Functional divergence of thyrotropin beta-subunit paralogs gives new insights into salmon smoltification metamorphosis

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Smoltification is a metamorphic event in salmon life history, which initiates downstream migration and pre-adapts juvenile salmon for seawater entry. While a number of reports concern thyroid hormones and smoltification, few and inconclusive studies have addressed the potential role of thyrotropin (TSH). TSH is composed of a α-subunit common to gonadotropins, and a β-subunit conferring hormone specificity. We report the presence and functional divergence of duplicated TSH β-subunit paralogs (tshβa and tshβb) in Atlantic salmon. Phylogeny and synteny analyses allowed us to infer that they originated from teleost-specific whole genome duplication. Expression profiles of both paralogs in the pituitary were measured by qPCR throughout smoltification in Atlantic salmon from the endangered Loire-Allier population raised in a conservation hatchery. This revealed a striking peak of tshβb expression in April, concomitant with downstream migration initiation, while tshβa expression remained relatively constant. In situ hybridization showed two distinct pituitary cell populations, tshβa cells in the anterior adenohypophysis, and tshβb cells near to the pituitary stalk, a location comparable to the pars tuberalis TSH cells involved in seasonal physiology and behaviour in birds and mammals. Functional divergence of tshβ paralogs in Atlantic salmon supports a specific role of tshβb in smoltification.

The Atlantic salmon (Salmo salar), like other salmonids, has a complex life cycle with the reproduction occurring in the upper part of the rivers, while the growth phase taking place in the ocean. A crucial life history transition, called smoltification (or parr-smolt transformation) initiates downstream migration and pre-adapts the juvenile salmon to seawater entry1-3. As recently reviewed4,5, smoltification may be viewed as a metamorphosis since it encompasses multiple morphological, physiological and behavioural changes allowing the transition from a life cycle stage and habitat to the next life stage in a different habitat (change of ecophase). Smoltification occurs during the juvenile stage, and therefore some authors refer to it as “second” or “secondary” metamorphosis in comparison to the larval metamorphosis that they refer to as “true” metamorphosis6. In amphibians, extensive research has demonstrated that larval metamorphosis is triggered mainly by thyroid hormones (TH, thyroxine T4 and triiodothyronine T3), the production of which is stimulated by a pituitary hormone, thyrotropin (or thyroid-stimulating hormone, TSH). TSH is comprised of two subunits, a common alpha subunit shared with the gonadotropins, luteinizing hormone and follicle-stimulating hormone, and a β-subunit conferring hormone specificity8.

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A histological study by Hoar first reported an activation of thyroid follicles in Atlantic salmon during smoltification. An increase in T4 plasma levels was then shown during smoltification in various salmonids (coho salmon, Oncorhynchus kisutch10,11, masu salmon, Oncorhynchus masou12; Atlantic salmon13), even though changes in T4 plasma levels were relatively limited compared to other hormones such as cortisol, insulin-like growth factor-1 and growth hormone14,15. Experimental hormonal treatments suggested that TH might be responsible for smoltification-related change in rheotaxis and swimming behaviour16 and olfactory imprinting17, but would be insufficient to induce preadaptation to osmoregulation in seawater18.

While many studies addressed TH, little is known on TSH and smoltification, and contradictory data have been obtained. In the Atlantic salmon, pituitary TSH cells were more numerous and had increased activity in presmols and smolts than in parr17, but no ultra-structural changes of TSH cells were observed in coho salmon during smoltification18. No change or a slight decrease in pituitary tshβ mRNA levels were measured in smolts as compared to parr in Atlantic salmon19,20 and coho salmon11. No variations in pituitary and plasma TSH protein levels were reported in coho salmon throughout smoltification14.

While extant amphibians, birds and mammals possess only a single TSH (a single tshβ gene), recent studies revealed the presence of duplicated tshβ paralogs in some other vertebrates21. Duplicated tshβ genes originated from whole genome duplication events that occurred in early vertebrates (“1R/2R”, for “1st and 2nd rounds of whole genome duplication”) and in early teleosts (“3R”, for “3rd round of whole genome duplication”)22. Thus, chondrichthians, such as the elephant shark, Callorhinichus milii, and basal sarcopterygians, such as the coelacanth, Latimeria chalumnae, have two tshβ paralogs issued from 2R (tshβ and tsh/β), while tetrapods have lost the tsh/β paralog and conserved only a single tshβ gene21. Tsh/β has also been lost in the actinopterygian lineage, but various teleost species possess two tshβ paralogs as a result of 3R-duplication of tshβ22,23, named in the present study tshβα and tshβb, according to the most common nomenclature “a and b” for teleost 3R-paralogs. In the present study, we searched for tshβ paralogs in the Atlantic salmon, considering also the additional genome duplication that occurred in the salmonid lineage (“4R”)24.

We revealed the presence and expression of two tshβ paralogs in Atlantic salmon and brought new knowledge on the evolutionary history of tshβ genes in salmonids. We investigated the potential involvement of the tshβ paralogs in smoltification, using the Atlantic salmon from the Loire-Allier basin as a model. This population is the last extant salmon population able to migrate long rivers in Western Europe and is currently endangered. Samplings were performed at the “Conservatoire National du Saumon Sauvage” (CNSS), Chanteuges, France, whom breeds wild brood stock and produces juvenile salmon which are released at different developmental stages, as part of a conservation programme. Quality and timing of smoltification are key issues for this population, as smolts need to achieve a 900 km-downstream migration before reaching the Loire estuary in a narrow window of suitable physiological and environmental conditions. This requires new research advances on environmental and neuroendocrine regulatory mechanisms of smoltification and initiation of downstream migration. With the demonstration of a striking expression of one of the tshβ paralogs, this study provides the first evidence of a peak expression of tsh during smoltification.

Results

Two thyrotropin beta subunit (tshβ) paralogs in the Atlantic salmon. Using the recent Atlantic salmon genome assembly (GCA_000233375.424, we retrieved two genes with two exons each, encoding complete TSH β-subunit sequences: one gene (named in the present study tshβα) located on the chromosome ssa22 and corresponding to the tshβ sequence previously isolated25, and the second gene (named in the present study tshβb) located on ssa15 and coding for another tshβ; this tshβb sequence encompassed two exons, as all vertebrate tshβ, and included a previously identified exon 121. In order to assess that the two tshβ paralogs are transcribed, cDNA sequences were successfully cloned using pituitary RNA from Atlantic salmon sampled during the parr-smolt transformation. A partial tshβα mRNA sequence (401 bp) was cloned and its sequence shared 100% identity with already characterized tshβ (AF060566)26. A tshβb mRNA sequence (517 bp) including the full length CDS was cloned (MG948546, this study); it presented 100% identity with the corresponding predicted tshβb sequence in the genome. A third putative tshβ locus was identified on ssa12, corresponding to a second paralog of tshβα. This locus includes only exon 2 with a frameshift mutation resulting in an early stop codon. Therefore this locus was identified as a tshβα-pseudogene (Supplementary Fig. S1A). In order to confirm this pseudogene, we cloned and sequenced the genomic sequence using DNA extracted from tests of an Imsa River (Norway) Atlantic salmon. The cloning confirmed the loss of exon 1 and the presence of the deletion in exon 2 leading to an early stop codon (Supplementary Fig. S1B). In addition, using Illumina short reads from an on-going sequencing project of the genome from a Loire-Allier Atlantic salmon, we aligned short reads against the two Atlantic salmon tshβ loci. For the pseudogene, read alignments confirmed the sequence of exon 2 with the presence of the deletion leading to the early stop codon. These results support the conclusion that the second tshβα paralog in Atlantic salmon is a pseudogene and that this loss is a common feature of Atlantic salmon populations.

Comparison of TSHβ deduced amino-acid sequences (Supplementary Fig. S2) showed that Atlantic salmon paralogs TSHβα and TSHβb shared 31.5% identity and 47.5% similarity and that both have conserved the twelve cysteine residues that have been shown to be required for proper folding and functional activity of TSH in mammals25. Both paralogs also shared the typical N-glycosylation site conserved among vertebrate glycoprotein hormone beta subunits; in addition, Atlantic salmon TSHβb presented a second N-glycosylation site located between the two first cysteine residues, as with the other teleost TSHβ26.

Phylogeny analysis of TSHβ. Molecular phylogeny analysis was performed on 38 gnathostome TSH β-subunit sequences and using lamprey, Petromyzon marinus, glycoprotein hormone β-subunit (GpHβ) as outgroup (Fig. 1; Supplementary Fig. S2). As previously shown27, most sequences grouped into a “classical TSHβ” clade, whereas a few chondrichthyan and basal sarcopterygian sequences formed a small TSHβ2 sister clade.
Among the TSHβ clade, the analysis also supported the two sister clades for teleost sequences resulting from teleost 3R, named here TSHβa and TSHβb following the teleost 3R paralogs nomenclature (previously named TSHβ and TSHβ321). The two Atlantic salmon TSHβ branched into the two teleost TSHβa and TSHβb clades, respectively (Fig. 1) allowing us to classify and name the Atlantic salmon TSHβ paralogs, TSHβa and TSHβb.

The pike, Esox lucius, representative species of a sister group of Salmoniforms, the Esociforms which have not undergone the salmonid 4R, also possessed the two teleost tshβ3R paralogs, encoding for TSHβa and TSHβb (Fig. 1). In contrast, we identified up to three tshβ genes in the genomes of rainbow trout, Oncorhynchus mykiss (GCA_002163495.1) and coho salmon (GCF_002021735.1): a single tshβb and two tshβa genes (tshβaα and tshβaβ according to the current nomenclature 23). They encode for three putative TSHβ (TSHβaα, TSHβaβ and TSHβb, Fig. 1, Supplementary Fig. S2).

Synteny analysis of tshβ genomic region. To further assess the origin and nomenclature of salmonid tshβ paralogs, we performed a synteny analysis (Fig. 2) of the tshβ genomic region of the pike and of two salmons, the Atlantic salmon and the rainbow trout. We used as a reference the tshβ genomic region of the spotted gar, Lepisosteus oculatus, holostean basal actinopterygian, which has not undergone the teleost 3R. Synteny analysis confirmed that the tshβ genomic region has been duplicated into two paralogons in the pike, in agreement with the teleost 3R, and further duplicated into four paralogons in the Atlantic salmon and rainbow trout, in agreement with the salmonid 4R. As an example, kcna10, one of tshβ neighbour genes, was present as a single gene in the spotted gar, as two paralogs in the pike, and as four paralogs in the Atlantic salmon and rainbow trout, reflecting full conservation of 3R- and 4R-duplicated paralogs (Fig. 2). Other tshβ neighbour genes, such as rplp2, tspan, or slc16a1, showed full conservation of 3R-duplicated paralogons, but incomplete conservation of 4R-duplicated paralogons leading to only three paralogons in salmonids (Fig. 2). Further paralog gene losses were observed for other neighbour genes, and in particular for ap4b1, with a single gene present in the pike and salmonids, as in the spotted gar, indicating losses of 3R- and 4R-duplicated paralogons (Fig. 2). With regards to tshβ, synteny analysis confirmed that tshβa and tshβb paralogons arose from the teleost 3R, as shown in the pike (Fig. 2).

Concerning tshβa, synteny analysis assessed that salmonid 4R further gave rise to tshβaα and tshβaβ paralogons, both conserved in the rainbow trout, while only tshβaα was conserved in the Atlantic salmon. Using Oxford Nanopore long reads from the on-going sequencing project of the genome from a Loire-Allier Atlantic salmon, we were able to further confirm the pseudogenization of tshβaβ. Long reads spanning the neighbouring genes of tshβaβ confirmed the lack of tshβaβ exon 1 and the presence only of tshβaβ exon 2 including the deletion leading

Figure 1. Consensus phylogenetic tree of TSHβ amino-acid sequences. Analysis was performed on 38 gnathostome TSHβ amino-acid sequences using the Maximum likelihood method, with 1000 bootstrap replicates. The tree was rooted using lamprey (Petromyzon marinus) GpHβ as outgroup. Bootstrap values are indicated at each node. The gnathostome TSHβ and TSHβ2 sister clades are highlighted in purple and yellow, and the teleost TSHβa and TSHβb sister clades, in blue and green, respectively. For sequence alignment, see Supplementary Fig. S2 and for sequence references, see Supplementary Table S1.
to an early stop codon. For simplicity, in this study we named the \( tsh_{\beta}a \) paralog conserved in the Atlantic salmon, \( tsh_{\beta}a \). Concerning the 4R-duplicated \( tsh_{\beta}b \) paralogs, synteny supported that only one paralog was conserved in the rainbow trout and Atlantic salmon, named here \( tsh_{\beta}b \).

**Differential tissue distribution of \( tsh_{\beta}a \) and \( tsh_{\beta}b \) transcripts in the Atlantic salmon.** We developed specific qPCRs for each Atlantic salmon \( tsh_{\beta}a \) and \( tsh_{\beta}b \) paralogs and compared the tissue distribution of their expression in smolts (Fig. 3). Both \( tsh_{\beta}a \) and \( tsh_{\beta}b \) paralogs were mainly expressed in the pituitary. While salmon \( tsh_{\beta}a \) transcript was exclusively found in the pituitary, \( tsh_{\beta}b \) was expressed also at low levels in various brain regions, and at lower but detectable levels in some peripheral tissues such as gills, kidney, liver, muscle, fat and gonads; \( tsh_{\beta}b \) transcripts were not detectable in heart, spleen and skin (Fig. 3).

**Evidence for the parr-smolt transformation during the experimental periods.** Three independent experiments were performed in 2013, 2014 and 2016 where fish were sampled from December/January until June in order to cover the smoltification period that occurs in early spring for the Loire-Allier Atlantic salmon population. Smoltification is classically described through typical morphological, behavioural and physiological changes. These series of changes were used to assess the occurrence of the smoltification.

**Morphological changes.** All juvenile salmon had visible parr marks at the first sampling time which progressively regressed throughout the parr-smolt transformation. Conversely, body silvering increased and darkening of the pectoral fins took place throughout the smoltification period. Photos were taken of each fish sampled in 2016 and representative photos are displayed in Supplementary Fig. S3. These changes are characteristic of the parr-smolt transformation (see review).

**Behavioural changes.** Rheotactic behaviour, positive or negative, was observed every day during the daylight hours throughout the experimentations. All parr exhibited positive rheotaxis at the beginning of the experiments and maintained this behaviour until the end of March. The inversion of rheotaxis from positive to negative, typical of smoltification and which triggers the onset of downstream migration, was observed during early April in all experiments. All fish had inverted to negative rheotaxis by April 4 (2013), April 9 (2014) or April 8 (2016). They maintained negative rheotaxis until the end of the experimental period (end of June). This inversion timing in early April is in agreement with the previous reports for the Loire-Allier salmon population.

**Physiological changes.** Gill Na\(^{+}\), K\(^{+}\)-ATPase (NKA) activity was measured in fish sampled in 2013 and 2014 (Fig. 4). Gill NKA activity was low in parr (February), increased from March to reach a peak in smolts in April/May and decreased in post-smolts in June. The increase of gill NKA activity is a typical physiological characteristic of smoltification which prepares smolts to transition from fresh water to sea water (for review).

**Peak expression of pituitary \( tsh_{\beta}b \) paralog during smoltification.** In 2013 and 2014 fish were sampled from February to June. Pituitary \( tsh_{\beta}a \) and \( tsh_{\beta}b \) mRNA levels were measured by qPCR. While the
expression profile of \( tsh\beta a \) remained relatively constant throughout the sampling period, a dramatic peak in the expression of the other paralog, \( tsh\beta b \), was measured in March-April, during the smoltification period (Fig. 4). Notably, the \( tsh\beta b \) expression peak was concomitant with the timing of rheotaxis inversion in both experiments (Fig. 4).

In order to confirm the differential regulation of the pituitary expression of \( tsh\beta a \) and \( tsh\beta b \) paralogs, we performed a third experiment from December 2015 to June 2016, with high frequency sampling during the smoltification period (Fig. 5). The results were in full agreement with 2013 and 2014 experiments. Pituitary expression of \( tsh\beta a \) remained stable throughout the experiment, while a large expression peak of \( tsh\beta b \) was recorded during the smoltification period. Pituitary \( tsh\beta b \) transcript levels started to rise during February, reached a dramatic peak in early April and then dropped at the end of April until June, to reach lower levels than in December (Fig. 5). It is noteworthy that qPCR results (Figs 4 and 5) were expressed as arbitrary units for each paralog; however comparison of quantification cycle values (Cq) between paralogs suggested lower pituitary levels of \( tsh\beta b \) than \( tsh\beta a \) at the beginning (December/February) and end (June) of the experiments (8 Cq mean difference), and even in April at the time of \( tsh\beta b \) peak (4 Cq mean difference). We observed again that the change in rheotaxis from positive to negative, a characteristic of smoltification, was concomitant with the expression peak of \( tsh\beta b \) paralog (Figs 4 and 5).

Distinct populations of \( tsh\beta a \)- and \( tsh\beta b \)-expressing cells in the Atlantic salmon pituitary. In order to get more insight into the pituitary expression of the two paralogs, we compared the localization of \( tsh\beta a \)
and tshβb transcripts by fluorescent in situ hybridization (FISH), using pituitaries from parr sampled in October and November, smolts sampled in April and post-smolts in June. Tshβa-expressing cells could be detected by FISH in parr and in smolts at all sampling times, while tshβb-expressing cells could be observed only in April. The lack of detection of tshβb-expressing cells by FISH in parr and in post-smolts is in agreement with the very low expression of this paralog outside of the smoltification period, as shown by qPCR.

Discussion

The present study revealed that two paralogous genes, named tshβa and tshβb based on phylogeny and synteny analyses, are expressed in the Atlantic salmon. The deduced protein sequences TSHβa corresponded to the subunit previously characterized in Atlantic salmon19, while TSHβb corresponded to a novel not yet investigated subunit. A third homologous gene sequence was identified as a pseudogene. Atlantic salmon TSHβa and b sequences shared the 12 cysteine residues and the N-glycosylation site conserved among vertebrate TSHβ. Yet, they were largely different with only 47.5% amino-acid similarity reflecting their divergence since the teleost 3R genome duplication event. Differently to TSHβa, Atlantic salmon TSHβb possessed a second glycosylation site, as previously observed for other teleost TSHβ21, which may confer differential biological properties. Recent work in mice has shown that tissue specific glycosylation of TSH, produced in pars distalis versus in pars tuberalis, induces differential bioactivity in the blood27,28.

Phylogeny and synteny analyses brought new advances on the evolutionary scenario of tshβ genes, as illustrated in Fig. 7. As previously indicated22, the duplicated paralogs tshβ3 and tshβ2 originated from vertebrate 2R, but only tshβ3 was conserved in the actinopterygian lineage as observed in an extant holostean, the spotted gar.
Figure 5. Profile of pituitary tshβa and tshβb transcripts in the Atlantic salmon throughout the smoltification period (experiment 2016). Under-yearling Atlantic salmon produced at CNSS were transferred in December to the experimental tanks under natural river water, temperature and photoperiod, and with circular water flow. Frequent fish samplings were made from December 2015 to June 2016. Messenger RNA levels of tshβa and tshβb paralogs were measured by qPCR, normalized to beta-actin as reference gene, and expressed as arbitrary units. Results are means ± s.e.m (n = 8 individual pituitaries per sampling group). Photoperiod and mean daily water temperature are indicated. Fish swimming behaviour and rheotaxis were observed during daytime. Fish positive (fish facing the water current) or negative (fish facing downstream) rheotaxis was observed during daytime. *Indicates date of inversion of fish rheotaxis from positive to negative.

Figure 6. Localization by FISH of tshβa and tshβb transcripts in the Atlantic salmon pituitary. Fluorescent in situ hybridization (FISH) of tshβa and tshβb was performed on 70 µm parasagittal sections of pituitaries of smolts sampled in April, 2017. FISH photos: tshβa and tshβb cells are labelled in green (FITC); cell nuclei are labelled in blue (DAPI); upper: tshβb labelling: Confocal Z-projection from 3 µm Z-stack; lower: tshβa labelling: Confocal Z-plan image. Diagram: representation of the localization of tshβa- and tshβb-expressing cell populations; tshβa-expressing cells are located in the rostral pars distalis (RPD) close to the prolactin follicles (grey circles), at the border with the proximal pars distalis (PPD); tshβb-expressing cells are less numerous and located in the dorsal PPD close to the pars nervosa (PN) of the pituitary stalk (black triangles). No FITC labelling was observed in the pars intermedia (PI). Controls were performed using FITC sense probes and showed no labelling (see Supplementary Fig. S4).
Teleost 3R duplicated tshβ into tshα and tshβ, both conserved in various extant teleosts. Salmonid 4R further duplicated tshα into tshα1 and tshαβ paralogs, both conserved in Oncorhynchus species; however, tshαβ is undergoing a loss in Atlantic salmon, where it was detected as a pseudogene. We confirmed the tshαβ gene has been conserved in the European eel (Anguilla anguilla) and the European eel, Anguilla anguilla (named tshβ and tshβ3 by the authors22).

As measured by qPCR in the Atlantic salmon, tshαβ was exclusively expressed in the pituitary, while tshβ was also expressed at lower levels in different brain regions, and in various peripheral tissues. A similar result was previously obtained in the European eel, with a pituitary-only expression of tshαβ and a more ubiquitous tissue distribution of tshβ (tshβα and tshββ respectively22). In amphibians, reptiles and birds, which possess a single tshβ gene, most studies have reported the expression of tshβ mRNA only in the pituitary (chicken26, bullfrog27, quail28; turtle29; duck30,31) while investigating a wide range of other tissues (brain, gonads, liver, thyroid, muscle, lung, heart, intestine, kidney and spleen). However in mammals, which also possess a single tshβ gene, most studies have reported the expression of tshβ transcript levels are higher than tshβ transcript levels. The expression of both tshαβ and tshββ paralogs in the pituitary has been previously reported in two other teleost species, the stickleback, Gasterosteus aculeatus (named tshβ1 and tshβ2 by the authors21) and the European eel.

We further investigated by FISH the respective localization of tshαβ and tshββ in the Atlantic salmon pituitary and revealed that the paralogs were expressed by distinct pituitary cell populations. Numerous tshαβ-expressing cells were located at the border between the RPD and antero-ventral PPD, while fewer tshββ-expressing cells were observed in the dorsal PPD close to the pituitary stalk. Early histological works in salmonids using radioiodoidecmy already reported the localization of TSH cells mainly in the RPD at the junction with PPD (Atlantic salmon;21; chinook salmon, Oncorhynchus tshawytscha22). Immunocytochemical studies, using an antibody against human TSHβ, revealed TSH cells in the ventral PPD adjacent to the RPD (chum salmon, Oncorhynchus keta and rainbow trout29,30). A similar localization was observed using an antibody raised against purified coho salmon TSH (rainbow trout21; chinook salmon30). In light of our present study, these previous investigations likely observed the localization of the abundant TSH/β.

Two distinct populations of TSH cells have also been described in the pituitary of birds and mammals, but both expressing the same single gene present in tetrapods (tshβ). The “classical” TSH cell population is located in the pars distalis (PD), while a less numerous TSH cell population is located in the pars tuberalis (PT) which surrounds the pituitary stalk (quail41; Soy sheep44; mice45; European hamster46). We may relate the localization of salmon tshαβ-expressing cells to that of amniote PD- and PT-tshβ cells, respectively, and infer that the
specific expression of \(tsh^a\) and \(tsh^b\) in these distinct pituitary cell populations would represent a typical case of subfunctionalization of duplicated paralogs.

The potential involvement of \(tsh^a\) and \(tsh^b\) paralogs in smoltification was investigated by measuring their pituitary expression profiles in juvenile Atlantic salmon from the Loire-Allier basin. Three independent sampling experiments were performed in 2013, 2014 and 2016 at CNSS. Classical smoltification-related changes such as body colouration, rheotaxis inversion and increased gill NKA activity were observed which indicate that these fish have undergone complete smolt development. Remarkably, a striking peak in the expression of \(tsh^b\), with no change in \(tsh^a\), was recorded in April, at the period of smoltification, in each yearly experiment. This is the first demonstration of a surge in pituitary \(tsh^b\) in relation to smoltification metamorphosis in salmonids. In contrast, previous studies reported slightly lower\(^{11,19}\) or no change\(^{20}\) in pituitary \(tsh^b\) transcript levels during smoltification; these investigations were in fact targeting the \(tsh^a\) paralog, the expression of which remains relatively stable as shown in the present study. The demonstration of a differential regulation of the pituitary expression of salmon \(tsh^a\) and \(tsh^b\) paralogs, with a specific peak of \(tsh^b\), revealed a marked functional divergence of the two paralogs, conferring a specific role in smoltification to \(tsh^b\) paralog.

In birds and mammals, TSH produced by PT cells plays a key role in the seasonal regulation of major steps of life cycles, including reproduction, migration and hibernation\(^{4,47,48}\). PT-TSH stimulates the expression of type 2 deiodinase (DIO2), which catalyses the conversion of T4 into the more biologically active T3, thus leading to the activation of TH-regulated brain functions (Fig. 8). In a recent study\(^{49}\), a salmonid specific 4R-issued DIO2 paralog (\(dio2b\)) has been identified in the Atlantic salmon, the expression of which increases in circumventricular brain area of cell proliferation, during experimental photoperiod-induced smoltification. The authors proposed a specific role of DIO2b in promotion of TH-dependent brain development during smoltification\(^{49}\).

In the line of PT-TSH role and action mechanism in amniotes, we propose that TSHb produced by dorsal PPD cells in Atlantic salmon may stimulate brain DIO2b expression and promote TH-activated brain functions related to smoltification (Fig. 8). As a support to this hypothesis, in our three yearly experiments, the expression peak of \(tsh^b\) paralog occurred simultaneously with smoltification-related changes in rheotaxis, which triggers the onset of downstream migration.

In the Atlantic salmon, \(tsh^b\) was expressed not only in the pituitary but also at lower levels in various brain regions, so that additional regulatory pathways may also occur locally in some brain regions. Such a local signalling between light sensors, TSH and DIO2, has been proposed in the \(saccus vasculosus\) for the photoperiodic regulation of reproduction in masu salmon\(^{46,61}\). In our study, \(tsh^b\) transcripts were detectable in the \(saccus vasculosus\) of the Atlantic salmon smolt but were at least 100 times less expressed than in the pituitary; \(tsh^a\) transcripts

![Figure 8. Proposed subfunctionalization of TSHa and TSHb, with potential role of TSHb in Atlantic salmon smoltification.](image)
were not detectable outside the pituitary. The expression of tsh/β paralog, not only in the pituitary, but in various brain regions and peripheral tissues observed in the present study warrants future investigations on the possible variations of tsh/β transcripts in these regions during smoltification.

In mammals, PT-TSH has been suggested to act not only on the basal hypothalamus but also to exert some paracrine effects in the pituitary22, so that we may also hypothesize pituitary actions of tsh/β paralog during salmon smoltification. Using a basal teleost, the European eel, as a model, we previously identified 3R-duplicated TSH receptor (TSHR) paralogs and revealed the specific expression of tsh/β paralog in various brain regions as well as in the pituitary, supporting brain and pituitary actions of TSH in teleosts21. Further studies should aim at investigating TSH receptor paralog number and tissue distribution in the Atlantic salmon.

In the present study, we propose a specific role of Atlantic salmon tsh/β paralog at smoltification, related to the change in rheotaxis and triggering of downstream migration. The remarkable expression peak of pituitary tsh/β demonstrated in our study may be of high relevance for deciphering the internal and environmental regulation of salmonid smoltification and initiation of downstream migration; especially concerning the endangered long-river Loire-Allier salmon population, understanding these mechanisms may provide new basis for its conservation. A specific role in migration of tsh/β paralog (named tsh/β by the authors22) was also suggested in the stickleback22, based on higher pituitary transcript levels of tsh/β in populations migrating to the sea, as compared to stream-resident populations, with no difference in tsh/β paralog (named tsh/β1 by the authors22) between the two ecotypes. The authors suggested that genetic differences in cis-regulatory regions of tsh/β gene may convey this adaptive divergence in migratory behaviour during stickleback radiation22,23. Similarly, there is large diversity of migration strategies among salmonid species and populations, from long-river anadromous to landlocked. Our present findings open new research avenues for comparing tsh/β expression during smoltification, as well as tsh/β regulatory genomic sequences, between salmonid species and ecotypes.

In conclusion, two tsh/β paralogs are expressed in the Atlantic salmon by distinct pituitary cell populations, and exhibit a striking functional divergence, with a large expression peak of tsh/β, but not of tsh/α, during smoltification. This is the first demonstration of a peak expression of tsh/β in salmonid smoltification. This involvement of the thyrotropic axis complies with the endocrine regulation of vertebrate metamorphosis typically observed in amphibian and flatfish. A specific role of tsh/β paralog is suggested in the onset of smoltification-related downstream migratory behaviour, possibly mediated by the stimulation of brain DIO2 and T3 production, as shown for PT-TSH involved in the seasonal regulation of life cycle traits in birds and mammals. The remarkable functional divergence of tsh/β and tsh/α in salmon may have represented selective forces for the conservation of these duplicated paralogs.

**Methods**

**Fish.** The study was carried on juvenile Atlantic salmon (Salmo salar) of the Loire-Allier population raised indoor under natural water, temperature, and photoperiod conditions, at the Conservatoire National du Saumon Sauvage (CNSS), Chanteuges, France (Agreement N° B43 056 005; according to the ARRETE N° DDCSPP/CS/2016/40), which breeds wild returning adult Atlantic salmon genitors caught at the Vichy dam, 620 km from the Loire estuary. The research project was performed in accordance with guidelines and regulations according to the protocol approved by Cuvier Ethic Committee France.

For each experimental year, 390 under-yearling fish were transferred in December into two circular tanks (3 m diameter; depth range 0.5 m) supplied with UV filtered natural running water from the Desges River (tributary of the Allier). Experiments were conducted from December to end of June, which spans the smoltification period that occurs in early spring for the Loire-Allier Atlantic salmon population26. Three independent experiments were performed (2013, 2014 and 2016). Water temperature was measured using probes (Johnson control, Colombes, France; TS 9101: accuracy ± 0.2 °C). An anti-clockwise flow was achieved by a tangentially oriented water inlet at the periphery of the tank and a central drain as previously described26. Photoperiod regime mimicked the natural photoperiod by using an outside light sensor that controlled the light above each tank. Each tank had a LedBulb (D 14–75 W E27 827 A67; Philips, Amsterdam, Netherlands) 3 m above the water surface. Fish were fed automatically with a custom fish diet (Turbot label Rouge, Le Gouessant, Lamballe, France) in excess five times a day at equal intervals during daylight hours. Fish swimming behaviour was visually observed during daytime: positive rheotaxis for fish facing the water current versus negative rheotaxis for fish swimming with the current26.

**Tissue collection.** Fish were anesthetized with an overdose of ms222 (0.4 ml/l, Sigma-Aldrich, St Louis, MI, USA). Photos were taken with a Canon EOS 1200D Digital SLR Camera with EF-S 18–55 mm f/3.5–5.6 III Lens in order to follow colouration changes, characteristics of smoltification. Fish were killed by decapitation. For qPCR analyses of the tissue distribution of tsh/α and tsh/β transcripts, 10 fish (5 males and 5 females) were sampled in March 2015; the following organs were individually collected and stored in RNA later (Ambion Inc, Austin, USA) at −20 °C until RNA extraction: retina, brain (dissected into olfactory bulbs, telencephalon, epiphysis, optic lobes, hypothalamus, saccus vasculosus, cerebellum, medulla oblongata), pituitary, as well as samples of gill filaments, kidney, liver, spleen, muscle, skin, abdominal fat, testis or ovary. For qPCR analyses of pituitary tsh/α and tsh/β expression profiles throughout smoltification, 20 fish (mixed sex) were sampled once a month from February to June in 2013 and 2014, and 8 fish (mixed sex) were sampled at more frequent intervals from December 2015 to June 2016; individual pituitaries were collected in RNA later and stored at −20 °C until RNA extraction. For fluorescence in situ hybridization of tsh/α- and tsh/β-expressing pituitary cells, 8 fish were sampled in April and 8 in June 2017; individual pituitaries were collected, fixed in paraformaldehyde (PFA) overnight at 4 °C, dehydrated in increasing series of ethanol (EtOH) concentration and stored in 98% methanol at −20 °C before further processing.
**Measurement of Na\(^{+}\), K\(^{-}\)-ATPase activity.** Gill samples of 10 fish per sampling times were collected in experiments 2013 and 2014. For each fish, four to six primary gill filaments were placed into 100 μl of ice-cold SEI buffer (250 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C for measurement of gill Na\(^{+}\)/K\(^{-}\)-ATPase (NKA) activity. NKA activity was determined with 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. Gill tissue was homogenized in 150 μl of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000 × 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 μmoles ADP mg protein\(^{-1}\) h\(^{-1}\). Protein concentrations are determined using BCA (bicinchoninic acid) Protein Assay (Pierce, www.piercenet.com, Rockford, IL, USA).

**Identification of tshβ paralogs in the Atlantic salmon.** Gene and transcript names are in lower case and italics (e.g. tshβ) and protein names are in upper case (e.g. TSHβ). Atlantic salmon tshβ loci were identified in the recent Atlantic salmon genome assembly (ICSASG_v2, GCA_000233375.4) after interrogation of the Atlantic salmon annotated gene database in GenBank. The presence of additional tshβ genes was investigated by blasting salmon tshβa and tshβb against the Atlantic salmon genome. Gene sequences were examined with CLC Main Workbench 8 (Qiagen Bioinformatics, Hilden, Germany) for prediction of exons, introns, coding sequence (CDS) and signal peptide.

**Cloning and sequencing of partial cDNA of Atlantic salmon tshβa and tshβb paralogs.** Cloning primers for Atlantic salmon tshβa and tshβb were designed on predicted mRNA sequences of corresponding genes (LOC100136355 and LOC106572976) using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, US) (Supplementary Table S3). PCR was performed using cDNA of smolt pituitaries collected in April using Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) for tshβa and Platinum Taq DNA polymerase (Invitrogen) for tshβb according to the manufacturer’s instructions. Purified PCR fragments were subcloned into PCRII vectors (Thermo-Fisher, Waltham, MA, USA) before sequencing by GATC Biotech (Brussels, Belgium). Plasmids were used for preparing cRNA probes (section fluorescence in situ hybridization).

**Cloning and sequencing of genomic DNA sequence of the second Atlantic salmon tshβa.** Cloning primers for the Atlantic salmon tshβa/β gene were designed, based on the alignment of salmons tshβa/β, on both sides of the coding sequence of the functional salmonid tshβa/β using Primer3 (Supplementary Table S3, Supplementary Figure s). Hatchery-produced Atlantic salmon from wild caught brood-fish from the Imsa River (Norway) were used. Genomic DNA from testis samples was extracted by alkaline lysis. Testis pieces were heated in alkaline buffer (25 mM NaOH, 0.2 mM EDTA) at 95 °C for 5 min with vortexing. Reaction was stopped by adding the neutralisation buffer (40 mm Tris-HCL). PCR was performed using Atlantic salmon genomic DNA, DNA polymerase (Invitrogen), according to the manufacturer’s instructions with a thermal cycle protocol including a 10 cycle touchdown phase (from 60 to 50 °C) followed by 40 cycles with annealing temperature at 50 °C. Purified PCR fragments were subcloned into PCRII vectors (Thermo-Fisher) before sequencing by GATC Biotech.

**Illumina libraries and sequencing.** Genomic DNA (gDNA) was extracted from snap-frozen liver from juvenile Loire-Allier Atlantic salmon using the Genomic-tip 100/G (Qiagen). DNA was subsequently sheared using a nebulizer (Life Technologies). Paired-end libraries were prepared from 5 μg of sheared DNA using the Paired-End Sequencing Sample Prep kit (Illumina Inc., San Diego, USA). For the library size selection step, the 400 bp band was cut from the agarose gel, purified and amplified by 10 PCR cycles. The resulting library was analyzed with a Bioanalyzer 2100 DNA 1000 series II chip (Agilent, Santa Clara, USA). All libraries were sequenced using an Illumina HiSeq2500 instrument with a read length of 2 × 151 nucleotides to a total of ~103.75 Gb of sequencing data and up to ~686.5 million reads.

**Nanopore libraries and sequencing.** Genomic DNA (gDNA) was extracted from snap-frozen liver, spleen and kidney from a juvenile Loire-Allier Atlantic salmon using the Genomic-tip 100/G (Qiagen). gDNA size was inspected in the TapeStation Genomic DNA system (Agilent) and found to be ~60 Kb. The gDNA was subsequently sheared to 10–20 Kb fragments using a g-tube (Covaris, Woburn, MA) before library preparation. The library preparation was performed using 1D Genomic DNA by ligation for either SQK-LSK108 or SQK-LSK109 kit (Oxford Nanopore technologies, Oxford, UK). All nanopore libraries were sequenced in a FLO-MIN106 R9.4.1 SpotOn Flow Cell attached to either MinION or GridION devices (Oxford Nanopore Technologies), generating 28.3 Gb of nanopore sequencing data divided over ~3.21 million reads.

**Alignment of Illuma and nanopore reads to tshβ paralogs.** Illuma reads were aligned to the tshβ paralogs using bowtie2 (version 2.2.5) while nanopore reads were aligned using minimap2 (version 2.5-r572) with the default Oxford nanopore parameters (map=ont). SAMtools (version 1.2) was used to remove unmapped reads from the SAM file, convert to BAM, sort and generate the index. The resulting BAM files were visualized using Integrative Genomics Viewer (IGV) (version 2.3.83).

**Phylogeny analysis.** Phylogeny analysis of 39 vertebrate TSHβ amino-acid sequences was performed using a part of dataset from, enriched with additional teleost TSHβ sequences, including TSHβ paralogs of salmonids identified in this study. New tshβ genes were either retrieved from GenBank or were identified by blasting (TBLASTN algorithm) genome assembly databases when genes were not annotated in GenBank. The amino-acid
sequences of TSHβ were deduced and signal peptides were predicted using CLC Main Workbench 8 (Qiagen). The sequence alignment was performed on CLC Main workbench 8 and manually adjusted. Phylogenetic tree was constructed using Maximum Likelihood algorithm with PhyML3.064 combined to the SMS model selection65 and SPR as tree improvement on ATGC browser (http://www.atgc-montpellier.fr/phyml/). Tree topology was assessed by bootstrapping on 1000 replicates.

Synteny analysis. Synteny analysis was performed on tshβ genomic region in actinopterygians, using a holostean, the spotted gar as a reference (LepOcu1 (GCA_000242695.1)). Comparisons were made with tshβ paralogs in the pike (Eluc_V3 (GCA_000721915.3)), and in two salmonid representatives, Atlantic salmon (ICASG_v2 (GCA_000233375.4)) and rainbow trout (Omyk_1.0 (GCA_002163495.1)). Neighbouring genes of tshβ loci were identified and compared manually using chromosome annotation. Blast analyses on the genomes were performed to search for un-annotated genes and additional paralogs. Genes fractionated, showing a frameshift mutation or missing exon were considered pseudogenes.

RNA extraction and cDNA synthesis. Total RNA was extracted by homogenizing tissues in TRIzol (Thermo-Fisher) according to the manufacturer’s protocol, using TissueLyser II (Qiagen). After a chloroform separation step, RNA was precipitated in ice cold isopropanol with 1 μl of glycobio (Ambion). Total RNA was treated with DNase I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. RNA concentration was measured using Nanopore 2000c/2000 (Thermo-Fisher). Reverse transcription was performed using 75 ng random hexamer primers (Invitrogen) and SuperScriptIII First Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer’s protocol. For pituitaries, 250 ng of total RNA were used and 750 ng for brain and peripheral tissues.

Quantitative RT-PCR. Specific quantitative real-time PCR (qPCR) primers for tshα and tshβ were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/)35 with forward and reverse primers on two different exons to prevent amplification of genomic DNA (Supplementary Table S3); primers were purchased from Eurofins scientific (Luxembourg). Specificity of the primers was controlled by sequencing PCR product. β-actin was used as reference gene using previously published primers62. Quantitative PCR assays were performed using LightCycler 1.2 (Roche Diagnostics) and LightCycler FastStart Master plus SYBR Green I kit (Roche Diagnostics). Each reaction contained: 4 μl of diluted cDNA template, 2 μl of SYBR green master mix and 1 μl of specific primers (500 nM final concentration). The following thermal cycling steps were used for each qPCR run: initial denaturation 94 °C for 10 min followed by 41 cycles of 10 s of denaturation at 95 °C, 5 s of annealing temperature (60 °C or 62 °C; Supplementary Table S3) and 6 s of elongation at 72 °C. The program ended by slowly increasing temperatures (0.1 °C/s) from 68–95 °C for amplification specificity controlled by melting curve analysis. Relative quantification was performed using standard curves created for each gene with serial dilutions of pooled pituitary cDNA. One dilution of the cDNA pool was added in each run as a calibrator. All samples were analysed in duplicates and each qPCR run contained a negative control using water in substitute for template cDNA. Calculations of sample concentrations were made using the Roche LightCycler 1.2 manufacturer’s software.

Fluorescence in situ hybridization (FISH). Antisense and sense cRNA probes for FISH were synthesized by in vitro transcription from tshα or tshβ plasmids using T7 and SP6 RNA polymerase (Promega, Madison, Wisconsin USA) and labelled with digoxigenin-11 UTP (Roche Diagnostics) at 37 °C for 2 h. Probes were purified using Nucleospin RNA cleanup kit (Machere-Nagal, Hoerdt, France) and controlled by gel electrophoresis. Probe length for tshα was 401 bp which nearly covers the 420 bp CDS sequence. Probe length for tshβ was 512 bp which covers the entire CDS sequence (Supplementary Fig. S5).

Whole fixed pituitaries were rehydrated (96, 70, 50, 25% EtOH), included in 3% RNase free agarose gel and sliced into 70 µm parasagittal sections using VT1000S Leica vibratome (Leica, Wetzlar, Germany). Sections were permeabilized using proteinase K (Sigma-Aldrich, 1 µg/ml in PBS with 0.1% Tween 20, PBST) for 45 min at 37 °C, then proteinase K was inactivated using glycine (Sigma-Aldrich, 2 mg/ml in PBST) for 30 min at room temperature (RT), followed by a post fixation step in 4% PFA for 15 min and washing in PBST.

Prior to FISH, sections were incubated with hybridization buffer (HB: 50% formamide, SSC 5X, 0.1% Tween 20, 15 ng/ml Heparin, Sigma-Aldrich; 80 µg/ml Torula yeast RNA, Sigma-Aldrich; pH 6.5) at 55 °C for 4 h. FISH was performed in fresh HB containing 300 ng/ml tshα/a probe or 600 ng/ml tshβ/b probe at 55 °C. After 18 h, sections were washed with a series of 4 different hybridization washes (HW): HW1 (50% formamide, SSC 5 X, 0.1 % Tween 20) 2 × 30 min, HW2 (50% HW1, 50% SSC 2 ×) 2 × 30 min, HW3 (SSC 2 ×, 1 % Tween 20) 2 × 30 min, HW4 (SSC 0.2 ×, 0.1 % Tween 20) 2 × 2 min. Sections were soaked in TNE buffer (10 mM Tris HCl pH 7.6, 500 mM NaCl, 1 mM EDTA) at 37 °C for 30 min then treated with RNase A (Sigma-Aldrich, 20 µg/ml in TNE buffer) for 30 min at 37 °C. Sections were washed in TNE buffer, 2 × 10 min at 37 °C followed by SSC 0.2 × 0.1 % Tween 20, 2 × 30 min at 55 °C. Sections were washed in PBST for 10 min with agitation at RT and incubated in PBST with 2% H2O2 for 30 min in order to inactivate endogenous peroxidases, followed by washes in PBST, 3 × 10 min with agitation. Blocking was performed using 1% Blocking Reagent (Roche Diagnostics) in Maleic acid buffer (MAB) for 2 h with agitation. Sections were incubated in blocking/MAB buffer with anti-digoxigenin peroxidase-conjugated antibody (1/250, Roche Diagnostics) overnight at 4 °C, and then washed in PBST for 2 h with agitation. Tyramide revelation was carried out using green FITC conjugated tyramide (Sigma-Aldrich, 1/200 in PBST with 0.01% H2O2) for 30 min in darkness at RT. After washing in PBST for 5 × 20 min, sections were let overnight in PBST at 4 °C in darkness. Cell nuclei were stained using DAPI staining (Sigma-Aldrich, 1/1000 in PBST) for 20 min at RT. Sections were mounted in Vectashield H-1000 Mounting Medium (Vector, Eurobio/ Abcys, CA, USA). Confocal images were obtained using a confocal microscope (Zeiss LSM710, Oberkochen, Germany). Channels were acquired sequentially to avoid signal crossover between the different filters. Images
were processed using the ZEN software (version 2009, Zeiss). Z-plan and Z-projection images were obtained using Image J software (Fiji software; http://rsbweb.nih.gov/ij/).

Data Availability

Data generated or analysed during this study are included in this article (and its Supplementary Information file).

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Author Contributions
M.S.F. and J.R. performed fish monitoring. M.S.F., G.M., A.G.L., S.D. and P.M. performed tissue sampling. M.S.F., G.M., A.G.L. and R.N.L. performed PCR and qPCR analyses. R.D. and E.S.Y. did Illumina and nanopore sequencing and read alignments. G.M. did phylogeny and synteny analyses. M.S.F., R.F. and F.A.W. performed FISH. S.D.M. performed NKA enzyme activity measurements. M.S.F., G.M., K.R. and S.D. wrote the main manuscript. M.S.F. and G.M. created the figures. S.D. and P.M. supervised the work. All authors reviewed the manuscript.

Additional Information
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