



Regional variation of gene regulation associated with storage lipid metabolism in American glass eels (*Anguilla rostrata*)



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ABSTRACT

Variation in gene regulation may be involved in the differences observed for life history traits within species. American eel (*Anguilla rostrata*) is well known to harbor distinct ecotypes within a single panmictic population. We examined the expression of genes involved in the regulation of appetite as well as lipid and glycogen among glass eels migrating to different locations on the Canadian east coast and captured at two different periods of upstream migration. Gene expression levels of three reference and five candidate genes were analyzed by real-time PCR with Taqman probes in recently captured wild glass eels. All gene transcripts were detected in glass eels. Of the five candidate genes, bile salt activated and triacylglycerol lipases were respectively 7.65 and 3.25 times more expressed in glass eels from the St. Lawrence estuary than in those from Nova Scotia, and there was no effect related to the two-week difference in capture date. These two genes explained 82.41% of the dissimilarity between the two rivers. In contrast, glycogen phosphorylase, ghrelin, and leptin receptor genes showed no significant differences in gene transcription. These results confirmed at the molecular level an observation that was recently made at the phenotypic level that glass eels from the St. Lawrence estuary have a greater capacity to use lipid reserves to sustain their metabolic needs. These observations add to the body of evidence supporting the hypothesis that regional phenotypic variation observed in American eel is determined early in life and that part of this variation is likely under genetic control.

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1. Introduction

American eel, *Anguilla rostrata*, is a facultative catadromous fish (i.e., it performs a non-obligatory trophic migration to fresh water). It is widely distributed throughout eastern North America and is associated with a unique reproductive site in the Sargasso Sea (Tsukamoto et al., 1998). Consequently, the species is composed of a single, genetically homogeneous panmictic population (Côté et al., 2013). The leptocephalus larvae migrate toward the continental slope where they metamorphose into glass eels (Tesch, 2003). Since the 1980s, a 99% decrease in glass eel recruitment has been observed in the St. Lawrence River, which used to support an important fishery in Canada (Cairns et al., 2014; Castonguay et al., 1994a, 1994b; MacGregor et al., 2008). This species was recently declared “threatened” in Canada (COSEWIC, 2012).

In contrast, recruitment has remained relatively stable along the Atlantic coast of Canada, and this regional discrepancy in recruitment despite panmixia has puzzled managers for many years. In addition

to regional recruitment variation, pronounced regional phenotypic variation has been documented in American eel. In particular, eels colonizing the St. Lawrence River and Lake Ontario are characterized as being much older and with a larger size at sexual maturity but with a slower growth rate compared to eels from the coastal areas, as well as being 100% females (Côté et al., 2015; Jessop, 2010). While it has been traditionally believed that such phenotypic variation is entirely controlled by the environment (i.e., reflecting pure phenotypic plasticity), there is growing evidence that regional functional genetic variation may also play a role.

Differences in growth and profiles of gene transcription have been documented between eels from the St. Lawrence River and from the Canadian Atlantic coast when reared in common environments for three years (Côté et al., 2009, 2014, 2015). In addition, latitudinal variations in RNA/DNA ratios have been observed, with both lower body condition and higher RNA/DNA ratios in high and low latitudes (Laflamme et al., 2012; 47–49°N [Eastern Canada] vs. 30–32°N [Florida–South Carolina]). Moreover, evidence for spatially varying selection resulting in regional variations in allele frequencies have been documented for genes involved in lipid and carbohydrate metabolism (Gagnaire et al., 2012). More recently, Pavey et al. (2015) provided strong evidence that the phenotypic differences observed between two ecotypes (fresh water from the St. Lawrence River vs. the brackish/salt water from the east coast of Canada) have a polygenic basis for genes that are mainly

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involved in vascular and morphological development, calcium ion regulation, growth, transcription factors, and olfactory receptors. In brief, the notion that regional phenotypic variation observed in American eel is only due to phenotypic plasticity is no longer tenable, and genetic variation resulting from spatially varying selection must therefore also be taken into consideration for a full understanding of the complexity of eel life history.

Glass eels rely on the energy stored during the larval stage to achieve their migration toward feeding habitats (Boëtius and Boëtius, 1989), which could be freshwater, estuarine, or marine (Arai, 2012; Cairns et al., 2004; Jessop et al., 2002; Lamson et al., 2006; Pavey et al., 2015). In a recent study on the energetic status of American glass eels captured along the east coast of Canada (Nova Scotia and the St. Lawrence estuary), Gaillard et al. (2015) showed that there was no relationship between energy status and salinity preference, but there were differences in energy storage strategies between capture sites. Glass eels captured in the St. Lawrence estuary had a higher level of glycogen and lower reserves of triacylglycerol than those captured on the Atlantic coast. There is also some evidence that glass eels resume feeding once they are totally pigmented, but some individuals may need to feed to complete their upstream migration (e.g., Harrison et al., 2014). Indeed, non-pigmented glass eels with full stomachs were observed in Grande Rivière Blanche (GRB), which is a tributary to the St. Lawrence estuary (C. Côté, pers. comm.), and non-pigmented glass eels captured in both GRB and Nova Scotia were feeding in the lab (Côté et al., 2009). How the relative expression for genes involved in lipid and carbohydrate metabolism as well as appetite regulation varies in glass eels from different sites needs to be clarified.

In this context, we tested 1) whether spatial or temporal differences in the expression of genes involved in lipid and glycogen metabolism differed between glass eels migrating to Nova Scotia on the Atlantic coast or in the St. Lawrence estuary; and 2) whether genes regulating appetite would be more expressed in glass eels captured in the St. Lawrence estuary, supporting the hypothesis of early feeding. To achieve these goals, we used real-time PCR to measure the transcription level of five candidate genes: triacylglycerol lipase (TAGL), bile salt activated lipase (BAL), glycogen phosphorylase (GPase), ghrelin (GHRL), and leptin receptor (LEP-R).

2. Materials and methods

2.1. Sampling

Glass eels were captured by a commercial elver fishery in the Mersey River (MR; 44°02' N, 64°42' W), Nova Scotia (Atlantic coast), on 26–28 March and 20–21 April 2012 and by members of our research team in Grande-Rivière-Blanche (GRB; 48°47' N, 67°41' W), Québec (St. Lawrence River), on 2–6 and 18–21 June 2012 (Boivin et al., 2015). These periods represent the early arrival of glass eels in this area (Côté et al., 2009). Glass eel captures began two hours before the nighttime high tide and lasted for three hours. Samplers waded into river mouths and captured eels using dip-nets and headlamps. Glass eels were transferred by car to the wet-lab facility at Maurice-Lamontagne Institute (IML; Fisheries and Oceans Canada) in buckets containing water from the estuary. The introduction and transfer of glass eels from Nova Scotia to Québec were done under conditions specified in the license obtained from Fisheries and Oceans Canada. A total of 40 individuals were sampled for the present study: 10 glass eels per river site and date of capture (MR, March; MR, April; GRB, early June; GRB, late June). Glass eels were anesthetized in 0.68 mM MS222 (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich). Total body length and wet mass were measured, and pigmentation stage was determined according to Haro and Krueger (1988). Pigmentation results are presented in Gaillard et al. (2015). Glass eels were individually stored at -20°C in RNALater until analyses.

2.2. Candidate and reference genes

Five candidate genes were studied: triacylglycerol lipase (TAGL), which catalyzes the breakdown of triacylglycerol in several tissues (Murray et al., 2003; Tocher and Sargent, 1984); bile salt activated lipase (BAL), the most important digestive lipase in teleosts (Iijima et al., 1998; Murray et al., 2003; Patton et al., 1975, 1977); glycogen phosphorylase (GPase), an enzyme that catalyzes the rate-limiting step in glycogenolysis (Brown and Cori, 1961); and ghrelin (GHRL) and leptin receptor (LEP-R), which are hormonal factors generally considered to regulate appetite (Lin et al., 2000; Unniappan and Peter, 2005). The use of only one reference gene in qPCR analysis is not recommended (Bustin et al., 2009), and reference gene expression 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), the reference genes chosen for the present work were acidic ribosomal protein (ARP) and cytochrome B (CytB); we also used elongation factor 1 (EF1), which was reported to be very stable in Atlantic salmon (Olsvik et al., 2005).

2.3. Whole body grinding

Glass eels were individually homogenized dry with liquid nitrogen using a Precellys Dual homogenizer coupled with a cooling system (Precellys, Bertin Technologies) in CKMix 50 R containing beads for hard tissue grinding. Samples were ground using three cycles of 26 s at 5800 rpm, with a 30 s time lapse separating each cycle. The resulting powder was held at -80°C until RNA extraction.

2.4. Total RNA extraction

Total RNA was extracted from 10 mg dry mass of homogenate powder using an RNeasy® Fibrous Tissue Kit (Quiagen Inc., ON, Canada) and was diluted to obtain a final concentration of $200\text{ ng }\mu\text{l}^{-1}$. Total RNA purity, quality, and concentration were determined using electrophoresis on 2% agarose gel stained with ethidium bromide (0.05 mg ml^{-1}) (Alpha Imager® HP System, Alpha-InnoTech, Alpha Imager 3400 software, ProteinSimple) and the 260 nm/280 nm absorbance ratio using a NanoVue Plus spectrophotometer (GE Healthcare, QC, Canada) before reverse transcription.

2.5. Reverse transcription

Reverse transcription was done using a Quantitect® Reverse Transcription Kit (Qiagen Inc., ON, Canada). The cDNA samples obtained were diluted to a final concentration of $20\text{ ng }\mu\text{l}^{-1}$, separated into aliquots, and kept frozen at -20°C until further analysis. cDNA integrity and concentrations were verified with a NanoVue Plus spectrophotometer. Reverse transcription efficiency was verified using serial dilutions of a pool of four RNA samples from different origins and dates of capture to ascertain slopes of -3.3 . qPCR analyses were performed in triplicate (Biorad MyiQ I cyclor, Bio-Rad Laboratories, Inc., ON, Canada) using IQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., ON, Canada) and an iCycler IQ™ Real-Time PCR on one reference gene (EF1). Linear regression of the serial dilution curve was done with MlciyiQ Software version 1.0 (Bio-Rad, USA), giving an efficiency of 94.5% ($y = -3.4603x + 10.341$; correlation coefficient: 0.997).

2.6. Specific sequences and design of Taqman primers and probes

For GHRL and reference genes, primers were designed based on available mRNA sequences from genus *Anguilla* found in the National Center for Biotechnology Information (NCBI) bank using Primer-Blast

(<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). Primers were ordered with Integrated DNA Technologies™ (Coralville, IA, USA).

A pool of randomly chosen cDNA samples (rivers and dates of capture) was used with primers for amplifications (all in duplicate) using polymerase chain reaction (PCR) with iCycler iQ™ Real-Time PCR (BioRad) using AmpliTaq Gold® 360 Master Mix Kit (Applied Biosystems®, CA, USA). The quality and integrity of each PCR product or amplicon were verified by electrophoresis on 2% agarose gel with ethidium bromide (0.05 mg ml⁻¹) containing a PCR marker (Sigma-Aldrich®, ON, Canada). Amplicons were purified on columns using a QIAquick PCR Purification Kit (Qiagen Inc., ON, Canada). Purified amplicons were sequenced in forward and reverse directions with associated primers and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, CA, USA). Unincorporated dye terminators from sequencing reactions were removed using the Ultra-Step® Dye Terminator Removal Kit (Omega Bio-Teck, GA, USA). Isolated fragments containing dye-labeled dideoxynucleotide triphosphates (ddNTPs) were dried for 20 min using SpeedVac (Savant AS160 Automatic) and suspended in formamide; fragments were analyzed using a 3130 Genetic Analyzer (Applied Biosystems®) and POP-7™ polymer (Life technologies™, ON, Canada). Amplification products obtained for GHRL, ARP, EF1, and CYTB were sequenced (Supplemental Table 1). Assembly and alignment verification were done with nucleotide blast in primer blast (<http://blast.ncbi.nlm.nih.gov>). The percentages of homology were about 99% between ARP and *Anguilla anguilla* acidic ribosomal phosphoprotein (GenBank accession no. **AY763793.1**), 99% between CytB and *A. rostrata* cytochrome b (GenBank accession no. **KJ546053.1**), 100% between EF1 and *Anguilla australis* elongation factor 1 (GenBank accession no. **HM367094.1**), and 99% between GHRL and *Anguilla japonica* mRNA prepo-ghrelin (GenBank accession no. **AB062427**).

For TAGL, BAL, GPase, and LEP-R, specific sequences from *A. rostrata* were obtained from the draft annotated American eel genome (Pavey et al. unpublished results). Putative CDS regions (Coding DNA Sequence) for each gene were manually verified with BLAST using the

SwissProt database based on homology with model organisms. TaqMan probes were designed using Primer Express 3.0 software (Applied Biosystems®) and were obtained from Life Technologies™ (Mairway, ON, Canada) (Table 1). All Taqman primers and probes gave stable qPCR efficiency with a coefficient of determination of 0.99 to 1 (Table 1).

2.7. Real-time PCR

Real-time PCR (qPCR) was performed in triplicate for each glass eel sample using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems®). A sample reaction volume of 10 µl was made up of 2 µl cDNA (10⁻² ng µl⁻¹), 5 µl TaqMan Advanced Mix (Life Technologies™, ON, Canada), 2.5 µl sterile water, and 0.5 µl TaqMan 20 µM of gene-specific primers and probe (Life Technologies™, ON, Canada). 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 AmpliTaq Gold® DNA Polymerase; and 2) 45 cycles of denaturing at 95 °C for 30 s and annealing/extend at 60 °C for 1 min. Cycle thresholds (C_T) were obtained using Expression Suite 1.0 software (Applied Biosystems®, Foster City, CA). qPCR efficiency was verified for each TaqMan probe (Table 1). A serial dilution of 10⁻¹ to 10⁻⁵ was made on a new pool of eight randomly selected cDNA samples. qPCR analyses for each TaqMan probe were performed on the pool with the same protocol described previously in which 2 µl cDNA (10⁻² ng µl⁻¹) was replaced by 2 µl of the cDNA pool.

Relative quantification of gene expression was calculated according to the 2^{-ΔΔC_T} method of Livak and Schmittgen (2001)

$$2^{-\Delta\Delta C_T} = 2^{-(\Delta C_{Te} - \Delta C_{Tc})} \quad (1)$$

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.

In our study, the calibrator was the group of glass eels sampled at MR in March 2012 (the more southern river, first date of capture). The suitability, stability, and validation of quantitative qPCR reference genes for

Table 1
Information on primers and probes. The GenBank sequences (www.ncbi.nlm.nih.gov/genbank/) used to design primers for sequencing specific sequences (EST) are shown as Forward (F) and reverse (R) primers with melt temperature. When cells are empty, specific coding DNA sequences (CDS) were obtained from the annotated American eel genome. Taqman forward (F) and reverse (R) primers and Taqman probes (P) used for real-time polymerase chain reaction (qPCR) analysis are provided with their melt temperature. qPCR efficiency (linear regression and coefficient of determination) are provided for each Taqman probe. See the Material and Methods section for descriptions of gene functions.

Gene (gene acronym)	Sequence used for primer design	Primer used for sequencing specific EST (melt temperature)	Taqman primers and probes used for measuring gene expressions (melt temperature)	qPCR efficiency
Reference genes				
Acidic ribosomal protein (ARP)	<i>A. anguilla</i> (GenBank accession no. AY763793)	F: GCCACGTGGAAGTCCAATA (57) R: CAGGAGTTTCTCCAGAGCGG (58)	F: TCTCCCTGCGTGCAAAGG (59) R: CTGCGCATCATGGTGTCT (58) P: TGGTGTGATGGGC (69)	y = -3.196x + 34.388 R ² = 0.9993
Cytochrome B (CytB)	<i>A. anguilla</i> (GenBank accession no. AF006714)	F: CATCTGCCGAGACGTCAACT (57) R: ATCTGCCCTCATGGAAGCAC (58)	F: TCATCTGCCGAGACGTCAAC (58) R: GAGGCCCATTTGCATGTAG (59) P: ATGGATGATTAATTCGC (68)	y = -3.1254x + 32.523 R ² = 0.9992
Elongation factor 1 (EF1)	<i>A. japonica</i> (GenBank accession no. AB593812)	F: CCTGAAGCCTGGTATGGTGG (58) R: TACGTTGCCACGACGGATT (57)	F: TTGCCCTGCCAAGCT (58) R: GGGACTCATGGTGCATTCC (59) P: ACCACTGAGTCAAGTC (68)	y = -3.3354x + 33.755 R ² = 0.9998
Target genes				
Triacylglycerol lipase (TAGL)	—	—	F: GCACCCGCTTCATGCTCT (60) R: ACCTTGCCCTGGTGTGTTG (59) P: ACCCGCAGAAAC (69)	y = -3.356x + 39.015 R ² = 0.9963
Bile salt activated lipase (BAL)	—	—	F: TGATGCCGTGCTGAAGATCA (59) R: GGTGTAGACTCCAGCCAGAGT (58) P: AGACCTGTGGCTCT (69)	y = -3.4627x + 40.166 R ² = 0.99972
Glycogen phosphorylase (GPase)	—	—	F: GCGTCCCGTCCACTTCTATG (59) R: AACCCATTGACCCCATCTG (58) P: CAGAGTGGAGCACAC (68)	y = -3.1649x + 36.873 R ² = 0.99959
Ghrelin (GHRL)	<i>A. japonica</i> (GenBank accession no. AB062427)	F: TCCAAGAGGCACTGGTTTC (57) R: CCATCTGAGTCTCGTCTGCC (57)	F: GCTTCAGACATGAGGCAGATGA (59) R: GGACGACAGCCAGCAGAT (60) P: CGCACCGCATAACAG (69)	y = -3.1767x + 41.44 R ² = 1
Leptin receptor (LEP-R)	—	—	F: GCGAGGCCCTTCTG (59) R: CTGGAGGTCTGGTCTAACTGT (58) P: TGCCTGGAGGGACT (69)	y = -3.1034x + 38.65 R ² = 0.99496

normalizing expression were verified with Expression Suite 1.0 software, 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 the expression.

2.8. Statistical analyses

The relative quantifications of gene expression ($2^{-\Delta\Delta C_T}$) for the five candidate genes were analyzed using two-way permutational multivariate analysis of variance (PERMANOVA; $p < 0.05$, 9999 permutations, type III of sums of squares, permutation of residuals under a reduced model) using the PERMANOVA+ add-on (v 1.02) in PRIMER (v6.1.1.12). River (two levels) and date (two levels) of capture were the two factors considered in analyses. Missing data (12 out of 560 data) were replaced by the mean of the subsample in a date–river group (García et al., 2015). A Bray–Curtis similarity matrix was completed for relative gene expression data. Before performing the two-way PERMANOVA (river, date) and the pair-wise tests on significant effects ($p < 0.05$, 9999 permutations, type III of sums of squares, unrestricted permutation of raw data), distance-based tests for homogeneity of multiple dispersions (PERMDISP; $p < 0.05$, 9999 permutations; deviation from centroid) were run for the two factors and their interaction to determine if data needed transformation (Table 2). Because all variables displayed homoscedasticity ($p > 0.05$), no transformations were required. When PERMANOVA detected a factor effect(s) on the whole data set, one-way PERMANOVA analyses ($p < 0.05$, 9999 permutations, type III of sums of squares, unrestricted permutation of raw data) were done for each variable according to the significant factor and the Bray–Curtis matrix was completed. PERMDISP analyses were performed for each variable to verify if transformation was required before running PERMANOVA analyses on individual variables (Table 2). When significant effects were detected, pair-wise tests were performed ($p < 0.05$, 9999 permutations, type III of sums of squares, unrestricted permutation of raw data).

To identify the relative contribution of each candidate gene to the differences between significant groups, one-way similarity percentage analysis (SIMPER; river) was run until 100% cumulative contribution. Only candidate genes contributing to more than 50% of differences among groups were considered. Finally, to explore dissimilarities between groups, multidimensional scaling plot analysis (MDS) was run and vectors of target genes that characterized the most the differences between groups were revealed. Vectors correspond to Spearman rank correlations.

Table 2

Results of permutational multivariate analysis of variance tests, PERMANOVA. A two-way PERMANOVA was first applied to the whole data set; depending on these results, a one-way PERMANOVA for coding genes was completed. Source tested, sum of squares of the test (SS), pseudo-F of the statistic, P(permutation), and the number of the unique permutations (unique perms) done for the test are presented. Bold letters indicate significant results. BAL: bile salt activated lipase, TAGL: triacylglycerol lipase, GPase: glycogen phosphorylase, GHRL: ghrelin, LEP-R: leptin receptor. Bold characters indicate significant results.

Database	Source	SS	Pseudo-F	P (perm)	Unique perms
Whole data set					
Relative gene expression	River	10,203	8.1713	0.0002	9951
	Date	2075	1.6619	0.1461	9943
	River × date	2909.5	2.3303	0.0557	9965
Single variable					
	BAL River	13,686	6.94410	0.0003	9953
	TAGL River	6144.3	4.06070	0.0091	9956
	GPase River	184.32	0.21166	0.8744	9940
	GHRL River	661.03	0.46196	0.6832	9940
	LEP-R River	127.89	0.23694	0.7619	9950

3. Results

The gene-stability measures for the three reference genes were 0.853, 0.886, and 0.713 for ARP, CytB, and EF1, respectively (calculated using ExpressionSuite software). The cycle thresholds (C_T) of the normalized reference gene (geometric mean of the three independent C_T for each sample) did not show any significant difference between the rivers and date of capture, ensuring a good standardization of the relative expression measures of candidate genes (Fig. 1). The qPCR efficiency for each target and reference gene varied from -3.10 (LEP-R) to -3.46 (BAL) (Table 1).

When the whole data set was analyzed with PERMANOVA, only the river factor could differentiate gene expression in glass eels (Table 2; whole data set). Pair-wise comparisons between GRB and MR confirmed that the differences in gene expression were explained by the river factor (pair-wise test between rivers: $t = 2.8586$, p (perm) = 0.0003, unique perms = 9953). The results of the multivariate analysis showed no dissimilarity in gene expression levels with respect to the date of arrival or the interaction river × date of arrival, although p was only slightly above 0.05 for the interaction factor (Table 2). MDS analysis also showed higher dissimilarities between individuals from GRB than between those from MR. Individuals from MR were more tightly grouped while individuals from GRB were more scattered on the horizontal axis of the plot (Fig. 2). The source of variations for GRB is explained by relative expression of BAL and TAGL (BAL and TAGL were highly correlated on the horizontal MDS axis with a correlation factor respectively of 0.97 and 0.88), while the source of variations for MR is explained by the greater discrepancies in the relative expression of the different candidate genes. To confirm the results of the projection on the MDS plot and to identify which candidate genes varied with river of origin, permutation tests were done gene by gene according to the river factor (Table 2; single variable). A highly significant river effect was detected for BAL and TAGL (Table 2). Indeed, the relative expression of genes coding for these two enzymes, which are involved in lipid metabolism, explained more than 80% of dissimilarities between MR and GRB (Table 3). BAL data alone explained about 60% of the dissimilarities.

To evaluate differences in the level of gene expression between MR and GRB, one-way PERMANOVA between rivers were performed for each candidate gene (Fig. 3). BAL gene expression was 7.4 times higher in glass eels from GRB compared to MR (Fig. 3A; 24 times higher than in the calibrator) and, in the absence of a date of capture effect, the BAL expression in MR was very close to the calibrator. Gene expression was calibrated based on the expression levels observed in the MR/March data (the most southern location and the earliest date of capture). TAGL gene expression was 3.3 times higher in GRB glass eels (7.65 higher than in the calibrator), and the expression in MR was again very close to the level of expression in the calibrator (Fig. 3B). The expressions of genes coding for GPase, GHRL, and LEP-R were very stable

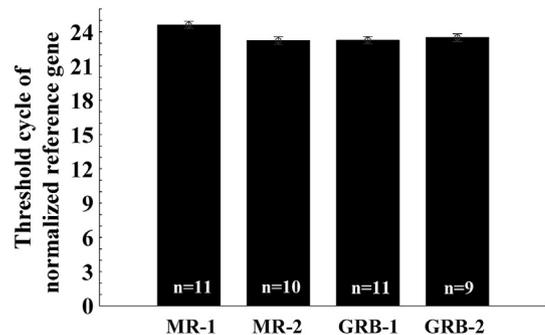


Fig. 1. Average threshold cycle (C_T) for the normalized reference gene (geometric mean of individual C_T obtained for the three reference genes) for the two rivers (MR: Mersey River; GRB: Grande-Rivière-Blanche) and the two periods of capture in each river (MR-1: 26–28 March; MR-2: 20–21 April 2012; GRB-1: 2–6 June; GRB-2: 8–21 June 2012).

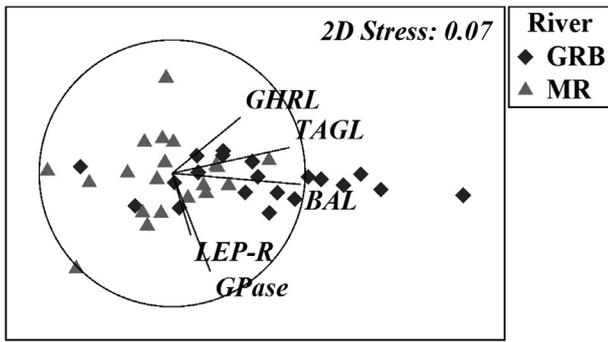


Fig. 2. Multidimensional scaling (MDS) between rivers including data from the 40 individuals (10 per river and date of capture) based on the Bray–Curtis dissimilarity matrix. River was the only significant factor detected by permutational multivariate analysis of variance (PERMANOVA). The stress values indicate the significance level of the representation. MDS illustrates how the measure of gene expression of whole glass eels differs between rivers on a two-dimensional plot and which variables characterize the difference between rivers. Vectors correspond to Spearman rank correlations. On the horizontal MDS axis, BAL $r = 0.97$, TAGL $r = 0.88$, GPase $r = 0.29$, GHRL $r = 0.52$, and LEP-R $r = 0.14$. On the vertical MDS axis, BAL $r = -0.09$, TAGL $r = 0.79$, GPase $r = -0.73$, GHRL $r = 0.43$, and LEP-R $r = -0.46$. GRB: Grande-Rivière-Blanche; MR: Mersey River. BAL: bile salt activated lipase, TAGL: triacylglycerol lipase, GPase: glycogen phosphorylase, GHRL: ghrelin, LEP-R: leptin receptor.

Table 3

Relative contribution (until 100% cumulative contribution) of candidate genes to differences between rivers by one-way similarity percentage analysis (SIMPER). Av. Abund.: average abundances over all samples, Av. Diss.: average contribution to the total dissimilarities, Contrib%: percentage contribution to the total dissimilarities, Cum%: cumulative percentage contribution to the total dissimilarities. BAL: bile salt activated lipase, TAGL: triacylglycerol lipase, GPase: glycogen phosphorylase, GHRL: ghrelin, LEP-R: leptin receptor. Bold text indicates a significant contribution of the target genes.

Candidate gene	Av. Abund. GRB	Av. Abund. MR	Av. Diss.%	Contrib.%	Cum.%
BAL	24.00	3.25	34.20	62.30	62.30
TAGL	7.65	2.33	11.04	20.12	82.41
GPase	1.93	1.59	3.79	6.91	89.32
GHRL	1.61	1.22	3.53	6.42	95.75
LEP-R	1.24	1.24	2.33	4.25	100.00
			Σ Diss.: 54.89%		

among glass eels from both origins, and expression was not significantly different from the expression measured in the calibrator group (Fig. 3C, D, E) despite tendencies for higher expression of GPase and GHRL in GRB. Indeed, the relative expression of these three candidate genes did not contribute to the dissimilarity between the two rivers (Tables 2, 3).

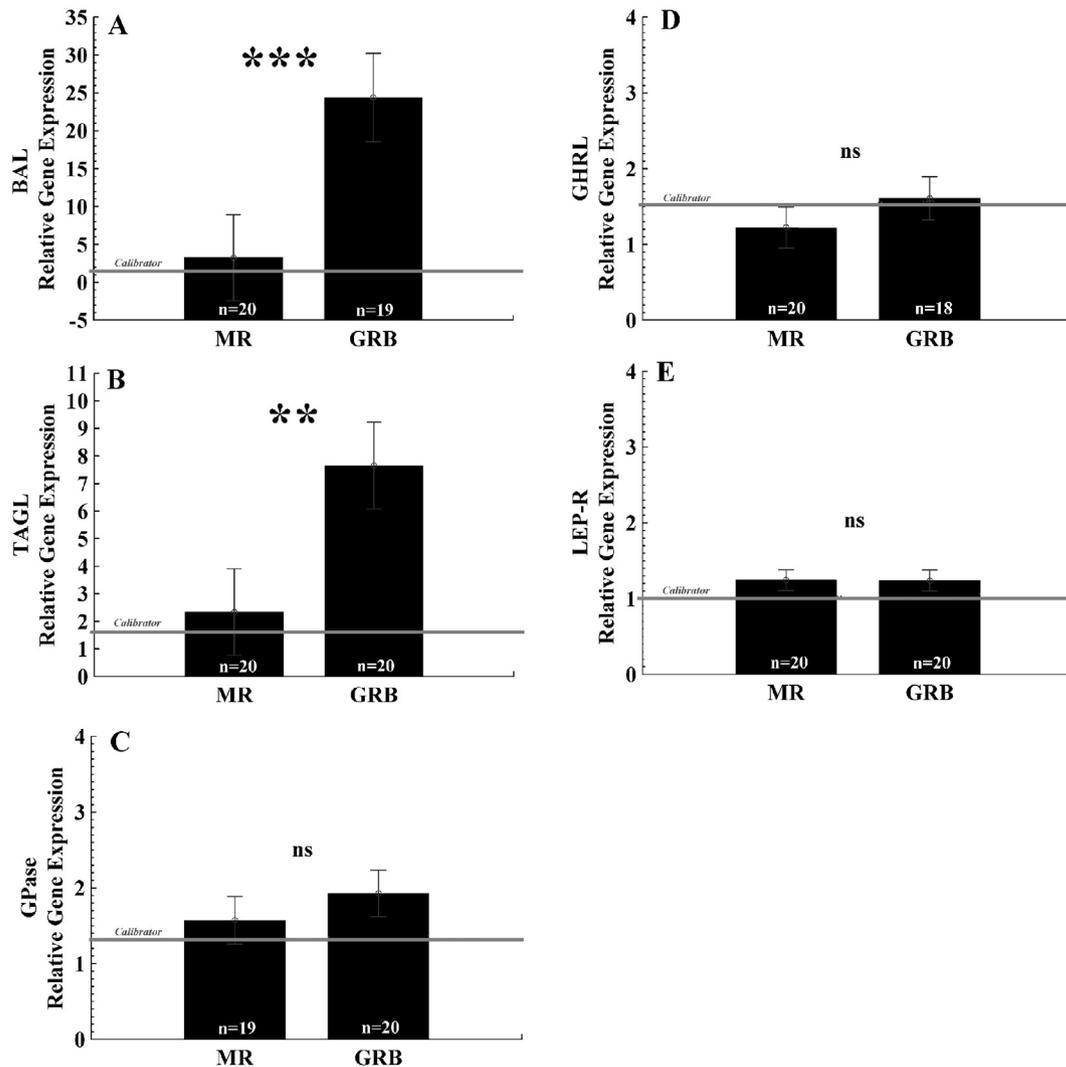


Fig. 3. Profiles (means \pm SE) of the relative gene expression of bile salt activated lipase (BAL), triacylglycerol lipase (TAGL), glycogen phosphorylase (GPase), ghrelin (GHRL), and leptin receptor (LEP-R) target genes in glass eels captured in the Mersey River (MR) and Grande-Rivière-Blanche (GRB). The horizontal lines show the level of relative gene expression of the calibrator group (MR, March 2012). Asterisks show the level of significance of the pair-wise test; **, $p < 0.01$, ***, $p < 0.005$, ns: $p > 0.05$. The total numbers of glass eels per river are also presented.

4. Discussion

4.1. Enzymes involved in lipid metabolism

Our results highlighted the importance of lipases in glass eel metabolism. The capacity for effective lipolysis is present in American glass eels since lipase enzymes were expressed. Among the candidate genes under study, TAGL and BAL explained most of the differences between rivers of capture. Both lipases were significantly more expressed in GRB glass eels, which were previously shown to have a 66% lower level of triacylglycerol content than glass eels from MR (Gaillard et al., 2015). These results confirm the hypothesis of local differences in the regulation of lipid metabolism between glass eels from different locations: GRB glass eels might use their lipid reserves more efficiently to sustain metabolism than those captured in Nova Scotia. Indeed, glycerol and fatty acids can be produced from the hydrolysis of triacylglycerol (e.g., Tocher, 2003).

We found no local differences regarding the expression of GPase, GHRL, or LEP-R, suggesting conservation of these functional traits. For TAGL and BAL, some factors affecting gene \times environment interactions, or others related to larval development or biochemistry, could be suggested as underlying the differences between GRB and MR. However, the absence of a “time effect” seems to refute the possibility of developmental differences. Because we used whole larvae instead of specific organs to measure gene expression, this may have made the detection of subtle differences more difficult.

BAL and TAGL were much more expressed in GRB. The differences we observed were present in glass eels upon arrival in their respective rivers. These results corroborate those of Côté et al. (2014), who documented that glass eels captured in either Nova Scotia or GRB had different expressions at two lipase EST (Expressed Sequence Tag) sites. Even after three months of growth under laboratory conditions, differences in expression were still present. Altogether, these results strongly support the presence of local differences in gene expression. Gagnaire et al. (2012) also reported different allele frequencies in glass eels captured from Florida to the St. Lawrence Estuary, including genes related to lipid metabolism (inhibition of phospholipase A2, acyl carrier activity, phospholipid-hydroperoxide glutathione peroxidase activity), and they suggested that temperature could be the environmental factor the most likely to induce such gene \times environment interactions. The most pronounced inter-individual variability shown by the MDS analysis was for GRB for lipase expression, and this may indicate a greater ability of GRB glass eels to respond to variations of environmental factors.

The expression of genes coding for lipase, trypsin, chymotrypsin, amylase, and phosphorylase A2 may also be modulated by dietary content in fish (e.g., Zambonino Infante and Cahu, 2001). Results from larval fishes are contradictory on this point, and species-specific responses are present. In European sea bass, *Dicentrarchus labrax*, sole, *Solea solea*, and red drum, *Sciaenops ocellatus*, lipase activity and expression were regulated by the source of the triglycerides (Buchet et al., 2000; Zambonino Infante and Cahu, 1999). In other species, such as winter flounder, *Pseudopleuronectes americanus*, diet did not affect BAL or TAG mRNA expression (Vagner et al., 2014). If we assume a diet effect on lipase gene expression in American glass eel, this could be the result of eating at the glass eel stage, as suggested by observations done by Côté (unpublished results), or it could be the effect of differences in dietary inputs at the leptocephalus stage. In such conditions, however, a time effect would have been expected.

For instance, lipase expression may also be related to developmental stage (Vagner et al., 2014; Zambonino Infante and Cahu, 2001), and variability in the capacity to digest lipids was observed within species in individuals of different ages (e.g., Glencross, 2009). Such age differences could explain the highest gene expression level observed for both lipases in GRB glass eels that arrived later on the migration site. However, this hypothesis does not seem to be supported since no temporal differences were observed either at MR or GRB (Gaillard et al., 2015).

Lipase activity may also be related to plasma levels of ketones, which act as mediators in lipolysis (Morais et al., 2004). GRB glass eels had significantly greater body contents of ketones than those from MR (4.2% vs. 1.7%, respectively; $p < 0.0001$, $F = 153.79$; unpublished data), but we do not know how this may affect lipase expression. Ketone bodies might represent an important fuel source during prolonged starvation from the conversion of excessive amounts of acetyl-CoA liberated by β -oxidation in fish (Figuerola et al., 2000; Heming and Paleczny, 1987; Ramos and Smith, 1978; Singer et al., 1990; Soengas and Aldegunde, 2002; Soengas et al., 1996, 1998).

4.2. Glycogen phosphorylase

We expected to find a more elevated GPase gene expression in MR glass eels because previous studies showed lower glycogen content in MR than in GRB glass eels (Gaillard et al., 2015). GPase, which is involved in the first step of glycogenolysis, was analyzed to confirm whether glass eels captured at MR had a greater ability to use glycogen stores to fulfill their energetic needs. Moon (1983) measured the activity of different glycolytic enzymes in immature starved American eels and showed that glycogen content remained constant and that the only enzyme showing differences in activity was GPase. In contrast to our expectations, higher allele frequencies for ESTs associated with gluconeogenesis were present in glass eels captured in GRB compared to those from the Atlantic coast (Pavey et al., 2015), indicating a decoupling between variations at the genotypic and phenotypic levels for this gene.

GPase is also involved in the energy supply for osmoregulation in fish gills. Tseng et al. (2007) showed that the expression of genes coding for different GPase isoforms differed according to environmental salinity. In isolated hepatocytes of *Clarias batrachus* and *Sebastes caurinus*, GPase activity increased with hyperosmotic conditions (Hallgren et al., 2003). Expression of GPase observed in both MR and GRB glass eels could then also be related to osmoregulation since glass eels are in transition between salt, brackish, or fresh water.

4.3. Ghrelin and leptin receptor

We hypothesized that when feeding is occurring, genes regulating feeding–appetite should be more expressed. GHRL and LEP-R do not provide direct evidence of feeding, but could be used as indicators of feeding based on previous work done on early stages of development in Japanese eel (Kurokawa et al., 2011). Both genes were expressed in glass eels, but their expression was similar among rivers and dates of arrival. In Japanese eel, gastric ghrelin hormone was immunocytochemically detected as early as the leptocephalus stage and was detected in several organs and tissues at the glass eel stage (esophagus, gastric gland, vascular system, kidney, and skin) (Kurokawa et al., 2002, 2011; Lee et al., 2015). Gastric ghrelin hormone appears to play a vital role in early development. Ghrelin isolated from *A. japonica* stimulated the release of GH and prolactin from *Oreochromis mossambicus* pituitary cells in vitro (Kaiya et al., 2003), thus ghrelin is likely associated with the control of feeding and intestinal motility in fish (e.g., Olsson et al., 2008; Volkoff et al., 2005). Changes in the distribution of ghrelin-producing cells between leptocephalus, glass eel, and elver stages have been suggested to be related to differences in dietary habits among the different developmental stages (Kurokawa et al., 2011). Because glass eels from both sites and dates of arrival differed in terms of pigmentation, length, glycogen, and lipid body contents (Boivin et al., 2015; Gaillard et al., 2015), none of these factors seem to affect the expression of these genes. Thus our hypothesis of feeding effects is not supported. Our observations would rather support the results on *A. japonica* by Lee et al. (2015), who found no relationship between ghrelin and glass eel growth. In adult European eel, four months of fasting did not induce any changes in the expression of LEP or LEP-R, and Morini et al.

(2015) suggested a role of this hormone in sexual maturation instead of feeding regulation.

For the first time, the expression of gene coding for leptin receptor was analyzed in glass eels. Unfortunately, we were unable to obtain primers that would allow the study of leptin transcription. In teleosts, LEP receptors are present in most tissues: they were detected in brain, pituitary, eyes, gills, skin, mesenteric fats, liver, ovaries, muscle, spleen, kidney, heart, intestine, and testes of fishes, including in other eel species (Gong et al., 2013, *Pelteobagrus fulvidraco*; Morini et al., 2015, *A. Anguilla* and *A. japonica*; Rønnestad et al., 2010, *Salmo salar*).

5. Conclusion

Our results show that lipolysis capacity clearly differs in glass eels migrating either to southern Nova Scotia or to the St. Lawrence estuary. Nothing in our results can relate these differences to growth or development stage, but differences in BAL and TAGL expression could be related to genetic differences between origins despite panmixia in American eel and caused by spatially varying selection (Gagnaire et al., 2012; Pavey et al., 2015). The high variability in the level of expression in GRB compared to MR glass eels supports a greater capacity for phenotypic plasticity related to the metabolism of lipids in estuarine glass eels. The advantage of such increased plasticity remains to be elucidated.

Because these two regions were previously associated with different ecotypes (Pavey et al., 2015), this could reflect genetic differences resulting in differential development and life history strategies during the glass eel stage. Glass eels migrating further north are better equipped to use their lipid reserves, which could be related to the longer migration route and to more advanced development. Since southern areas are mainly characterized by male production while the St. Lawrence system harbors nearly 100% females, this ability to use lipids may be sex related. However, this remains to be investigated in more detail.

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