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ARTICLE

River-Specific Gene Expression Patterns Associated with Habitat Selection for Key Hormone-Coding Genes in Glass Eel-Stage American Eels

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Abstract

The glass eel stage of the American Eel *Anguilla rostrata* marks the onset of the catadromous migration into estuarine or freshwater habitats, and the endocrine mechanisms underlying this habitat selection are still not well understood. Using a candidate genes approach, the aim of this study was to test for different patterns of gene expression related to (1) salinity preferences and/or (2) capture site to predict physiological differences between migratory behaviors. We performed analyses revealing the expression of genes coding for key hormonal factors or their receptors on glass eel-stage American Eels collected at the mouths of three rivers on the east coast of Canada (Grande-Rivière-Blanche, St. Lawrence estuary; Rivière-Saint-Jean, Gaspé Peninsula; and the Mersey River, Nova Scotia); eels from the three systems displayed different salinity preferences (brackish water/salt water/freshwater) under laboratory conditions. Transcripts from genes coding for prolactin (PRL), thyroid-stimulating hormone β subunit, type-2 iodothyronine deiodinase (DIO-2), thyroid hormone receptors α_a and α_b (THRα_a and THRα_b), growth hormone (GH), insulin-like growth factor 1 (IGF-1), and their respective receptors (GH-R_1 and IGF-1R) were all detected in glass eels. No differences in the expression patterns were detected pertaining to salinity preference, but strong differences were found among rivers. Rivière-Saint-Jean glass eels, which were the longest and the least pigmented among the three rivers, were characterized by the highest expression of PRL, DIO-2, and THRα_b. Those from Grande-Rivière-Blanche showed an increase in IGF-1R. Glass eels captured from these two rivers also exhibited the highest expression of GH and GH-R_1. Overall, these results confirm gene × environment interactions at the gene expression level when glass eels settle into their continental habitat. As such, our results also support the concept of the presence of different ecotypes in the Atlantic Canadian coast and in the estuary and Gulf of St. Lawrence.

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The glass eel stage of the American Eel *Anguilla rostrata* marks the end of the oceanic migration of the leptocephalus larvae and the onset of catadromous migration into estuarine or freshwater (FW) habitats (Jessop et al. 2002; Tesch 2003; Cairns et al. 2004; Pavey et al. 2015). The range of American Eel distribution along the coast of North America is large and covers tropical, temperate, and subarctic areas. Facultative catadromy (nonobligatory trophic migration to FW) has only recently been documented in eels; Tsukamoto et al. (1998) were the first to describe a "sea eel" ecophenotype, with ecophenotype defined as an ecological subunit that is adapted to a particular habitat under environmental influences (Turesson 1922). In glass eel-stage European Eels *A. anguilla*, facultative catadromy has been suggested to be under endocrine control through phenotypic plasticity mechanisms, and different ecophenotypes (FW versus brackish water [BW]/salt water [SW]) were associated with differences in osmoregulatory ability (Edeline et al. 2005b). The FW type exhibited a salinity preference for FW and colonized river habitats. Glass eels exhibiting FW preference were also characterized by high locomotor activity and poor growth performance traits that were described as promoting the colonization of FW (Edeline et al. 2005b). This "FW type" of glass eel-stage European Eel also had high thyroid gland activity (higher plasma levels of thyroxine compared to triiodothyronine) and high thyroid hormone levels relative to glass eels of the "SW type" (Edeline et al. 2004). Edeline et al. (2005a) also showed that immersion in thyroxine enhanced locomotor activity, while immersion in thiourea modified rheotaxis, supporting an active role of thyroid hormones during glass eel migrations. In contrast to those with a FW preference, the BW/SW type of glass eel-stage European Eel exhibited BW/SW preference, low locomotor activity, high growth performance (Edeline et al. 2005b), and low thyroid activity with low thyroid hormone levels, especially thyroxine (Edeline et al. 2004). This ecophenotype colonized marine and estuarine habitats (Edeline et al. 2009). Weak thyroid activity was hypothesized to affect sensitivity to olfactory cues, intestine development, and rheotaxis (Edeline 2005). Based on results obtained for the European Eel, Edeline et al. (2009) proposed that a strategy related to condition was underlying the expression of different ecophenotypes. Thus, it is predicted from these previous studies that high energetic status and high thyroid activity but a low level of growth hormone (GH) secretion should characterize the FW ecotype, while low energetic status and low thyroid activity but a high level of GH secretion should characterize the BW/SW ecotype (Edeline et al. 2005a, 2005b, 2009).

A recent population genomics study on American Eels showed that yellow and silver eels colonizing FW and BW/SW in eastern Canada can be genetically distinguished and reclassified with high accuracy, supporting the occurrence of a genetic basis for the different ecotypes in this species (Pavey et al. 2015). In contrast to an ecophenotype, an ecotype is an ecological subunit that is morphologically, physiologically, and genetically adapted to a habitat and, if transplanted into a different habitat, its differences would be retained, as they are fixed genetically (Turesson 1922). Moreover, rearing of glass eel-stage American Eels in different salinity conditions also resulted in different growth performance according to their geographic origin, confirming this notion in young stages (Coté et al. 2009). In addition, transcriptomic differences were observed for polygenic traits and genes involved in many physiological functions related to both salinity rearing conditions and glass eel origin (Coté et al. 2014). However, using American Eel glass eels captured at different locations on the Canadian east coast, Boivin et al. (2015) showed that although most did not make a choice between FW and SW, they usually preferred FW regardless of their geographic origin.

The aim of this study was to test whether differences in hormones and/or hormone receptor genes are associated with differences in the settlement habitat of glass eel-stage American Eels. We studied gene expression tools, an approach that was successfully used previously both in American Eel glass eels (Gaillard et al. 2015, 2016) and in embryonic and larval European Eels (Politis et al. 2017), and we studied glass eels colonizing rivers previously associated with different American Eel ecotypes (Mersey River on the Atlantic coast [BW/SW ecotype]; Rivière-Saint-Jean, Gulf of St. Lawrence [FW ecotype]; and Grande-Rivière-Blanche, St. Lawrence estuary [FW ecotype]; Pavey et al. 2015) and according to their salinity preference (Boivin et al. 2015). Since the St. Lawrence estuary glass eels were associated with the FW ecotype (Pavey et al. 2015), we hypothesized that expression of prolactin (PRL), a hyperosmoregulatory hormone for FW adaptation, and coding genes for the thyroidal axis would be higher than that in glass eels from the Atlantic coast (BW/SW ecotype). We also hypothesized that higher expression of the somatotropic axis would be present in glass eels from the southern location (smaller, heavier glass eels) compared to the northern locations. Considering the model for European Eels (Edeline et al. 2009), we predicted that American Eel glass eels with a FW preference would have a higher expression of thyroid function and lower expression of coding genes for the somatotropic axis compared to those exhibiting a BW/SW preference. Finally, using glass eels exhibiting different salinity preferences, we employed gene expression analysis tools to test whether the endocrine model underlying the expression of salinity preference suggested by Edeline (2005), Edeline et al. (2009) for glass eel-stage European Eels could apply to American Eels.

METHODS

Sampling.— Glass eels were captured during the new and full moon at their earliest arrival in the estuaries of three east coast Canadian rivers at different upstream distances relative to the Sargasso Sea (Figure 1): Mersey River, Nova Scotia (March 26–28 and April 20–21, 2012; $n = 3{,}209$); Rivière-Saint-Jean, Québec (May 16–21 and May 28–June 3, 2012; $n = 636$); and Grande-Rivière-Blanche, Québec (June 2–6 and June 18–21, 2012; $n = 1{,}657$). At the Mersey River, glass eels were captured with fish nets by a commercial elver fishery; at Grande-Rivière-Blanche, we captured glass eels with fish nets; and at Rivière-Saint-Jean, glass eels were captured in the river estuary with two trap nets operated by the Ministère des Forets, de la Faune, et des Parcs (see Boivin et al. 2015). Sampling was identical for glass eels captured in each of the three rivers.

On the day after capture, glass eels were transferred to the Maurice-Lamontagne Institute according to the procedures of the Canadian Council on Animal Care to assess salinity preference (Boivin et al. 2015). They were placed in a thermostatic chamber and kept unfed (see Boivin et al. 2015 for detailed information about the experimental system). After 48 h of acclimation, behavioral experiments took place. The experimental setup was used to assess FW preference (percentage of eels that chose FW), SW preference (percentage of eels that chose SW), and BW preference (percentage of eels that remained in BW). Three glass tanks (31.5 × 27 × 61 cm) provided triplicate measurements for each experiment. Acclimation salinity and salinity into the experimental tanks were both 18‰. Two funnels connected to filtering flasks were inserted into each tank. Freshwater and seawater (salinity = 33‰) were gravity-delivered into the neck of the flasks at a rate of 180 mL/min, offering a binary choice between flows of FW and SW. An overflow drain allowed any excess water to be evacuated throughout the experiments. Charcoal-filtered, dechlorinated tap water was used as FW, while BW and SW were prepared by adding either FW or synthetic salts (Instant Ocean) to sand-filtered St. Lawrence estuary

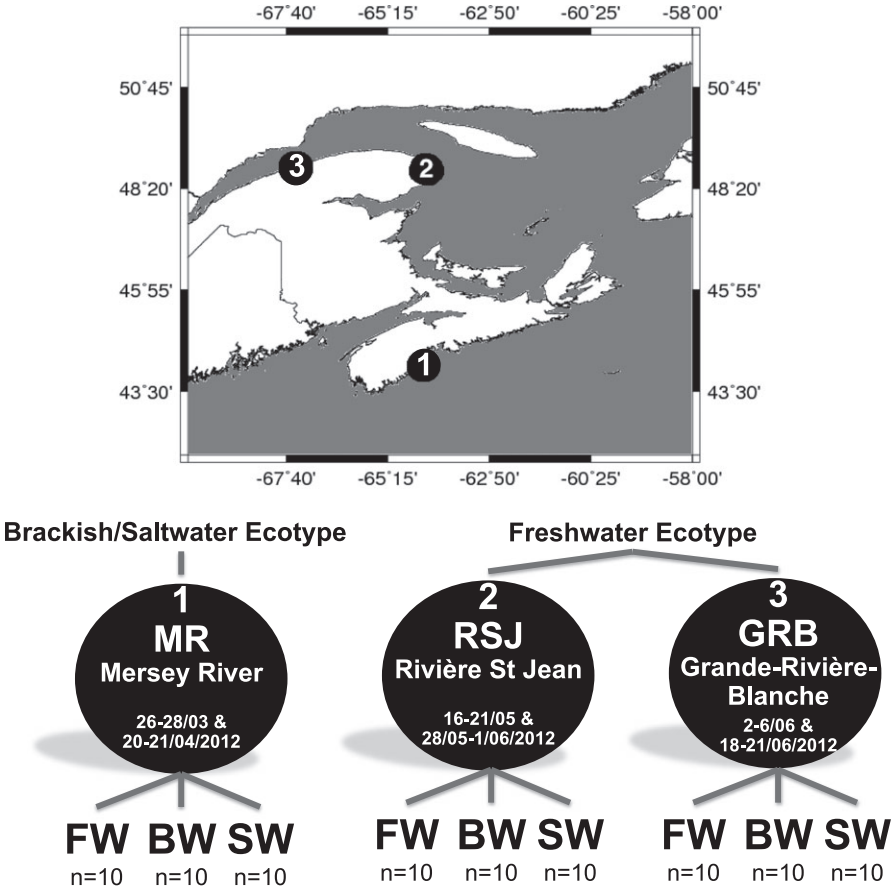

FIGURE 1. Map showing sites where glass eel-stage American Eels were sampled: (1) Mersey River (MR), Nova Scotia (brackish-water [BW]/saltwater [SW] ecotype); (2) Rivière-Saint-Jean (RSJ), Gulf of St. Lawrence, Québec (freshwater [FW] ecotype); and (3) Grande-Rivière-Blanche (GRB), St. Lawrence River, Québec (FW ecotype). The dates of capture and a schema representing the experimental design (10 glass eels per river and per salinity preference [FW, BW, or SW]; Boivin et al. 2015) are also shown.

water (salinity = 20–25‰). See Boivin et al. (2015) for more detailed information about evaluation of the experimental bias of the apparatus. For all experiments, an average ± SD of 71 ± 34 individuals, selected to ensure a sufficient number of replicates, was placed at the beginning of each experiment in the BW-filled waiting chamber for an acclimation period of 30 min, after which the water flows were activated for a 30-min experimental period. Both the acclimation and experimental periods were conducted in darkness in order to minimize the stress associated with the manipulations and because glass eels are mainly active at night in natural habitats. A preference experiment was run for each glass eel arrival (two per river; see Figure 1), and each experiment was performed in triplicate tanks (see Boivin et al. 2015). At the end of the experiments, all glass eels that chose FW from the triplicate experimental tanks were pooled, as were those that chose SW and those that remained in BW. From these, 10 "FW," 10 "SW," and 10 "BW" glass eels (for a total of 20 per salinity preference per river) were individually anesthetized in 0.68-mM tricaine methanesulfonate (Sigma-Aldrich). For this study, we used 10 individuals classified as FW, SW, or BW from each river, resulting in a total of 90 glass eels sampled for further analyses (Figure 1). Total body length (from the tip of the snout to the tip of the caudal fin; nearest 1 mm) and wet mass (nearest 1 mg) were measured. Pigmentation stage was determined according to Haro and Krueger (1988). Glass eels were rinsed with BW, gently blotted dry, transferred to 1.5-mL tubes filled two-thirds full with RNAlater (Sigma-Aldrich, Oakville, Ontario), and kept frozen overnight at 4°C before being stored at −20°C until molecular analyses were performed.

Candidate and reference genes.— Nine candidate genes were studied: thyroid-stimulating hormone β subunit (TSH-β), type-2 iodothyronine deiodinase (DIO-2; enzyme code 1.21.99.4; IUBMB 1992), thyroid hormone receptors α_a and α_b (THRα_a and THRα_b), PRL, GH and its receptor 1 (GH-R_1), and insulin-like growth factor 1 (IGF-1) and its receptor (IGF-1R). The TSH β subunit is a proxy of TSH that stimulates the thyroid gland to produce thyroxine (Han et al. 2004; MacKenzie et al. 2009); DIO-2 is the major isoform that converts the pro-hormone thyroxine into bioactive triiodothyronine (e.g., Gomes et al. 2014); and THRα_a and THRα_b mediate the biological activity of thyroid hormones binding with triiodothyronine (e.g., Gomes et al. 2014). Prolactin is well known for its central role in FW osmoregulatory processes (ionic and osmotic balance; Manzon 2002; Sakamoto and McCormick 2006). Growth hormone stimulates growth through IGF-1 activation, has a direct effect on growing tissues, and regulates lipid mobilization (e.g., Dai et al. 2015). The receptor GH-R_1 binds specifically with GH to initiate the actions of GH (Ozaki et al. 2006a, 2006b). In fishes, IGF-1 mediates the action of GH, which promotes somatic growth during cellular differentiation and mitogenesis processes in muscle tissues, during development of the nervous system, and in bones, and it has a critical role in neural induction by binding with IGF-1R, which triggers its actions (Perrot et al. 1999; Escobar et al. 2011; Dai et al. 2015). Growth hormone and IGF-1 are also associated with SW acclimation (e.g., Sakamoto and McCormick 2006).

The quantitative PCR (qPCR) analysis must be normalized using internal standards—the reference genes—for which transcription is assumed to be constant. The use of only one reference gene in qPCR analyses is not recommended (Bustin et al. 2009), and the expression of reference genes can vary from one tissue to another (Olsvik et al. 2005). Vandesompele et al. (2002) recommended the use of three reference genes for the reliable normalization of a pool of normal tissues in order to avoid relatively large errors caused by the use of one reference gene. Based on previous eel studies (Weltzien et al. 2005; Gaillard et al. 2016), the reference genes chosen for the present work were acidic ribosomal protein (ARP), cytochrome b (Cyt b), and elongation factor 1 (EF1).

Whole-body grinding.— Glass eels were individually dry-homogenized with liquid nitrogen using a Precellys dual homogenizer coupled with a cooling system (Precellys; Bertin Technologies, Rockville, Maryland) in CKMix 50 R containing beads for hard-tissue grinding. Samples were ground using three cycles of 26 s at 5,800 revolutions/min, and cycles were separated by 30 s. The resulting powder was held at −80°C until RNA extraction.

Total RNA extraction.— The RNA was extracted from 10 mg (dry mass) of homogenate powder using the RNeasy Fibrous Tissue Kit (Qiagen, Inc., Mississauga, Ontario) and was diluted to obtain a final concentration of 200 ng/μL. The purity, quality, and concentration of RNA were determined using electrophoresis on 2% agarose gel stained with ethidium bromide (0.05 mg/mL; Alpha Imager HP System, Alpha-Innotech; Alpha Imager 3400 software, Protein Simple) and the 260/280 absorbance ratio (NanoVue Plus spectrophotometer; GE Healthcare, Pittsburgh, Pennsylvania).

Reverse transcription.— Reverse transcription was performed in duplicate using the Quantitect Reverse Transcription Kit (Qiagen). The complementary DNA (cDNA) samples obtained were diluted to a final concentration of 20 ng/μL, separated into aliquots, and kept frozen at −20°C until further analysis. The integrity and concentrations of cDNA were verified using a NanoVue Plus spectrophotometer. Reverse transcriptase efficiency was verified using serial dilutions of a pool of four RNA samples from different origins and dates of capture and compared with the ideal slope of −3.3. Quantitative PCR

analyses were performed in triplicate (Bio-Rad MyiQ iCycler; Bio-Rad Laboratories, Inc., Mississauga, Ontario) using IQ SYBR Green Supermix (Bio-Rad) and an iCycler iQ Real-Time PCR on one reference gene (EF1) and one candidate gene (PRL). Linear regression of the serial dilution curves was performed with MyiQ software version 1.0 (Bio-Rad), giving an efficiency of 94.5% for the reference gene ($y = -3.4603x + 10.341$; $r = 0.997$) and 97.4% for the candidate gene ($y = -3.3187x + 22.533$; $r = 0.935$).

Specific sequences and design of TaqMan primers and probes.— Except for the IGF-1R sequence, which was obtained from the draft annotated American Eel genome (Pavey et al. 2016), the messenger RNA (mRNA) sequences for the reference and target genes were not available for American Eels in the GenBank databases. Therefore, oligonucleotide primers were designed using Primer-BLAST (Basic Local Alignment Search Tool) for each reference and candidate gene of interest based on available mRNA sequences from genus *Anguilla* found in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/ accessed November 23, 2015). Primers were ordered from Integrated DNA Technologies (Coralville, Iowa) and diluted to 20 μM before use. GenBank numbers for sequences were found on the NCBI Web site; forward and reverse primer sequences are reported in Table 1. A pool of randomly chosen cDNA samples (rivers and salinity preferences) was used with primers for amplifications (all in duplicate) by PCR with iCycler iQ Real-Time PCR (Bio-Rad) using the AmpliTaq Gold 360 Master Mix Kit (Applied Biosystems, Inc., Foster City, California). The quality and integrity of each PCR product or amplicon were verified by electrophoresis on 2% agarose gels with ethidium bromide (0.05 mg/mL) containing a PCR marker (Sigma-Aldrich). Single fragments were obtained for all reference and candidate genes except THRα_a, for which two fragments were obtained. Both THRα_a amplicons were isolated with the Ezna Gel Extraction Kit (Omega Bio-Tek, Norcross, Georgia) before purification and were thereafter treated separately. Amplicons were purified on columns using the QIAquick PCR Purification Kit (Qiagen), and purified amplicons were sequenced in forward and reverse directions with associated primers and the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Unincorporated dye terminators from sequencing reactions were removed using the Ultra-Step Dye Terminator Removal Kit (Omega Bio-Tek). Isolated fragments containing dye-labeled dideoxynucleotide triphosphates were dried for 20 min using a Speed Vac (Savant AS 160 Automatic) and suspended in formamide; fragments were analyzed using an Applied Biosystems 3130 Genetic Analyzer and POP-7 polymer (Life Technologies, Burlington, Ontario). Sequence assembly and alignment verification were achieved with Sequencher version 5.2.4 (Genes Codes Corp., Ann Arbor, Michigan).

Alignments between the sequence obtained and the sequence used for primer design were performed for each gene. The supplemental table (available in the online version of this manuscript) reports specific sequences obtained for each gene. Except for IGF-1R, the percentages of identity between sequences obtained from glass eels and sequences from GenBank are also presented in the Supplement. For THRα_a, the longest sequence that showed the highest homology score was retained for the study. TaqMan probes were designed using Primer Express version 3.0 (Applied Biosystems) and were obtained from Life Technologies (Table 1).

Real-time PCR assays and quantification.— Quantitative PCR was performed in triplicate on glass eel samples with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The 10-μL volume for each reaction was made up of 2 μL of cDNA (10^{-2} ng/μL), 5 μL of TaqMan Advanced Mix (Life Technologies), 2.5 μL of sterile water, and 0.5 μL of TaqMan probe specific to a gene (Life Technologies). Thermal cycling of qPCR consisted of two steps: (1) 2 min at 50°C for optimal AmpErase uracil-N-glycosylase activity followed by 10 min at 95°C to activate the AmpliTaq Gold DNA Polymerase, and (2) 45 cycles of denaturing at 95°C for 30 s and annealing/extension at 60°C for 1 min. Cycle thresholds (C_T) were obtained using Expression Suite version 1.0 (Applied Biosystems).

Relative quantification of gene expression was calculated according to the $2^{-\Delta\Delta C_T}$ method of Livak and Schmittgen (2001),

$$2^{-\Delta\Delta C_T} = 2^{-(\Delta C_{Te} - \Delta C_{Tc})}$$

where $C_{Te} = C_{T,candidate\ gene} - C_{T,reference\ genes}$ for sample x; and $C_{Tc} = C_{T,candidate\ gene} - C_{T,reference\ genes}$ for the calibrator (see below).

In this study, the calibrator was the group of glass eels sampled from the Mersey River (the southernmost study river) that exhibited a BW preference (absence of choice for either FW or SW). The calibrator always represents the 1.0-fold expression level, and other individuals and groups are expressed in these units. Nondetectable expressions (threshold cycles greater than 38) were given the same C_T value of 40 instead of eliminating fish that showed no expression, thus avoiding an overestimation of the global expression level. For this reason, normalization for PRL and TSH-β was above 1.0.

Quantitative PCR validation.— Quantitative PCR efficiency was verified for each TaqMan probe (slopes close to −3.3; all $R^2 > 0.98$; Table 1). A serial dilution of 10^{-1} to 10^{-5} (or, when the signal was low, 4^{-1} to 4^{-5} [IGF-1 and THRα_a] or 2^{-1} to 2^{-5} [PRL and TSH-β]) was performed

TABLE 1. List of GenBank accession numbers, eel species, and sequences of forward (F) and reverse (R) primers, TaqMan primers (F, R), and TaqMan probes (P) for the reference and candidate genes. Quantitative PCR (qPCR) efficiencies (linear regression equation and R^2) are also presented.

Coding gene	Species and GenBank accession number	Forward and reverse primers used for sequencing	TaqMan forward, reverse, and probes used for qPCR	qPCR efficiency
Acidic ribosomal protein (ARP)	European Eel *Anguilla anguilla* (AY763793)	F: GCCACGTGGAAGTCCAACTA R: CAGGAGTTTCTCCAGAGCGG	F: TCTCCCTGCGTGCAAAGG P: TGGTGCTGATGGGC R: CTTGCGCATCATGGTGTTCT	$y = -3.1958x + 34.388$ ($R^2 = 0.99926$)
Cytochrome b (Cyt b)	European Eel (AF006714)	F: CATCTGCCGAGACGTCAACT R: ATCTGCCCTCATGGAAGCAC	F: TCATCTGCCGAGACGTCAAC P: ATGGATGATTAATTCGC R: GAGGCCCCATTTGCATGTAG	$y = -3.1254x + 32.523$ ($R^2 = 0.99922$)
Elongation factor 1 (EF1)	Japanese Eel *A. japonica* (AB593812)	F: CCTGAAGCCTGGTATGGTGG R: TACGTTGCCACGACGGATTT	F: TTGCCCCTGCCAACGT P: ACCACTGAGGTCAAGTC R: GGGACTCATGGTGCATTTCC	$y = -3.3354x + 33.755$ ($R^2 = 0.99974$)
Prolactin (PRL)	European Eel (X69149)	F: CTTCCCTCCAAACCCCTCAC R: CCTTGGAGGAGGAGCCAATC	F: GGCGCCATCTACAGCAAAAC P: AGGGAACTGCAGGACC R: CCCAGAGCTCAGGCTGTTG	$y = -3.2675x + 29.078$ ($R^2 = 0.98724$)
Thyroid-stimulating hormone β subunit (TSH-β)	Japanese Eel (AY158008.1)	F: GCCCTCCAAGATGAGAGTGG R: GCAGACTGGAGGCTCTTACC	F: GCAGGACAGGTTCTCTCCATCT P: CCTGTGGACTACACGCT R: TCACACTCTGGTTTCTCCACGTA	$y = -3.2842x + 28.009$ ($R^2 = 0.98643$)
Thyroid hormone receptor α_a ($THR\alpha_a$)	Japanese Eel (AB678206.1)	F: TCGTTTTGTTGCAGGGGTTG R: CTTTCATTGGCAAGCTCCCG	F: CGATGCAGAAAGTGGTGATACC P: TGAGACCGACCGCTC R: CAAATCTCCACTGCTCTTGTCTCA	$y = -3.6152x + 24.467$ ($R^2 = 0.99738$)
Thyroid hormone receptor α_b ($THR\alpha_b$)	Japanese Eel (AB678207.1)	F: CTCACGTGCGTGGAGAAGAT R: GTCTGCTTCACACCTCCTGG	F: GCTGGCGTTCGAACACTACA P: AACTACCGCAAGCACA R: TGGGCCAGAAGTGAGGAATG	$y = -3.1052x + 40.300$ ($R^2 = 0.99847$)
Type-2 iodothyronine deiodinase (DIO-2)	Japanese Eel (AB199797.1)	F: AAGCTGGGCTTCAGTAGCAG R: CCTCTGAGCAGCCAGAACTC	F: CCTGCTGGACTTTGCGTCAT P: CGACCGTCCTTTAGTG R: GTGGCCGAGCCAAAGTTG	$y = -3.3820x + 40.970$ ($R^2 = 0.99624$)
Growth hormone (GH)	European Eel (AY616666.1)	F: CCCAGGTTAAGGGGCTGATG R: CAAGCCCAATCCCTCACACA	F: ATCTCCTCAGCCCTGATCCA P: TCATGGGTGTATCCTC R: AAGCATCGCTCAGGGTCTTC	$y = -3.1047x + 25.839$ ($R^2 = 0.99967$)
Growth hormone receptor 1 ($GH\text{-}R_1$)	Japanese Eel (AB180476.1)	F: CATCTTCACCCCTTGCCTGT R: TACTGGGTCCAGACGGATGT	F: GGGTCTTCTTCGCCACTAGCT P: CTGCCCAGCGACTG R: TGACCGAGTAGTCCGGACACT	$y = -3.3030x + 38.281$ ($R^2 = 0.99985$)
Insulin-like growth factor 1 (IGF-1)	Japanese Eel (AB353115.1)	F: GCTGCAGTTTGTGTGTGGAG R: TCTGATGCACCTCCTTGCAG	F: CAGGCTATGGATCCAGCTCAA P: ACGGTCACACAATCG R: GCAGCACTCGTCGACTATGC	$y = -3.3309x + 25.418$ ($R^2 = 0.99202$)
Insulin-like growth factor 1 receptor (IGF-1R)	American Eel *A. rostrata*	S. A. Pavey, unpublished data	F: CGAGCGCATCGAGTTCCT P: AACGAGGCGTCCGTC R: GGTGGCAGTTGAACTCCTTCA	$y = -3.2673x + 40.298$ ($R^2 = 0.99755$)

on a new pool of eight randomly selected cDNA samples within samples of different origin and salinity preference. The qPCR analyses for each TaqMan probe were performed on the pool with the same protocol described above, except that the 2 μL of cDNA (10^{-2} ng/μL) were replaced by 2 μL of the pooled cDNA. Suitability, stability, and validation of qPCR reference genes were verified with Expression Suite version 1.0, where the score was calculated according to Vandesompele et al. (2002). The score is a gene stability measure in qPCR analyses that may vary with tissues: the lower the score, the more stable is the expression. The gene stability measures for the three reference genes were 0.786, 0.836, and 0.714 for ARP, Cyt b, and EF1, respectively (calculated using Expression Suite software). The three reference genes exhibited very little variation among subsamples (Supplementary Figure 1).

Le Cren condition index and statistical analyses.— The Le Cren condition index (K_n) was calculated as described by Gaillard et al. (2015). Linear regressions of $\log_{10}$ transformed length and wet mass were conducted using data from all individuals that expressed a salinity preference. The constants were determined from the regression line obtained ($y = -5.4551 + 2.6137x$; $r^2 = 0.50$; $n = 1{,}143$). The residual distribution of K_n was verified by fitting a Henry line ($F = 1{,}786.65$; df = 1, 1,140; $P < 0.0001$; $r^2 = 0.61$). As demonstrated by Gaillard et al. (2015), K_n is both a condition index and an indicator of triacylglycerol and glycogen content. Because Gaillard et al. (2015) already showed that K_n, wet mass, length, and pigmentation stage did not differ according to salinity preference, only the presence of a river effect was tested for these variables.

The relative quantification of gene expression ($2^{-\Delta\Delta C_T}$) for the nine candidate genes was analyzed using two-way permutational multivariate ANOVA (PERMANOVA; $\alpha < 0.05$, 9,999 permutations, type III sums of squares) with the PERMANOVA+ add-on (version 1.02) in PRIMER version 6.1.1.12. The two fixed factors were river (levels = 3) and salinity (levels = 3). Missing data (29 of 810) were replaced by the mean of the subsample in a salinity preference–river group (García et al. 2015). Distance-based tests for homogeneity of multiple dispersions (PERMDISP) were verified for the two factors to determine whether data needed transformation, and we found that no transformations were required (river: $F = 1.7571$, $P_{perm} = 0.2421$; salinity: $F = 1.1642$, $P_{perm} = 0.3832$). Thus, a Bray–Curtis similarity matrix was constructed for relative gene expression data. Even though this type of statistical approach was first developed for ecological studies, it is now used in gene expression (e.g., Maurice et al. 2013) or metagenomic studies (e.g., Tamaki et al. 2011). When PERMANOVA tests detected a factor effect, pairwise comparisons were conducted. Finally, to explore dissimilarities between groups, we performed multidimensional scaling (MDS) plot analysis. Similarity percentage (SIMPER) analysis was also run to identify the relative contribution of each gene and biological trait to the differences observed within one factor. Because SIMPER results were similar to those obtained with the MDS (results not shown), only the MDS results are presented. One-way ANOVAs ($\alpha = 0.05$) were run to test for specific differences in gene expression for each candidate gene or biological trait using STATISTICA version 10.0 (http://www.statsoft.com accessed November 21, 2015) when PERMANOVA indicated significant factor effects. The ANOVAs were followed by Tukey's multiple comparison tests ($P < 0.05$). Normality and homoscedasticity of data were verified with the Kolmogorov–Smirnov test and Levene's test, respectively. Pigmentation index data were analyzed with the nonparametric Kruskal–Wallis test.

RESULTS

Gene Expression and Salinity Preference

At the glass eel stage, no salinity preference was associated with the expression of any candidate gene since no factor or interaction effects were detected (salinity preference: pseudo-$F = 0.92891$, df = 2, $P_{perm} > 0.05$; salinity preference × river: pseudo-$F = 1.2209$, df = 4, $P_{perm} > 0.05$). However, patterns of gene expression differed among the three rivers (river: df = 2, pseudo-$F = 4.1295$, $P_{perm} < 0.0005$). Differences were most apparent between Rivière-Saint-Jean and Mersey River glass eels (pairwise tests, Grande-Rivière-Blanche and Mersey River: $t = 1.5866$, $P_{perm} < 0.05$; Grande-Rivière-Blanche and Rivière-Saint-Jean: $t = 2.0927$, $P_{perm} < 0.005$; Mersey River and Rivière-Saint-Jean: $t = 2.3426$, $P_{perm} < 0.001$), which was also confirmed by the MDS analysis (Figure 2). Differences in the expression of PRL, DIO-2, and GH on the MDS horizontal axis and the expression of TSH-β and IGF-1R explained most of the differences among rivers. The expression of TSH-β and that of IGF-1R were strongly positively correlated, and a positive correlation was also observed between DIO-2 and PRL (Figure 2A, C). The MDS results suggested that high expression of DIO-2 and PRL characterized the Rivière-Saint-Jean glass eels, while those from Grande-Rivière-Blanche were characterized by stronger expression of IGF-1R, TSH-β, and GH (Figure 2A, C).

Expression of PRL was 10.3 times higher in Rivière-Saint-Jean glass eels than in the calibrator (Mersey River eels with BW preference), while PRL expression in Mersey River and Grande-Rivière-Blanche glass eels was similar and close to the expression level observed in the calibrator group (Figure 3A). Expression of DIO-2 was 2.7 times

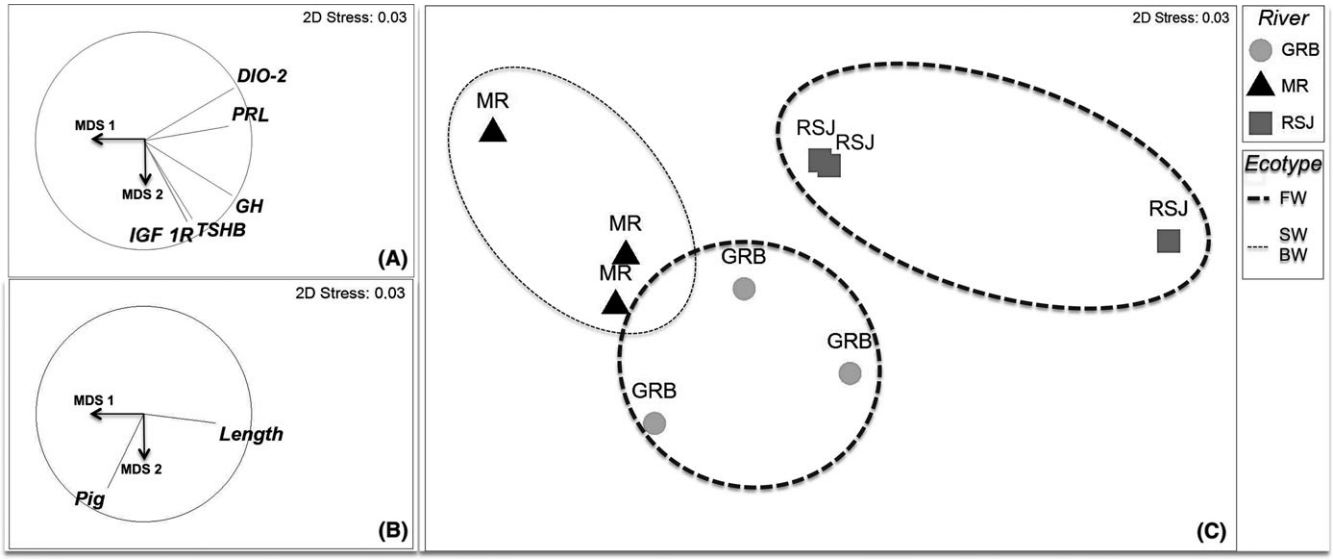

FIGURE 2. Multidimensional scaling (MDS) of Bray–Curtis similarities from the gene expressions database and associated vector plots for glass eel-stage American Eels from three rivers (GRB = Grande-Rivière-Blanche; MR = Mersey River; RSJ = Rivière-Saint-Jean). Only vector plots that contributed the most to dissimilarities between rivers are shown (Spearman correlation coefficient $\rho > 0.60$ on one MDS axis). Vector plots characterize the grouping on the MDS plot: direction of the vectors is dictated by **(A)** elevated gene expressions and **(B)** elevated biological trait measurements. **(C)** Each river was averaged in the MDS by the factor river × salinity to maximize readability on the two-dimensional ordination. Bold dashed ellipses indicate the freshwater (FW) ecotype; thin dashed ellipse indicates the saltwater/brackish-water (SW/BW) ecotype. Spearman correlations of the vector plots were as follows: (A) $\rho = -0.84$ for type-2 iodothyronine deiodinase (DIO-2), −0.93 for prolactin (PRL), −0.71 for growth hormone (GH), −0.40 for thyroid-stimulating hormone β subunit (TSH-β), and −0.30 for insulin-like growth factor 1 receptor (IGF-1R) on the horizontal axis of MDS 1; $\rho = -0.37$ for DIO-2, −0.09 for PRL, 0.55 for GH, −0.62 for TSH-β, and 0.82 for IGF-1R on the vertical axis of MDS 2; and (B) $\rho = -0.64$ for length and 0.75 for pigmentation stage (Pig) on MDS 1; $\rho = -0.74$ for length and −0.11 for pigmentation stage on MDS 2.

higher in Rivière-Saint-Jean glass eels compared to the calibrator group, and this expression level was significantly greater than those observed in glass eels from the two other rivers (although DIO-2 expression was 1.7 times higher in Grande-Rivière-Blanche glass eels than in Mersey River glass eels; Figure 3B). Growth hormone expression was 2.0 and 2.4 times higher in the glass eels from Rivière-Saint-Jean and Grande-Rivière-Blanche compared to the calibrator group and was significantly higher than the value observed in Mersey River glass eels (Figure 3C). Expression of IGF1-R was 1.3 times higher in Grande-Rivière-Blanche glass eels compared to the calibrator group, and this level was significantly higher than observed in glass eels from the other two rivers (Figure 3D). Contrary to our prediction, the expression of TSH-β did not differ among glass eels from the three rivers (Figure 3E). Expression of $THR\alpha_b$ in Rivière-Saint-Jean glass eels was slightly more elevated (1.64) than in the calibrator group and significantly higher than levels measured in Mersey River and Grande-Rivière-Blanche glass eels (Figure 3F). Finally, the expression of $GH\text{-}R_1$ was significantly higher (1.3 times) in Rivière-Saint-Jean glass eels compared to glass eels from the Mersey River, with intermediate expression levels in Grande-Rivière-Blanche eels (Figure 3G). Again, expression in the Mersey River glass eels was very close to that of the calibrator. The expression of $THR\alpha_a$ (Figure 3H) and IGF-1 (Figure 3I) was similar among rivers.

Phenotypic Traits

The longest glass eels were captured in Grande-Rivière-Blanche and Rivière-Saint-Jean (Figure 4A). The Rivière-Saint-Jean and Mersey River glass eels were 1.2 times heavier than those captured in Grande-Rivière-Blanche and had a higher condition index (Figure 4B, C). Glass eels captured in Rivière-Saint-Jean were generally nonpigmented (pigmentation index close to 1), while pigmentation at the lateral line was present in both Mersey River and Grande-Rivière-Blanche glass eels (median of the pigmentation index ranged from 2 to 4; Figure 4D). The MDS analysis indicated that phenotypic differences among glass eels from the three rivers were largely associated with variations in length and pigmentation status.

DISCUSSION

The first goal of this study was to investigate whether the endocrine model underlying salinity preference

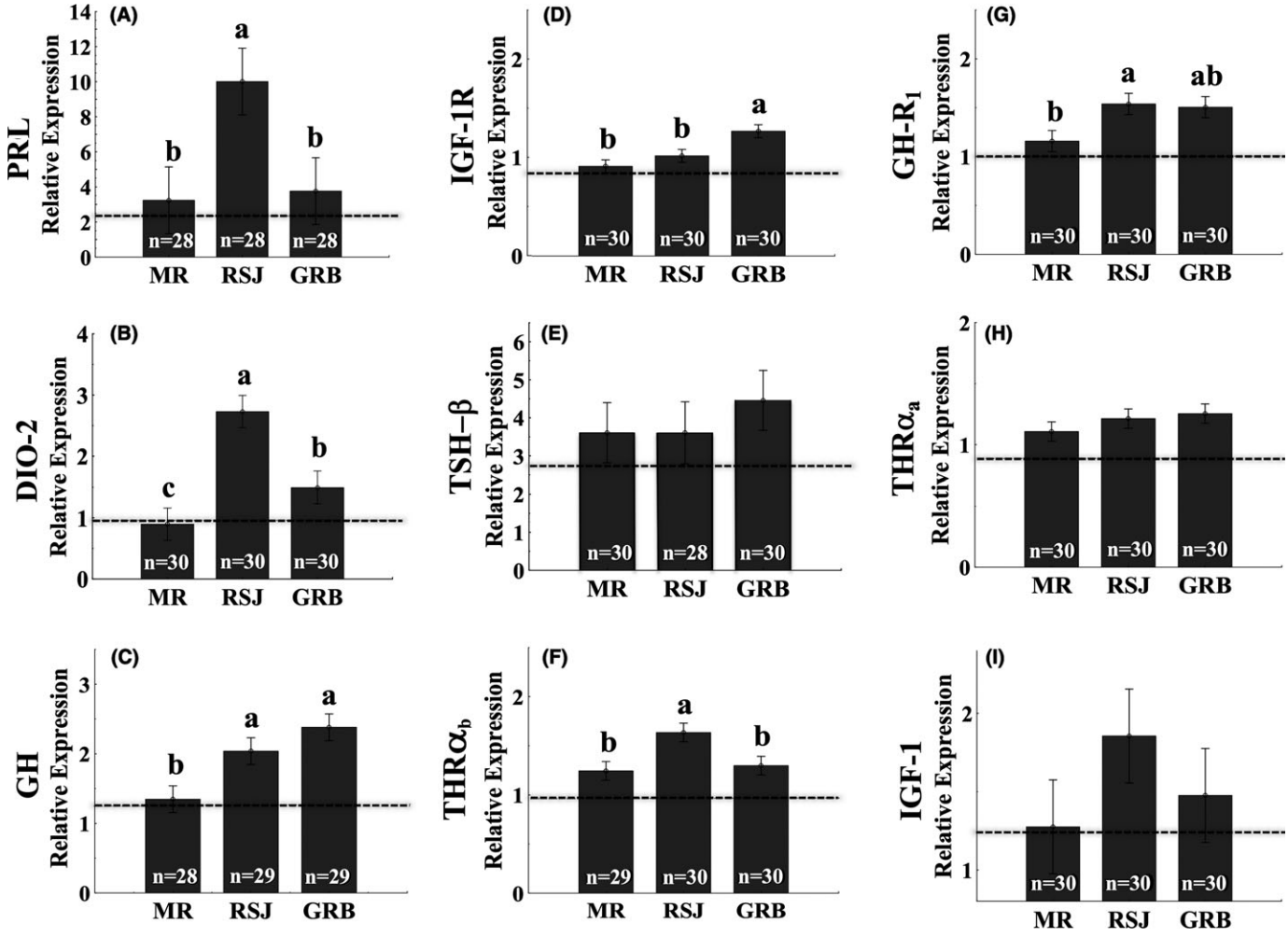

FIGURE 3. Relative changes in gene expression (mean ± SE) of nine candidate genes in glass eel-stage American Eels captured from the Mersey River (MR), Rivière-Saint-Jean (RSJ), and Grande-Rivière-Blanche (GRB): **(A)** prolactin (PRL), **(B)** type-2 iodothyronine deiodinase (DIO-2), **(C)** growth hormone (GH), **(D)** insulin-like growth factor 1 receptor (IGF-1R), **(E)** thyroid-stimulating hormone β subunit (TSH-β), **(F)** thyroid hormone receptor α_b (THRα_b), **(G)** growth hormone receptor 1 (GH-R_1), **(H)** thyroid hormone receptor α_a (THRα_a), and **(I)** insulin-like growth factor 1 (IGF-1). The dashed horizontal lines indicate the normalized values for the calibrator group (MR glass eels with a preference for brackish water). Different letters indicate significant differences among rivers (Tukey multiple comparison tests: $\alpha = 0.05$).

suggested by Edeline et al. (2009) for European Eel glass eels could apply to American Eels. A second goal was to test whether there were different hormone gene expression patterns associated with the different habitats colonized by glass eels. To achieve these goals, comparative analyses of gene expression were performed according to the glass eels' salinity preference and site of capture. Although we found limited support for different expression patterns according to salinity preference, pronounced differences in gene expression were observed among rivers of origin. However, the observed patterns did not generally correspond to our working hypotheses and predictions. Thus, we found no difference in the expression of genes coding for the thyroxine–triiodothyronine axis or for the GH–GH-R–IGF-1–IGF1-R axis according to salinity preference. These results, coupled with those demonstrating the absence of differences related to energy storage status according to salinity preference and the presence of strong differences between glass eels of differing origins at the gene expression and cellular levels (Gaillard et al. 2015, 2016), confirm that the "hypothetical endocrine mechanism for the control of glass eel migratory plasticity" suggested for glass eel-stage European Eels (Edeline et al. 2009) does not apply to American Eels.

Differential Pattern of Gene Expression among Sites of Capture

The presence of different American Eel ecotypes in the Maritimes and in the St. Lawrence River is supported by strong evidence based on growth and sex

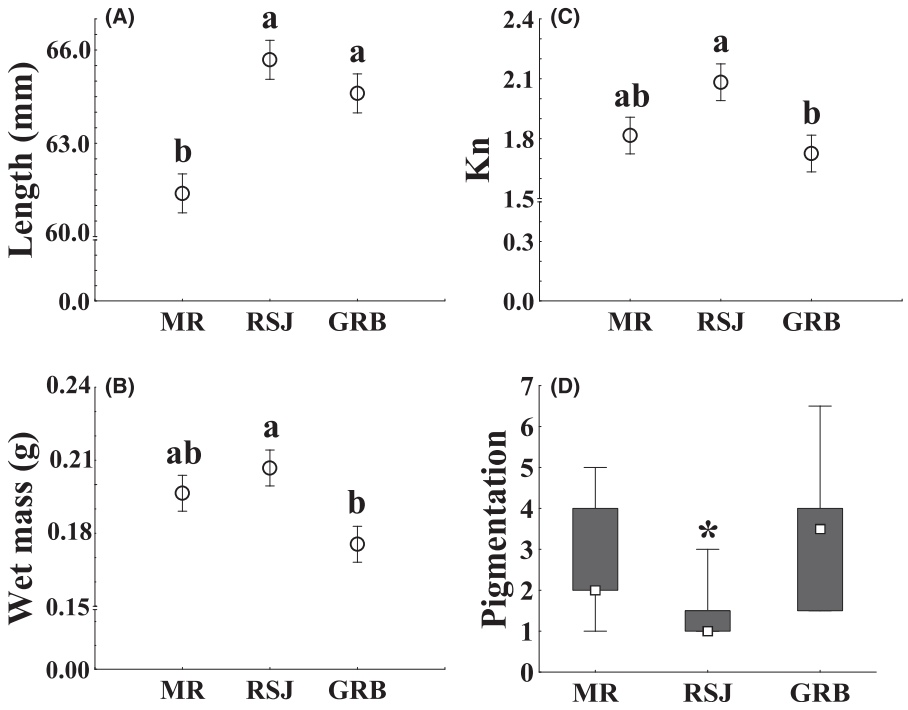

FIGURE 4. Biological trait measurements in glass eel-stage American Eels captured from the Mersey River (MR), Rivière-Saint-Jean (RSJ), and Grande-Rivière-Blanche (GRB): **(A)** length (mean ± SE), **(B)** wet mass (mean ± SE), **(C)** LeCren condition index (K_n; mean ± SE), and **(D)** pigmentation stage (box plot depicts the median, 25th and 75th percentiles, and minimum and maximum values). The asterisk (nonparametric analysis) and different letters (parametric statistical analysis) indicate significant differences among rivers.

determination phenotypic attributes (Coté et al. 2015) and a genome-wide association study (Pavey et al. 2015). Based on genome-wide genotypic differences, Pavey et al. (2015) associated (1) the St. Lawrence River ecotype to a FW ecotype and (2) the Atlantic Canada ecotype to a BW/SW ecotype. Thus, both intra- and inter-ecotype differences were found, the most pronounced being between the Mersey River and Rivière-Saint-Jean, which each harbored a different ecotype. Rivière-Saint-Jean glass eels were characterized by the highest expression of PRL, DIO-2, and THRα_b, a hormonal gene expression pattern that would be expected for a FW ecotype. They were also the least pigmented, indicating that they were the least developed. Expression of DIO-2 was also higher in Grande-Rivière-Blanche glass eels than in Mersey River glass eels. Considering that both Rivière-Saint-Jean and Grande-Rivière-Blanche contain the FW ecotype, their higher DIO-2 expression compared to the calibrator group could be associated with FW colonization. Even though there was no specific difference in the expression of TSH-β, MDS analysis discriminated Grande-Rivière-Blanche glass eels through their expression of this hypothalamic factor, which stimulates thyroxine production. The results of the MDS analysis showed a clear correlation between the PRL and DIO-2 expression patterns; thus, a link between activation of transcripts from the thyroidal axis and the FW ecotype cannot be dismissed.

Glass eels seem to be prepared for the osmotic challenge posed by the SW–FW transition, given that they have twice as many PRL cells compared to leptocephali (in Japanese Eels *A. japonica*; Arakawa et al. 1992) and they develop a multi-layered esophageal mucosa (in European Eels; Ciccotti et al. 1993). Considering the important role of PRL in FW adaptation (Sudo et al. 2013), it is not surprising to observe a higher expression level in Rivière-Saint-Jean glass eels than in Mersey River glass eels. However, we expected a similarly high level of PRL in Grande-Rivière-Blanche glass eels, especially since Coté et al. (2014) observed differences in gene expression for two unique transcripts associated with PRL function between Grande-Rivière-Blanche glass eels and those from the Mersey River prior to their river entrance, but this was not the case for PRL here. However, these results showed that activation of PRL transcripts was high in unpigmented FW glass eels that just achieved metamorphosis from leptocephali into glass eels. This finding is in agreement with a previous study by Arakawa et al. (1992).

Complex patterns for coding genes of the somatotropic axis were observed in glass eels from the three rivers. Higher levels of GH and GH-R_1 expression were

observed in the FW ecotype, and those glass eels were longer than those captured in the Mersey River. Recently, Politis et al. (2017) studied the larval stage of European Eels and showed that GH expression was higher at a higher temperature. Here, temperature may not play such a role, as temperatures in northern areas (Grande-Rivière-Blanche and Rivière-Saint-Jean) are expected to be colder than in the Nova Scotia littoral zone. Expression of the IGF-1 receptor was also higher in Grande-Rivière-Blanche glass eels. Although not statistically different, IGF-1 expression also tended to be more elevated in these two rivers. Previous studies on American Eels found different effects of origin on the growth of glass eels and yellow eels (Coté et al. 2009, 2015; Boivin et al. 2015). For example, glass eels from the BW/SW ecotype had a higher wet mass and grew faster in controlled conditions, regardless of the salinity of rearing water, than glass eels from the FW ecotype (Coté et al. 2009, 2015; Boivin et al. 2015). Thus, a stimulated hormone-encoding gene for the somatotropic axis would have been expected in Mersey River glass eels. On the contrary, all indicators remained low compared to glass eels from the two other rivers caught at their arrival in the river system. Perhaps stimulation of transcripts of the somatotropic axis occurs later after the entrance into the rivers and/or with the resumption of food in the BW/SW ecotype. Indeed, Pavey et al. (2015) showed enrichment in allelic frequencies of growth factor receptor binding at the yellow eel stage.

Many factors could be involved in the regulation of growth; determining the exact role of the observed response must await more detailed studies on tissue-specific functions even though tissue puncture is delicate at this stage. Indeed, fasting should affect the somatotropic axis in a dissimilar fashion between ecotypes, and these differences could also reflect differences in osmoregulatory and energy storage strategies. In fact, GH could regulate lipid mobilization by increasing lipid depletion from adipose tissues in fasting fish (e.g., Dai et al. 2015). Recently, Gaillard et al. (2016) detected a 25-fold higher expression of lipolysis enzymes in Grande-Rivière-Blanche glass eels than in Mersey River glass eels and demonstrated that glass eels from the FW ecotype had a better ability to efficiently mobilize lipid storage at recruitment. A possible activation of GH transcripts could be related to lipid depletion. However, the presence of different patterns in GH and GH-R expression certainly supports the presence of transcriptomic differences in the FW and SW eel ecotypes, despite the fact that they belong to a single panmictic population (Coté et al. 2013).

No Effect of Salinity Preference on Gene Expression

The absence of a salinity preference effect on gene expression may seem counterintuitive considering the abundant literature describing the environmental influence on salinity preference of European Eel glass eels (Creutzberg 1961; Tosi et al. 1988); Gulf Killifish *Fundulus grandis* juveniles (Miller et al. 1983); Chum Salmon *Oncorhynchus keta*, Pink Salmon *O. gorbuscha*, Chinook Salmon *O. tshawytscha*, Sockeye Salmon *O. nerka*, and Coho Salmon *O. kisutch* presmolts (McInerney 1964; Otto and McInerney 1970); and Gulf Grunion *Leuresthes sardina* postlarvae (Reynolds and Thomson 1974). Considering that the American Eel is very euryhaline, salinity preferences could be seen as an index of migratory capacity similar to the smoltification transformation (Otto and McInerney 1970) or to genetic cognitive capacities, as suggested for European Eel glass eels (Podgorniak et al. 2015). Overall, the presence of very different endocrine gene expression patterns observed in glass eels captured on the Canadian east coast adds to the growing volume of evidence that both phenotypic plasticity and spatially varying selection processes are present in this species. Divergent lipolysis capacity in glass eel-stage American Eels has also been demonstrated for individuals captured at the same time and the same sites (Gaillard et al. 2016). Gene × environment and origin × salinity effects can influence growth patterns and the expression of genes representing many functional groups in American Eel glass eels arriving on the Canadian east coast (Coté et al. 2009, 2014). Latitudinal variations in RNA/DNA ratios have been detected throughout the entire distribution range (Laflamme et al. 2012), and evidence of differing patterns of selection along environmental gradients (spatially varying selection) was inferred to explain shifts in allele frequencies involved in metabolism (e.g., lipid and sugar metabolism, development of respiratory function, and development of heart muscle) within the time frame of a single generation despite the panmictic reproduction mode in American Eels (Gagnaire et al. 2012; Pavey et al. 2015).

Gene Expression of the Thyroid Axis and American Eel Development

Since the development of glass eels captured from Rivière-Saint-Jean seemed to be less advanced than that of glass eels from the other two rivers, the higher expressions of thyroid activity indicators could be related to developmental processes. The thyroid axis has been shown to be involved in fish metamorphosis (Power et al. 2001; Sudo et al. 2014). Specifically, iodothyronine deiodinases have been shown to control developmental phases in teleost fishes (e.g., Jarque and Piña 2014). Kawakami et al. (2013) showed higher expressions of $THR\alpha_a$ and $THR\alpha_b$ during the larval stage of the Japanese Eel, with a decrease at the onset of metamorphosis followed by a peak at later stages of metamorphosis. In the Whitespotted Conger *Conger myriaster*, the expression of these two

THRs peaked at metamorphosis, and the expression of the isoform THRα_a was higher than that of THRα_b. Moreover, triiodothyronine in Japanese Eels was primarily triggered during metamorphosis, while thyroxine peaked at the end of the metamorphosis and at the glass eel stage (Yamano et al. 2007).

In American Eels, hormonal concentration at the yellow eel stage indicated that the eels that were colonizing rivers had the highest thyroxine concentrations (Castonguay et al. 1990). Considering that both Edeline et al. (2004) and Castonguay et al. (1990) showed only slight differences in triiodothyronine concentrations between FW and BW/marine glass eels and yellow eels, this may argue for a role related to metamorphosis to explain high expression levels of genes related to the thyroid axis in Rivière-Saint-Jean glass eels. However, the role of development processes involved in the hormone-encoding genes for the thyroid axis remains unclear considering the variations in response of gene expression profiles for this axis among our study rivers along with variations in the stage of pigmentation. Moreover, the number of individuals prevented us from including the time of capture for consideration in the present study. This is an important question for future studies.

Gene Expression Profiles of the Thyrotropic Axis

We analyzed different indicators of the thyrotropic axis, and we expected similar differences in gene expression along the whole axis (from TSH to the THRs), which was not the case. The different portions of this axis are regulated by different mechanisms (for a review, see Orozco and Valverde-R 2005), and it could be relevant to attempt to identify the level of regulation at which differences between ecotypes occur. Indeed, Sudo et al. (2014) found increased thyroid hormone levels without an increase in the expression of TSH-β during Japanese Eel metamorphosis (Sudo et al. 2014). Body concentration measurements of circulating thyroid hormones would have provided suitable information. Unfortunately, our attempts to perform such measures were unsuccessful in the sense that levels were always below the detection threshold of the radioimmunoassay kit used, even when we worked with pools of four individuals that were shorter and younger and had less plasma than European Eel glass eels at their entrance into the rivers.

Conclusions

This study highlights that endocrine mechanisms underlying recruitment in glass eels depend on their ecotype and vary along geographic sites, since ecotypes are adapted to live under varying environmental conditions. Our results show that the larger size of the FW ecotype could be explained by their GH/GH-R_1 and IGF-1R gene expression and that high GH/GH-R_1 and high DIO-2 mRNA levels could be important traits allowing this ecotype to reach remote and colder Canadian estuaries. The regulatory mechanisms of the thyroidal and somatotropic axes that could explain the different geographic hormonal gene expression patterns as well as how these differences are programmed during the glass eel stage remain to be elucidated. The demonstration of differential molecular phenotypes between ecotypes and rivers at the recruitment stage supports the view that the origin of glass eels potentially used in stocking practices should be taken into account in management decisions. Namely, the choice of the geographic site to be used in resettlement programs should consider ecotypic variation (e.g., FW versus BW/SW ecotypes; sensu Pavey et al. 2015) in order to respect the ecological and genetic integrity of the supplemented contingents.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.