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 harbour 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.

Key words: ecotypes, ghrelin, glycogen phosphorylase, leptin receptor, lipase, mRNA expression, transcriptomics

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 througout 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 larger size at sexual maturity but 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. 2015, 2014, 2009). 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 (freshwater 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 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 towards feeding habitats (Boëtius & 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 anaesthetized 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 & 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 & 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 & Cori 1961); and ghrelin (GHRL) and leptin receptor (LEP-R), which are hormonal factors generally considered to regulate appetite (Lin et al. 2000, Unniappan & 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 (Weltzein 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 ng μl^{-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 ng μl^{-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 cycler, 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 MIciyiQ 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 Analyser (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 *Anguilla rostrata* cytochrome b (GenBank accession no. **KJ546053.1**), 100% between EFI 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 *Anguilla 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 uM 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; 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 $2^{-\Delta\Delta C_T}$ method of Livak & 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 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 (Garcia 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.

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. 1). 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 among glass eels from both origins, and expression was not significantly different from the expression measured in the calibrator group (Fig. 3C, 3D, 3E) 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).

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 × 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 × 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 & 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 & 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 & 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 (Figueroa et al. 2000; Heming & Paleczny 1987; Ramos & Smith 1978; Singer et al. 1990; Soengas & Aldegunde 2002; Soengas et al. 1998, 1996).

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

311 first step of glycogenolysis, was analyzed to confirm whether glass eels captured at MR had a greater ability to
312 use glycogen stores to fulfill their energetic needs. Moon (1983) measured the activity of different glycolytic
313 enzymes in immature starved American eels and showed that glycogen content remained constant and that the
314 only enzyme showing differences in activity was GPase. In contrast to our expectations, higher allele
315 frequencies for ESTs associated with gluconeogenesis were present in glass eels captured in GRB compared to
316 those from the Atlantic coast (Pavey et al. 2015), indicating a decoupling between variations at the genotypic
317 and phenotypic levels for this gene.

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

323 *4.3. Ghrelin and leptin receptor*

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

343 For the first time, the expression of gene coding for leptin receptor was analyzed in glass eels. Unfortunately,
344 we were unable to obtain primers that would allow the study of leptin transcription. In teleosts, LEP receptors

345 are present in most tissues: they were detected in brain, pituitary, eyes, gills, skin, mesenteric fats, liver,
346 ovaries, muscle, spleen, kidney, heart, intestine, and testes of fishes, including in other eel species (Gong et al.
347 2013, *Pelteobagrus fulvidraco*; Morini et al. 2015, *A. Anguilla* and *A. japonica*; Rønnestad et al. 2010, *Salmo*
348 *salar*).

349 5. Conclusion

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

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

363

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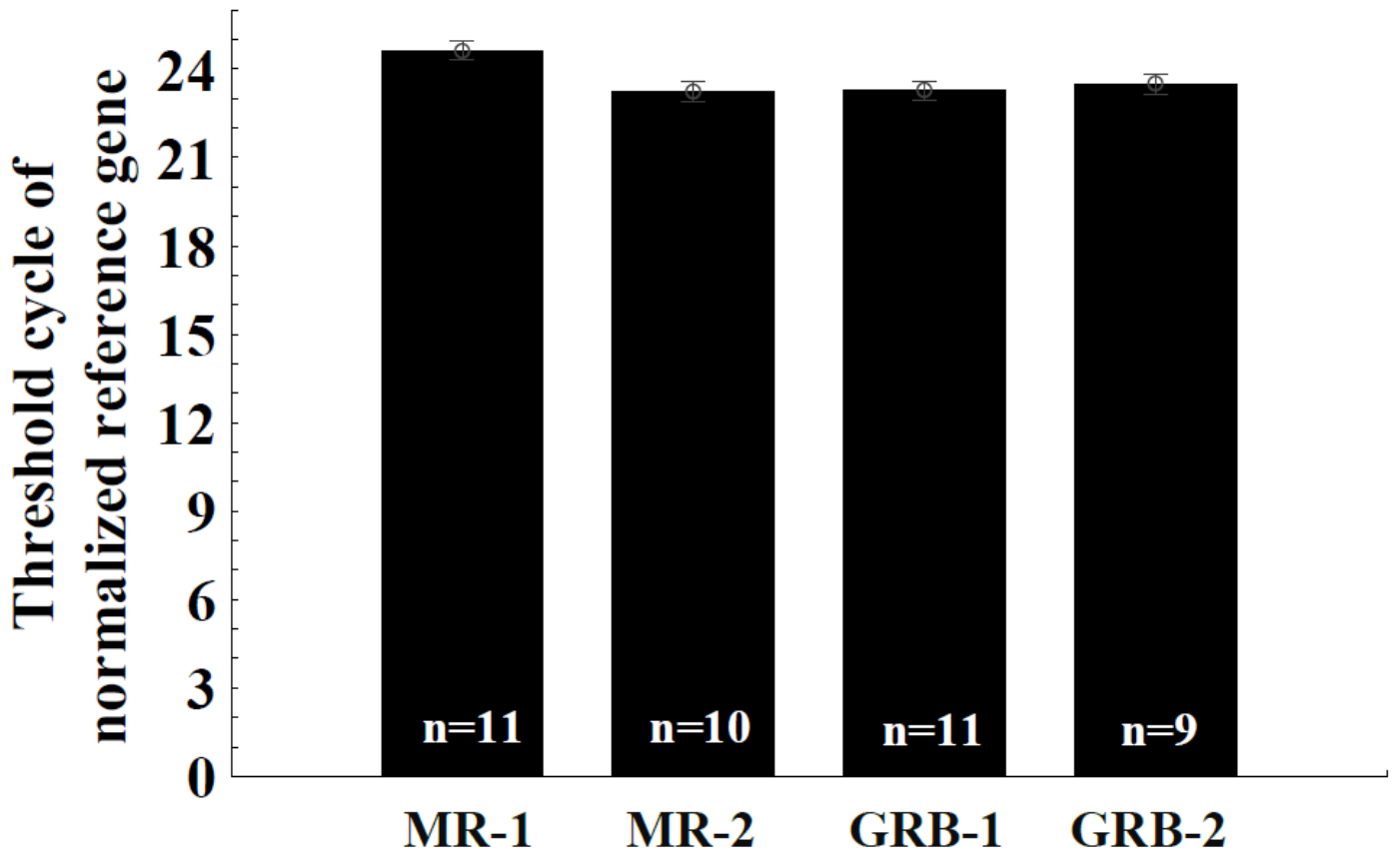
Figure 1. Average threshold cycle (C_T) for the normalized reference gene (geometric mean of individual C_T obtained for the three reference genes) for the two rivers (MR: Mersey River; GRB: Grande-Rivière-Blanche) and the two periods of capture in each river (MR-1: 26–28 March; MR-2: 20–21 April 2012;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

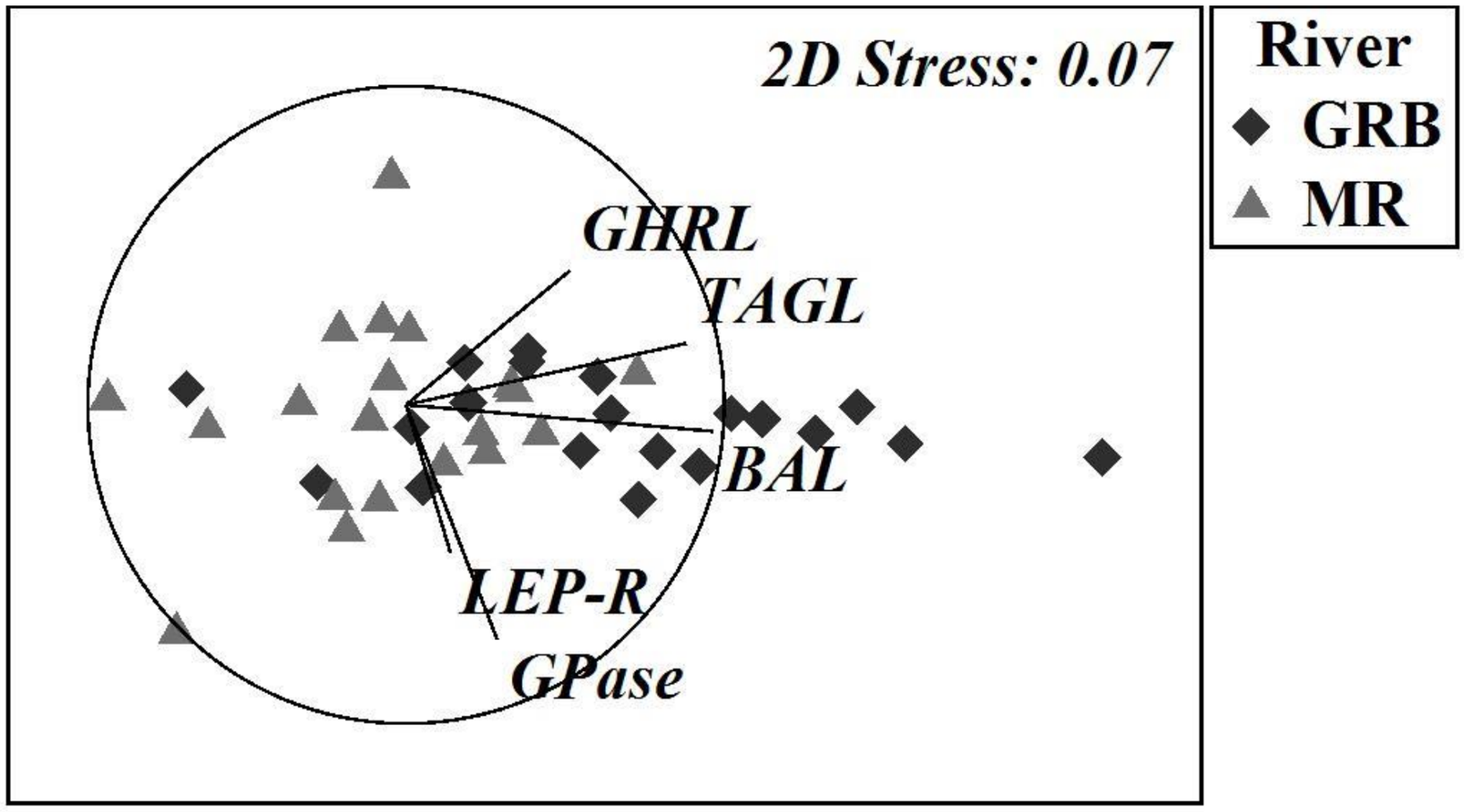
Figure 2. Multidimentional scaling (MDS) between rivers including data from the 40 individuals (10 per river and date of capture) based on the Bray-Curtis dissimilarity matrix. River was the only significant factor detected by permutational multivariate analysis of variance (PERMANOVA). The stress values 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, 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, 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.

Figure 3. Profiles (means ± SE) of the relative gene expression of bile salt activated lipase (BAL), triacyglycerol 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.


Threshold cycle of normalized reference gene
0
3
6
9
12
15
18
21
24
n=11
n=10
n=11
n=9
MR-1
MR-2
GRB-1
GRB-2

565
566

567



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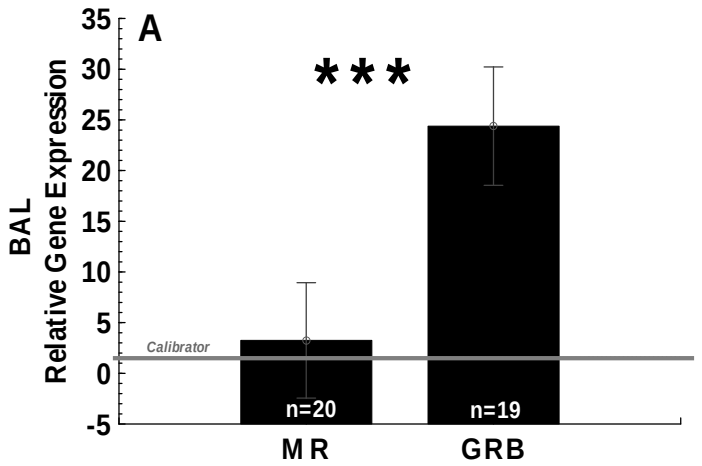
A
***
BAL
Relative Gene Expression
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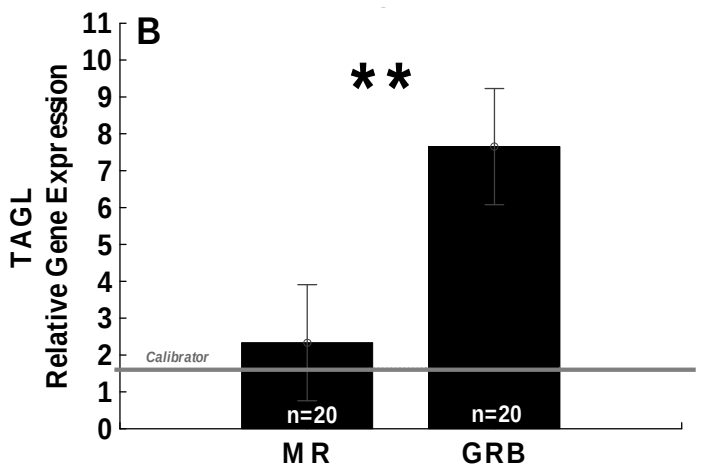
-5
Calibrator
n=20
n=19
M R
GRB


B
**
TAGL
Relative Gene Exprression
11
10
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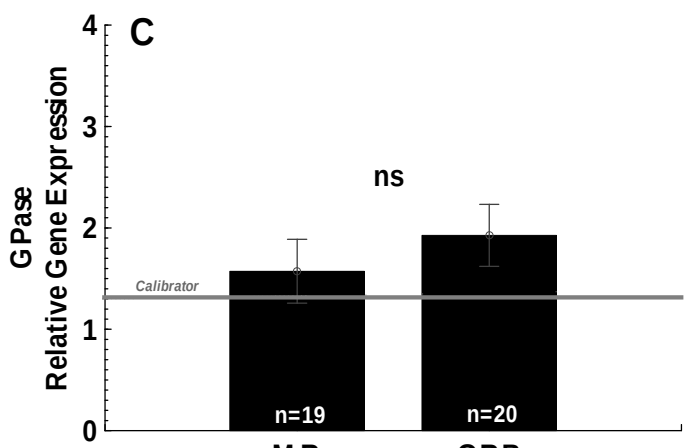
8
7
6
5
4
3
2
1
0
Calibrator
n=20
n=20
M R
GRB


C
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GPase
Relative Gene Exprression
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3
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Calibrator
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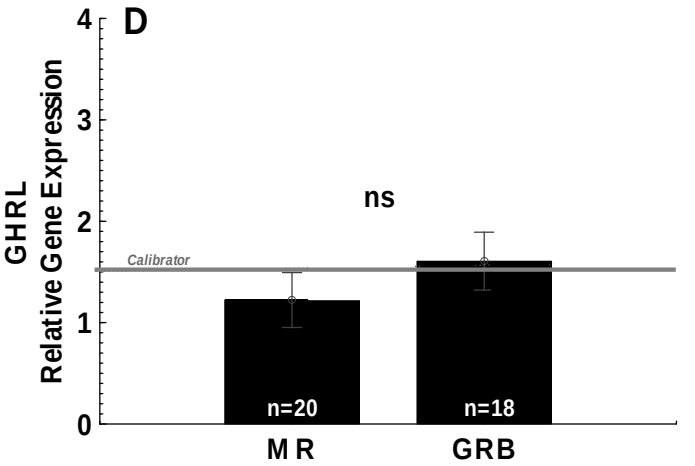
n=20
M R
GRB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GHRL
Relative Gene Expression
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M R
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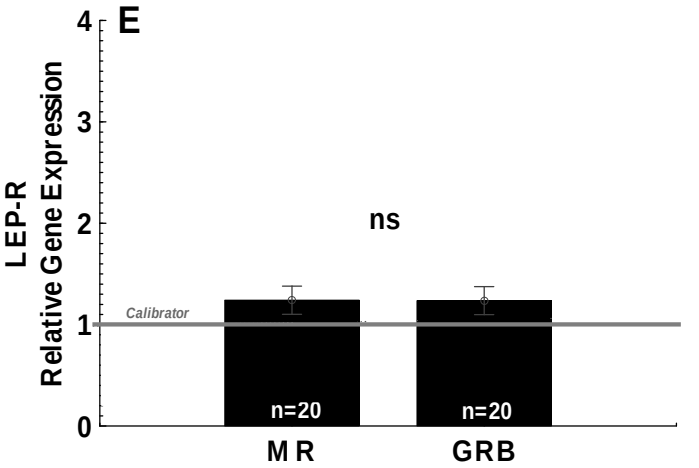
E
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LEP-R
Relative Gene Expression
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Calibrator
n=20
n=20
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569

570 Table 1

571 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)
572 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
573 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
574 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 measure gene expressions (Melt temperature)**	**qPCR efficiency**
REFERENCE GENES				
Acidic ribosomal protein (ARP)	*A.anguilla* (GenBank accession no. **AY763793**)	F: GCCACGTGGAAGTCCAACTA (57) R: CAGGAGTTTCTCCAGAGCGG (58)	F: TCTCCCTGCGTGCAAAGG (59) R: CTTGCGCATCATGGTGTTCT (58) P: TGGTGCTGATGGGC (69)	y = -3.196x + 34.388 R^2 = 0.9993
Cytochrome B (CytB)	*A.anguilla* (GenBank accession no. **AF006714**)	F: CATCTGCCGAGACGTCAACT (57) R: ATCTGCCCTCATGGAAGCAC (58)	F: TCATCTGCCGAGACGTCAAC (58) R: GAGGCCCCATTTGCATGTAG(59) P: ATGGATGATTAATTCGC (68)	y = -3.1254x + 32.523 R^2 = 0.9992
Elongation factor 1 (EF1)	*A.japonica* (GenBank accession no. **AB593812**)	F: CCTGAAGCCTGGTATGGTGG (58) R: TACGTTGCCACGACGGATTT (57)	F: TTGCCCCTGCCAACGT (58) R: GGGACTCATGGTGCATTTCC (59) P: ACCACTGAGGTCAAGTC (68)	y = -3.3354x + 33.755 R^2 = 0.9998

TARGET GENES				
Triacylglycerol lipase (TAGL)	—	—	F: GCACCCGCTTCATGCTCTT (60) R: ACCTTGCCTGGTGGTTGTTG (59) P: ACCCGGCAGAACC (69)	y = -3.356x + 39.015 R^2 = 0.9963
Bile salt activated lipase (BAL)	—	—	F: TGATGCCGTGTCTGAAGATCA (59) R: GGTGTAGACTCCCAGCCAGAGT (58) P: AGACCCTGTGGCTCT (69)	y = -3.4627x + 40.166 R^2 = 0.99972
Glycogen phosphorylase (GPase)	—	—	F: GCGTCCCGTCCACTTCTATG (59) R: AACCCATTTGACCCCATCTG (58) P: CAGAGTGGAGCACCAC (68)	y = -3.1649x + 36.873 R^2 = 0.99959
Ghrelin (GHRL)	*A.japonica* (GenBank accession no. **AB062427**)	F: TCCAAGAGGCACTGGGTTTC (57) R: CCATCTGAGTCTCGTCTGCC (57)	F: GCTTCAGACATGAGGCAGATGA (59) R: GGACGCAGACCAGCAGGAT (60) P: CGCACCGCATACAG (69)	y = -3.1767x + 41.44 R^2 = 1
Leptin receptor (LEP-R)	—	—	F: GCGAGGGCCCCTTCTG (59) R: CTGGAGGTCTGGTGCTTAACTGT (58) P: TGCGTGGAGGGACT (69)	y = -3.1034x + 38.65 R^2 = 0.99496

575

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(perm), 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**	**10203**	**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**	**13686**	**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

584 Table 3

585 Relative contribution (until 100% cumulative contribution) of candidate genes to differences between rivers by
586 one-way similarity percentage analysis (SIMPER). Av. Abund.: average abundances over all samples, Av.
587 Diss.: average contribution to the total dissimilarities, Contrib%: percentage contribution to the total
588 dissimilarities, Cum%: cumulative percentage contribution to the total dissimilarities. BAL: bile salt activated
589 lipase, TAGL: triacylglycerol lipase, GPase: glycogen phosphorylase, GHRL: ghrelin, LEP-R: leptin receptor.
590 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%			

591

592 Supplemental Table 1. Sequences of the amplification products obtained for ARP, CytB, EF1, and GHRL. For TAGL, BAL, GPase, and LEP-R, specific sequences from *Anguilla rostrata* were obtained

593 from a draft annotated American eel genome (Pavey et al., unpublished results).

Gene (Gene acronym)	**Specific EDS or CDS of *Anguilla rostrata* (number of base pairs)**
Acidic ribosomal protein (ARP)	TGCCACGTGGAAGTCCAACTATTTTATGAAAATCATCCAACTCTTGGATGAGTACCCCAAGTGCTTCATTGTGGGGGCCGACAATGTGGGCTCCAAGCAG ATGCAGACCATCCGCCTCTCCCTGCGTGCAAAGGCTGTGGTGCTGATGGGCAAGAACACCATGATGCGCAAGGCCATCCGTGGCCATCTGGAGAACAAC CCCGCTCTGGAGAAACTCCTGAAATCCCTATTTTCCAAAGCCCGAAGGCTTTGCATATGTAATTTGCCATAAAACACCTTTTTGCCAAGAAGGTAAATTC CTC (302bp)
Cytochrome B (CytB)	TTCATCTGCCGAGACGTCAACTATGGATGATTAATTCGCAACCTACATGCAAATGGGGCCTCATTCTTCTTTATCTGCCTATACCTTCACATTGCCCGAGG ACTTTACTACGGCTCATATCTTTACAAAGAAACATGAAACATTGGAGTCGTATTATTCCTATTAGTAATAATAACAGCATTCGTKGGGTATGTGCTTCCAT GAGGGCAGATAAAGAA (218bp)
Elongation factor 1 (EF1)	TTCCTGAAGCCTGGTATGGTGGTGACCTTTGCCCCTGCCAACGTGACCACTGAGGTCAAGTCTGTGGAAATGCACCATGAGTCCCTGCCTGAGGCTCTTC CCGGTGACAATGTTGGCTTCAACGTCAAGAACGTCTCTGTCAAGGAAATCCGTCGTGGCAACGTAAA (167bp)
Ghrelin (GHRL)	TTCCAAGAGGCACTGGGTTTCCTCTTAAAGTGCAAAACCCCACTGTGAGCTTCAGACATGAGGCAGATGAAACGCACCGCATACAGCATCCTGCTGGTCT GCGTCCTGGCGCTGTGGATGGACTCTGTCCAGGCTGGCTCCAGCTTCCTCAGCCCCTCACAGAGACCGCAGGGGAAGGATAAGAAGCCTCCCAGGGTTG GCAGACGAGACTCAGATGGAA (220bp)
Triacylglycerol lipase (TAGL)	CTGGGCTGTTTCACGGACGACGTTCCCTGGGCGGGCACCACCGAGCGGCCAATCGCCAAGCTCCCCTGGAGCCCAGAGAAGATAGGCACCCGCTTCATG CTCTTCACCCGGCAGAACCCCAACAACCACCAGGCAAGGTCCACTTTACAGGAGATCACAACAAAAGAGGACATCCTTCTGGCATCGAATTACAACGGG ACCAGGAAGACGCGCTTCATTACCCACGGCTACGTCGACAAAGGGGATGAAAACTGGCTGATTGACATGTGCAAGCTGATGCTCCAAGTGGAGGACATC AACTGCATCTGCGTGGACTGGAAGAAGGGAGGCCGGACCCTGTACACGCAGAGCGCCAGCAACATCCGCGTCATCGGGGCCCAGATGGCCTACATGAT CCAGCTGTTCCAGACGCTGTACCAGCAGAGGCCCGAGTCGGTCCACATCATTGGACACAGCCTGGGGGCGCACTGTGCAGGCGAGGCCGGGCGCAGGA CCCCAAACCTGGGCCGCATCACCGGTCTGGATCCTGCCGAGCCGTACTTCCAGGGCTGCCCCAGCCTGGTGCGCCTGGACCCCAGCGACGCCAAGTTCGT

	GGACGTCATTCACACAGACGCGAAACCCATGATTCCCTATCTTGGGATGGGAATGGCTCAGGCCGTCGGCCATCTTGACTTCTACCCTAACGGAGGGGA GCACATGCCTGGATGCGACAAGAACCTCATCTCTCAGATTGTGGACATCGACGGCATTTGGGAA (756bp)
Bile salt activated lipase (BAL)	AACATCTGGGAGATCGCTGACCGGGGGAACGTCATTGTGGTTACGCTGGGGTACCGCGTGGGCACCCTGGGGTTCCTCAGCAGCGGCGATGCCAGCGGG CCAGGGAATTATGGGCTGTGGGACCAGCACGCCGGCATCGCCTGGGTGCACCGGAACATCAGGGCCTTCGGAGGCGACCCCGACAACATCACCGTCTTC GGCGAGTCCGCCGGGGCCGCCAGCGTCAACTTCCAGCTCCTCTCTCCCAAAAACAGGGGTCTGATCCGCAGGGGCATCTCTCAGAGCGGGGTGGCCCTC AGCCCCTGGGCCGTGAACAGGAACCCGCGGGTGCTGGCAGAGGAGATCGCCATTAAGGTGGGCTGTCCCACAGATGAGAAGATGATGCCGTGTCTGAA GATCACAGACCCTGTGGCTCTCACTCTGGCTGGGAGTCTACACCTGAAGGGCTCTCCATCCAACCCTCTCCTGTTCAACCTGCTGCTGGCCCCCGTGATCG ACGGGGACTTCCTGCCGGACGAGCCGGGAAACCTGTTCCACAACGCCGCCGACATCGACTACATCGTAGGCATCAACAACATGGACGGCCACCTCTTCT GCGGCATCGACGTGCCCTCGATCAACCAGCCCCTG (630bp)
Glycogen phosphorylase (GPase)	CCCTGGGATAAGTTCTACATGGGCCGAACCCTGCAGAACACCATGGTAAACCTGGCGCTGGAGAATGCCTGCGACGAGGCCACGTACCAGCTCGGCCTT GACATGGAGGAGCTGGAGGACATGGAGGAGGATGCTGGCTTGGGAAATGGAGGCTTGGGACGTCTGGCGGCCTGTTTTCTGGACTCCATGGCATCACTG GGGCTGGCGGCCTACGGCTATGGCATCCGCTATGAGTTTGGCATCTTCAACCAGAAAATCTGCAACGGCTGGCAGGTGGAGGAGGCAGACGATTGGCTG CGCTACGGCAACCCCTGGGAGAAGGCGCGCCCCGAGTACATGCGTCCCGTCCACTTCTATGGCAGAGTGGAGCACCACCCAGATGGGGTCAAATGGGTT GACACTCAGGTAGTCTTGGCCCTGCCTTACGACACCCCGGTCCCCGGGTACAGGAACAACGTGGTCAACACCATGAGGCTGTGGTCTGCCAAGGCACCC TGCGAGTTCAACCTGAAAGACTTTAACATCGGCGGCTATATCCAGGCTGTGCTGGACAGAAACCTGGCTGAGAACATCTCCCGTGTGCTGTACCCCAATG ACAACTTCTTCGAGGGGAAGGAGCTGCGTCTGAAGCAGGAGTACTTTGTGGTGGCCGCCACCCTTCAGGACATCATCCGCCGCTTCAAGGCCTCCAAGTT TGGCTCCAAGGAAGTCGTGCGTACGGACTTCGCTGCCCTGCCCGACAAGGTTGCCATCCAGCTGAACGACACCCACCCCGCCATGGCGATCCCCGAGCT GATGAGGATCCTGGTAGACGAGGAGAAGCT (824bp)
Leptin receptor (LEP-R)	ACCTTTCTGCCGGATGCTCATCTAAGTATCTGGTGGAAGAGGCCTGAACTGCCTGTCTATGAGCTCCAGTTTGAGGTCCGGTACTCAGTGGACAAAGATG ACACACTGCAAAAGGTAATCAGGTCCGTGTCCAATCAGTCAGCCGTGGTCCCGGTTGTGGATCCGTGTGTGGTGTACACCATTCAGGCACGCTGCAAGC GGCTTGCTGGACCTGGATTCTGGAGTGATTGGAGCAGCCCCTACTATACCACCATTAATAATATCAAAGCACCTGAACAGGGACCTGATTTCTGGAGAGT ACTTCAGGAATATCCCAAACTGAATCAGACACATGTCACTCTTCTCTTCACGCTTTCGCAGAGCGAGGGCCCCTTCTGTTGCGTGGAGGGACTTACAGTT AAGCACCAGACCTCCAGGGGCTCTGTTTGGTCGGAGAATTTAGGCCGTGTCTCCACCTACTCCTTTTCCTGGACTGAAGATGTCCACACTGTCACTGTCCT GGCCCACAATGCCCTTGGATCTTCAACCAAGAACAGCAACATGACACTGACCAGACACACCAAATCGCAATCCGTCCACTCGTTTAGCTCAATGATGGT

AAACAGCAGCTGTGTGGCACTCTCCTGGACCCTGTTCCCAAACAGCTCTGCTCCGTCCTCATTTGTAATCCAGTGGAGCGGCCAAAGCAGGAGCAGGCA
GCAGGACAAGCAGGGGGGGAGAGTGAAGTGGGTGCGGGTTCCCCCCAACAACCGTGCCTTTCACCTACATGAAACCTTCTTTGCCTCAGAGGAATACCA
GTTTATTTTGTATCCAATATTTGAGAATACCGAAGGAGAGCCCATCTACGCCAAAGGCAAGCCTGAAGACAGGGGGCGCCCCAGGGGAGACCATGCGGC
CTACATGCTCCTGCTGATTATCACCTTCCTGTCAGTGGTCTTGTTTGTGACACTCGCCGTCTCCCAGAATCAAATGAGGAAGCTGGTGTGGAAAGATGTTC
CCAACCCCAACAACTGCTCCTG (1019bp)

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