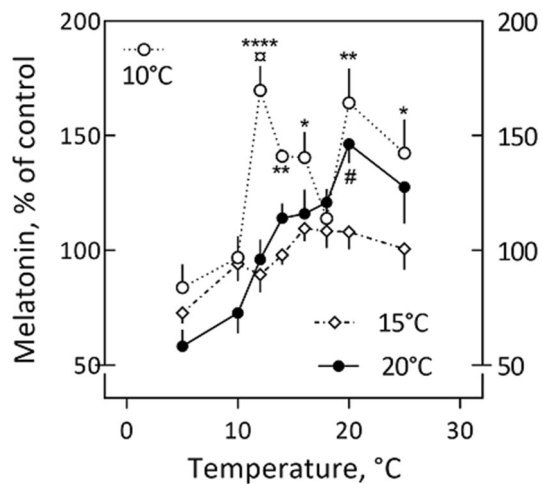


Fig. 7 Impact of temperature acclimation on the in vitro pineal melatonin response to temperature challenges. Pineal organs were acclimated to temperatures of 10, 15 and 20 °C, respectively, for 3 weeks before being challenged as indicated in Fig. 6. Two days before the temperature challenge the pineal glands were placed in the dark at noon, and 6 h later the medium was collected for melatonin quantification. The values obtained served as reference values for the normalization of the data obtained after the temperature challenge (i.e., 2 days later). The two-way ANOVA indicated (i) significant differences in the response to temperature in each group, (ii) between groups acclimated to different temperatures, (iii) and a significant interaction between the temperature challenge and acclimation condition (details in Table 4). Mean \pm SEM, $n = 7-8$. *Post-hoc* Sidak's test of comparison of individual means between the different groups indicated significant differences (* or α or # $P < 0.05$, ** $P < 0.005$, **** $P < 0.0001$); 10 °C compared to 15 °C (*), 10 °C compared to 20 °C (α) and 15 °C compared to 20 °C (#)

decreased in May (Fig. 3). Such a peak coincides with the beginning of the migratory movements.

Smolts usually also display a phase of decrease of the condition factor (K) (McCormick and Saunders 1987; Stefansson et al. 2008). Here we observed that K tended to decrease in March, independent of the photoperiod and temperature conditions (Fig. 2). Throughout the experiment, the high temperature induced a stimulatory effect on both mass and fork length at early stages of the smoltification process; except for the WPT + group, which displayed a significant weight decrease in June that was reflected by a decrease in K. Although our experimental design did not allow maintaining a 4 °C temperature difference throughout the entire study, temperature alone cannot explain this decrease, because it did not occur in the NPT + treatment. It must then be assumed that the decrease of K in June was due to the combined effects of a winter photoperiod and a high temperature. In agreement with potential interaction of photoperiod and temperature, previous studies have reported a correlation between the decrease in K and maintenance of a winter photoperiod (Björnsson et al. 1989), and variations in K depending on the light conditions (Björnsson et al. 1989;

Saunders et al. 1985). In contrast, when the temperature increase of May was advanced to February, K increased in fish maintained under a short (9L/15D) photoperiod, while it decreased in those under a natural photoperiod (McCormick et al. 2002). These discrepancies might be explained by the differences in experimental protocols and differential responses to photoperiod and temperature by different *S. salar* populations. Whatever it might be, these results highlight the importance of the photoperiod/temperature interaction and its timing; it is possible that some stages of the photoperiod-regulated mechanisms are more sensitive to temperature than others.

Development of gill NKA is a crucial step in smoltification (McCormick 2013). *S. salar* from the Loire Allier displayed gill NKA of 10–15 $\mu\text{mol ADP/mg/h}$ between February and March (Boeuf et al. 1989 and current study), which is typical of other smolt studies and indicates that at least partial smolt development had occurred in the present study. It has been suggested that temperature elevation induced a higher initial rate of increase in the gill NKA without affecting the timing of peak levels (McCormick et al. 2002). In the present study, the peak of activity was reached in April whatever the condition, i.e., the fish had similar timing of smolt development under either the natural or constant winter photoperiod, with or without a temperature increase. The major difference among groups was the amplitude of the elevation (lower in the WPT, WPT + and NPT + conditions, vs. the NPT condition).

Previous investigations indicated photoperiod plays a central role in the regulation of smoltification (Björnsson et al. 2000; Boeuf 1993; Hoar 1988; McCormick 2001, 2013; Stefansson et al. 2008). Berge et al. (1995) reported that a progressive increase in photoperiod is a necessary condition to induce smolt characteristics in *S. salar*; and a prior winter (short day) photoperiod regime is necessary for longer photoperiods (seemingly exceeding 16 h of light, at least in northern habitats) to trigger smolting (Strand et al. 2018). Our data thus contrast somewhat with these findings, because smoltification, at least in terms of gill NKA, was observed also in the WPT groups. It is possible that not all processes that accompany smoltification rely solely on increasing photoperiod. In *O. kisutch* multiple factors interact to dictate migration timing, and the specific set of factors that govern migration varies substantially among populations (Spence and Dick 2013). This might explain why *S. salar* from the Loire/Allier, but reared from hatching at a different location (5° latitude further north) and under different conditions than those of the current study, displayed a later occurrence of the peak of smolting (May), and a lower gill NKA at the peak (Bernard et al. 2018). It is also possible that a putative endogenous annual clock can initiate the parr-smolt transformation. Such an endogenous clock would explain the increase in gill NKA that occurred in the WPT

groups in the present study and has been observed in other studies using short day conditions (McCormick et al. 2002). The presence of such a circannual clock has been suggested from studies on *S. salar* from the Baltic Sea (Eriksson and Lundqvist 1982), and gains support from our data on thyroid hormones (see below).

Gill NKA and salinity tolerance return to presmolt levels if fish are prevented from entering the ocean (Duston et al. 1991; Hoar 1988). Here, the April-to-May decrease in NKA was accelerated in the groups subjected to increased temperature (NPT+ and WPT+) irrespective of photoperiod. Such a decrease has been observed in salmonids exposed to elevated temperatures in the wild and in captivity (Bernard et al. 2019; Duston et al. 1991; Handeland et al. 2004; McCormick et al. 1999), and with a compromise or loss of hypo-osmoregulatory capacities related to the smolt development. A previous study also performed with the Loire-Allier *S. salar* strain, but reared in northern latitude (5°), showed that a shift from natural to elevated temperature (a delta of 5 °C reached in 3 days, applied at different stages of the smoltification period) caused a decrease of gill NKA, but also of cortisol, GH and insulin-like growth factor 1 levels (Bernard et al. 2019). These hormones are known to increase during smoltification and to interact with each other to promote the development of hypo-osmoregulatory capacity (e.g., by promoting the increase of gill NKA and the proliferation of the cells (ionocytes) specialized in ion transport; McCormick 2013). The limited amount of plasma available did not allow us to quantify these hormones in the present study; however, our data emphasize an important role of temperature in smolt osmoregulation processes, at least with regard to gill NKA. Since the increase above natural temperature was ~2 °C at the time gill NKA started its decrease, we can assume that this difference was enough to induce a faster loss; it is also possible that the previous temperature history of the fish, or the presence of a circannual clock (as hypothesized above), conditioned this response.

Melatonin and thyroid hormones

Impact of photoperiod

The current investigation in *S. salar* adds to previous studies reporting daily variations in plasma melatonin (Porter et al. 2001, 1998; Randall et al. 1995) and in plasma T3 and T4 (Ebbesson et al. 2008) in this species. Under natural photoperiod and temperature, melatonin titers were high at night and low during day as expected. Accordingly, the shape of the oscillations changed from December to April with the changing photoperiod. Plasma T3 and T4 daily levels (in April) were high during day and low at night. While T4 increased in the morning and decreased in the afternoon, T3 reached maximum values in the afternoon, and decreased

from the onset of the night until beginnings of the next day. This phase delay between the T4 and T3 daily profiles is consistent with the idea that the conversion of T4 into its active form T3 by iodothyronine deiodinase (Little et al. 2013) occurs at a higher rate during the second half of the day and first half of the night, as also suggested from the daily profile of the T3/T4 ratio.

The observation that the melatonin and thyroid hormone daily profiles were ~180° out of phase (Supplementary material Fig. 8) is consistent with previous data showing that long-term administration of T4 decreased nocturnal plasma melatonin concentrations in *S. salar* (Kulczykowska et al. 2004). A negative correlation between thyroid hormones and melatonin secretion has also been established in *C. auratus* (Jung et al. 2016), *Clarias gariepinus* (Premabati et al. 2018) and *Sparus aurata* (Montoya et al. 2010). In *C. auratus*, melatonin treatment decreased plasma T3 and T4 levels and thyroid hormone receptor mRNA expression; in parallel, glucose and cortisol levels were also decreased (Jung et al. 2016). Melatonin treatment is known to reduce plasma cortisol levels in *C. auratus*, *O. mykiss*, *S. salar*, *O. kisutch* and European seabass *Dicentrarchus labrax* (Azpeleta et al. 2010; Conde-Sieira et al. 2014; Herrero et al. 2007; Mardones et al. 2018). Conversely, cortisol inhibits melatonin synthesis and secretion in *O. mykiss* (Benyassi et al. 2001; López-Patiño et al. 2014) and Mozambique tilapia *Oreochromis mossambicus* (Nikaido et al. 2010), supporting the idea that both are part of a negative feedback loop. In *S. salar*, the daily plasma rhythm of cortisol is 180° out of phase with the melatonin rhythm (Ebbesson et al. 2008; Huang et al. 2010; Thorpe et al. 1987). Considering that (i) melatonin modulates GH secretion in *O. mykiss* (Falcón et al. 2003) and (ii) the cortisol and the GH/IGF-I axis interact to stimulate salinity tolerance (McCormick 2013), it is possible that part of the effects of photoperiod on the smoltification process results from feedback loops involving the time-keeper, melatonin, on the one hand, and GH, T3/T4 and cortisol, on the other hand. It is noteworthy that thyroid hormones and melatonin might regulate similar processes in salmonids, because melatonin receptors (in *O. mykiss*) and Dio2b expression (in *S. salar*) target similar brain areas (Lorgen et al. 2015), particularly those related to the temperature dependent regulation of energy metabolism and locomotor activity in which both hormones are involved (Falcón and Zohar 2018; Little 2016; Little et al. 2013).

Maintaining the fish under sustained winter photoperiod (WPT) did not change plasma free T3 and T4 profiles. This suggests that the photoperiod had no significant impact on the shape of their oscillations. It has been hypothesized that (i) schedule feeding time impacts daily titers and profile of T4 (*C. auratus*: Spieler and Noeske 1984; red drum: *Sciaenops ocellatus*: Leiner and Mackenzie 2003); (ii) endogenous feeding rhythms are important synchronizers of thyroid

hormones in *O. mykiss* (Boujard and Leatherland 1992), and (iii) feeding-entrained and light-entrained oscillators interact to determine the phase of the T4 rhythm (Leiner and MacKenzie 2003). In the present study, feeding intervals were adjusted based on the increase of natural photoperiod and were kept the same for control and experimental groups. It is possible that feeding time was a predominant factor conditioning T3 and T4 daily levels, but a conjunction of factors such as feeding and diel activity rhythms (Youngson 1989) and/or the previous photoperiod history of the fish could also have conditioned the response. Assuming photoperiod is central to the start and further regulation of smoltification (Björnsson et al. 2000; McCormick 2013; Stefansson et al. 2008), the absence of effect of photoperiod on T3 and T4 would suggest that thyroid hormones do not play a central role in the timing of this process. If ever photoperiod had an effect in T3 and/or T4 in winter or early spring (not analyzed in this study), this did not seem to impact the timing of the smoltification aspects observed in the present work. Finally, it is also possible that the T3 and T4 daily profiles were driven by a yet unidentified photoperiod-independent oscillator.

Impact of temperature

Previous studies reported that temperature has a marked effect on melatonin secretion in fish (Falcón et al. 1996, 1994; Falcón and Collin 1989; García-Allegue et al. 2001; Iigo and Aida 1995; Masuda et al. 2003; Max and Menaker 1992; Porter et al. 2001; Vera et al. 2007; Zachmann et al. 1992). We highlight that, differently from most of the studies cited above, in the present study the elevated temperature was not constant but followed the natural daily temperature oscillation. Here we observed that the imposed increase in temperature resulted in a higher nocturnal production of melatonin (except in April in the WPT+ fish). The shape of the oscillations depended on the photoperiod and temperature conditions to which the fish were acclimated. It is noteworthy that under natural or winter photoperiod the amplitude of the nocturnal melatonin peak remained more or less the same, although the fish experienced a natural decrease in temperature from December to February, and a natural increase from February to April. Altogether, this suggests a complex interaction of photoperiod and temperature in shaping the melatonin rhythm, in which the previous photoperiod and temperature history of the fish may be important (discussed in detail below).

Increasing the temperature did not change the shape of the T3 rhythm, under either natural (NPT+) or winter (WPT+) conditions; the titers were, however, lower compared to their respective NPT or WPT counterparts (depending on the time of day). This is despite the fact that at the time of the experiment (April) the difference in temperature

was relatively small (2 °C compared to the initial ~4 °C). Independent of the photoperiod condition, plasma T4 levels remained unchanged. Interestingly, the daily oscillations also remained unchanged with the elevated temperature but only under the winter photoperiod (WPT+); under the natural photoperiod (NPT+) it appeared dampened, mainly because of a nocturnal elevation not seen in the NPT group. In *O. mykiss* several surges of T4 have been observed during a LD cycle at some times of the year but not at others (Laidley and Leatherland 1988), in addition to displaying great inter-individual variability (Gomez et al. 1997). It is possible that the 2 °C increase obtained in April was enough to induce the observed differences in T3/T4 titers and oscillations. It is also possible that the previous photoperiod history of the fish conditioned the response to temperature in April, because a change in T4 was observed in fish that experienced a natural change in photoperiod but not in those maintained under a constant winter regime. These differences impacted the T3/T4 ratio (Fig. 4, Supplementary material Fig. 9), and it is probable that the temperature elevation resulted in a lower T4 to T3 conversion, or possibly increased T4 secretion.

Overall, we provide evidence that in juvenile Atlantic salmon, increased temperature changed the shape of the melatonin oscillations, which relies on the concomitant action of photoperiod. Altering one of these parameters changes the phase relationship between the melatonin rhythm and the LD variations in other circulating hormones, as evidenced here for T3 and T4. This is likely to alter the temporal harmony between the biological events that rely on these hormones for their appropriate regulation.

Pineal melatonin secretion and thermal history

The pineal gland of fish is able to respond to rapid changes in temperature within a few milliseconds via its nervous output (*O. mykiss*: Tabata and Meissl 1993) or a few hours via its hormonal (melatonin) output (*O. mykiss*: Thibault et al. 1993; northern pike *Esox lucius*: Falcón et al. 1994; *S. salar*: this study). There are reasons to believe that the response depends on the previous thermal history of the fish: (i) the natural seasonal variations in temperature (a ~5 °C decrease from December to February and a 5–6 °C increase from February to April), had no visible effect on the shape of the melatonin daily profile, while (ii) the forced temperature increase did change the profile (in February or April); (iii) in culture, isolated pineal organs sampled at different times of the year responded differently to temperature challenges including at a 1 year interval (here the response recorded in October of the first year was different from the response obtained the following October; Fig. 6); (iv) temperature challenges induced different responses in pineal glands previously acclimated at different temperatures for 3 weeks (all other conditions being identical: time and conditions of the

experiment, animals group and strain, age, previous photoperiod and temperature history).

The complexity of the responses obtained (one or two maxima, or a flat bell-shaped curve), indicates the mechanisms involved are also complex. The penultimate and crucial step in the photoperiodic control of melatonin secretion is the conversion of serotonin into *N*-acetylserotonin by the arylalkylamine-*N*-acetyltransferase 2 enzyme (AANAT2; EC 2.3.1.87). Darkness allows activation of the enzyme and light leads to its degradation (Falcón et al. 2010). Temperature also acts on AANAT2 activity within the photoreceptor cells. At the molecular level, the AANAT2 response curve to temperature challenges is bell-shaped and the response reflects the thermal preferences of the species (Cazaméa-Catalan et al. 2012, 2013). This is different from the response of a majority of enzymes (from either fish or other vertebrates), wherein activity increases up to 37 °C and decreases at higher temperatures in *in vitro* assays. AANAT2 thermal stability and catalytic efficiency, both depend on the enzyme amino-acid sequence (Cazaméa-Catalan et al. 2012, 2013, 2014); however, in the case of salmonids there is a mismatch between the response curves to temperature obtained from recombinant enzymes or pineal homogenates on the one hand (Cazaméa-Catalan et al. 2012, 2013, 2014) and cultured pineal glands on the other hand (Nisembaum et al. 2015; Thibault et al. 1993). The complex effects of temperature such as those described herein must necessarily involve some kind of sensing at the cellular level, associated to an intracellular transduction mechanism. Previous investigations in *O. mykiss* (Nisembaum et al. 2015) indicate that Transient Receptor Potential Channels of the Vanilloid subfamily 1 and 4 (TRPV1 and TRPV4) expressed by the pineal photoreceptor cells mediate the effects of temperature on melatonin secretion. This, together with the observations that the pineal organ of fish is involved in behavioral thermoregulation (Kavaliers 1982a, 1982b; Kavaliers and Ralph 1980) and TRPV1 and TRPV4 participate in the coordination of temperature sensing during the behavioral fever in the Chilean *S. salar* (Boltana et al. 2018) may prompt future investigations on the role the pineal gland and TRP channels play in temperature sensing and regulation.

Conclusions

The present data highlight the importance of environmental influences on the timing of smoltification in salmon species particularly in those performing long downstream migrations, as is the case of the Loire/Allier basin salmon population, in which downstream migration starts about 800 km from the sea. Our data suggest that the best development of hypo-osmoregulatory capacity was obtained under natural environmental conditions. Increasing temperature and/

or maintaining winter photoperiod alters fish growth and appropriate development of gill NKA activity, thus affecting the ‘window’ during which fish may quickly regulate ion homeostasis in response to increased salinity. The increase in temperature also alters the phase relationship between rhythmic components of the fish endocrine system (here melatonin and thyroid hormones), indicating different mechanisms may contribute to controlling their rhythmic expression. The water temperature in the Loire/Allier basin has increased by ~2 °C in the past three decades (Gosse et al. 2008; Martin et al. 2012), and disadvantageous alterations of smoltification may have already begun, as seen in the past decade through the swimming activity and the identification of ‘late migrant’ smolts (Imbert et al. 2013; Martin et al. 2012). Late migration may compromise the successful entry of smolts into the sea, olfactory imprinting (which is indispensable for return to the home river (Hansen and Jonsson 1991), and survival in the ocean (Hansen and Jonsson 1989; McCormick et al. 2009)). A further rise of up to 4 °C, as predicted for the end of this century (Moatar et al. 2010), is likely to substantially compromise the survival of smolts of the Loire/Allier *S. salar* and other populations at the southern edge of their distribution or those with long freshwater migrations. Indeed, in addition to the ongoing global warming, *S. salar* faces other anthropogenic threats, including dams, artificial light at night, chemical pollution from the cities and agriculture, and illegal overfishing. The present study is a step forward in providing critical information on the environmental factors and mechanisms involved in controlling the timing of major life history events of salmon.

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Author contributions JF, PM: conceptualization; PM: setup of fish facilities and rearing; LGN, PM, MF, LB, EM, JF: sampling; JF, LGN, LB, SDM: data collection and analysis; JF, LGN, SDM: writing; JF: funding acquisition. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government.

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