1   **Alternative migratory tactics in brown trout (*Salmo trutta*) are**

2   **underpinned by divergent regulation of metabolic but not neurological**

3   **genes**

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19   **Running title:** RNA-seq of alternative brown trout morphs

Abstract

The occurrence of alternative morphs within populations is common but the underlying molecular mechanisms remain poorly understood. Many animals, for example, exhibit facultative migration, where two or more alternative migratory tactics (AMTs) coexist within populations. In certain salmonid species, some individuals remain in natal rivers all their lives, whilst others (in particular, females) migrate to sea for a period of marine growth. Here we performed transcriptional profiling ("RNA-seq") of the brain and liver of male and female brown trout to understand the genes and processes that differentiate migratory and residency morphs (AMT-associated genes) and how they may differ in expression between the sexes. We found tissue-specific differences with greater number of genes expressed differentially in the liver (n = 867 genes) compared to the brain (n = 10) between the morphs. Genes with increased expression in resident livers were enriched for Gene Ontology terms associated with metabolic processes, highlighting key molecular-genetic pathways underlying the energetic requirements associated with divergent migratory tactics. In contrast, smolt-biased genes were enriched for biological processes such as response to cytokines, suggestive of possible immune function differences between smolts and residents. Finally, we identified evidence of sex-biased gene expression for AMT-associated genes in the liver (n = 18) but not the brain. Collectively, our results provide insights into tissue-specific gene expression underlying the production of alternative life-histories within and between the sexes, and point towards a key role for metabolic processes in the liver in mediating divergent physiological trajectories of migrants versus residents.

Keywords: alternative life histories, phenotypic plasticity, salmonids, smoltification, sex bias

Introduction

Discrete phenotypic variation, where individuals from the same population develop into two or more morphs, is common in nature (Roff, 1996; Chevin and Lande, 2013). Developmental switches between alternative phenotypes, such as males versus females (Van Dooren and Leimar, 2003), insect castes (Schwander *et al.*, 2010), trophic polymorphisms (Skulason and Smith, 1995), colour polymorphisms (Roulin, 2004), horn polyphenisms (Moczek and Nijhout, 2002) or mating types (Brockmann and Taborsky, 2008; Schunter et al. 2014) can reflect genetic or environmental determination, or some interaction between the two (Roff, 1996; Tomkins and Hazel, 2007; Chevin and Lande, 2013). Recent technical advances in genomics and bioinformatics (Alvarez, Schrey and Richards, 2015; Todd, Black and Gemmell, 2016) allow for novel insights into the molecular mechanisms and developmental plasticity underpinning the production of alternative phenotypes (Smith-Gill, 1983; West-Eberhard, 2003).

Alternative migratory tactics (AMTs), where both migratory and resident individuals coexist within a population (also known as partial, or facultative, migration), represent a particularly interesting type of discrete phenotypic variation that occurs in all major vertebrate groups and many invertebrates (Chapman *et al.*, 2011). The adoption of AMTs is triggered by environmental cues in interaction with genetically-inherited thresholds, with various selective mechanisms involved in the maintenance of AMTs and associated phenotypic variation across time and space (Tomkins and Hazel, 2007; Pulido, 2011; Buoro, Gimenez and Prévost, 2012). Anthropogenic influences can also drive rapid shifts in AMT frequencies via microevolution or phenotypic plasticity (Pulido, 2007; Thériault *et al.*, 2008; McCleave and Edeline, 2009; Phillis *et al.*, 2016), hence understanding the proximate and ultimate drivers of migration versus residency is a topic of increasing conservation and management relevance (Railsback, Harvey and White, 2014).

67   Diadromous migrations between fresh and saltwater habitats occur in many fishes (McDowall,

68   1997), which, on top of the rigours of migration itself, involve the added challenge of coping

69   with transitions between different osmotic environments. For example, salmonid fishes

70   (salmon, trout, charr and relatives) often undertake anadromous migrations between freshwater

71   spawning and marine feeding habitats. Species, populations and individuals vary markedly in

72   rates of anadromy, as well as in the spatial extent and duration of marine migrations (Klemetsen

73   *et al.*, 2003; Quinn and Myers, 2004). In populations where both anadromous and resident

74   individuals occur, they freely interbreed and offspring inherit a propensity for one or other

75   tactic as a polygenic threshold trait (Dodson *et al.*, 2013; Sloat *et al.*, 2014; Kendall *et al.*, 2015;

76   Ferguson *et al.*, 2019). The benefits of anadromy, which include enhanced growth in the marine

77   environment and avoidance of seasonally harsh freshwater conditions, are finely balanced

78   against the costs, which include increased energetic expenditure, potentially greater mortality

79   risks and less scope for early maturation (Hendry and Stearns, 2004; Pavlov and Savvaitova,

80   2008; Curry *et al.*, 2010; Dodson *et al.*, 2013; Sloat and Reeves, 2014; Kendall *et al.*, 2015;

81   Ferguson, 2017; Ferguson *et al.*, 2019; Nevoux *et al.*, 2019). The life-history trade-offs

82   involved also vary between the sexes, with females typically gaining more in reproductive

83   success terms from the larger potential body sizes afforded by marine feeding. Females are

84   thus more likely to adopt an anadromy tactic and males tend to retain a residency tactic, with

85   males also exhibiting a broader range of ages and sizes at first reproduction (Jonsson and

86   Jonsson, 1993).

87   Many genes are likely involved in the initial decision to adopt a migratory instead of a residency

88   tactic (Hecht *et al.*, 2013, 2015), potentially including some major effect loci (reviewed by

89   Ferguson *et al.*, 2019). Studies on rainbow/steelhead trout (*Oncorhynchus mykiss*), for

90   example, have identified a migration-associated region (MAR) on chromosome *Omy5* that

91   might harbour a "master control switch" for AMTs (Nichols *et al.*, 2008; Leitwein, Garza and

92   Pearse, 2016; Pearse and Campbell, 2018; Arostegui *et al.*, 2019; Kelson *et al.*, 2019). This

93   MAR involves a 55-Mb double-inversion that acts as a "supergene" controlling sex-specific

94   migratory tendencies via sex-dependent dominance (Pearse *et al.*, 2019). Once an individual

95   has "committed" to a particular AMT – a decision which might occur months or even years in

96   advance of actual migration, depending on the species – a series of molecular changes are set

97   in motion that channel residents and (future) migrants along divergent developmental

98   trajectories. Migrants and residents can only be differentiated externally at the smoltification

99   stage, when migrants undergo morphological and physiological transformations in preparation

100   for transition to the marine environment. However, internal differences in patterns of energy

101   acquisition and allocation to competing functions, such as metabolism, growth and lipid

102   storage, are apparent much earlier in the life history (reviewed in Dodson *et al.*, 2013; Ferguson

103   *et al.*, 2019).

104   Complex gene regulatory mechanisms, perhaps involving one or more master regulators

105   (Aubin-Horth, Letcher and Hofmann, 2009) and a series of epigenetic modifications (Baerwald

106   *et al.*, 2016), likely control this phenotypic divergence. Theory on the evolution of

107   developmental plasticity indeed suggests that morph-specific gene expression can decouple

108   developmental pathways and reduce pleiotropic correlations among traits expressed in each

109   morph (Snell-Rood *et al.*, 2010). For example, in *O. mykiss,* differential gene expression

110   between migrant and resident offspring is evident in the brain a full year before smoltification

111   (McKinney *et al.*, 2015). As smoltification approaches, migrants undergo phenotypic

112   "remodelling", including changes in body shape and colour, behavioural changes and hormonal

113   plus enzyme alterations (McCormick, 2012) that are underpinned by transcriptomic differences

114   (Aykanat, Thrower and Heath, 2011; Sutherland *et al.*, 2014; Houde *et al.*, 2019). Complex

115   genetic networks are involved that might include clusters of genes on particular chromosomes,

116   e.g. *Omy12* in *O. mykiss* (Hecht *et al.*, 2012, 2013). Environmental-dependent methylation

changes might also play a regulatory role in seawater acclimation of smolts (Morán *et al.*,
2013). Residents, in contrast, increasingly allocate more energy and resources to maturation
processes, with males in particular often maturing earlier than (resident or anadromous)
females (Jonsson and Jonsson, 1993; McMillan *et al.*, 2012; Archer *et al.*, 2019, 2020).

Here we use RNA sequencing ("RNA-seq") to explore differential gene expression between
migrant and resident brown trout (*Salmo trutta*), a species exhibiting facultative anadromy as
well as flexible freshwater migration strategies (Ferguson, 2017; Ferguson *et al.*, 2019; Nevoux
et al., 2019). Previous studies of AMTs in salmonids have focussed on the brain as a key organ
that might regulate divergent phenotypic trajectories by integrating internal and external
signals, and found differential expression in genes associated with metabolism, morphology,
olfactory imprinting, osmoregulation and sexual maturation (McKinney *et al.*, 2015; Hale *et
al.*, 2016, 2018). In brown trout, genomic studies have found loci associated with variation in
the distance of migration (Lemopoulos *et al.*, 2018, 2019), while previous transcriptomic work
using cDNA microarrays using a restricted number of genes identified gene expression
differences in the liver associated with migratory versus sedentary lifestyles (Giger *et al.*, 2006;
2008). We therefore focussed on the liver and brain as candidate organs mediating AMTs and
smoltification processes in brown trout. Our first objective was to identify genes that are
differentially expressed between individuals classified as immature smolts (anadromous
migratory tactic) or mature non-smolts (residents, i.e. freshwater maturation tactic), here
defined as putative "AMT-associated genes", and to distinguish these from genes that are
differentially expressed as a result of the general stress of a transition from fresh to salt
water. Second, we examined whether putative AMT-associated genes demonstrated conserved
or tissue-specific expression profiles. Lastly, we asked whether genes differentially expressed
between smolts and residents were also differentially expressed between males and females,
given their divergent phenotypic trajectories with respect to migration.

Materials and Methods

Laboratory rearing of fish

Fish in our study were reared from the egg stage in a controlled laboratory environment as part of a broader study on genetic and environmental drivers of facultative anadromy (Archer *et al.*, 2019, 2020). Here, we sampled fish from a single population background and environment-of-rearing (i.e., single rearing tank, see Archer *et al.,*2019,2020 for full details on fish husbandry) to avoid confounding effects in the gene expression data.

The fish were offspring of wild-born parents caught in November 2015 in Tawnyard Lough, a small upland lake in the western reaches of the Erriff catchment in the west of Ireland (53° 37′ 0.00″ N: 09° 40′ 17.10″ W). The lake is fed primarily by the Glendavoch river, within which there is extensive suitable spawning habitat for *Salmo trutta*, along with a series of smaller spawning tributaries. Tawnyard Lough produces a large run of anadromous smolts annually (Gargan *et al.*, 2016) but some juveniles remain within the lake and never go to sea, instead maturing in freshwater. It was not possible to distinguish clearly whether the wild-born parents in our study had been to sea or not, but the Tawnyard population, in general, shows high rates of anadromy (Gargan *et al.*, 2016; Archer *et al.*, 2019, 2020). Thus, we expected a genetic predisposition towards high rates of smolting in our tank-reared fish, but also for some individuals to adopt a freshwater maturation (residency) tactic.

Determination of smolt status

Over 22 months of tank rearing, the fish were routinely assessed for morphological indicators of smoltification, following the criteria of Tanguy *et al.*, (1994). Smolts undergo a change in colouration from dark to silvered flanks, lose colour in the fins and lose their parr marks (the dark traverse bands on the side of young salmonids). They also have pronounced lateral lines and a more fusiform body shape than parr. Sixteen putative smolts were thus identified in early

166   summer 2018 (at age 2+). Such morphological indicators are not always a reliable guide to

167   saltwater tolerance capabilities in this highly phenotypically variable species (Klemetsen,

168   2013). We, therefore, subjected our putative smolts to a saltwater tolerance test to assess their

169   hypo-osmoregulation capacities. Fifteen putative residents (fish showing no outward signs of

170   smolting) were also subjected to the same saltwater tolerance test, to confirm their non-smolt

171   status.

172   The trials involved transferring fish from their freshwater tank into another tank at a salinity of

173   30 parts per thousand (ppt) for 24-hours, a period sufficient to induce hypo-osmoregulation in

174   euryhaline species (Schultz and McCormick, 2012), and then measuring their plasma chloride

175   levels. Low plasma chloride concentrations indicate physiological tolerance to salt water (full

176   details provided in Archer *et al*. 2019, 2020). A salinity of 30 ppt was used rather than full

177   oceanic salinity (35-40 ppt) to minimise unnecessary stress on the fish, and also because wild

178   brown trout often spend large amounts of time in estuaries during seaward migration (Ferguson

179   *et al.*, 2019; Nevoux *et al.*, 2019). Immediately after 24-hr saltwater exposure, the fish were

180   euthanized with an overdose of Tricaine mesylate (MS-222) and a blood sample was collected

181   from the caudal vein using a 21 G needle and a 2.6 ml heparinized syringe. Blood samples were

182   transferred to 2 ml Eppendorf tubes and centrifuged at 8,000 rpm for 3 min to separate the

183   plasma from the erythrocytes and other cellular components. The plasma supernatant was then

184   siphoned off via pipette into a new 1ml Eppendorf tube and stored at −80°C before being

185   measured for plasma chloride concentration (mmol/L) by coulometric titration using a Jenway

186   PCLM3 chloride meter (FishVet Group, Oranmore, Ireland).

187   Given their expected poorer hypo-osmoregulatory abilities, residents were expected to

188   experience higher stress levels than smolts as a result of this acute exposure to saltwater. Thus,

189   comparing gene expression differences between smolts and residents after saltwater testing

190   might confound life history (AMT) differences with stress differences (Fig. 1). To explore this,

191 we also sampled (see below) an additional group of residents (n = 9) who were not subjected

192 to the saltwater challenge but remained within their original freshwater tank and hence

193 experienced similar, or slightly lower, stress levels than the smolts (who might have

194 experienced mild osmotic stress during the saltwater trial).

195 Fish from all three groups (putative smolts exposed to salt water, putative residents exposed to

196 salt water, putative residents not exposed to salt water) were terminally sampled in early

197 summer 2018 and dissected to determine maturation status and extract liver and brain tissue

198 for transcriptional profiling. Before tissue extraction, every fish was measured for fork length

199 (to the nearest mm) and weight (to the nearest g) and a small section of tail fin tissue was

200 removed using a sterilized scalpel and stored in 100% ethanol for subsequent DNA analysis

201 (to determine genotypic sex, see below). Each dissection session took place at the same time

202 each morning in an attempt to minimise among-individual variation in gene expression related

203 to circadian rhythms. Visual gonad inspections were performed first and then whole brains and

204 livers were removed and transferred into individual sterile 2ml Eppendorf tubes containing 1ml

205 of RNALater solution. Samples were incubated at 4°C for 24 hours to allow RNALater to

206 permeate through tissues, and then transferred to -80°C storage.

207 Females were classified as sexually mature if the body cavity was filled with identifiable eggs,

208 whereas males were classed as sexually mature if they had enlarged, white testes, or were

209 running milt. A fish was classified as immature if gonads were undeveloped (see Archer *et al*.

210 2019 for full details). This maturation status information, coupled with the morphological and

211 physiological evidence for smolting, then allowed us to define three final groups of individuals

212 for whom smolt status (morphological signs of smolting present, low plasma chloride,

213 reproductively immature) or resident status (morphological signs of smolting absent, high

214 plasma chloride if saltwater tested, reproductively mature) could be confidently assigned. This

215 yielded seven smolts, all of which had been saltwater tested ("smolt-SW"; n=7), nine saltwater-

216 tested residents ("resident-SW"; n=9) and nine freshwater (non-saltwater-tested) residents

217 ("resident-FW"; n=9). Fish in our smolt-SW group had lower average plasma chloride

218 concentrations (mean = 129.6 mmol/L, standard deviation, sd = 12.6) compared to fish in our

219 resident-SW group (mean = 159.2 mmol/L, sd = 13.9; unpaired t-test: t = 4.4, $P < 0.001$, df =

220 13.49), confirming the former had adopted a migratory tactic.

221 *RNA extractions*

222 Total RNA was extracted from each individual brain and liver sample using TRI Reagent

223 (Sigma, Aldrich) and subsequently purified with the GenElute™ Mammalian Total RNA

224 MiniPrep kits (Sigma Aldrich), following manufacturer's instructions. We DNase-treated each

225 sample using the Turbo DNA-free kit (Qiagen, UK) to remove residual DNA. We initially

226 assessed the quality and quantity of purified RNA using the Nanodrop 2000 (Thermo Fisher,

227 USA) and the Qubit RNA Broad Range assay kit with a Qubit 4 Fluorometer (Thermo Fisher,

228 USA), respectively. Finally, we calculated a relative integrity number (RIN) per sample using

229 an Agilent TapeStation 4200 (Agilent, USA) to ensure purified RNA was not degraded and we

230 only sent samples with a RIN of seven or higher (Sigurgeirsson, Emanuelsson and Lundeberg,

231 2014) for subsequent library preparation and sequencing.

232 *Library preparation and sequencing*

233 We shipped samples (n = 49; 24 livers and 25 brains) to Novogene Europe (Cambridge, UK)

234 for mRNA-enriched library preparation and sequencing. In brief, DNase-treated RNA quality

235 was secondarily assessed by TapeStation at Novogene to ensure quality was high and no

236 degradation occurred during transport. For each sample, an individual mRNA-enriched library

237 was generated using the NEBNext Ultra II RNA library preparation kit (NEB, UK). Individual

238 libraries were barcoded with specific identifiers, multiplexed and sequencing (150bp paired-

239 end (PE)) was performed on an Illumina Novaseq6000 generating a total of ~1.29 billion PE

240   reads. For liver samples, the total number of PE reads were ~590 million (mean: ~24.6 million,

241   min: ~20.3 million, max: ~35.4 million) while for brain samples ~699 million reads were

242   generated (mean: ~27.9, min: ~20.4 million, max: ~42.4 million). Sample information,

243   including the total number of paired-end reads generated per sample, is provided in Supporting

244   information Table S1.

245   *Sequence data quality assessment*

246   To assess the quality of our raw reads, we used FastQC (version 0.11.3; Andrews, 2010) to

247   identify the presence of sequences with low base quality and/or adapter contamination. Overall,

248   FastQC indicated high sequence quality. Following this, we estimated transcript abundance for

249   each sample through quasi-aligning reads with Salmon (version 1.2.0; Patro *et al.*, 2017)

250   against predicted transcripts (cDNA sequences) from the publicly available *Salmo trutta*

251   reference genome assembly (fSalTru1.1, INSC Assembly GCA_9010011651.1) obtained from

252   Ensembl. Using these estimates, we generated summarised gene-level counts per individual

253   sample using tximport (version 3.10; Soneson *et al* 2015), which were then imported into

254   DESeq2 (version 3.10; Love, Huber and Anders, 2014) for differential expression analysis. For

255   the purpose of reanalysis, gene-level counts are provided in Supporting information Table S2.

256   For differential expression and Gene Ontology enrichment analyses, we used modified scripts

257   initially developed by Colgan *et al.* (2019).

258   *Differential expression analyses*

259   To first get a broad sense of global differences in transcriptional profiles across our three

260   groups, we ran a principal component analysis (PCA) on variance stabilising transformed gene-

261   level counts using DESeq2. Given expected differences in transcriptional profiles between

262   tissues, we performed independent PCAs for liver and brain samples using the plotPCA

263   function in DESeq2.

264   For each tissue, we next performed gene-level tests for differential expression between

265   comparisons of interest using DESeq2. We first defined a global model with main effects of

266   "phenotype" – a two level factor indicating whether the individual fish was a smolt or resident

267   – and "testing environment" – a two level factor indicating whether the fish was exposed to the

268   24-hour saltwater test just prior to terminal sampling (salt), or not (fresh), as explanatory

269   variables. Note that phenotype and testing-environment are not perfectly crossed, as all smolts

270   were salt-water tested (which was necessary to confirm their smolt status in physiological

271   terms), whereas the residents fell under either salt or fresh for testing environment (Fig.1). This

272   global model was then compared against two reduced models, one which contained only a main

273   effect of environment, and one which contained only a main effect of phenotype. This enabled

274   us to generate P values, on a gene-by-gene basis, for the effects of phenotype and environment.

275   We then defined differentially expressed genes as those where the absolute log fold change

276   was greater than one, and the Benjamini-Hochberg adjusted P values were less than 0.05. This,

277   therefore, yielded two lists of genes: those differentially expressed between smolts and

278   residents, and those differentially expressed between salt- versus freshwater testing-

279   environment.

280   To isolate the effect of phenotype *per se*, we identified the unique subset of genes that were

281   differentially expressed between smolts and residents only and not also between osmotic

282   testing environments. Hereafter we refer to these genes as "AMT-associated genes". Similarly,

283   genes that were differentially expressed between salt- versus freshwater testing-environments,

284   but not also between smolts and residents, were classified as "osmotic environment response

285   genes". Genes that were differentially expressed between both comparisons were labelled as

286   "stress genes". For each set of genes, we also used heatmaps and scatter plots (using the ggplot2

287   package, version 3.3.2 in R) to explore and illustrate the patterns of (z-score normalised) gene

288   expression with respect to our three groups, i.e. smolt-SW, resident-SW and resident-FW, for

289   livers and brains separately.

290   Finally, we tested for each tissue whether any of our AMT-associated genes or osmotic

291   environment response genes were also differentially expressed between males and females. To

292   achieve this, a DESeq2 model with a main effect of sex was compared against a null model,

293   with no effects. We defined significantly differentially expressed genes as before (i.e. absolute

294   logFC > 1 between males and females and BH-adjusted $P < 0.05$). We then identified which

295   of these sex-biased genes had also been classified as AMT-associated genes or osmotic

296   environment response genes in the above analyses.

297   ***Gene Ontology term enrichment analyses***

298   For each gene in the *S. trutta* reference genome, we used the functional annotations assigned

299   to the corresponding orthologs in the *Danio rerio* (zebrafish) genome, obtained from Ensembl

300   BioMart (Kinsella *et al.*, 2011), as very little functional information exists for brown trout

301   genes. For both the smolt-biased and resident-biased AMT-associated genes, we tested for

302   enrichment of Gene Ontology (GO) terms using Fisher's exact tests in topGO (version 3.10,

303   Alexa *et al.* 2006), applying the "weight01" algorithm and a node size of 50. We corrected for

304   multiple testing using the Benjamini-Hochberg method to generate adjusted P values. As a

305   means of understanding more general processes enriched with differentially expressed genes,

306   we also subset higher level GO terms of significantly enriched GO terms (e.g. Level Two GO

307   terms that are child terms of each of the three main GO subcategories of 'Biological Process',

308   'Molecular Function' and 'Cellular Component').

309

Results

Distinct AMT-associated transcriptional profile in liver but not brain

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311

312 For the livers, PCA demonstrated a clear separation of samples based on gene expression with

313 respect to our three groups, i.e. combinations of phenotype and testing-environment (Fig. 2A).

314 The first principal component (PC1) accounted for 21% of the total variance and primarily

315 separated resident-SW individuals, who likely experienced high osmotic stress, from resident-

316 FW and smolt-SW individuals, each of whom presumably experienced low osmotic stress. In

317 contrast, PC2, which accounted for 15% of the total variance, primarily separated smolt-SW

318 individuals from resident-FW individuals, with resident-SW individuals being intermediate

319 (Fig. 2A). The combination of PC1 and PC2 separates smolts from residents, with smolts

320 having high values for both PC1 and PC2, but residents having lower PC2 and a range of PC1

321 values. In contrast, the PCA on the brain samples did not produce clear separation among the

322 three groups based on the first two principal components (Fig. 2B), nor PC4-6 (result not

323 shown).

Genes in liver differentially expressed between migrants and residents and are enriched for distinct GO terms

324

325

326 We identified 1,670 genes that were significantly differentially expressed (LRT, BH-adjusted

327 $P < 0.05$) between the livers of smolts and residents. A total of 3,426 genes were differentially

328 expressed between fish sampled in salt- and freshwater testing-environments (global model

329 versus reduced model with phenotype effect only). We found 867 genes that were uniquely

330 differentially expressed between the phenotypes but not osmotic testing-environments, which

331 we designate as potential AMT-associated genes. The output of both models, as well as the

332 different subcategories of genes, are provided in Supporting information Table S3.

333 Of the 867 putative AMT-associated genes (Fig. 3), we identified 430 genes with increased

334 expression in residents relative to smolts, while 437 genes showed increased expression in

335 smolts relative to residents (Supporting information Table S3). The resident-biased genes were

336 enriched (BH-adjusted $P < 0.05$) for 21 GO terms, including 13 terms related to "biological

337 process" (Fig. 4) with the majority categorized under "metabolic processes" (Supporting

338 information Table S4). The three most significant terms were steroid biosynthesis process

339 (GO:0006694), oxidation-reduction process (GO:0055114) and cholesterol metabolic process

340 (GO:0008610) (Supporting information Table S4). In contrast, the 437 genes with smolt-biased

341 expression were significantly enriched for 14 GO terms. The majority of these enriched terms

342 (n = 12) were "biological process"-related GO terms with the three most significant GO terms

343 being response to cytokine (GO:0034097), positive regulation of cell differentiation

344 (GO:0045597) and endothelial cell migration (GO:0043542)(Fig. 4). These terms form part of

345 GO term hierarchies with higher level terms, such as cellular processes, response to stimulus

346 and metabolic processes (Supporting information Table S4).

347 *Fewer transcriptional changes in brain underlying AMTs*

348 We identified 53 genes that were significantly differentially expressed between the brains of

349 smolts and residents (Supporting information Table S3C). We found 716 genes that were

350 differentially expressed between salt- and freshwater testing-environments (Supporting

351 information Table S3D), and 43 genes that overlapped between both comparisons. Thus, ten

352 genes were uniquely differentially expressed between the phenotypes but not testing-

353 environments (i.e. brain AMT-associated genes).

354 Seven of these genes exhibited elevated expression in smolts compared to residents; Sox18

355 (ENSSTUG00000006705), protein phosphatase PTC7 (ENSSTUG00000008660), E3

356 ubiquitin-protein ligase RNF38-like (ENSSTUG00000023791), N-acetyltransferase 8-like

357 gene (ENSSTUG00000014735), BUB3 mitotic checkpoint protein (ENSSTUG00000036930),

358   a zinc finger protein 239-like (ENSSTUG00000002250) and an uncharacterised gene

359   (ENSSTUG00000047265). There is an annotated ortholog for this latter gene in *Salmo salar*

360   where it has been described as an ankyrin-3-like (ENSSSAG00000039215). The three genes

361   with increased expression in the brains of residents compared to smolts were a zinc finger and

362   BTB domain-containing protein (ENSSTUG00000030799), sperm tail PG-rich repeat

363   containing protein (ENSSTUG00000039823) and plexin-A1(ENSSTUG00000046884). These

364   genes were not significantly enriched (BH-adjusted P > 0.05) for any specific GO term.

365   Furthermore, no overlap was identified between brain and liver AMT-associated genes.

Sex-biased gene expression in certain AMT-associated genes

367   We identified evidence of sex-biased gene expression in the liver with 103 genes significantly

368   differentially expressed between males and females (Supporting information Table S5). Of

369   these genes, 12 were also differentially expressed between smolts and residents. We found no

370   significant enrichment (BH-adjusted P > 0.05) for GO terms for these 12 genes. The analysed

371   transcriptome as a whole comprised 44,366 transcribed genes, of which 0.23% (103/44,366)

372   exhibited sex-biased expression, whereas just over 1% of our AMT-associated liver genes

373   (12/867) exhibited sex-biased expression. Thus, there was a statistically significant excess of

374   sex-biased AMT-associated genes in comparison to sex-biased non-AMT-associated genes

375   (chi-squared = 45.7, df = 1, P <0.001). Amongst these 12 sex-biased AMT-associated liver

376   genes, six exhibited elevated expression in residents relative to smolts and six demonstrated

377   smolt-biased expression. The majority of these genes (n = 9) were also elevated in females

378   relative to males, with the sex differences being typically larger in smolts than residents (Fig.

379   5). The remaining three sex-biased AMT-associated liver genes had higher expression in male

380   residents compared to female residents.

381   We identified eight genes in the brain that exhibited sex-biased gene expression. However, we

382   found no overlap between these genes and the ten AMT-associated genes that differed between

383   the brains of smolts and residents.

Discussion

385   Here we compared whole-genome transcriptomic profiles of sea-migratory versus resident

386   brown trout during the presumed migration period, to investigate molecular processes

387   underpinning the generation of well-integrated, ecologically-important, alternative phenotypes.

388   Our study revealed three key findings: 1) extensive transcriptional differences between

389   migratory and resident brown trout for genes underlying metabolic, cellular and immune

390   processes (particularly genes enriched for GO terms associated with metabolic processes,

391   which had the lowest adjusted P values; Fig. 4); 2) tissue differences in expression profiles

392   between migrants and residents with distinct life-stage specific patterns evident in the liver but

393   not brain; and 3) sex-biased expression differences in genes associated with AMTs. The genes

394   we identify may be downstream targets of master regulatory genes proposed to trigger AMTs

395   and allow for the transition between aquatic environments; alternatively, they may represent

396   non-target effects of physiological interactions unrelated to migration.

397   Animal migration can take place over long distances and last prolonged periods requiring

398   dynamic changes in behavioural, physiological, morphological and biochemical aspects of an

399   organism's phenotype. In metabolic terms, migration utilises substantial energetic resources

400   (Wikelski *et al.*, 2003), which likely require tight regulation during such extended periods of

401   metabolic stress. A key organ involved in such regulation is the liver. Here we identified

402   distinct transcriptional profiles between migrants and residents with genes differentially

403   expressed between these alternative phenotypes enriched for Gene Ontology terms involved in

404   a range of key metabolic processes (Fig. 4), including steroid biosynthesis, cholesterol

405   biosynthesis, phospholipid and fatty acid metabolism, with a general reduction in expression

406   of such genes within migrants compared to residents. These findings are in line with previous

407   molecular and biochemical research performed on other salmonids that identified changes in

408   glycogen stores due to the energetic demands of migration, as well as changes in gene

409   expression underlying lipid remodelling during transition from a freshwater to a saltwater

410   environment (Gillard *et al.*, 2018). On top of changes in genes associated with lipid

411   metabolism, we also identified additional metabolic processes that differ between migrants and

412   residents. Such changes may also be related to differences in reproductive maturity status,

413   whereby energetic demands vary due to residents being fully mature reproductively, while for

414   migrants (who were the same age but immature), reduced expression of metabolic genes may

415   be associated with suppressed reproductive development.

416   The above findings tally with previous work on brown trout, and on other salmonids exhibiting

417   AMTs, that has established links between various aspects of an individual's physiological

418   condition and its migratory status (reviewed by Dodson *et al.*, 2013; Sloat *et al.*, 2014; Kendall

419   *et al.*, 2015; Ferguson *et al.*, 2017, 2019). For example, energy limitation owing to poor food

420   availability is associated with higher rates, or earlier ages of, migration (Olsson *et al.*, 2006;

421   Wysujack *et al.*, 2009; O'Neal and Stanford, 2011; Morán *et al.*, 2013; Jones, Bergman and

422   Greenberg, 2015; Archer *et al.*, 2019, 2020; Shry *et al.*, 2019), and the energy value of food

423   may also be important (Kendall *et al.*, 2015). Metabolic rate, and how it links to growth

424   efficiency and energy allocation to competing functions (e.g. lipid storage; (Jonsson and

425   Jonsson, 2005; Boel *et al.*, 2014)), is further believed to underpin these associations (Forseth

426   *et al.*, 1999; McCarthy, 2000; Seppänen, Piironen and Huuskonen, 2010; Norin and Malte,

427   2011; Sloat and Reeves, 2014; Archer *et al*. *In Press*). Our study found the most significantly

428   elevated gene in smolts was *serine/threonine-protein kinase* (*SBK1*), which elevated

429   expression levels in an *O. mykiss* line selectively bred for fast growth have been linked with

430   growth hormone-mediated physiological pathways (Cleveland, Gao and Leeds, 2020).

431   Although faster growing parr have a higher propensity to adopt residency, or to mature earlier,

432   in many salmonid species, the relationships between growth/body size and AMTs are not

433   always consistent (reviewed by (Dodson *et al.*, 2013; Ferguson *et al.*, 2019). Another gene of

434   interest was *desert hedgehog gene* (*DHH*) that differed in expression between migrants and

435   residents. Genes in the hedgehog pathway have been associated with tissue

436   growth/regeneration and developmental changes in teleost fishes (Sims, Eble and Iovine, 2009;

437   Marí-Beffa and Murciano, 2010), including aspects of fin growth (Iovine 2007). This is

438   noteworthy as migrants go through a series of morphological changes (e.g. body streamlining,

439   changes in fin shape and colour) during smoltification.

440   As physiological influences on, or consequences of, life history decisions, may be mediated by

441   hormones, e.g. glucocorticoids (Peiman *et al.*, 2017; but see Jain-Schlaepfer *et al.*, 2018), or

442   by antioxidant capacity (Birnie-Gauvin *et al.*, 2017), our results implicate the liver as an

443   important organ involved in the genetic regulation of these processes. This bolster and expands

444   upon earlier work on *S. trutta*, which based on cDNA microarrays screens of liver samples,

445   found that almost 21% of 900 screened genes were differentially expressed between sedentary

446   and migratory populations (Giger *et al.*, 2008, but also see Giger *et al*., 2006). These included

447   the candidate genes *endozepine* and *transaldolase 1* that are involved in lipid metabolism in

448   the liver, which were upregulated in residents, and the constitutive heat-shock protein HSC70-

449   1, which was upregulated in smolts (Giger *et al*., 2008). Our study also found that *transaldolase*

450   *1* was upregulated in the livers of residents when compared to the livers of smolts, further

451   supporting these findings.

452   Aside from differences in genes associated with metabolism, smolt-biased genes were enriched

453   for Gene Ontology terms associated with cell proliferation and regulation, and response to

454   stimulus. Migrants face a number of threats when moving between environments, including

exhaustion, predation, as well as pathogens and disease. While immunity is metabolically costly to constitutively express and actively use, it is hypothesised that migrants will boost their immune potential in preparation for novel pathogen encounter during migration (Møller and Erritzøe, 1998; Buehler, Tieleman and Piersma, 2010). Within our study, the most significantly enriched Gene Ontology term for smolt-biased genes was response to cytokines, which suggests changes in the immune profile of migrants. Cytokines are a broad range of small proteins with immunomodulatory capacities (Kishimoto, Taga and Akira, 1994). While the higher hierarchical GO term for response to cyktokines was 'response to stimulus', rather than "immune system process", cytokines are originally produced in response to antigens and hence a link to immune function can be inferred here. Genes falling under the GO term response to cytokines included a number of immune genes with elevated expression in the liver of migrants including *macrophage mannose receptor 1*, *macrophage capping protein*, *LDL receptor related protein 2*, *interleukin 13-receptor*, *lipopolysaccharide-induced tumor necrosis factor alpha*, as well as seven genes with putative roles in the complement pathway, a key component of the innate immune system that functions in pathogen removal, opsonization and promotion of inflammation. While the fish in our study were not maintained in sterile conditions and we would expect some form of background immune expression, differences in immune regulation between alternative life-history phenotypes may be associated with preparation for travelling to, and residing, in a marine environment, which presents different pathogenic threats to those found in freshwater. Alternatively, given the energetic trade-offs between immunity and reproduction (Roff, 1992), increased immune potential in migrants may be indirectly related to reduced reproductive investment.

In comparison to the liver, we identified fewer differentially expressed genes in the brain indicating unique tissue-specific profiles that differ between alternative life-history phenotypes. This was surprising, given the brain's proposed role as a control centre for

480   salmonid AMTs via hormonal cascades (Aubin-Horth, Letcher and Hofmann, 2009; Dodson

481   *et al.*, 2013). Indeed, previous research on rainbow trout (*Oncorhynchus mykiss*) (McKinney

482   *et al*. 2015, Hale *et al*. 2016, 2018) identified larger transcriptional differences in the brain

483   between migrants and residents, associated with a range of physiological processes, including

484   phototransduction and circadian rhythm. Here, although we found no enrichment of specific

485   biological processes, there were candidate genes of interest. For example, of the ten genes

486   differentially expressed between migrants and residents, three were annotated as transcription

487   factors, including two zinc finger domain-containing proteins and transcription Sox18A.

488   Transcription factors, such as *vgll3* and *six6* (Barson *et al.*, 2015; Czorlich *et al.*, 2018), have

489   been linked recently to the age of maturity of *Salmo salar*, demonstrating the importance such

490   genes play in the expression of complex phenotypes. While the exact function of the

491   transcription factors identified in our present study are currently unknown, future functional

492   studies may elucidate their role, if any, in migration within brown trout or other species.

493   For certain facultative salmonids, such as brown trout and rainbow trout, sexes can differ in

494   their rates of anadromy, with females often gaining more, in fitness terms, from anadromy than

495   males (Gross, Coleman and McDowall, 1988; Ohms *et al.*, 2013; García-Vega, Sanz-Ronda

496   and Fuentes-Pérez, 2017; Huusko *et al.,* 2018). Within a given life history tactic (e.g. anadromy

497   or residency), the sexes can also differ in behaviour, morphology and physiology; for example,

498   anadromous females may spend longer at sea than anadromous males (Thorstad et al. 2016); f.

499   Given these expected phenotypic differences between the sexes, we examined genes that were

500   differentially expressed both between males and females but also life-history phenotypes and

501   found 12 such genes that were differentially expressed in the liver (out of a total of 103 sex-

502   biased liver genes). While we identified no enrichment of Gene Ontology terms for these 12

503   genes, one possibility is that they play indirect roles in sex-specific maturation processes

504   (Rossignol, Dodson and Guderley, 2011), given that our smolts were all immature and our

505   residents mature. In mice (*Mus musculus*), for instance, glucocorticoid receptor genes in

506   hepatocytes regulate the expression of sets of genes involved in growth and sexual maturation

507   (Engblom *et al.*, 2007). Sex-biased gene expression in the liver has been previously shown for

508   other salmonids, such as brook charr, *Salvelinus fontinalis* (Sutherland *et al.*, 2018). The lack

509   of observed sex biases in gene expression in the brains of brown trout in our study is surprising

510   given the noted behavioural and hormonal differences between the sexes. Given the cellular

511   complexity of the brain in comparison to the liver, which is more homogenous, our sequencing

512   of the entire brain may have reduced the ability to detect subtle, sub-region specific, differences

513   in gene expression between the sexes. Future studies will benefit from more targeted profiling

514   of specific sections of the brain to elucidate fine-scale differences between the sexes.

515   Developmental switches in salmonids (Thorpe *et al.*, 1998; Mangel and Satterthwaite, 2008;

516   Dodson *et al.*, 2013; Arostegui *et al.*, 2019) are thought to be triggered by differential

517   expression of one or more 'master regulator' genes early in ontogeny (at least in *O. mykiss*);

518   these in turn orchestrate divergent physiological cascades, likely hormonally-mediated, that

519   result in AMTs (Aubin-Horth, Letcher and Hofmann, 2009; Dodson *et al.*, 2013). A major

520   challenge in these studies is in distinguishing cause from effect. On the one hand, observed

521   gene expression differences between tactics could be the actual cause of physiological changes

522   that originally triggered alternative phenotypes, with these 'decision genes' potentially

523   remaining switched on (in one tactic) from that point onwards. On the other hand, they may

524   simply reflect 'downstream' physiological consequences of earlier activation of master

525   regulators that are only expressed during an early sensitivity window (Dodson *et al.*, 2013;

526   Ferguson *et al.*, 2019). Studies that examine gene expression at older ages (e.g. our current

527   study; Giger *et al.*, 2006, 2008; Seear *et al.*, 2010; Aykanat, Thrower and Heath, 2011; Norman,

528   Ferguson and Danzmann, 2014; Sutherland *et al.*, 2014) might be more likely to identify genes

529   whose differential regulation follow from, rather than causes, the adoption of AMTs. This may

530   be particularly true for organs such as the liver, kidney or gills, which play diverse roles in

531   physiologically responding to hormonal cascades that likely begin in the brain, for example via

532   the light-brain-pituitary axis (Ebbesson *et al.*, 2003). Identifying the actual master regulators is

533   much more difficult and future studies will benefit from time-series of gene expression and

534   profiling of associated phenotypic changes across ontogeny (Dodson *et al.*, 2013).

535   Interestingly, in a comparison of two-year old *O. mykiss* smolts against same-age residents,

536   Baerwald *et al.* (2016) found 57 differentially methylated regions, over half of which were in

537   transcriptional regulatory regions. This suggests a role for epigenetic regulation of gene

538   expression in mediating AMTs, but it remains unknown when these methylation changes are

539   triggered and by what mechanisms.

540   **Conclusions**

541   Our study represents an important step towards understanding molecular mechanisms

542   underlying alternative life history tactics and the regulatory roles played by different organs.

543   Our list of candidate genes showing differential expression between migratory phenotypes

544   and/or the sexes in brown trout could be considered by future salmonid studies that aim to

545   disentangle molecular processes activating developmental switches from those that follow

546   from their activation. This knowledge should inform efforts to conserve wild or cultured

547   populations of facultatively migratory salmonids and other taxa of ecological or economic

548   importance (e.g. Robinson *et al.*, 2016) and their capacity for evolutionary or plastic responses

549   to anthropogenic change (Mangel and Satterthwaite, 2008; Thériault *et al.*, 2008; Railsback,

550   Harvey and White, 2014).

551

562 **Ethical statement**

563 All fish were euthanized humanely at the end of the laboratory rearing phase under license.

564 The study and all associated procedures were carried out with ethical approval from Health

565 Products Regulatory Authority (HPRA) Ireland, under HPRA project license AE19130/P034,

566 and HPRA individual licenses AE19130/1087, AE19130/I200, AE19130/I201, and

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895   **Data availability**

896   Raw sequence data files are deposited in the NCBI short read archive (Accession ID:

897   PRJNA670837). Scripts underpinning the analysis of transcript estimation, differential

898   expression, and Gene Ontology term enrichment are for the purpose of review included in

899   Supporting information. Upon publication, scripts will also be hosted on a public repository on

900   Github. Raw sequence counts for each sample are provided in the Supporting information.
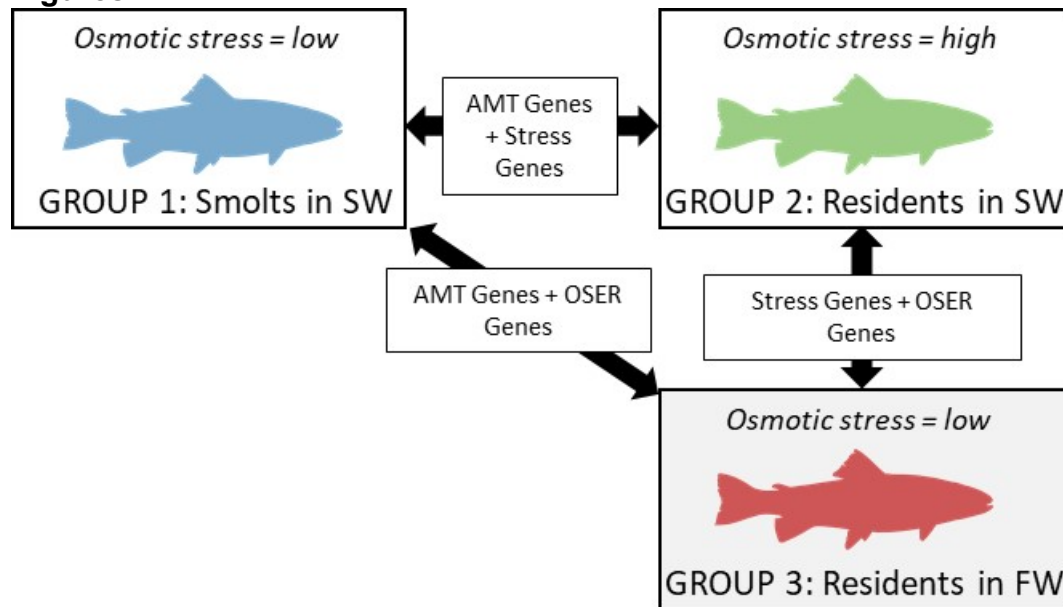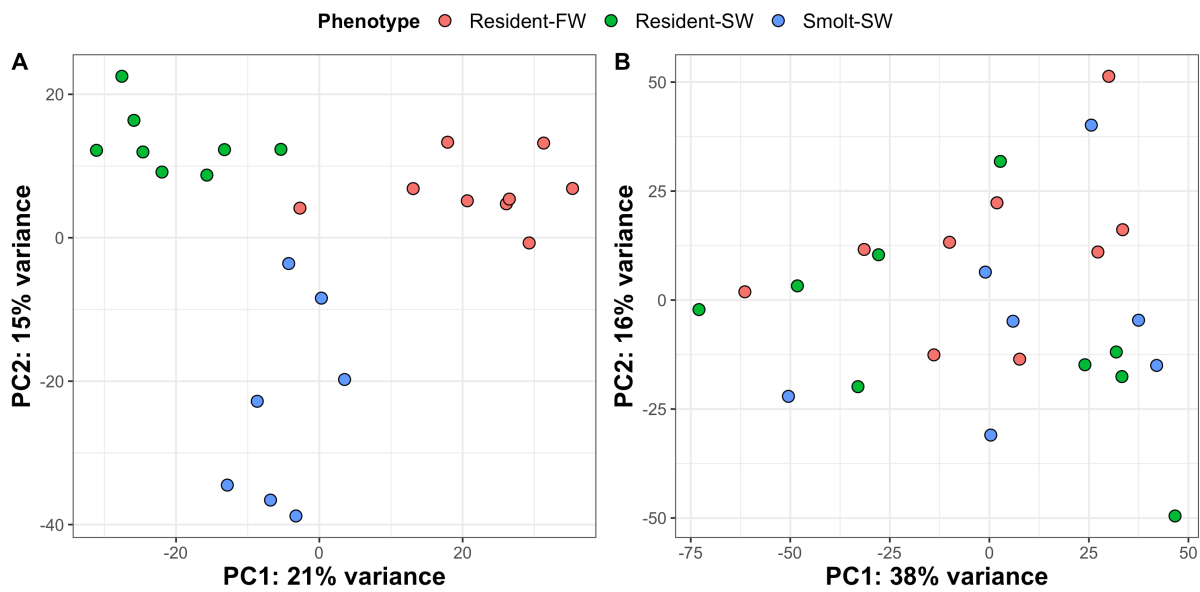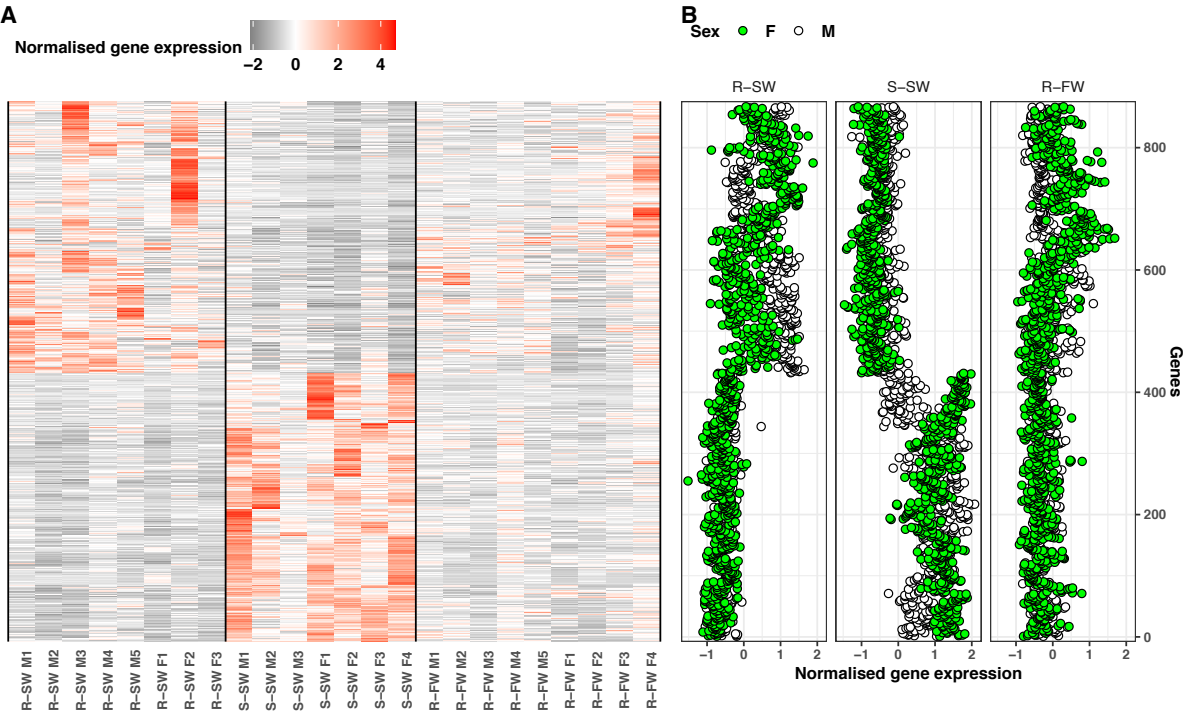
Figures

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Fig. 1. Identification of putative AMT genes in brown trout. Panel Schematic representation of experimental design and associated logic. Livers and brains of trout deemed to have adopted an anadromous tactic (smolts) were RNA-sampled immediately following a 24-hr saltwater (SW) challenge (group 1 = smolts-SW). Livers and brains of a second group of trout deemed to have adopted a resident tactic were sampled following the same SW challenge (group 2 = residents-SW), while a third group were sampled at the same time in fresh water (FW) without having undergone a saltwater challenge (group 3 = residents-FW). Note that smolts could not be tested in FW prior to being subjected to a SW challenge (which is required to define a fish as a smolt or not) because liver and brain sampling is terminal for the fish. Because smolts are by definition physiologically tolerant of SW, group 1 experienced mild osmotic stress. Residents are physiologically intolerant of SW but well suited to life in FW, hence group 2 experienced high osmotic stress while group 3 experience low osmotic stress. Black arrows represent genes that are differentially expressed between each pairwise comparison. AMT genes refer to those that are differentially expressed between the alternative life history tactics and potentially encompass genes involved in morphology, behaviour, physiology (including saltwater tolerance) and reproduction. Stress genes here refer to those for which differential expression between osmotic environments reflects the general stress of transitioning from FW to SW. Here OSER genes (osmotic environmental response genes) refers to genes which would be differentially expressed in response to different environmental salinity.

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Fig. 2. Distinct transcriptional profiles between resident and smolt livers but not brains.
Scatterplots displaying the results of a principal component analysis (PCA) for normalised gene
expression values for **(A)** livers and **(B)** brains collected from three groups differing in
phenotype and/or environment. The proportion of variance within gene expression explained
by principal component 1 (PC1) and 2 (PC2) in displayed on the x- and y-axes, repectively.
Here each group is a categorical variable corresponding to the combination of the individuals
phenotype (life history tactic) and osmotic environment the individual experienced for 24-hrs
just prior to terminal sampling (salt water or fresh water).

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Fig. 3. Putative AMT genes expressed in the brown trout liver. (A) Heatmap displaying the normalised gene-level expression estimates for 867 genes that were uniquely differentially expressed between the livers of resident and migrant brown trout. Each row represents a single gene (y-axis) while for each sample (x-axis), the sample name provides information on phenotype (R = "resident", S = "smolt"), environment of sampling (SW = "saltwater", FW = "freshwater") and sex (M = "male", F = "female). **(B)** Scatterplots showing the mean normalised gene expression for each sex within each of three groups (R-SW = "resident saltwater", S-SW = "smolt saltwater", R-FW = "resident freshwater"). Each point represents an individual gene and is coloured by sex (green = "female", white = "male").
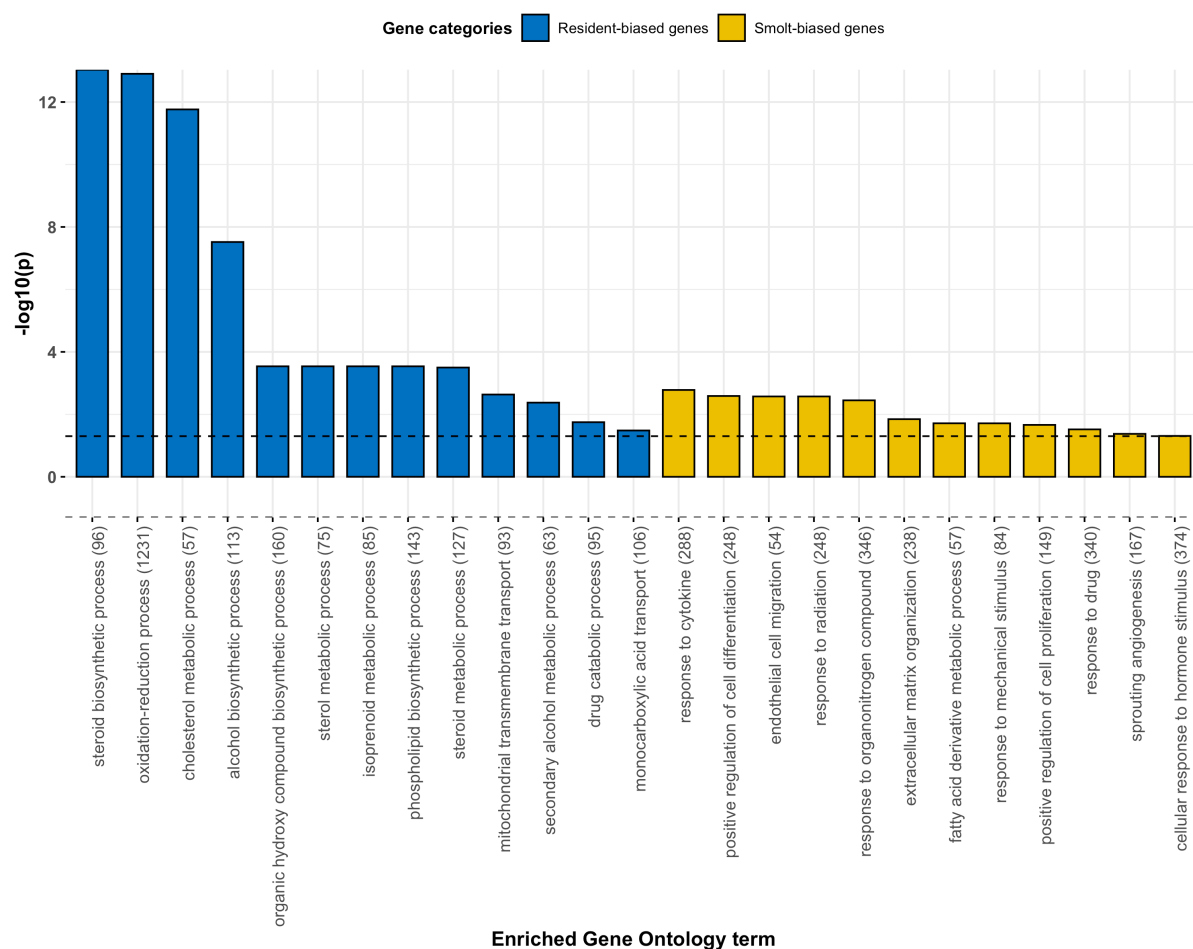
Fig. 4. Gene Ontology enrichment analysis of putative AMT genes in brown trout liver.
Enriched Gene Ontology associated with biological process-related terms for genes differentially expressed between alternative life histories. Each bar represents the -log10 of p value (Fisher's Exact test) for each GO term with description of GO term, as well as the total number of annotated genes per term provided. The black vertical dashed line represents a -log10(p) value equivalent to a P value = 0.05 threshold of significance.
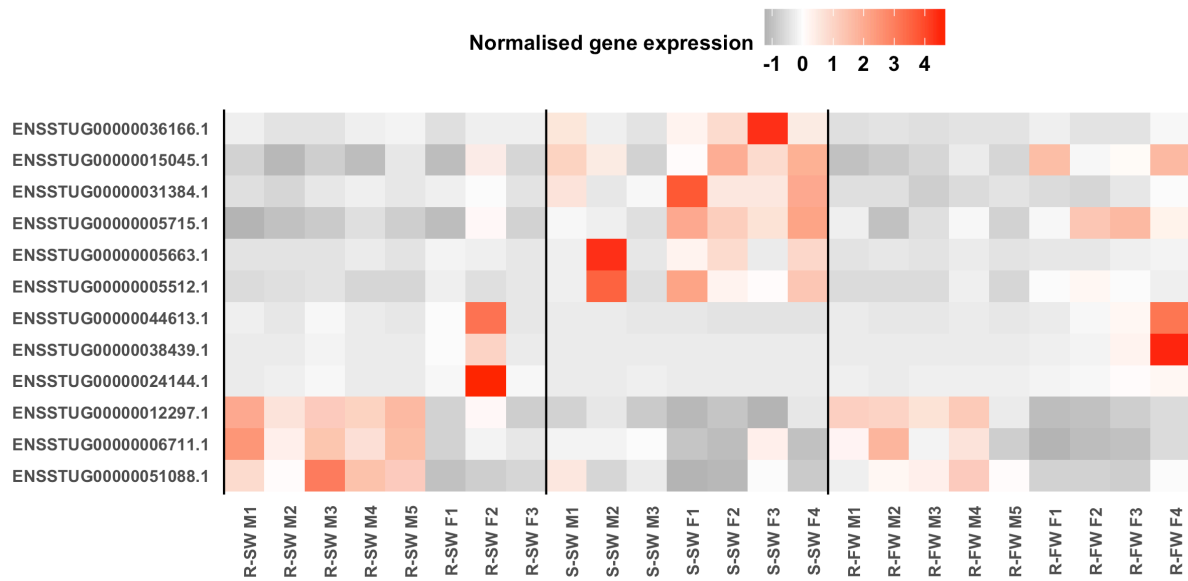
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Fig. 5. Sex-biased expression of putative AMT genes. Heatmap displaying the normalised gene-level expression estimates for 12 putative AMT genes that also demonstrated sex-biased gene expression within the liver. For each gene, the Ensembl gene ID is provided (y-axis) while for each sample (x-axis), the sample name provides information on phenotype (R = "resident", S = "smolt"), environment of sampling (SW = "saltwater", FW = "freshwater") and sex (M = "male", F = "female").