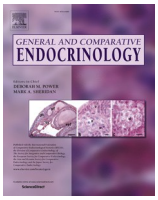
General and Comparative Endocrinology 331 (2023) 114160

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Growth regulation in brook charr *Salvelinus fontinalis*

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ARTICLE INFO

Keywords:
Selection
Phenotype
Growth
Transcriptomics
gh/igf-1 axis
Appetite regulation

ABSTRACT

Fish growth can be modulated through genetic selection. However, it is not known whether growth regulatory mechanisms modulated by genetic selection can provide information about phenotypic growth variations among families or populations. Following a five-generation breeding program that selected for the absence of early sexual maturity and increased growth in brook charr we aimed to understand how the genetic selection process modifies the growth regulatory pathway of brook charr at the molecular level. To achieve this, we studied the regulation of growth traits at three different levels: 1) between lines—one under selection, the other not, 2) among-families expressing differences in average growth phenotypes, which we termed family performance, and 3) among individuals within families that expressed extreme growth phenotypes, which we termed slow- and fast-growing. At age 1+, individuals from four of the highest performing and four of the lowest performing families in terms of growth were sampled in both the control and selected lines. The gene expression levels of three reference and ten target genes were analyzed by real-time PCR. Results showed that better growth performance (in terms of weight and length at age) in the selected line was associated with an upregulation in the expression of genes involved in the growth hormone (GH)/insulin growth factor-1 (IGF-1) axis, including the *igf-1 receptor* in pituitary; the *gh-1 receptor* and *igf-1* in liver; and *ghr* and *igf-1r* in white muscle. When looking at gene expression within families, family performance and individual phenotypes were associated with upregulations of the *leptin receptor* and *neuropeptid Y*—genes related to appetite regulation—in the slower-growing phenotypes. However, other genes related to appetite (*ghrelin, somatostatin*) or involved in muscle growth (*myosin heavy chain, myogenin*) were not differentially expressed. This study highlights how transcriptomics may improve our understanding of the roles of different key endocrine steps that regulate physiological performance. Large variations in growth still exist in the selected line, indicating that the full genetic selection potential has not been reached.

1. Introduction

Fish reproductive success in nature is determined not only by the number of offspring that an individual produces, but also by how many offspring survive to reproductive maturity (Clutton-Brock, 1988). Large males, which may be preferred by females, can dominate competitors in contests for mates or breeding territories, and large females can produce more and larger offspring than small ones (Perry et al., 2005; Anderson et al., 2010). Aquaculture production aims to produce large fish that invest in growth instead of reproduction and that best adapt to the captive environment (Gjedrem, 2005; Sauvage et al., 2010; Bastien et al., 2011). Selective breeding exploits the substantial genetic variation that is present for desirable traits. Thus, a high growth rate as well as the absence of early sexual maturity are the most used criteria since energy is preferentially invested in growth rather than in gamete production (e.g., Nilsson, 1990; Bastien et al., 2011).

Growth in teleosts is controlled at the endocrine level, mainly by the growth hormone (GH) / insulin factor 1 (IGF-1) axis (Björnsson, 1997; Wood et al., 2005; Duan et al., 2010; Vélez et al., 2017). GH, which is synthesized by the pituitary gland, regulates many functions, including somatic growth, energy metabolism, reproduction, digestion, osmoregulation, and immune function (Kawaguchi et al., 2013). The release and

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https://doi.org/10.1016/j.ygcen.2022.114160
Received 26 January 2022; Received in revised form 12 September 2022; Accepted 2 November 2022
Available online 7 November 2022

synthesis of GH in the brain and peripheral tissues are i) stimulated by neuroendocrine factors, such as neuropeptide Y (NPY) (Aldegunde and Mancebo, 2006) and ghrelin (GRL) (Rønnestad et al., 2017; Perelló-Amorós et al., 2018), and ii) inhibited by somatostatin (SRIF, mainly synthesized in the brain) (Nelson and Sheridan, 2005; Very and Sheridan, 2007; Sheridan and Hagemeister, 2010; Volkoff et al., 2010). GH stimulates the production of liver IGF-1 (Volkoff et al., 2010), so its physiological effects are usually indirect, via IGF-1 actions. Appetite regulation is another key process controlling growth and is regulated by hormones also acting as appetite stimulators (i.e., orexigenic factors NPY and GRL) (Breton et al., 1989; Cerdá-Reverter and Larhammar, 2000; Rønnestad et al., 2017) or appetite inhibitors (i.e., anorectic factors: leptin LEP) (Hoskins and Volkoff, 2012; Dar et al., 2018). The continued production of muscle fibres is another important process that controls fish growth (Ahammad et al., 2015). Teleosts are unique among vertebrates because of their continued growth due to the continuous production of muscle fibres from birth to death (Ahammad et al., 2015). Some key myofibrillar proteins such as myosin, actin, tropomyosin, and troponin are specifically expressed in muscle tissue and are involved in its contraction (Skaara and Regenstein, 1990; Zhang et al., 2011). Other myogenic factors involved in tissue differentiation and maturation processes, such as myosin heavy-chain (MHC) and myogenic regulatory factor (MRF4), are key for understanding growth-regulating mechanisms (Vélez et al., 2016).

Few studies have focused on how the selection process affects growth regulation in brook charr *Salvelinus fontinalis*. Sauvage et al. (2010) reported that selective breeding led to a 4.16% difference in expressed genes between the control and domesticated lines at the juvenile stage. In particular, they observed that genes involved in growth pathways (e.g., transforming growth factor b and T complex protein 1) were generally more highly expressed in the selected line than in the control line. Studies in other salmonids, such *Oncorhynchus kisutch* and *O. mykiss,* evaluated the effects of domestication on growth (Devlin et al., 2009; Tymchuk et al., 2009) and the relationship between genomics and selection in aquaculture based on the study of divergence and genome size (Hessen et al., 2010; Pankova et al., 2017). However, no one has looked at the growth regulation pathways occurring in different brook charr tissues, and that is the objective of the study presented here.

Selective breeding is particularly well-developed for brook charr, for which several studies have been carried out with both anadromous (seawater migratory) and resident fish (Laval strain, Québec). Perry et al. (2004) showed that higher fertility was associated with higher mean fry length, suggesting that stabilizing selection for juvenile length occurred prior to yolk sac resorption. Furthermore, parental-based genetic variance for early size traits appears to be partially segregated at the embryo–fry boundary, with maternal genetic variance being high prior to yolk-sac resorption and relatively low thereafter (Perry et al., 2004, 2005). Significant heritability for traits related to the accumulation and use of energy reserves was found in two out of three different strains used by the Québec fish-farming industry, which include the Laval strain (Crespel et al., 2013). Domestication has led to large increases in fish weight: for *S. fontinalis* from the same Laval strain, the weight of selected fish at age 22 months increased by 23% between the F1 and F2 generations and by 32% from F2 to F3 (Bastien et al., 2011).

The main goal of this study was to understand how the genetic selection process modifies the growth regulatory pathway of brook charr at the molecular level. To achieve this, we studied the regulation of growth traits at three different levels: 1) between lines—one under selection, the other not, 2) among-families expressing differences in average growth phenotypes, which we termed family performance, and 3) among individuals within families that expressed extreme growth phenotypes, which we termed slow- and fast-growing. We tested the hypothesis that selection enhanced the differential expressions of genes involved in the GH/IGF-1 axis and in appetite control as well as in muscle growth between slow- and fast-growing phenotypes and family performance. We also aimed at identifying molecular indicators that could be implemented in a selection program to enhance sustainable production for brook charr aquaculture.

2. Methodology

2.1. Selection process

A selective breeding program described by Bastien et al. (2011) and Sauvage et al. (2010) was initiated in 1994 using wild *S. fontinalis* from the Laval River (Québec; 48.449° N, 68.059° W). Briefly, a combined between- and within-family selection protocol was applied based on 1) the absence of precocious sexual maturation at 22 months and 2) growth performance in sexually immature fish. A control line was created by the arbitrary selection of equal numbers of fish from each family for every generation (i.e., domestication to culture conditions, but no intentional selection) (Bastien et al., 2011). This line was maintained over the same period, which allowed us to perform studies aimed at tracking temporal genetic and phenotypic changes occurring in selected vs. control lines reared in the exact same environment. It is important to note that this control group—even if it was not selected by the criteria of growth and absence of maturity—can be considered as domesticated across generations (non-directed selection). Fish were healthy throughout this study, and we encountered no problems in maintaining all families and lines.

2.2. Rearing conditions

Fertilized eggs were incubated in darkness. Each family was incubated separately in individual trays with screened bottoms that allowed the upwelling of water through the egg layers during incubation and the inflow from the upstream side during fry rearing. Water temperature followed the natural winter decrease but was not allowed to drop below 4°C. At hatching, temperature was gradually increased by 1°C per week to reach 8°C, providing optimal conditions for first feeding. At the beginning of June, when natural water conditions reached 8°C, no further temperature adjustments were made, and fish were reared under natural temperature and photoperiod conditions in flow-through dechlorinated fresh water in our wet lab facilities (maximal temperature: 15°C in September; minimal temperature 3°C in February). Each family was maintained in its individual tray until fish reached a size that allowed family identification by fin clippings (eight possible marks combining adipose, right and left pelvic fins). Families (control and selected combined) with different markings were randomly pooled in five 250 L rearing tanks and 4 months later in five 500 L tanks. Fish from six families were placed in each 250 L tank (400 individuals per family, 2400 ind. tank^{-1}, mean charge of 9.2 kg m^{-3}), and then in 500 L tanks (200 ind. per family; 1200 ind. tank^{-1}, mean charge of 10.8 kg m^{-3}).

Fish were fed commercial pellets eight times per day at the beginning of exogenous feeding (March) with a gradual decrease to reach one meal per day by November. We calculated rations (based on fish size and prevailing temperature conditions) so that the food supplied was overestimated (commercial charts were designed for rainbow trout) and to avoid having an excess of unfed food that would decrease water quality. We stopped supplying pellets when fish stopped eating, thus satiety was ensured. Fish were hand fed each morning except in winter (December to end of March), when they were fed twice a week. Fish were weighed at regular intervals and fin markings were verified. With this information, feeding rations were modified and care was taken not to exceed a rearing load greater than 30 kg m^{-3}. Fish numbers were reduced when this was the case, with no attempt to keep the highest-performing fish, and family pools in the different tanks were randomly modified except to avoid having similar family fin marks in the same tank.

2.3. Sampling

We used the progeny of 13 families from the selection line and 16 families from the control lines, both from the F5 generation (n = 4471

individuals: 2078 selected and 2393 control). Individuals from the four families with the largest mean weight (high-performing families) and the four families with the lowest mean weight (low-performing families) were sampled in both the control and selected lines (Table 1, Fig. 1). Fish from each family were weighed (± 0.1 g) and measured (± 0.1 cm) in July at the age of 7 months, in November at 11 months, and in June at 18 months (1+) (Supplementary Fig. 1); the phenotypes at 18 months of age were used to rank individuals and families for this study. Fish were not fed for 24 h and then were anaesthetized (3-aminobenzoic acid ethyl ester, 0.16 g L^{-1}) prior to measurements (length and weight). Fulton's condition factor (K) (Fulton, 1904) was calculated as

$$K = \left(W\,L^{-3}\right)^{*}\,100$$

where W is the weight in g and L is the fork length in cm.

For each family (Table 1), the eight heaviest (fast-growing individuals) and the eight lightest (slow-growing individuals) juveniles were sacrificed by severing the spinal cord and used for further molecular analyses (Table 2, Fig. 1). The pituitary gland, brain, liver, and white muscle were immediately removed and placed in sterile tubes, frozen in liquid nitrogen, and stored at −80°C pending analyses.

2.4. Total RNA and cDNA synthesis

For each fish, liver and brain total RNA were extracted from 30 mg wet weight of tissue. For pituitary RNA extraction, a pool of eight individuals from a same group was used because of the gland's small size (Fig. 1), which prevented analysis at the individual level. RNA extractions were performed using the RNeasy Plus Universal Mini Kit (liver, pituitary, and brain; Qiagen, Inc., Mississauga, ON, Canada) and RNeasy Fibrous Tissue Kit (muscle; Qiagen, Inc., Mississauga, ON, Canada). Extracted RNA was diluted to a final concentration of 200 ng μL^{-1}. RNA purity, quality, and concentration were measured by SYBRSafe DNA Gel Stain 2% agarose gel electrophoresis (Alpha Imager HP System, Alpha-Innotech, Alpha Software, Invitrogen, Inc., CA, USA) with an absorbance ratio of 260/280 (NanoVue Plus spectrophotometer, GE Healthcare, Pittsburgh, PA, USA). Reverse transcription of mRNA into complementary DNA (cDNA) was performed in duplicate for each sample and then pooled using the Quantitect Reverse Transcription Kit (Qiagen, Inc., Mississauga, ON, Canada). cDNA was diluted to a final concentration of 200 ng μL^{-1}, separated into aliquots, and kept frozen at −20°C until further analysis. cDNA integrity and concentrations (1.8–2.0) were verified using a NanoVue Plus spectrophotometer. The efficiency of reverse transcription was verified by quantitative polymerase chain reaction (qPCR) using serial dilutions of a representative pool of cDNA samples collected from different sampling sites and compared to the ideal slope of −3.3.

Table 1
Growth characteristics of *Salvelinus fontinalis* 1+ families used in the study.

Group	Family	n	Weight (g)	Length (cm)	Condition (K)
CL	C19	142	6.14 ± 1.82	8.82 ± 0.79	0.87 ± 0.13
	C16	119	7.19 ± 2.23	9.34 ± 0.99	0.86 ± 0.14
	C1	164	7.21 ± 3.63	8.92 ± 1.31	0.95 ± 0.23
	C18	135	7.43 ± 2.76	9.07 ± 1.10	0.96 ± 0.18
CH	C22	99	9.59 ± 2.45	10.02 ± 0.91	0.93 ± 0.10
	C5	199	10.50 ± 3.42	10.36 ± 1.17	0.91 ± 0.12
	C17	156	10.77 ± 3.66	10.54 ± 1.27	0.89 ± 0.11
	C10	179	11.20 ± 4.00	10.50 ± 1.30	0.93 ± 0.11
SL	S1	147	8.14 ± 2.49	9.62 ± 1.07	0.89 ± 0.08
	S19	164	10.25 ± 3.94	10.21 ± 1.35	0.92 ± 0.17
	S5	157	10.59 ± 3.63	10.55 ± 1.25	0.87 ± 0.10
	S3	178	10.98 ± 4.35	10.25 ± 1.49	0.98 ± 0.19
SH	S11	195	12.97 ± 4.42	10.93 ± 1.31	0.96 ± 0.11
	S12	95	13.27± 3.61	11.06 ± 1.07	0.96 ± 0.11
	S8	184	14.01 ± 4.81	11.11 ± 1.37	0.98 ± 0.10
	S13	176	16.09 ± 6.61	11.72 ± 1.69	0.95 ± 0.11

Groups were formed according to line (C = control; S = selected) and family performance (L = low; H = high).

2.5. Primer design for target genes

To evaluate the impact of selection on the growth regulation pathway, the expressions of the genes present in different tissues were quantified in each sampled fish (except for the pituitary gland for which we used family pools). These different tissues included brain (target genes *npy, lep-r*), pituitary (target genes *gh, ghr-1, igf-1r, grl, srifr*), liver (target genes *igf-1, ghr-1*), and muscle (target genes *igf1r, ghr-1, mhc, myog*).

The first step was to obtain the DNA sequences for *S. fontinalis* since sequences were not available for this species. We designed primers from Artic charr *Salvelinus alpinus* and rainbow trout *Oncorhynchus mykiss* sequences (Table 3) to perform PCR and amplify products of interest in *S. fontinalis*. PCR was performed in 25 µL reactions containing 12.5 µL of AmpliTaq Gold 360 (Applied Biosystems), 0.5 µL of 360 GC enhancer (Applied Biosystems), 2.5 µL of cDNA, 1.25 µL each of forward and reverse primer (20 mM), and 7 µL of nuclease-free H_2O. Reactions were amplified under a thermal profile of 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min and 20 s, followed by 7 min at 72°C. PCR products were then tested by gel electrophoresis on 2% agarose gels. The amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen) and in forward and reverse sequences using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). For each gene, the sequence obtained was compared with the sequence used for primer design with the BLAST® software (Altschul et al., 1990). Sequence lengths and percentages of similarity to the reference sequences are presented in Table 3.

2.6. Measurement of gene expression by qPCR

Gene expression was measured by qPCR using the TaqMan technology, which involved designing primers and probes specific to brook charr based on the gene sequences obtained in the step described above. For pituitary analyses, IDT PrimeTime probes (Table 4) were designed using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA, USA). For brain, liver, and muscle, TaqMan probes (Table 5) were designed using the Primer Express software version 3.0 (Applied Biosystems). For all samples, qPCR gene expression was performed in triplicate using a QuantStudio 3 Real Time PCR System (Applied Biosystems). Each reaction consisted of 2 µL of diluted cDNA, 5 µL of TaqMan Fast Advanced Mix, 0.5 µL of Custom TaqMan Gene Expression Assay, and 2.5 µL of sterile water, for a total volume of 10 µL.

The thermal cycling of qPCR was done in two steps: (1) 2 min at 50°C for optimal AmpErase uracil-N-glycosylase activity followed by 20 s at 95°C to activate DNA polymerase, and (2) 45 denaturation cycles for 1 s at 95°C and annealing / extension for 20 s at 60°C. Cycle thresholds (CT) were obtained with the QuantStudio Design Analysis software (ThermoFisher Connect). The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001), with C_T being a threshold cycle:

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

where C_{Te} = C_T of the candidate gene - C_T of the reference genes for sample x, and C_{Tc} = C_T of the target gene - C_T of the reference genes for the calibrator.

In this study, the calibrator was the CLS group (control line + low-performing families + slow-growing phenotype). The stability of reference gene expressions between groups was verified with Expression Suite version 1.0, where the score was calculated according to Vandesompele et al. (2002). The reference genes were *18s*, *β-actin,* and *ef1α,* and the best score combination obtained with the QuantStudio Analysis software was kept for each tissue. For accurate averaging of the control

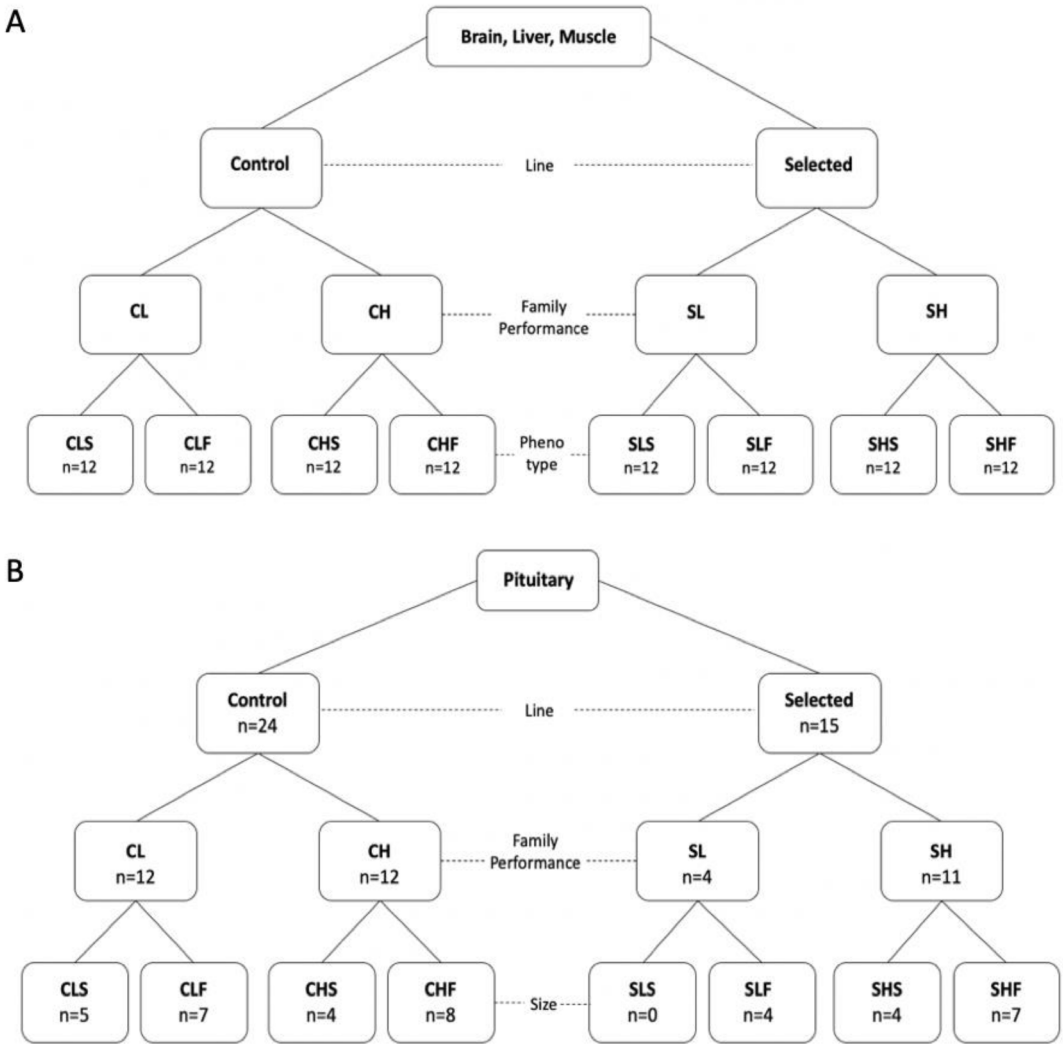

Fig. 1. Diagram showing the experimental design for different tissues used to evaluate the effect of line, family performance, and size (phenotype). Line: Control (C), Selected (S); Family performance: Low (L), High (H); Phenotype: Slow-growing (S), Fast-growing (F). A: brain, liver, and muscle. These tissues were not pooled, so each phenotype group represents four families (three slow-growing individuals per family, n = 12; three fast-growing individuals per family, n = 12). B: pituitary. For pituitary analysis, each family is represented by a pool of eight individuals, so only family differences were assessed.

Table 2
Mean weight, length, and condition factor of *Salvelinus fontinalis* at 18 months.

Line	Family performance	Phenotype	Group	n	Weight (g)	Length (cm)	Condition
Control	Low	Slow-growing	CLS	32	5.03 ± 1.39	8.18 ± 0.71	0.90 ± 0.05
		Fast-growing	CLF	32	12.91 ± 3.03	11.24 ± 0.82	0.90 ± 0.11
	High	Slow-growing	CHS	32	6.95 ± 1.04	9.11 ± 0.48	0.92 ± 0.11
		Fast-growing	CHF	32	19.06 ± 3.85	12.90 ± 0.78	0.88 ± 0.09
Selected	Low	Slow-growing	SLS	32	6.33 ± 0.95	8.59 ± 0.54	1.00 ± 0.09
		Fast-growing	SLF	32	18.57 ± 3.42	12.49 ± 0.87	0.95 ± 0.10
	High	Slow-growing	SHS	32	6.62 ± 1.43	8.88 ± 0.62	0.94 ± 0.12
		Fast-growing	SHF	32	25.59 ± 11.18	13.99 ± 1.27	0.90 ± 0.09

Line: C = control, S = selected; Family performance L = low, H = high; Phenotype: S = slow, F = fast; Group designation: line, family performance, phenotype; n = number of individuals.

genes, we used the geometric mean instead of the arithmetic mean because the former better controls for possible outlier values and abundance differences between the different genes (Vandesompele et al., 2002).

2.7. Statistical analyses

2.7.1. Length, weight, and condition

One-way ANOVAs ($\alpha < 0.05$) were used to compare family lengths, weights, and Fulton condition factors within lines. This allowed us to compare growth performance among families and to select those families used for gene expression as well as the slow- and fast- growing individuals within each family. Data normality was verified using the

Table 3
Information on sequences and primers obtained for *Salvelinus fontinalis*. For each studied gene, we present the species of origin of the sequences used to amplify the gene in brook charr, the designed PCR primers, the amplicon size (number of base pairs [bp]), and the percentage of similarity obtained between the original sequence and the *S. fontinalis* amplified sequence.

Gene	Sequence used for primer design (accession no.)	Designed primers (5′-3′)	*S. fontinalis* PCR amplicon size (bp)	*S. fontinalis* sequence similarity
grl	*Salvelinus alpinus* (XM_023995867)	F – ACTGATGCTGTGTACTCTGGC R – CTCTCAATGTCTCGCCGACC	223	97%
srifr	*Oncorhynchus mykiss* (NM_001124534)	F – GGGAAAAGACACCGGTTGGA R – TGGTGTTGCCTGTTAGACCC	273	98%
lepr	*Salvelinus alpinus* (XM_024004689)	F – CAGTTAGCTACATGTCGGGGA R – GCCGATTTCCCAGTAGCTGA	209	97%
ghr-1	*Oncorhynchus mykiss* (AY861675)	F – TTGCTGATACGGGTCGAACAT R – GAGGGTCTGGTTCCACGATG	431	99%
igf-1	*Oncorhynchus mykiss* (M95183.1)	F – TCAAGAGTGCGATGTGCTGT R – TTCGGTAGTTCCTTCCCCCT	301	100%
mhc	*Salvelinus alpinus* (XM_023984421)	F – GTTGAGGATCCGAGTGCAGGT R – CGGGAACAGCTCAGGGATAAC	506	99%

Abbreviations are as follows: *grl*: ghrelin; *srifr*: somatostatin receptor; *lepr*: leptin receptor; *ghr-1*: growth hormone receptor 1; *igf-1*: insulin-like growth factor 1; *mhc*: myosin heavy chain.

Table 4
qPCR IDT assays used for transcriptomics in *Salvelinus fontinalis* pituitary. Genes (*18s*: 18s ribosomal; *β-actine:* beta actin; *ef1-α*: elongation factor 1 alpha; *gh*: growth hormone; *ghr-1*: *gh* receptor 1; *igf-1r*: *igf-1* receptor; *grl*: ghreline; *srifr*: somatostatin), primers (5′-3′) (F [forward], R [reverse], P [probe]), bp (number of base pairs), and Tm (melting temperature;°C) are given.

Gene	Primer (5′-3′)	bp	Tm
18s	F – CAAGACGAACGAAAGCGAAAG	21	62
	P – AACGAAAGTCGGAGGTTCGAAGACG	25	68
	R – AGATACCGTCGTAGTTCCGA	20	62
β-actine	F – AGAGAGGTATCCTGACTCTGAAG	23	62
	P – CACCAACTGGGACGACATGGAGAA	24	68
	R – CATCACACCTTCCTACAACGAG	22	62
ef1-α	F – ATCGGCGGTATTGGAACAG	19	62
	P – CCTGAAGGCCGGTATGATCGTCAC	24	68
	R – GTGAAGTCTGTGGAGATGCA	20	62
gh	F – GTCGCTAAGACAGGCTCTTG	20	62
	P – CGTCTACAGAGTGCAGTTGGCCTC	24	68
	R – AAGGTCGAGACCTACCTGAC	20	62
ghr-1	F – CCCACTGCCCCCTGTATCT	19	62
	P – CTTCAGAAGGAGGCTGTTTTGC	22	71
	R – ACCATGGTGGAAGGAG	16	50
igf-1r	F – CAGCCTCATCACTGTACTCTTC	22	61
	P – AAAGAGGAACAGTGACAGGCTGGG	24	68
	R – CTCAGGGTTGACAGAAGCATAG	22	61
grl	F – CCCAGAAACCACAGGGTAAA	20	61
	P – TTGGTCGGCGAGACATTGAAAGCT	24	68
	R – TTTGTCTTCCTGGTGAAGGG	20	61
srifr	F – CTTAGCTCACAGTAGGAGAAACC	23	62
	P – AATAGACAACATGGCCGCCAATGG	24	67
	R – GACTAGCAACTACCCAGCATAC	22	62

Table 5
qPCR TaqMan assays used for *Salvelinus fontinalis* transcriptomics in the brain, liver, and muscle. Genes (*18s*: 18s ribosomal; *β-actin:* beta actin; *ef1-α*: elongation factor 1 alpha; *npy*: neuropeptide Y; *lepr*: leptin receptor; *igf-1*: insulin growth like factor 1; *ghr-1*: *gh* receptor 1; *igf-1r*: *igf-1* receptor; *mhc*: myosin heavy chain; *myo*: myogenin), primers (5′-3′) (F [forward], R [reverse], P [probe]), bp (number of base pairs), and Tm (melting temperature;°C) are given.

Gene	Primer (5′-3′)	bp	Tm
18s	F – GATCCATTGGAGGGCAAGTCT	21	59
	P – TGCCAGCAGCCGC	13	69
	R – GATACGCTATTGGAGCTGGAATTAC	25	58
β-actin	F – GGTCGTCCCAGGCATCAG	18	59
	P – ATGGTTGGGATGGGC	15	69
	R – CGTCTCCCACGTAGCTGTCTT	21	58
ef1α	F – GCCCCTCCAGGATGTCTACA	20	59
	P – AATCGGCGGTATTGGA	16	69
	R – ACGGCCCACGGGTACTG	17	59
npy	F – TGCTGAAGAGCTGGCCAAAT	20	60
	P – CTATACCGCGCTCAGAC	17	70
	R – TCTGTCTCGTGATCAGATTGATGTAG	26	58
lepr	F – CAGCATTCTGACATTGCTTTAACA	24	58
	P – TATGGTCTACAACAGTAGCTT	21	68
	R – CACCAATTCAAGGGCGGATA	20	59
igf-1	F – CGGTCACATAACCGTGGTATTG	22	59
	P – CGAGTGCTGCTTCC	14	70
	R – GCCGCAGCTCGCAACT	16	59
ghr-1	F – CCCACTGCCCCCTGTATCT	19	62
	P – CTTCAGAAGGAGGCTGTTTTGC	22	71
	R – ACCATGGTGGAAGGAG	16	50
igf-1r	F – TCCTCAGTGGGACCCTTCTG	20	59
	P – CCGCCGGACTATAG	14	69
	R – GGACCATGAAGCCCAGTAGGT	21	59
mhc	F – CAAACCACATTGAACACCATCAG	23	59
	P – CACCACACTAGAACTGT	17	69
	R – GGGTTAAGCTTTATTGATACAGGAAGTG	28	60
myog	F – CCTTGGGCCTGCAAGCT	17	58
	P – TGCAAACGCAAGACT	15	69
	R – CGCTTTTCGTCGGTCCAT	18	58

Kolmogorov-Smirnov test and homoscedasticity was tested using the Levene test (Statistica, version 6.1.478, Statsoft). When ANOVA revealed significant differences between groups, we used post-hoc HSD Tukey tests if homoscedasticity was verified and multiple-range Games-Howell tests if there was heteroscedasticity.

2.7.2. Gene expression

Data outliers for *grl* (n = 1), *srifr* (n = 2), pituitary *ghr-1* (n = 1), white muscle *myog* (n = 1), and weight (n = 1) were removed before running analyses. For gene expression in all tissues except pituitary, n was the number of individuals (six individuals per family; Fig. 1A). In the pituitary, the statistical n was the number of families per line and not the number of individuals. Because we had to pool individuals to obtain enough biological material (eight individuals per family; Fig. 1B), no family effect was assessed. Prior to analyses, the following data transformations were applied to achieved normality: log transformations for pituitary *grl* and *srifr* and liver *ghr-1*; Box-Cox transformations for pituitary *igf-1r* and *ghr-1*, brain *npy* and *lepr*, liver *igf-1*, and white muscle *igf-1*r, *ghr-1,* and *mhc*. For each selected gene in the pituitary, separate linear mixed models (LMM) or linear models (LM) were built that related gene expression to length, condition (Fulton index), and line (control or selected) (R version 4.0.5 package lme4). Family identity was first included in all non-pituitary models as a random effect. Models were simplified by a backward elimination procedure, where the least significant term (based on *P*-value) was sequentially removed until all remaining variables were significant (*i.e.*, $P < 0.05$, confirmed by a Likelihood Ratio Test). Body weight was not included in the models due to its strong positive correlation with length (R = 0.937). Marginal and conditional R-squared values were obtained using the rsquared function from the piecewiseSEM R package (Lefcheck, 2016).

Gene expressions were compared using two-way nested ANOVAs (factors: line and family; individual performance nested in "family"). Normality and homoscedasticity were tested and a posteriori tests were run as previously described. Finally, the relationships between growth variables (weight, length, and condition) were analyzed using simple linear regressions.

3. Results

3.1. Weight, length, and condition differences between control and selected lines

On average, fish from the selected line were 37.21% heavier than fish from the control line (11.95 g ± 4.57 vs 8.71 g ± 3.36; $F_{(1,4470)} = 740.42$; $p < 0.001$; Fig. 2A and B). Their length (10.76 cm ± 1.38) was also 11.54% greater than the control line (9.65 cm ± 1.26; $F_{(1,4470)} = 793.96$; $p < 0.001$; Fig. 2C and D). However, the condition factor of control line fish was significantly (albeit only slightly) higher than that of the selected line (0.93 ± 0.14 vs 0.92 ± 0.12; $F_{(1,4470)} = 6.98$; $p = 0.0083$; Fig. 2E and F).

3.2. Weight, length, and condition differences among families

Even though the selection process lasted five generations, family effects were still very present in both the selected and control lines. In the selected line, weights of the best- and the least-performing families differed by 49.42% ($F_{(12,2077)} = 35.79$; $p < 0.001$) while they differed by 14.82% in the control line ($F_{(15,2392)} = 32.76$; $p < 0.001$). It is noteworthy that the family with the lowest weight in the selected line was significantly different from the rest of the selected families, but not significantly different from those control line families that had average and low performance (Fig. 2A and B). This same family effect was also observed for length in the selected line: the largest family was significantly bigger—by 7.63%—than the smallest family ($F_{(12,2077)} = 24.96$; $p < 0.001$), and this was even more evident in the control line, where the difference was 14.62% ($F_{(15,2392)} = 35.34$; $p < 0.001$; Fig. 2C and D).

Weight and length were significantly positively correlated in the selected line ($F_{(1,2076)} = 12814.51$; $p < 0.001$; Length = 0.28 * Weight + 7.32; $R = 0.927$) as well as in the control line ($F_{(1,2392)} = 16228.92$; $p < 0.001$; Length = 0.3493 * Weight + 6.60; $R = 0.933$). However, significant albeit very slight correlations were found between condition factor and weight ($F_{(1,4469)} = 15.03$; $p < 0.001$, $R = 0.057$) and between condition and length ($F_{(1,4469)} = 238.36$; $p < 0.001$, $R = 0.225$).

3.3. Gene expression

3.3.1. Selection and family performance–based effects on gene expression

In the brain, *npy* and *lepr* gene expressions were not different

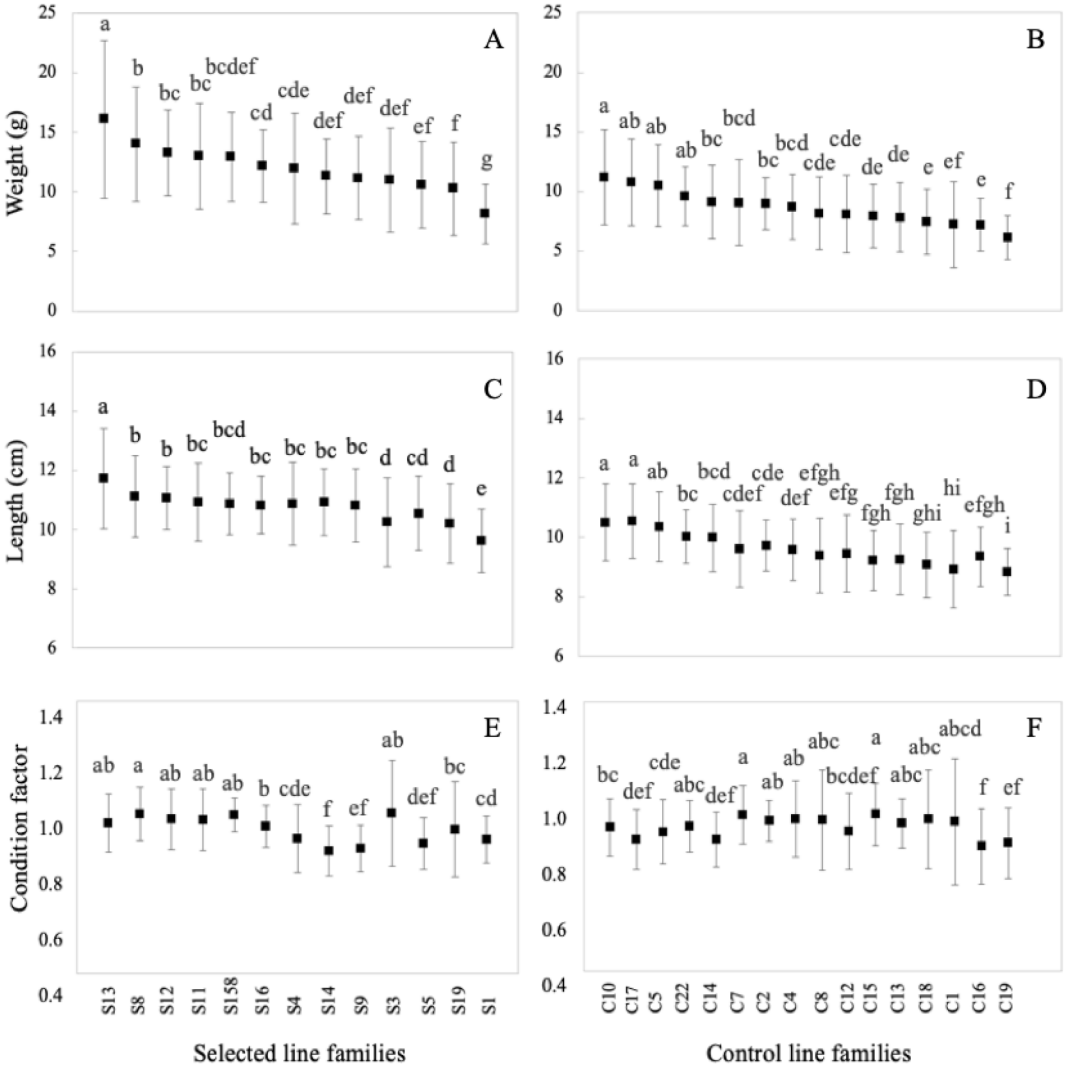

Fig. 2. Weight (g), length (cm), and condition factor of 1+ *Salvelinus fontinalis* families from control or selected lines. Significant differences ($p < 0.05$) within each panel are shown by different letters. Data are presented as mean ± SD.

between lines (Table 6), but *lepr* expression was significantly higher in low-performing families ($F_{(1,44)} = 6.85$; $p = 0.012$); no family effect was found in *npy* (Table 6). The expression of these two genes was not linked to length or condition (Table 6). Pituitary *grl*, *srifr, gh,* and *ghr-1* gene expressions were not different between lines (Table 6). The expression of these genes was not significantly linked to length or condition (Table 6). While pituitary *igf-1r* gene expression was not different between lines ($p = 0.80$), it was significantly positively linked to length (Table 6).

In liver, *ghr-1* and *igf-1* gene expressions were significantly higher in the selected line compared to the control line (Table 6, Fig. 3A and C), but no family effect was found (Table 6). Expression of the *ghr-1* gene was negatively impacted by condition but not by length (Table 6, Fig. 3B). On the contrary, *igf-1* gene expression was positively associated with length but not with condition (Table 6, Fig. 3D). In white muscle, the relative expressions of *mhc, ghr-1,* and *myog* were not significantly different between lines (Table 6), while *igf-1r* gene expression was significantly higher in the selected line (Table 6, Fig. 3E). *Mhc*, *ghr-1,* and *igf-1r* gene expressions were not different among families, but we found a significant family effect in *myog* gene expression (Table 6). Nevertheless, the complementary ANOVA analysis did not show significant differences among families with low and high performance ($F = 2.8$, $p = 0.09$). Relative expressions of *mhc, myog,* and *igf-1r* were not impacted by length (Table 6), but *ghr-1* expression significantly increased with length (Table 6, Fig. 3F). None of the genes quantified in white muscle (*mhc*, *ghr-1, myog, igf-1r*) were linked to condition (Table 6).

Table 6
Effect of body length, condition, and line (selected or control) on the relative expression of genes related to the growth regulation pathway in 1+ *Salvelinus fontinalis* in different sampled tissues (liver, brain, pituitary, and muscle). Only final linear models (LM) and linear mixed models (LMM) including at least one significant effect are presented in this table (final models containing no significant effects are presented as supplementary material). Total n = 96 for all tissues except pituitary (total n = 39). Family was included in all models except pituitary as a random effect. For pituitary, family was the statistical unit. Estimates in bold are significant. *lepr* = leptin receptor; *ghr-1* = growth hormone receptor 1; *igf-1* = insulin-like growth factor 1*; igf-1r* = insulin-like growth factor 1 receptor; *myog* = myogenin.*

	Estimate	SE	Variance	P-value
Liver				
ghr-1 ($R^{2=}$ 0.136)				
Condition	1.964	0.865		**0.026**
Length	0.064	0.037		0.09
Line (selected)	0.548	0.156		**0.001**
Family (random effect)			0.069	0.20
igf-1 ($R^2 = 0.236$)**				
Condition	1.939	1.069		0.07
Length	0.148	0.044		**0.001**
Line (selected)	0.681	0.183		**<0.001**
Family (random effect)			0.075	0.35
Brain				
lepr (Marginal $R^2 = 0.000$; Conditional R2 = 0.223)**				
Condition	0.149	1.140		0.92
Length	0.068	0.045		0.13
Line (selected)	0.219	0.297		0.43
Family (random effect)			0.219	**0.019**
Pituitary				
igf-1r ($R^2 = 0.141$) **				
Condition	4.062	3.922		0.31
Length	0.207	0.093		**0.034**
Line (selected)	0.095	0.372		0.80
Muscle				
ghr-1 ($R^2 = 0.061$)**				
Condition	0.437	1.148		0.70
Length	0.117	0.048		**0.016**
Line (selected)	0.019	0.213		0.93
Family (random effect)			0.087	0.38
igf-1r ($R^2 = 0.049$)**				
Condition	0.352	1.211		0.77
Length	0.028	0.049		0.56
Line (selected)	0.442	0.202		**0.031**
Family (random effect)			0.001	1.00
myog (Marginal $R^2 = 0.000$, Conditional $R^2 = 0.160$)				
Condition	0.111	0.958		0.92
Length	0.040	0.039		0.31
Line (selected)	0.004	0.249		0.99
Family (random effect)			0.112	**0.038**

* A log transformation was applied to achieve normality
** A Box-Cox transformation was applied to achieve normality

3.3.2. Individual performance–based effects

Phenotypes were compared between low- and high-performing families; selected and control families were combined since no significant line effect was found (Supplementary Table 1). Brain *npy* and *lepr* gene expressions were higher in slow-growing individuals than in fast-growing individuals from both low- and high-performing families (respectively $F_{(1,91)} = 5.26$; $p = 0.02$; $F_{(1,91)} = 6.70$; $p < 0.001$) (Fig. 4A and B).

In liver, the relative expression of *ghr-1* showed no line × family interactions ($F_{(1,91)} = 0.20$; $p = 0.64$), but it differed according to both family and individual performances. The *ghr-1* expression was higher in fast-growing juveniles from high-performing families ($F_{(2,91)} = 24.31$; $p < 0.001$), but no difference was observed in low-performing families ($F_{(2,91)} = 1.37$; $p = 0.24$) (Fig. 4C). The same nested effect was observed for liver *igf-1*, with no line × family interactions ($F_{(1,\ 91)} = 0.87$; $p = 0.35$), a higher expression in the fast-growing juveniles in high-performing families ($F_{(2,91)} = 15.75$; $p < 0.001$), and no differences in low-performing families ($F_{(2,91)} = 2.17$; $p = 0.14$) (Fig. 4D).

In muscle, *ghr-1* expression showed no line × family interactions ($F_{(1,91)} = 0.33$; $p = 0.56$). A nested effect was observed, with higher expression in the fast-growing juveniles from families with both low and high performance ($F_{(1,91)} = 4.23$; $p = 0.01$) (Fig. 4E). No nested effect or interactions were observed in the relative expression of *igf-1r* ($F_{(1,91)} = 2.6$; $p = 0.07$), *mhc* ($F_{(1,91)} = 2.42$; $p = 0.09$), or *myog* ($F_{(1,91)} = 0.01$; $p = 0.98$).

4. Discussion

In this study, we tested the hypothesis that selection enhanced the differential expression of genes involved in the GH/IGF-1 axis, in appetite control, and in muscle growth between fish with slow- and fast-growing phenotypes and between families with different growth performance. When comparing the selected and control lines, we predicted differences on endocrine traits, and when comparing family and individual traits, we predicted differences resulting from physiological and endocrine traits. As expected, fish from the selected line were heavier and larger than those from the control line. However, our results did not support our main hypothesis—that selection enhanced differential expressions of the examined target genes involved in the GH/IGF-1 axis between slow- and fast-growing phenotypes. Indeed, growth performance in the selected line was associated with a higher relative expression of liver *igf-1* and muscle *igf1-r*, but not with genes controlling appetite or muscle growth. However, some genes related to appetite control or muscle growth were linked to family performance and individual phenotypes, raising interesting questions about factors underlying non-selection-based phenotypic variations.

4.1. Selection-based effects on gene expression

Our results showed an upregulation of the GH/IGF-1 axis, starting with pituitary *igf-1r* and followed by liver *ghr-1*, liver *igf-1*, muscle *ghr-1,* and muscle *igf-1r* in the selected line, clearly indicating an effect of selection on this axis (Fig. 5). The only gene we examined on this axis that

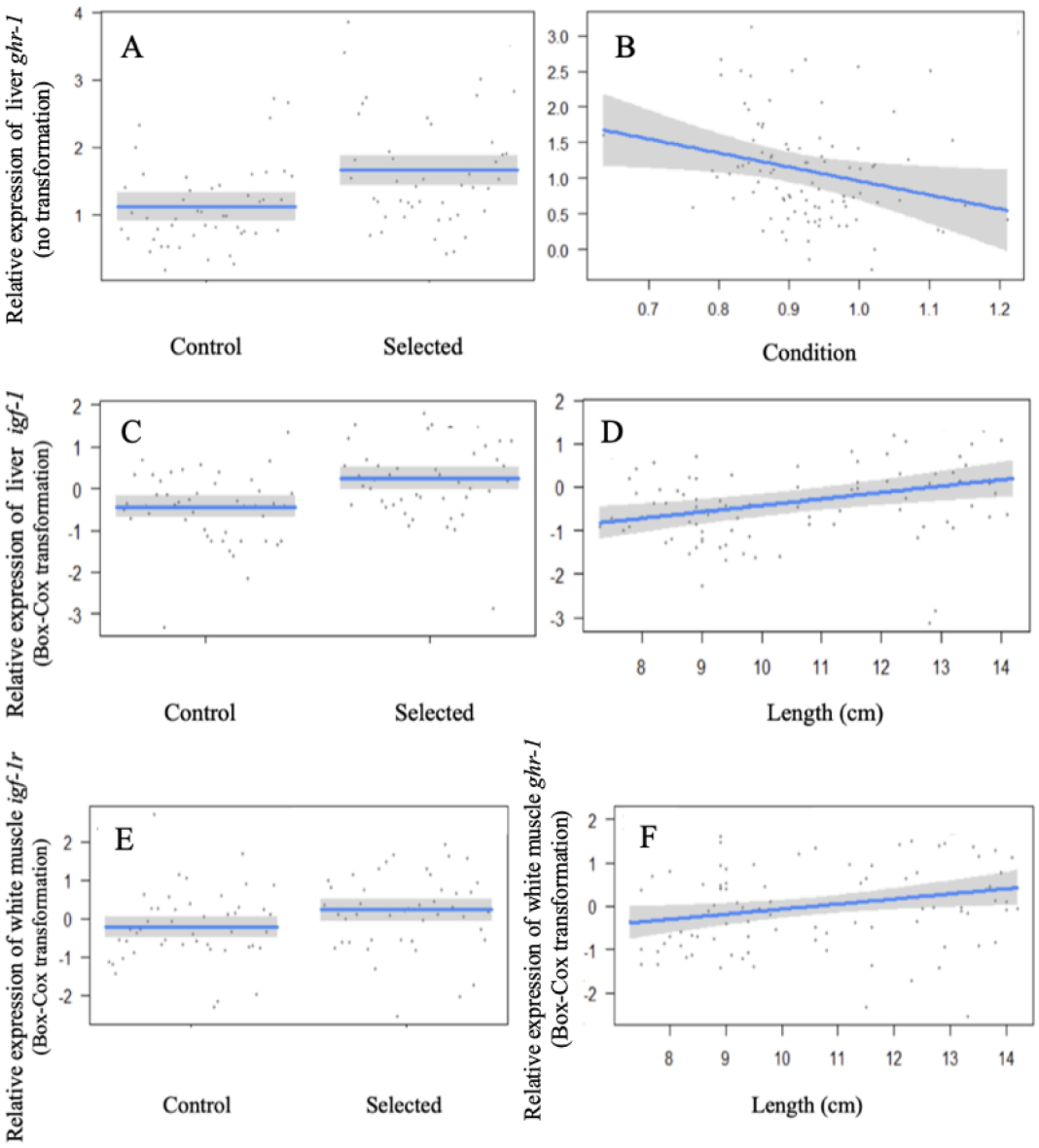

Fig. 3. Line effects on the relative gene expressions in 1+ *Salvelinus fontinalis*. A: liver *ghr-1* from control and selected lines (data were not transformed). B: liver *ghr* correlated with condition from control and selection lines combined (data were not transformed). C: liver *igf-1* from control and selected lines (transformed data). D: liver *igf-1* correlated with length (cm) from control and selection lines combined (transformed data). E: muscle *igf-1r* from control and selected lines (transformed data). F: muscle *ghr-1* correlated with length (cm) from control and selection lines combined (transformed data). Blue lines represent means and shaded areas SD.

was not upregulated in the selected line was pituitary *gh*. While this may be explained by the limited statistical power for the analysis of pituitary gene expression considering that samples were pooled in the analysis, we nevertheless consider this unlikely because differences in pituitary *igf-1r* were detected.

In other fast-growing salmonids that had followed a simple selection process based only on mass, the upregulation of liver *igf-1* and muscle *igf-1r* combined with positive growth correlation is well known (Fleming et al., 2002; Devlin et al., 2009; Tymchuk et al., 2009). It appears that selection based on growth and the absence of early sexual maturation in brook charr also enhanced weight gain via upregulation of the *gh/igf-1* axis. It is noteworthy that the upregulation of liver *ghr-1* promotes the synthesis of *igf-1* in the liver. Indeed, mRNA levels of *igf-1*, *igf-1r,* and *gh* had already been identified as genes of interest for promoting growth in the same strain of *S. fontinalis* (Sauvage et al., 2012). Such upregulation was shown to enhance lipid catabolism to obtain energy for growth in Atlantic salmon *Salmo salar* (Hevrøy et al., 2015), which could explain the improved condition factor in the selected line. Even though the selection process did not modify the expression of pituitary *srifr,* this does not mean that a reduction in the production of SRIF, an inhibitor of growth hormone synthesis, could not be occurring. It should be noted that the effects of SRIF on *gh* expression are limited and conflicting (Wang et al., 2016).

We showed that 1+ *S. fontinalis* juveniles reared under the same conditions, including temperature and food rations, displayed a differential modulation of the *gh/igf-1* axis, which may have been enhanced by the selection process itself and not modulated by the influence of rearing variables such as stress (Meier et al., 2009; Nakano et al., 2013), feeding, or fasting (Chauvigné et al., 2003; Fukada et al., 2004; Norbeck et al., 2007; Bower et al., 2008; Walock et al., 2012), diet composition (Gomez-Requeni et al., 2005; Hack et al., 2018), or temperature (Hevrøy et al., 2013), as previously documented in other salmonids. It is important to note that temperature is one of the most dominant factors influencing key biological functions in fish—including food ingestion—that decrease at higher or lower temperatures (Assan et al., 2021). Winter temperature did not slow growth (Supplementary Fig. 2), and

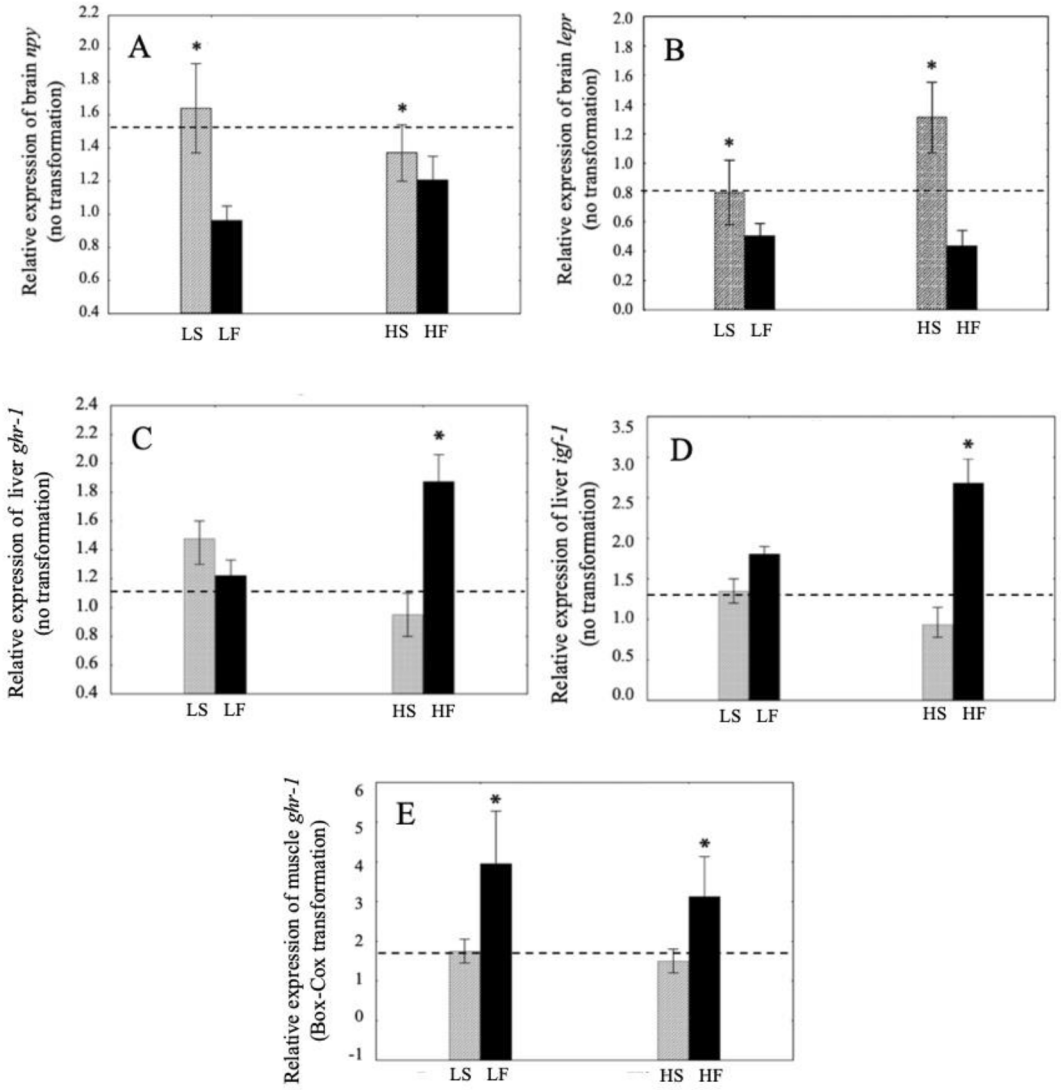

Fig. 4. Relative gene expressions in 1+ *Salvelinus fontinalis* between individual phenotypes (slow- and fast-growing individuals) and family performance (low- and high-performing families). The results for gene expressions when nested ANOVAs indicated significant differences in individual performance (individual phenotype nested in family performance) are presented here, and asterisks indicate statistical differences between the slow- (LS and HS) and fast- (LF and HF) growing individuals within a family group (LS and LF: low performing families; HS and HF: high performing families). Dashed lines represent the relative expression of the CLS calibrator group (control line + low performance + slow growing).

even though specific family growth rate (SGR) was generally lower from 7 to 11 months of age (July to November) than from 11 to 18 months (November to June), rankings remained roughly the same and confirmed that phenotype differences were consistent between lines through time and representative of the phenotypes measured in 18-month-old fish.

We expected to find a difference in appetite control (*npy* in particular), but found no difference in relative gene expression between control and selected lines. Yet, it was previously reported that selection had an impact on food intake in Atlantic salmon, promoting faster growth and also improving the efficient utilization of proteins and energy (Gjedrem and Baranski, 2009). Again, we did not find any differences in the relative expression of muscle *mhc* and *myog* between lines. However, the absence of gene expression may not necessarily mean an absence of protein activity. It should also be noted that these two genes are only involved in the final stages of myocyte development (differentiation and maturation) (Evans et al., 2014). Differences could have been present in the expression of muscle genes involved in the first stage of activation, such as nuclear antigen in proliferating cells (*pcna*) or in cell proliferation with the expression of different transcription factors, such as *Sox8*, *Myf5*, *MyoD2,* and *Pax7* (Vélez et al., 2017), which activate intracellular transduction cascades via *igf-1* receptors (Dupont and LeRoith, 2001; Hack et al., 2018).

4.2. Family performance–based effects on gene expression

Contrary to what we observed between selected and control lines, family performance was related to the expressions of both *lepr* and *myog* for both the selection and control lines. Relative *lepr* expression was upregulated in low-performing families, suggesting suppressed food intake and increased metabolism, resulting in increased energy expenditure and weight loss (Klok et al., 2007; Volkoff, 2016; Blanco and Soengas, 2021). In contrast, high-performing families did not show any difference in *lepr* expression but rather an upregulation of muscle *ghr* that could enhance growth; this has been widely demonstrated in several teleosts (Picha et al., 2008; Hevrøy et al., 2013, 2015; Vélez et al., 2017).

4.3. Individual performance–based effects

In slow-growing juveniles, differences in appetite regulation may be

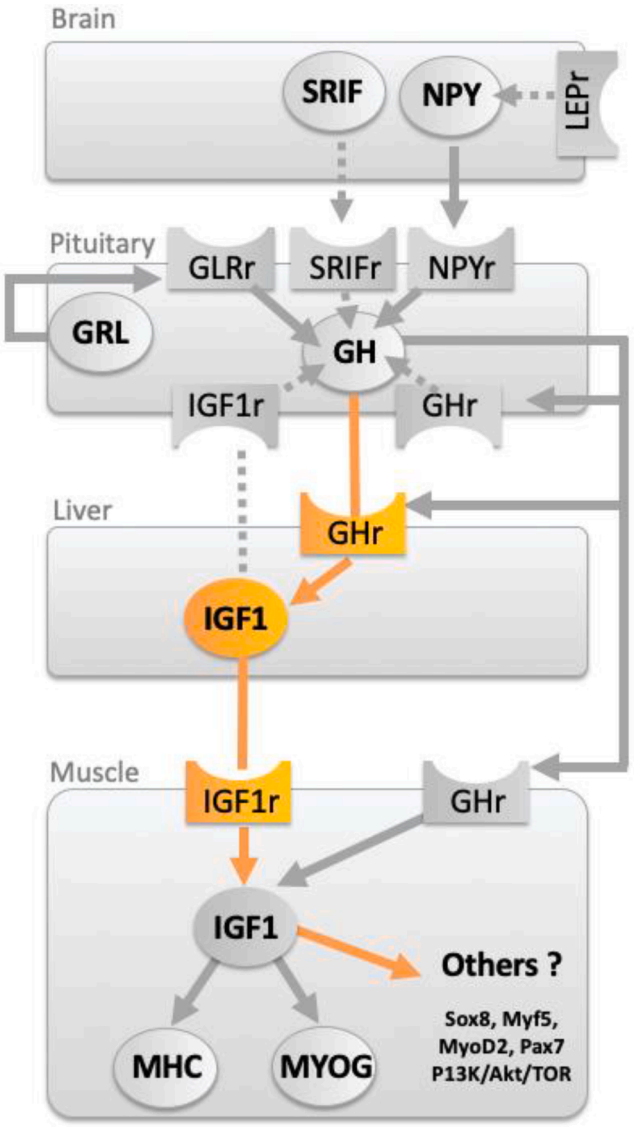

Fig. 5. Endocrine growth regulation pathway of 1+ *Salvelinus fontinalis* in the selected line. This schema represents gene expression, not protein activity. Genes presented in orange indicate mRNA upregulation. Solid arrows indicate stimulation and dashed arrows represent inhibitory actions. *npy* = neuropeptid Y; *lepr* = leptin receptor; *grl* = ghrelin; *srifr* = somatostatin receptor; *gh* = growth hormone; *ghr-1* = growth hormone receptor 1; *igf-1* = insulin like growth factor 1; *igf-1r* = insulin like growth factor 1 receptor; *mhc* = myosin heavy chain; *myog* = myogenin.

due to *lepr* upregulation (Fig. 6) since the binding of leptin to *lepr* activates the Jak/STAT intracellular signaling pathways, which decreases food intake by down regulating other neuropeptides such as NPY (Blanco and Soengas, 2021; Volkoff et al., 2003). Conversely, the expression of *npy* was also upregulated in slow-growing fish, although the exact mechanism of action triggered after leptin binding to *lepr* is unknown in teleost fish (Blanco and Soengas, 2021). The relative weight of these two mechanisms on appetite regulation cannot be assessed without food intake experiments, which should certainly be a focus in further studies. Also, we cannot refute the possibility of differences in appetite or food consumption that may have occurred among individuals or families throughout the experiment. Nevertheless, we are confident that maintaining an equal load in each rearing tank, feeding to satiation, and grouping the families differently at regular intervals helped to maintain dominance and family hierarchy at the lowest possible levels. Despite these precautions, and as in any similar studies of this type on salmonids, we cannot rule out that size variation could partly be the result of aggressive interactions, with some fish not feeding maximally, which would result in reduced growth rates.

We found no indication of differences related to muscle growth regulation. In future studies, it would be relevant to look for differences in the PI3/Akt/TOR pathway (the central mediator in the nutrient sensing protein pathway and precursor of many myogenic factors), which is only activated by feeding. Upregulation of this pathway was recently reported in fast-growing *O. mykiss* (Cleveland et al., 2020). Modifications in the trajectory of growth antagonist genes (e.g., precursors to the alpha subunits of Meprin A) (Valente et al., 2013; Evans et al., 2014), which were strongly expressed in the Laval control line in brook charr (Sauvage et al., 2010), should also be assessed.

4.4. Impact of selection on general growth across generations

In the first generations following initiation of the selective breeding programs with wild breeders brought into captivity, Bastien et al. (2011) found that mean weight in the selected line increased by 23.1% after the first generation, by 32.1% after the second, and by 4% after the third. In our study, the combined selection showed that fish from the fifth generation of the selected line showed a weight gain of 37.2% compared to those from the control line. In other salmonids, it is known that genetic improvements produce permanent gains (Gjedrem and Baranski, 2009). Our results are consistent with those of previous studies, such as Kause et al. (2005), who showed that combined selection improved *O. mykiss* growth by 7% per generation over two generations, and Gjerde and Korsvoll (1999) reported that Atlantic salmon after six generations showed improvements in growth rate of 83.9% overall (14% per generation) and a 12.5% reduction in the frequency of early sexual maturity.

Surprisingly, the condition factor in brook charr juveniles was lower in the selected line than in the control line due to variable gains in weight and length in the selected line. This could be explained by different regulations in the mechanisms related to energy reserves, as has been mentioned for *igf-1* and lipid catabolism. A strongly significant positive correlation between condition factor and total lipid content in Atlantic salmon suggests that condition factor can be used to indicate the state of energy reserves rather than as an indicator of growth (Herbinger and Friars, 1991; Sutton et al., 2000).

5. Conclusions

Selection for the absence of early maturation combined with selection for high growth rate resulted in an upregulation of the *gh/igf-1* axis with no effect on the expression of genes related to appetite control or muscle growth. In contrast, phenotype differences in both the selected and control lines within families resulted in different expressions of genes related to appetite regulation. Slow-growing fish were characterized by an upregulation of brain *lepr* and a downregulation of the *gh/igf-1* axis. Overall, our results show that *lepr* could be used as a physiological indicator of growth related to phenotypic variation and family performance. Liver *igf-1* as well as muscle *ghr* and *igf-1r* gene expressions could be considered as indicators of good growth among brook charr lines. The role of the receptors, which can only be studied with the transcriptomic approach, should be included in future studies because of their importance in the growth regulation pathway. Further research is needed to investigate which genes involved in muscle growth could be stimulated through *gh/igf-1* axis upregulation. By identifying the molecular mechanisms by which *gh/igf-1* signaling is modulated at the endocrine level (paracrine and autocrine), we should be able to better understand growth patterns that optimize growth strategies in commercial fish production. Finally, large weight and length variations still exist in the selected line, indicating that the full genetic selection potential had not been reached after five generations.

Supplementary data to this article can be found online at https://doi.

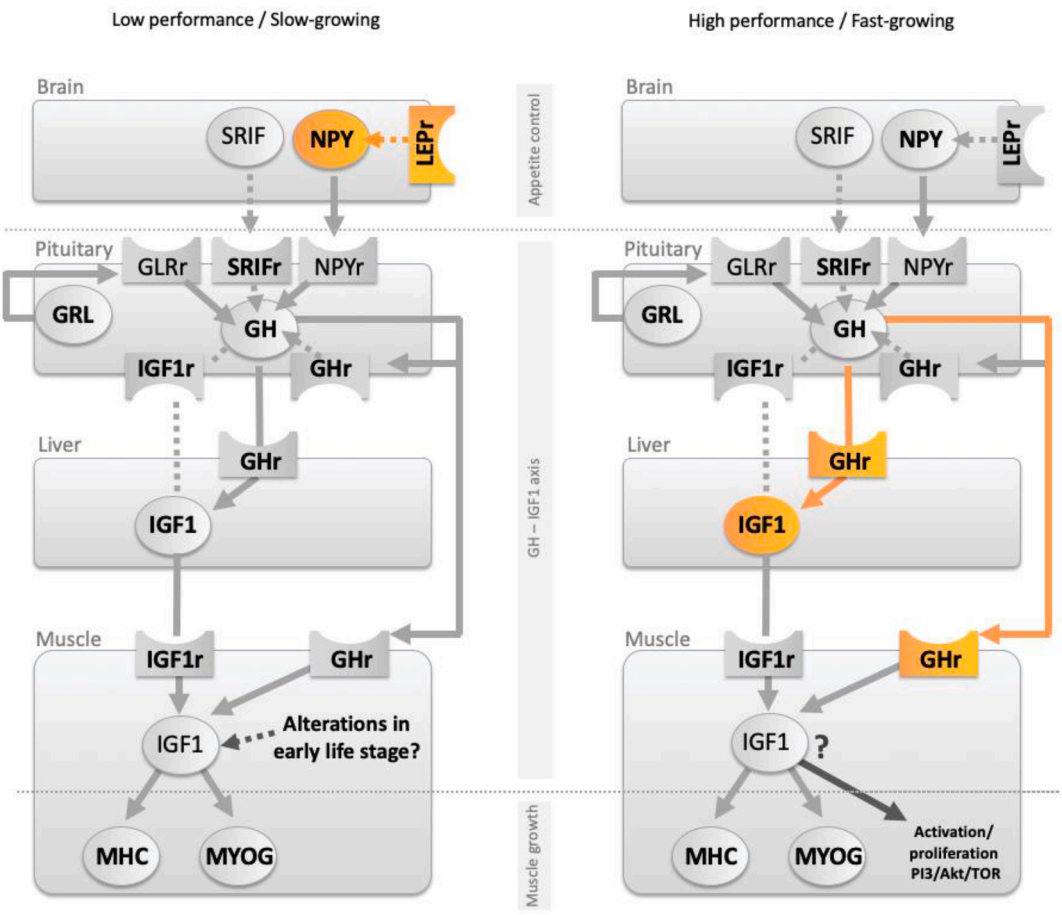

Fig. 6. Endocrine growth regulation pathway of 1+ *Salvelinus fontinalis* from (left) low-performing families / slow-growing phenotype and (right) high-performing families / fast-growing phenotype. This schema represents gene expression, not protein activity. Genes presented in orange indicate mRNA upregulation. Solid arrows indicate stimulation and dashed arrows represent inhibitory actions. *npy* = neuropeptid Y; *lepr* = leptin receptor; *grl* = ghrelin; *srifr* = somatostatin receptor; *gh* = growth hormone; *ghr* = growth hormone receptor; *igf-1* = insulin-like growth factor 1; *igf-1r* = insulin-like growth factor 1 receptor; *mhc* = myosin heavy chain; *myog* = myogenin.

org/10.1016/j.ygcen.2022.114160.

Data availability

Data will be made available on request.

Acknowledgements

We are grateful to all colleagues who provided technical and scientific assistance in the laboratory. Special thanks to Renée Gagné (Institut des sciences de la mer de Rimouski) who contributed to laboratory training, and Geneviève Parent, Éric Parent, and Grégoire Cortial (Fisheries and Oceans Canada), who helped with sequencing. We also thank the journal editor M.A. Sheridan and two anonymous reviewers for their constructive input on a previous version of this manuscript. This research was supported by the Conseil de Recherche en Sciences Naturelles et en Génie du Canada (CRSNG, subvention à la découverte N° RGPIN-2019-05739) and by the *Ressources Aquatiques Québec* research network.

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