RNA-seq reveals differential gene expression in the brains of juvenile resident and migratory smolt rainbow trout (*Oncorhynchus mykiss)*

Matthew C. Hale[a,b], Garrett J. McKinney[a,1], Frank P. Thrower[c], and Krista M. Nichols[a,d*]

[a] Department of Biological Sciences, Purdue University, 915 W. State Street, West Lafayette, Indiana, 47906, USA

[b] Department of Biology, Texas Christian University, 2800 S. University Drive, Fort Worth, Texas, 76133, USA

[c] Ted Stevens Marine Research Institute, Alaska Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Juneau, Alaska, USA

[d] Conservation Biology Division, Northwest Fisheries Science Center, National Marine Fisheries Service, NOAA, 2725 Montlake Blvd E, Seattle, Washington, 98112, USA

[1]Present address: School of Aquatic and Fisheries Sciences, University of Washington, 1122 NE Boat Street, Seattle, Washington, 98105, USA

*Corresponding author: +1 206-302-2470; krista.nichols@noaa.gov

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Abstract

Many migratory traits are heritable, but there is a paucity of evidence identifying the molecular mechanisms underlying differentiation in alternative migratory tactics, or in linking variation in gene expression to migratory behaviors. To that end, we examined differential gene expression in the brain transcriptome between young steelhead trout that had undergone the smoltification process, and resident rainbow trout (*Oncorhynchus mykiss*) from Sashin Creek, Alaska. Samples were sequenced from two time points: immediately before (at 20 months of age) and during (2 years of age) the presumed peak of smoltification. Smolt and resident individuals came from two genetic crosses, one where both parents were migratory, and another where both parents were residents. A total of 533 (1.9%) genes were differentially expressed between crosses, or between smolt and resident samples. These genes include some candidate migratory genes (such as *POMC*), as well as genes with no previous known involvement in the migratory process. Progeny from resident parents showed more upregulated genes than progeny from migrant parents at both time points. Pathway analysis showed enrichment in 227 biological pathways between cross type, and 171 biological pathways were enriched between residents and smolts. Enriched pathways had connections to many biofunctions, and most were only enriched in one contrast. However, pathways connected to phototransduction were enriched between both cross type and migratory tactics in 11 out of 12 contrasts, suggesting there are fundamental differences in how smolts and residents process light in the brain. The genes and pathways described herein constitute an *a priori* candidate list for future studies of migration in other populations of *O. mykiss*, and other migratory species.

45

46 1. Introduction

47 Migration, the long distance movement of animals to take advantage of temporary
48 resources, has captured the attention of scientists and lay people alike (Dingle, 1991).
49 Adaptation to a migratory lifestyle allows individuals to take advantage of temporary
50 resources not available to resident conspecifics. Migration is a key behavior in several
51 taxonomic groups including insects, fishes, birds, and mammals, and plays a critical role
52 in the exchange of nutrients between different ecological systems (Wilcove and Wikelski,
53 2008). In many migratory species individuals within a population vary in their propensity
54 to migrate (Dingle and Drake, 2007). These alternative migratory tactics (AMTs) include
55 individuals that migrate, as well as others that remain resident. Typically, migratory
56 behaviors are triggered by environmental cues, such as increased photoperiod, which
57 promote a series of changes in the physiology, behavior, and morphology of migrants that
58 are required before migration can commence (Dingle and Drake, 2007). Such large-scale
59 changes demand the integration of many biochemical, molecular, and physiological
60 processes, that are not required of resident individuals.

61

62 Many traits that vary between resident and migrant individuals have a strong heritable
63 component (e.g. Pulido and Berthold, 2003; Pulido et al., 2001; Thrower et al., 2004;
64 Hecht et al., 2015). However, there is little information at the molecular level regarding
65 the genetic basis of migratory related traits (reviewed in Liedvogel et al., 2011; but see
66 Tarka et al., 2010; Bensch et al., 2009 and Zhan et al., 2011). This lack of knowledge is
67 due in part to limited information on the roles of the environment on determining

68 migration, and limited genomic resources (most migratory species are non-model
69 species), which makes finding associations between genes and migratory behaviors
70 difficult. In addition, migrant and resident individuals rarely exhibit obvious
71 morphological differences before migration, complicating the identification of AMTs
72 until the onset or duration of migration, at which point sampling individuals may be
73 difficult.
74
75 The salmonids (salmon, trout, and char) are an exemplary taxonomic group for research
76 on migration. AMTs in salmonids are manifested in clear morphological, physiological,
77 and behavioral differences both before, and during migration (Hoar, 1976). Most
78 salmonids hatch in fresh water, and spend a variable portion of time in their natal rivers
79 and streams. Migratory individuals then transform to saltwater adapted smolts, via a
80 process known as smoltification, before migrating to the ocean (Folmar and Dickhoff,
81 1980; Hoar, 1976; Thorpe, 1994). Therefore, the decision to migrate or stay resident must
82 be made before the individual begins its migration (Hoar, 1976). Salmonids that migrate
83 to the ocean face increased predation risk, but also benefit from an abundance of
84 resources compared to individuals that stay in fresh water (Quinn, 2005).
85
86 In salmonids, traits such as body shape, and skin silvering that vary between AMTs show
87 polygenic patterns of inheritance as has been determined by QTL approaches (Nichols et
88 al., 2008; Le Bras et al., 2011; Hecht et al., 2012), and by genome wide association
89 studies (GWAS) (Limborg et al., 2011; Martinez et al., 2011; Narum et al., 2011; Hale et
90 al., 2013; Hecht et al., 2013). However, phenotypic variation can also be determined by

differences in gene expression. For example, expression variation in candidate genes has been linked to downstream migratory behavior, and preparation for smoltification in juvenile brown trout (*Salmo trutta*; Giger et al., 2008), juvenile rainbow trout (*Onchorhynchus mykiss*; Aykanat et al., 2011; Hecht et al., 2014), juvenile Atlantic salmon (*S. salmo*; Seear et al., 2010; Robertson and McCormick, 2012), and juvenile brook trout (*Salvelinus fontinalis*; Boulet et al., 2012). Migratory variation is also associated with differences in gene expression between sexually mature residents and migrants in rainbow trout (Sutherland et al., 2014) and Atlantic salmon (Aubin-Horth et al., 2009; Perrier et al., 2013). However, these studies have used microarray or real time qPCR methods and so are limited to small gene sets. An attractive alternative is to use next generation RNA sequencing (RNA-seq) methods that aim to sequence the majority of mRNAs being expressed in the tissue of interest at the time of sampling. Such methods allow both common and rarely expressed transcripts to be sampled (Wang et al., 2009).

In this study, we examine differential gene expression in *O. mykiss* derived from a natural system in Sashin Creek, Alaska. This population has become a model system in studying the genetic basis of migration, as quantitative genetics (Thrower et al., 2004; Hecht et al., 2015), QTL (Hecht et al., 2012), Genome Wide Association Studies (GWAS, Hale et al., 2013), population genomic approaches (Hale et al., 2013), and studies of gene expression (Aykanat et al., 2011; Hecht et al., 2014) all confirm that the development of AMTs has a genetic component. Previous studies of gene expression between AMTs have mostly focused on the kidney and gill tissues due to their crucial roles in osmoregulation when smolts move from freshwater to saltwater (e.g. Boulet et al., 2012; Sutherland et al.,

114 2014; Giger et al., 2008; but see Aubin-Horth et al., 2005, 2009; Robertson and
115 McCormick, 2012; Hecht et al., 2014). However, the brain is also a key organ in the
116 development of AMTs in salmonids, as it integrates environmental cues such as changes
117 in photoperiod (Davie et al*.,* 2009), with the production and regulation of hormones
118 involved in the smoltification process (Bjornsson et al*.,* 2011; Kitano et al*.,* 2010). We
119 evaluated differential gene expression of the brain transcriptome between progeny
120 produced from migratory and non-migratory parents at two time points in the second year
121 of life: 1) during February prior to migration, when fish have not differentiated into
122 identifiable AMTs, and 2) during June, when smoltification peaks and downstream
123 migration ensues. Our findings contribute to the identification of transcripts and pathways
124 that are differentially regulated between smolts and residents. Ultimately, this will help
125 identify the key genes involved in the development of AMTs in *O. mykiss*. To that end,
126 the aims of the study herein were two fold: 1) To evaluate whether the brain
127 transcriptome shows patterns of differential gene expression in progeny produced from
128 the different resident vs. migrant parents, and 2) to evaluate differential gene expression
129 during smoltification in the Sashin Creek *O. mykiss* population (June) in individuals with
130 known ecotype.
131

132 ## 2. Materials and methods

133 *2.1 Sample collection and preparation*

134 *Oncorhynchus mykiss* were sampled from F_1 generation crosses between male and female
135 anadromous (A) steelhead trout from Sashin Creek, Alaska to produce an anadromous (A
136 x A) cross type and between male and female resident (R) rainbow trout from Sashin

137 Lake Alaska to produce a resident (R x R) cross type. Crosses were initiated in May
138 2010. Parents of crosses were captured either at a weir at the outlet of Sashin Creek upon
139 their return from ocean migration (anadromous adults) or by fyke net at the outlet of
140 Sashin Lake (resident adults). Adults were anesthetized with either MS-222 (50 mg/L;
141 Argent Chemicals, Redmond, WA) or clove oil (25 mg/L), and gametes were expressed
142 into storage bags by applying pressure on the abdomen cavity. Gametes were stored at 4
143 °C for not more than 24 hours before single pair matings were conducted at the Little
144 Port Walter Marine Station. Fertilized eggs were reared in total darkness in recirculating
145 incubators using water from Sashin Creek. Once fish had reached the swim-up stage of
146 development (utilization of yolk proteins), each family was thinned to equal densities and
147 placed in an outdoor 'micro' raceway (170 L) for continued development. Flow-through
148 water in the raceways came from Sashin Creek, and the fish were maintained under
149 natural photoperiod. At age 1, approximately 50 fish from each of 7 families of each
150 cross type were combined into larger vertical raceways (~25.5 m^2), whereby each cross
151 type was reared in equal densities in two different raceways. For this study, fish were
152 sampled at two time points: February 2012 and in June 2012. Ten fish were sampled at
153 both time points and from both cross types to give a total of 40 fish. Fish were euthanized
154 with a lethal dose of MS-222 (500 mg/L; Argent Chemicals, Redmond, WA), and fork
155 lengths and weights were taken at each sampling. The February samples were taken
156 before individuals had differentiated into AMTs, thus contrasts for the February sample
157 were simply between cross types. In June, at age 2, AMTs became obvious and all
158 individuals from each of the cross types were scored for life history. Smolts were
159 identified by the presence of silvery reflective skin, a lack of parr marks, and by having a

more streamlined fusiform shape (Hoar, 1976). Male residents were identified by applying pressure to the abdominal cavity causing the expression of milt, and a lack of visual signs of undergoing smoltification. As no female resident had reached sexual maturity, females were identified by an absence of signs of smoltification, and a condition factor (weight/length^3 x 100000) greater than 1.0 (Thrower et al., 2004; Hecht et al., 2012). Some fish had an indeterminate life history i.e. could become either a migrant or a resident) such fish were placed in a fourth group "indeterminate" and were not used in this study. At this June time point, a subset of individuals from each life history type was selected for dissection and gene expression analysis, as described below.

At sampling, whole brains (for RNA-seq) and liver (for DNA extraction for sexing) samples were dissected, immediately placed in RNAlater (Ambion, Grand Island, NY) and stored at -80°C. A total of 23 brain samples were chosen for RNA extraction, 8 from February and 15 from June. The February samples consisted of 4 each from A x A and R x R crosses. The June samples consisted of seven from A x A and eight from R x R, consisting of three smolts and four resident samples from the A x A cross and four each of smolts and resident samples from the R x R cross. RNA was extracted using a combination of Trizol (Invitrogen, Carlsbad, CA) and RNeasy columns (Qiagen, Valencia, CA). Briefly, standard Trizol extraction methods were followed until organic phase separation after the addition of chloroform. The nucleic acid phase was then added to an RNeasy column and extraction proceeded as described by Qiagen. RNA was eluted in Qiagen Elution Buffer EB and checked for integrity on a 1.5% agarose gel. DNA was extracted (from liver tissue) following methods described in Wasko et al. (2003) with

183 slight modification (Hecht et al., 2012). DNA samples were used for sexing fish by
184 genotyping the *OmyY1* marker, as described by Brunelli et al. (2008).
185
186 Library construction for sequencing was conducted at Purdue University's High-
187 throughput Genomics Unit using TruSeq RNA preparation kits (Illumina, San Diego,
188 CA). Individual libraries were barcoded and run on five lanes using the Illumina
189 HiSeq2000 platform. Illumina (v 3 chemistry) paired-end 100bp sequencing chemistry
190 was used. Two of the five lanes consisted of a pool of cDNA with equal amounts from
191 each of the 23 samples. Samples on the other three lanes were split between all February
192 samples (lane 1) and all June samples (lanes two and three). Since there was no lane
193 effect on the number of reads per sample (2-way ANOVA, $p > 0.05$), reads from each
194 sample from all five lanes were pooled for analyses.
195
196 *2.2. Bioinformatics*
197 Sequences were quality trimmed (i.e. sequencing adaptors removed, sequence ends with
198 quality scores less than 20 were trimmed, and sequences of less than 30 base pairs were
199 removed) using Trimmomatic v.0.17 (Lohse et al., 2012). Trimmed reads were mapped
200 to a brain and embryo transcriptome assembly constructed from *O. mykiss* samples from
201 four earlier developmental time points from Sashin Creek, Alaska (McKinney et al.,
202 2015) using RSEM (RNA-seq by Expectation-Maximization) v.1.1.19 (Li and Dewey,
203 2011).
204

In order to simplify read mapping, and to reduce the noise associated with mapping to isotigs, contigs were collapsed into gene-level contigs (components). Gene level counts generated by RSEM were used for estimating gene expression using EdgeR (Robinson et al., 2010). Components were filtered to include those that had greater than one count per million (cpm > 1) in at least three of the eight samples from February, or in seven out of 15 individuals (June). Remaining component read counts were then normalized using the trimmed mean of values method (TMM) in EdgeR to account for differences in library size. To lessen the chance that sequencing errors or differences in sequence coverage contributed to estimates of gene expression, we further limited our results to only include components with a minimum of 10 sequences for both treatments.

2.3. Measuring differences in gene expression

Two approaches were taken to evaluate differential gene regulation between progeny of the A x A and R x R cross types, and between the AMTs within cross types that were expressed only in the June time point. First, we evaluated differential gene expression on a transcript by transcript basis using general linear models while accounting for sex, and correcting for multiple testing by applying a Benjamini Hochberg false discovery rate (FDR). Second, we evaluated whether specific biofunctions, molecular mechanisms, and pathways were enriched for differentially expressed genes. Both analyses are described below.

General linear models (GLMs) were used to identify differentially expressed genes between treatments in EdgeR. A total of eight contrasts were performed, two models

were constructed: 1) to test differences between cross type within each time point and within each sex (i.e. $y_{ijk} = \mu + S_i + T_j + C_k + S_iT_jC_k + e_{ijk}$, where μ is the sample mean, y is the $\log_2$ comparison between samples, S is the sex of the sample, T is the time point (i.e. February or June), C is the cross type (i.e. R x R or A x A), $S_iT_jC_k$ is the three-way interaction between these variables, and e is the error); and 2) to test the differences between migrant and resident samples within the June time point for each sex and each cross type (i.e. $y_{ijk} = \mu + S_i + A_j + C_k + S_iA_jC_k + e_{ijk}$ where μ is the sample mean, y is the $\log_2$ comparison between samples, S is the sex of the sample, A is the migratory phenotype of the sample (resident or migrant), C is the cross of the sample (i.e. R x R or A x A), $S_iA_jC_k$ is the three-way interaction between these variables, and e is the error). For all models a Benjamini Hochberg false discovery rate FDR correction was applied in EdgeR (alpha = 0.05; Benjamini and Hochberg, 1995).

Pathway and network analyses using Ingenuity Pathway analysis (IPA; Redwood City, CA) software have been shown to be useful in determining biological pathways that are enriched for genes differentially expressed between treatments (McKinney et al., 2015). Contigs that met filtering criteria were mapped to the zebrafish and human ortholog ENSEMBL databases using BLASTn and BLASTx with a minimum e-value of 1.00E -05. If multiple contigs mapped to the same Gene ID, IPA removed duplicates by retaining the match with the lowest e-value. Human and zebrafish orthologs were then compared to the Ingenuity Knowledge Base and significantly altered pathways and biofunctions (Gene Ontology terms) were determined using a Fisher's exact test ($p < 0.05$) and a minimum expression fold difference between the phenotypes of 1.8. In comparisons between cross

type, a Z score ≥ 2.0 signifies upregulation of that pathway in R x R samples relative to A x A samples, a Z score ≤ -2.0 signifies upregulation of that pathway in A x A samples relative to R x R samples. In comparisons between phenotypes, a Z score ≥ 2.0 signifies upregulation of that pathway in residents compared to smolts. A Z score ≤ -2.0 signifies upregulation of that pathway in smolts compared to residents. As IPA software is built from mammalian pathways, human gene and protein nomenclature will be used throughout.

2.4. Identification of pituitary transcripts

As whole brain samples were taken it is possible that the pituitary was included in some samples and not others. The pituitary is the site of synthesis of several peptides including Growth Hormone 1, Prolactin, and Pro-opiomelanocortin (*POMC*) that have been found to be associated with smoltification and salmonids. Therefore, the potential inclusion of the pituitary in some samples and not others could lead to differences in gene expression. We used two approaches to test for the inclusion of the pituitary: 1) by identifying transcripts presented in a previous study that specifically measured transcripts expressed in the pituitary of *O. mykiss* (Gahr et al., 2007), and 2) by comparing gene expression between the samples herein and brain and pituitary specific RNA-seq libraries from *O. mykiss* downloaded from NCBI's SRA database (accessions ERX297518 (brain) and ERX297516 (pituitary)). For the second approach, RSEM was used to align reads from these libraries to the *O. mykiss* transcriptome used in this study (McKinney et al., 2015). Transcripts per million (TPM) was then used to cluster patterns of gene expression between samples in three ways: 1) using the pituitary specific transcripts identified by

274 Gahr et al. (2007), 2) comparing all the filtered transcripts from this study with the brain
275 and pituitary specific databases (this was done separately for the February samples, the
276 June A x A samples, and the June R x R samples), and 3) comparing differentially
277 expressed transcripts in this study with the same transcripts from the brain and pituitary
278 specific databases from NCBI SRA (again, this was done separately for the February,
279 June A x A, and June R x R samples). All cluster analysis was conducted in R v 3.2.1.
280
281 *2.5. Gene mapping*
282 Differentially expressed genes that passed sequence count threshold were mapped against
283 the *O. mykiss* genome (Berthelot et al., 2014). All genes that showed differential gene
284 expression in at least one of the comparisons were compared (using BLASTn) at the
285 sequence level with the published *O. mykiss* genome sequence. BLASTn settings were a
286 minimum e-value of $1E^{-10}$, minimum alignment length of 100 base pairs and a maximum
287 of 2 mismatches. Any gene that produced multiple hits to different regions of the genome
288 were removed.
289
290 *2.6. Quantitative PCR validation*
291 Ten different genes were chosen for validation using quantitative real time PCR (qPCR).
292 All samples used for RNA-seq were included in the validation. Samples were run in
293 triplicate and β-actin (expression was not statistically different between contrasts of
294 interest) was used as a reference gene. All primers were run with a non-template control
295 in triplicate. Primers were designed in Primer 3 (Rozen and Skaletsky, 2000), and, where
296 possible, were designed to span exon-intron boundaries so that potential contamination

297 from genomic DNA could be observed (see Supplementary file 1 for primer sequences).
298 A melt curve analysis was performed on each gene to evaluate whether non-target
299 products were amplified during PCR. A set of serial dilutions was performed on each
300 gene to determine PCR efficiency. Quantitative PCR was performed on an ABI
301 StepOnePlus (Alameda, CA) using SYBR green master mix following manufacture's
302 recommended protocols. Amplification conditions were 50 °C for 2 minutes, 95 °C for 10
303 minutes, and then 40 cycles of 95 °C for 15 seconds and 60° C for 1 minute. If any
304 sample produced variation in the cycle threshold (C_t) greater than 0.5 then that sample
305 was repeated. Cycle threshold values were normalized to β-actin to estimate the $\otimes\otimes C_t$
306 value following Pfaffl (2001). Significant differences in expression between cross type or
307 migratory phenotype were tested on $\Delta\Delta C_t$ values using one-way ANOVA (performed in
308 SAS v.9.2 (SAS Statistical Institute, Cary, NC).
309

310 ## 3. Results

311 *3.1. Summary of sample collection*

312 In this study, seven families from each of the A x A and R x R cross types were reared to
313 age 2 (June 2012) in a common environment, at which point life history was determined.
314 Individual families originated from four anadromous females, five anadromous males,
315 four resident females, and six resident males, whereby some families were either
316 maternal or paternal half siblings with one other family (gametes for males and females
317 were not split for more than two families). The proportion of each life history produced
318 from each of the cross types varied, but each of the cross types produced all life history
319 categories (i.e. mature, resident, smolts, and indeterminate). The A x A cross produced a

320 greater proportion of smolts than the R x R cross (29.89 % brood smolts in A x A cross,
321 18.3% brood smolts in R x R cross), while the R x R cross produced a large number of
322 fish that were indeterminate in their life history (34% of A x A cross, 46.2% of R x R
323 cross; Figure 1). The mean monthly water temperature (ambient temperatures of Sashin
324 Creek, which supplied water to the rearing systems herein) from fertilization through age
325 2 (May 2010 through June 2012) ranged from 1.4°C in the coldest winter month (March
326 2011 and March 2012) to 13.1°C in the warmest summer month (August 2010; see
327 Supplementary file 2).
328
329 *3.2. Sequencing and analysis*
330 Five lanes of 100bp Illumina paired-end sequencing produced 511,810,102 quality
331 filtering paired reads. These reads were aligned to a reference transcriptome that
332 consisted of 513,933 contigs (N50 = 1,733 bp; McKinney et al., 2015). Components that
333 failed to meet minimum count thresholds were removed, leaving a total of 28,616
334 components, hereafter referred to as genes that passed count thresholds. The number of
335 paired-end reads mapped varied from 5.23 million to 21.08 million per individual
336 (Supplementary file 3). A total of 19,072 (70.4%) genes produced a significant BLAST
337 hit to one of four databases used in Integrative Pathway Analysis software for annotation.
338 For pathway analysis, 12,095 genes were available once duplicates were removed.
339 Principal component analysis was used to cluster samples on similar patterns of gene
340 expression (tpm) for the filtered gene set. Supplementary file 4 show there was limited
341 clustering of the February or June samples for both cross types. However, Supplementary
342 File 4: 4b and 4c shows that smolts cluster more tightly than residents suggesting patterns

343 of gene expression are more variable between resident samples than between smolts.
344 Similar results were obtained by cluster analysis of differentially expressed genes shown
345 in Figures 2a-2c. Figure 2b shows how smolt samples form a separate cluster from the
346 resident samples suggesting separation of gene expression based on phenotype. Samples
347 from the February time point (Figure 2a) also formed discrete clusters based on cross
348 type, with the exception of one sample (RxR_1). Cluster analysis strongly suggested that
349 one sample (Feb RxR_1) contained the pituitary as well as the whole brain (see
350 Supplementary file 5 figures 5a). Specifically, three transcripts (*POMC*, Growth hormone
351 1, and prolactin) were expressed in sample RxR_1 at similar levels to the pituitary
352 sample. Therefore, we removed transcripts identified in Gahr et al (2007) as being mostly
353 expressed by the pituitary from this sample.
354
355 *3.3. Differential gene expression: Cross type analysis*
356 A total of 533 genes were differentially expressed in at least one contrast. Of these, 232
357 (181 annotated) genes were differentially expressed in the brain between A x A and R x
358 R individuals in at least one comparison. This number varied between 40 in February
359 females to 104 in February males. There was a general pattern of a greater number of
360 genes upregulated in the R x R samples compared to the A x A progeny (varied from
361 59% (56 out of 95 in the June males), to 100% (40 out of 40 in February female samples;
362 see Figure 3)). Of the 139 differentially expressed genes in the February samples, three
363 unannotated genes were differentially expressed in both males and females between cross
364 types, one of which was upregulated in the R x R samples, the other two were
365 upregulated in R x R samples in the female samples, and in the A x A samples in the

366 males (see Supplementary file 6: S6.1 for a list of annotated differentially expressed
367 genes). Of the 159 genes that were differentially expressed in the June samples, eight
368 were differentially expressed in both sexes. Five of eight were upregulated in R x R
369 samples of both sexes, and 3 upregulated in A x A samples in both males and females
370 (see Supplementary file 6: S6.1 for a list of annotated differentially expressed genes).
371 Log_2 fold change, p values, and FDR corrected p values of all genes that passed filtering
372 criteria are given in Supplementary file 6 (S6.4 and S6.5).
373
374 *3.4. Differential gene expression: Life history type analysis*
375 RNA-seq identified a total of 284 differentially expressed genes, of which 154 were
376 annotated. This number varied between 46 and 108 genes (FDR corrected p value 0.05)
377 that were differentially expressed in the brain between resident and smolt samples within
378 cross type and sex. There were 57 differentially expressed genes in female A x A samples
379 between resident and smolt samples, of which 20 genes were upregulated in resident
380 samples and 36 were upregulated in smolts. A total of 46 genes were differentially
381 expressed in male A x A samples between resident and smolt samples (of which 20 were
382 upregulated in residents and 26 were upregulated in smolts). In the samples from the R x
383 R cross 108 genes were differentially expressed between resident females and female
384 smolts of which 19 were upregulated in resident and 89 were upregulated in smolts. A
385 total of 106 genes were differentially expressed between resident male R x R individuals
386 and male R x R smolts, of which 55 were upregulated in resident individuals and 51 were
387 upregulated in smolts (see Figure 4, Supplementary file 6: S6.2 for annotated genes
388 upregulated in the R x R and A x A samples).

389

390 *3.5. Biofunction analysis: Cross type analysis*

391 A total of 227 biofunctions (full list in Supplementary file 7) were enriched between
392 cross type in at least one treatment. A total of 144 biofunctions were enriched in the
393 February samples and 114 biofunctions were enriched in the June samples. The number
394 of enriched biofunctions varied from 28 in the June female samples to 104 in the June
395 males. Two biofunctions, neonatal death, and perinatal death were enriched between
396 cross type in all four treatments. Most enriched biofunctions had more differentially
397 expressed genes in the R x R samples than the A x A in all treatments, apart from the
398 June female samples. The most enriched biofunctions with more differentially expressed
399 genes in the February R x R samples were organismal development ($Z = 4.747$, $p <$
400 0.001), cell-to-cell signaling and interaction ($Z = 3.423$ $p < 0.001$) and hematological
401 system development and function ($Z = 3.423$, $p < 0.001$). The most enriched biofunctions
402 with more differentially expressed genes in the A x A February samples were organismal
403 death ($Z = -5.377$; $p < 0.001$), perinatal death ($Z = -4.574$; $p < 0.001$) and neonatal death
404 ($Z = -4.374$; $p < 0.001$). The most enriched biofunctions with more differentially
405 expressed genes in the R x R June samples were cell death and survival ($Z = 4.41$; $p <$
406 0.001), organismal development ($Z = 4.381$; $p < 0.001$), and vision system development
407 and function ($Z = 3.412$; $p < 0.001$). The most enriched biofunctions with more
408 differentially expressed genes in the A x A June samples were cellular assembly and
409 organization ($Z = -6.59$; $p < 0.001$), perinatal death ($Z = -4.49$; $p < 0.001$), and neonatal
410 death ($Z = -4.20$; $p < 0.001$).

411

412 *3.6. Biofunction analysis: Life history type analysis*

413 A total of 171 biofunctions were enriched between smolts and residents in at least one
414 contrast. One biofunction (organismal survival) was consistently enriched between all
415 four contrasts. The A x A samples produced more enriched biofunctions between resident
416 and smolts than the R x R samples (136 versus 42). In the A x A samples the enriched
417 biofunctions with more differentially expressed genes in residents were quantity of cells
418 ($Z = 3.33$; $p < 0.001$), transport of molecules ($Z = 3.26$; $p < 0.001$), and chemotaxis of cells
419 ($Z = 3.20$; $p < 0.001$). The most enriched biofunctions with more differentially expressed
420 genes in the smolts were organismal death ($Z = -5.05$; $p < 0.001$), perinatal death ($Z = -$
421 4.76; $p < 0.001$), and neonatal death ($Z = -4.14$; $p < 0.001$). Whereas in the R x R
422 samples, the most enriched biofunctions with more differentially expressed genes in
423 residents were degeneration of cells ($Z = 3.68$; $p < 0.001$), degeneration of photoreceptors
424 ($Z = 3.55$; $p < 0.001$), and retinal degeneration ($Z = 3.06$; $p < 0.001$). The most enriched
425 biofunctions in the R x R samples with more differentially expressed genes in the smolts
426 were quantity of photoreceptors ($Z = -3.33$; $p < 0.001$), visual system development ($Z = -$
427 3.37; $p < 0.001$), and quantity of cells ($Z = -2.85$; $p < 0.001$). See Supplementary file 8 for
428 a complete list of all biofunctions that were enriched between residents and smolts.

429

430 *3.7. Pathway analysis: Cross type analysis*

431 Pathway analysis incorporates differentially expressed genes into networks and pathways
432 that can give a more complete picture of the biological or physiological differences
433 between contrasts. We identified 100 altered canonical pathways ($p < 0.05$) between A x
434 A and R x R samples at different time points. Within contrasts, the total number of

435 altered pathways varied from 22 in the June male samples, to 39 in the February male
436 samples. A single pathway, phototransduction, was altered in all four contrasts (i.e.
437 altered between A x A and R x R samples for both time points, and both males and
438 females), indicating a consistent difference in gene expression related to photoreceptors
439 and their signaling. cAMP-mediated signaling was significantly altered in three contrasts
440 (only in February females was it not altered). A total of 21 other pathways were altered in
441 more than two contrasts. The top altered canonical pathways between A x A and R x R
442 samples is provided in Table 1 for the February samples, and Table 2 for the June
443 samples (see Supplementary file 9 for all altered canonical pathways between cross type
444 for both February, and June samples).
445
446 *3.8. Pathway analysis: Life history type analysis*
447 A total of 65 canonical pathways were altered between smolts and residents. This number
448 varied from 27 in the R x R females to 15 in the A x A males. No pathways were
449 significantly altered in all contrasts, but seven pathways (phototransduction, bupropion
450 degradation, acetone degradation, intrinsic prothrombin activation, LXR/RXR activation,
451 atherosclerosis signaling, and serotonin receptor signaling) were significantly altered in
452 three of the four contrasts. Four additional pathways (cAMP-mediated signaling,
453 mitochondrial L-carnithine shuttle pathway, retinoate biosynthesis and cardiac adrenergic
454 signaling) were significantly altered in two of the four contrasts. The top altered
455 canonical pathways between smolt and mature samples is provided in Table 3 for the R x
456 R samples, and Table 4 for the A x A samples (see Supplementary file 10 for all altered
457 canonical pathways between AMTs for both cross types).

458

459 *3.9. Genome mapping*

460 We used sequence similarity to determine the position of differentially expressed genes in
461 the *O. mykiss* genome. A total of 285 out of 535 (53.3%) differentially expressed genes
462 were placed in a unique position on the *O. mykiss* genome, of which 205 were annotated.
463 Every chromosome contained at least one differentially expressed gene except Omy25.
464 Full details of mapping differentially expressed genes are provided in Supplementary file
465 11.

466

467 *3.10. Quantitative PCR validation*

468 Eight of the ten genes selected for validation amplified a single product of the expected
469 size and were amplified in all samples used for RNA-seq. Two genes (recoverin and
470 rhodopsin) produced multiple PCR products despite extensive optimization, suggesting
471 gene duplication (salmonids went through a whole genome duplication event (Allendorf
472 and Thorgaard 1984)). All eight genes showed consistent patterns of expression between
473 the qPCR data and the RNA-seq data in the February samples between A x A and R x R
474 samples (Figure 5; correlation = 0.975, $p < 0.001$). For the June A x A samples, there
475 were consistent patterns of expression between qPCR and RNA-seq data (correlation =
476 0.809, $p = 0.05$) although genes (*emex* and *IGM*) showed different patterns of expression
477 between both datasets, although note that these patterns were not statistically significant
478 ($F = 0.185$, $p = 0.685$ and $F = 1.819$, $p = 0.326$ for *IGM* from RNA-seq and qPCR
479 respectively; and $F = 0.543$, $p = 0.491$ and $F = 0.179$ $p = 0.689$ for *emex* from RNA-seq
480 and qPCR respectively). For the June R x R samples there were consistent patterns of

481 expression between the RNA-seq and qPCR data (correlation = 0.859, p = 0.02).
482 However, one gene, *PRL* showed different patterns of expression in the qPCR and RNA-
483 seq data. *PRL* was upregulated in smolt samples in the qPCR data (F = 9.46, p = 0.021),
484 but downregulated in smolts in the RNA-seq data (F = 10.1, p = 0.019).
485
486 **4. Discussion**
487 Previous studies documenting differential gene expression between salmonid AMTs have
488 mostly targeted the gill and kidney transcriptomes due to their roles in maintaining
489 osmoregulatory balance during saltwater adaptation (e.g. Giger et al., 2008; Aykanat et
490 al., 2011; Norman et al., 2014; Sutherland et al., 2014; but see Aubin-Horth et al., 2009;
491 Seear et al., 2010; Robertson and McCormick, 2012; Hecht et al., 2015). These studies in
492 concert with hormone assays conclude that the upregulation of growth hormone (GH),
493 thyroid hormones (THs), and cortisol are essential components of the smoltification
494 process (Folmar and Dickhoff, 1980; Dickhoff et al., 1997; Stefansson et al., 2008;
495 reviewed in McCormick, 2013). The production of these hormones is under the control of
496 the light-brain-pituitary axis. Changes in photoperiod during spring are detected by the
497 retina and pineal organ, which relay this information to the preoptic area (POA) of the
498 brain. The POA then communicates with the pituitary stimulating the release of GH,
499 which in turn causes an increase in the production of THs, cortisol, and other hormones.
500 Despite the importance of the brain in the smoltification process, previous studies have
501 been limited to a relatively small gene set (Aubin-Horth et al., 2009; Seear et al., 2010;
502 Robertson and McCormick, 2012; Hecht et al., 2014). To that end, we took a
503 transcriptome wide view of gene expression in the brain between AMTs before

504 smoltification begins and after its completion. The genes and pathways that were altered
505 between AMTs elucidate the molecular mechanisms that control the large-scale changes
506 required during seawater adaptation.
507
508 *4.1 Candidate genes associated with the development of AMTs*
509 The smoltification process culminates in physiological adaptation to seawater. A key
510 change in this physiological transformation is an increase in length relative to weight
511 giving migrants a lower condition factor than residents (Thorpe and Metcalfe, 1998;
512 Hoar, 1976; Dickhoff et al., 1997; Dodson et al., 2013). Thyroid hormones are known to
513 regulate many developmental functions in salmonids, and the link between thyroid
514 hormones (THs) and smoltification is well described (Young et al., 1989; Ebbesson et al.,
515 2000; Ebbesson et al., 2003). In the study herein, thyroid hormone receptor alpha
516 (*THRA*), was upregulated in R x R males from the February time point (p value = 0.0005,
517 but note this is not significant after FDR correction). *THRA* was a component of three
518 pathways enriched between cross-type in the February males. These pathways
519 (organismal survival, developmental disorder, and organismal development) are all
520 connected to developmental processes, suggesting large-scale differences in cellular
521 development in the brain between A x A and R x R samples in the winter leading up to
522 smoltification. Upregulation of *THRA* could have an effect on concentrations of thyroid
523 stimulating hormone (TSH). High concentrations of TSH have been reported in smolts
524 compared to residents in several salmonid species (Sullivan et al., 1983; Larsen et al.,
525 2011), as well as in the blood plasma of marine three-spined sticklebacks (Kitano et al.,
526 2010) compared to freshwater conspecifics. TSH stimulates the production of

triiodothyronine (T_3), and thyroxine (T_4), which increase during smoltification (Dickhoff et al., 1997; Larsen et al., 2011). Another candidate gene connected to regulation of thyroid hormones (thyroid hormone response, *THRSP*) was upregulated in residents compared to smolts from the female A x A samples (FDR corrected p value = 0.04). Several canonical pathways connected to thyroid activity and thyroid hormone regulation were altered between contrasts. The pathway thyroid receptor – retinoid X receptor (TR-RXR) was altered between crosses in the female February samples. TR-RXR is important in the release of T_3. Thyroid Receptor (TR) is normally found as a heterodimer with a retinoid x repressor. The binding of T_3 to the TR component of the heterodimer causes RXR to be released (Li et al., 2002). Additional altered pathways connected to thyroid hormone production included thyroid hormone metabolism I (via deiodination) (altered between cross types in the February female samples, and June male samples), and thyroid hormone biosynthesis (altered between cross types in the June female samples). Aubin-Horth et al. (2009) found upregulation of type II iodothyronine deiodinase-encoding gene (*DIO2*) in the brains of early migrating versus late migrating Atlantic salmon, providing additional evidence that transcripts connected to thyroid function are important in preparation for migration.. Note that in the study herein *DIO2* was upregulated in smolts in the June R x R male contrast ($p = 0.012$, $\log_2$ fold change = -2.045), but this was not significant after FDR correction.

The production and circulation of cortisol is an important part of the smoltification process (Madsen, 1990; McCormick et al., 2002; Hoar, 1976). Although cortisol is not a peptide hormone, Pro-opiomelanocortin (*POMC*) is a precursor to melanocortin receptor

550 2 (*MCR2*), and *MCR2* has been found to regulate the release of cortisol from the head
551 kidney (Holmqvist et al., 1994; Holloway et al., 1994). In the study herein, *POMC* was
552 upregulated in the brain of A x A smolts compared to residents. This upregulation may
553 suggest that cortisol levels in A x A smolts are higher than A x A residents. However, a
554 study by Yada et al. (2014) found no difference in the concentration of corticoid
555 receptors in the brains of smolt and resident *O. mykiss* during the smoltification process.
556 Indeed, although glucocorticoid receptors were present in our transcriptome (as
557 determined by BLAST; see Supplementary file 6, S6.3) at no point were they
558 differentially expressed between cross type or AMTs. These results suggest that the
559 products of *POMC* are likely targeting other organs (Shrimpton and McCormick, 1998).
560 In addition, Aubin-Horth et al. (2009) found *POMC* to be upregulated in the brains of
561 mature sneaker male Atlantic salmon compared to early migrants. Therefore, the role of
562 *POMC* expression in the brain could also be involved in precocious sexual maturation.
563
564 In sum, there is evidence for differential expression of candidate genes between cross
565 types and AMTs. However, the results are somewhat contradictory with respect to the
566 development of AMTs, as *THRA* was upregulated in the brains of the February R x R
567 samples, but studies in circulating levels of these proteins and transcripts in other tissues
568 suggest that thyroid hormones (and their receptors) promote the development of
569 anadromy. Why this is the case is unclear, however, samples from the February time
570 point did not show external evidence of smolting, nor did they express gametes. Although
571 the migratory fate of these animals may have already been determined, we cannot
572 characterize the February samples as different AMTs. Therefore, making conclusions

between patterns of gene expression in the February samples, and whether any up- or down-regulated genes are connected to the development of AMTs in *O. mykiss* is unwise. In addition, it is possible that the function of these genes in the brain differs from their function in other tissues in which they have been previously described.

4.2. Identification of pituitary transcripts

The potential inclusion of the pituitary in some, but not all samples could cause differential expression of pituitary produced transcripts such as *GH*, prolactin, and *POMC*. Cluster analysis clearly showed that one sample (R x R_1) from the February time point included the pituitary, whereas all other samples did not, and therefore, transcripts that are mostly produced by the pituitary (such as *GH*, *POMC*, and *PRL*) were removed from the February R x R comparison. Many of the transcripts produced by the pituitary are known to have an important effect on the process of smoltification, especially growth hormone (Bjornsson et al., 2011; Dickhoff et al., 1997; Stefansson et al., 1991; McCormick et al., 2002) and PRL (Prunet et al., 1985). Although the primary site of production for these transcripts is the pituitary, other studies have found the differential expression of GH in the brain between AMTS in salmonids. For example, Aubin-Horth et al. (2009) found GH to be differentially expressed between Atlantic salmon AMTs in the brain, and McKinney et al. (2015) found GH to be differentially expressed between cross type in four-month old *O. mykiss* in the brain. Furthermore, GH TPM values in the other samples herein were not zero (see Figure 2 and Supplementary file 6 for expression information for GH in other samples). These results strongly suggest that GH mRNA is not restricted to the pituitary, but instead is present in the whole brain

more broadly. However, because of inconsistencies in sampling (i.e. the almost certain inclusion of the pituitary in one sample) we took a conservative approach and removed these transcripts from the February time point.

4.3. Lipid metabolism and migration

Pathway analysis provided insight into how differentially regulated genes were interconnected during and preceding the development of AMTs. Several biofunctions were enriched between AMTs in both cross types, including cardiovascular and hematological systems, lipid metabolism, organismal development, and skeletal development. Of these pathways a role for lipid metabolism during the smoltification process has previously been identified (Thorpe and Metcalfe, 1998; McMillan et al., 2012). Smolts go through a period of altered metabolism resulting in a lower condition factor compared to resident conspecifics. Seven pathways connected to lipid metabolism were enriched between AMTs in the A x A females (of which four were also enriched between AMTs in the A x A male samples). Many of the genes within these pathways were upregulated in residents compared to smolts, but some (e.g. *SSTR2*, and *npy*) were upregulated in smolts. Somatostatins are produced by endocrine neurons within the hypothalamus, and suppress the release of GH from the pituitary (Sheridan et al., 1998; Sheridan and Hagemeister, 2010). The upregulation of a somatostatin receptor (*SSTR2*) in the brains of female smolts from the A x A cross may indicate suppression of the release of several hormones in the brain. In mammalian model organisms the expression of *SSTR2* is high in the pineal organ, which we have sampled in our whole brain extractions (Mato et al., 1997; Champier et al., 2003). If this upregulation of *SSTR2* is suppressing

active release of GH in the pituitary, it may be because these smolts have reached the threshold required for smoltification. Although we are unaware of any studies documenting upregulation of *SSTR2* in the brains of any salmonid, previous research has found upregulation of *SSTR2* in blood plasma both before and during seawater acclimatization in coho (*O. kisutch*) and Chinook salmon (*O. tshawytscha*, Sheridan et al*.,* 1998).

During smoltification, changes occur in the production and metabolism of lipids (Sheridan, 1989). Regulation of appetite is one mechanism that could lead to variation in amounts of lipid between AMTs. In the study herein, *neuropeptide Y* (a gene with known involvement in controlling appetite) was upregulated in smolts compared to residents in the female R x R samples. Studies in goldfish (*Carassius auratus*) have also found upregulation of *npy* in response to a lack of food (Bernier et al., 2004). *npy* is expressed in several regions of the brain, including the POA of both Chinook and coho salmon (*Oncorhynchus tshawytscha* and *O. kisutch*) and was found to increase after fasting (Silverstein et al., 1998). Variation between AMTs in the regulation of appetite could (in part) explain why residents have higher levels of lipids compared to smolts. Smolts typically go through a period of fasting immediately before smoltification (Pankhurst et al., 2008; McCormick et al., 1998; Jørgensen et al*.,* 2013). Indeed, reducing the amount of food available prior to the smoltification period causes an increase in the number of smolts in Atlantic salmon (Thorpe et al., 1990), Chinook salmon (Larsen et al., 2006; Larsen et al., 2013), rainbow trout (Tipping and Byrne 1996), and brown trout (Pirhonen and Forsman, 1999). Migration necessitates high energetic cost. Smolts must alter their

642 metabolism to cope with the energetic demands of migration. It has been proposed that
643 maintaining variation in AMTs is a strategy to overcome limited resources in natal
644 freshwater environments. Individuals that are small or underweight will gain a greater
645 advantage from migrating, whereas larger fish compete well with conspecifics and so do
646 not need to migrate (Forseth et al., 1999; Sloat et al., 2014). The upregulation of *npy* in
647 smolts compared to residents could be one molecular mechanism that regulates variation
648 in metabolism between AMTs.
649
650 *4.4 Phototransduction and migration*
651 One of the most compelling findings from our pathway analysis is the consistent
652 enrichment of pathways connected to phototransduction, both in comparisons between
653 cross types and between AMTs. Even more compelling is that differential regulation of
654 phototransduction pathways is also observed between migratory and resident cross types
655 in the first year of life (McKinney et al., 2015). Although multiple environmental factors
656 have an effect on the development of AMTs in salmonids (Narum et al., 2011; Dodson et
657 al., 2013), the annual increase in day length in spring is the key environmental change
658 that initiates the smoltification process (Stefansson et al., 2007). Exposing developing
659 fish to continuous light interferes with smoltification, producing individuals with
660 compromised seawater survival, reduced skin silvering, and a high condition factor
661 (McCormick et al., 1987; Stefansson et al., 2007; Ebbesson et al., 2007). Although the
662 exact mechanisms behind this are unclear, exposing fish to constant light suppresses the
663 extension and development of new retinal fibers into the POA (Ebbesson et al., 2007).
664 This leads to miscommunication between the brain and the pituitary, and therefore a

665 reduction in the production of GH, and a reduction in the amount of THs, cortisol, and
666 other hormones. In this study, at the gene level, rhodopsin (*RHO*), recoverin (*RCVRN*),
667 and retinol binding proteins were upregulated in June in the brains of smolts compared to
668 residents from both A x A and R x R crosses. At the pathway level, quantity of
669 photoreceptors was enriched between AMTs in both the A x A and R x R crosses,
670 suggesting there are fundamental differences in gene expression that regulates the
671 number of photoreceptors in the brain between AMTs. Figure 6 shows a network built
672 from genes involved in the phototransduction, and visual cycle pathways. Thirty of 35
673 molecules are upregulated in smolts compared to residents (R x R females), and many of
674 the same genes (such as recoverin, and various retinol binding proteins) are also
675 upregulated in smolts compared to residents in the R x R male samples. The saccus
676 vasculosus (an area of the brain that is rich in photoreceptive cells) in fish is important in
677 interpreting changes in photoperiod (Nakane et al., 2013). Nakane et al. (2013)
678 determined that the saccus vasculosus of Masu salmon (*O. masou*) expressed genes
679 related to phototransduction (such as rhodopsin, and various opsins). As we sampled
680 whole brains, it is impossible to determine whether the upregulation in smolts of genes
681 connected to phototransduction are due to differential expression in the saccus
682 vasculosus, the POA, or in the brain more broadly. It is compelling that many of the
683 genes differentially expressed specifically in the saccus vasculosus as reported by Nakane
684 et al. (2013) are also differentially expressed here. Further studies that determine where
685 in the brain phototransduction genes are expressed, and how these differences lead to
686 variation in brain neuron distribution and abundance, would aid in our understanding of
687 how brain development varies between AMTs.

Registering changes in photoperiod also has an effect on concentration of melatonin, which has been found to influence migratory behavior in both birds (Schneider et al., 1994) and salmon (Ojima and Iwata, 2009). In this study, pathways associated with the degradation of melatonin are altered between smolts and residents in R x R males, and A x A females. Several genes within the degradation of melatonin pathway (such as *cyp2F1*, *UGT2A1*, and *MPO*) are upregulated in smolts compared to residents in both the R x R male and A x A female samples. Melatonin is an inhibitor of myeloperoxidase (*MPO*), therefore the upregulation of *MPO* in smolt samples suggests a reduction of melatonin in smolts at the time of sampling. Other studies in *O. mykiss* have found upregulation of melatonin related genes (especially arylakylamine N-acetyltransferase; *AANAT*) when smolts are placed in salt water (Lopez-Patino et al., 2011). Moreover, experimental increases in the concentration of melatonin have been shown to improve osmoregulatory function in seawater in *O. mykiss* (Sangiao-Alvarellos et al., 2007), decrease mortality during smoltification in *S. salar* (Porter et al., 1998), and affect the timing of smoltification in Masu salmon (Iigo et al., 2005). Melatonin production has also been shown to regulate circadian rhythm genes such as *Clock* and *Period*, and these genes have been found to influence the development of salmonid AMTs (O'Malley et al., 2007; Bromage et al., 2001). Although we did not find differential expression of any candidate circadian rhythm genes, the canonical pathway circadian rhythm signaling, was altered between residents and smolts in the female R x R comparison, suggesting that other genes involved in these processes are differentially regulated in the brain. The pineal organ is a key site of melatonin production (Ekstrom and Meissl, 1997), and is

711 located on the roof of the brain. Therefore, our whole brain extractions included the
712 pineal organ. Although as already mentioned the brain is not a homogenous structure, and
713 patterns of gene expression vary between its different components.
714
715 **5. Conclusions**
716 Here, we describe many genes and pathways that have different expression patterns
717 between smolting and non-smolting individuals. The R x R and A x A crosses both
718 produced residents and smolts in the June time point. However, the genes and pathways
719 that are differentially expressed between smolts and residents differ between cross types.
720 This is unlike studies of freshwater adaptation in three-spined sticklebacks (*Gasterosteus*
721 *aculeatus*), where multiple studies have found evidence of parallel evolution involving
722 the same regions of the genome (e.g. Peichel et al*.,* 2001; Colosimo et al*.,* 2005;
723 Hohenlohe et al*.,* 2010). A natural extension of the work presented herein is to investigate
724 the migratory and resident transcriptomes of *O. mykiss* in other populations. Are the same
725 genes and pathways significantly altered as presented here? What about other species of
726 salmonids? How applicable the expressed genes and pathways described herein will be in
727 understanding the genetic basis of migration in other taxonomic groups is unclear. AMTs
728 exhibited by birds, mammals, and insects often do not differ in such dramatic
729 physiological processes as in salmonids. However, there are still parallels in growth,
730 morphology, and metabolism that are necessary to undertake long distance migrations.
731 As the genetic and molecular determinants of migration are studied in parallel taxonomic
732 groups, we may better understand these alternative life histories have evolved, and the
733 proximate mechanisms promoting very different developmental trajectories.

734

735 **Sequence data**

736 Quality filtered paired end sequence data used in estimating gene expression is deposited
737 in the Short Read Achieve repository in GenBank, accession number PRJNA269115.

738

739 **Supplementary files**

740 Supplementary file 1: Primer sequences for qPCR analysis of RNA-seq validation genes
741 Supplementary file 2: Average monthly water temperature in degrees centigrade from the
742 start of sampling (June 2010) to June 2012.
743 Supplementary file 3: Sex and phenotype information for the samples sequenced when
744 available. Filtered reads is the number of million reads that passed quality filtering.
745 Mapped reads is the number of million reads that mapped to the transcriptome.
746 Supplementary file 4: Principal component analyses constructed from transcripts per
747 million (tpm) for all genes that met filtering criteria (see methods). Plot A shows the first
748 two principal components for all samples in the study herein. Open circles donate
749 February A x A samples, closed circles February R x R samples, open triangles represent
750 the June A x A samples, closed triangles represent the June R x R samples. Plot B shows
751 the first two principal components for the June samples, open circles represent the A x A
752 samples, closed circles represent the R x R samples. Plot C shows the first two principal
753 components for the June samples, closed circles represent resident samples, open circles
754 migratory samples.

755 Supplementary file 5: Cluster analysis on transcripts identified in Gahr et al. (2007) and
756 differentially expressed genes in the current study. Sequences from the SRA database
757 were included to test for the presence of pituitary expressed transcripts.
758 Supplementary file 6: Gene expression information from both annotated and unannotated
759 genes. S6.1 Genes that were differentially expressed between cross type. S6.2 Genes that
760 were differentially expressed between migratory tactics. S6.3 All annotated genes in the
761 assembly. S6.4 Estimates of gene expression between cross type for the February time
762 point. S6.5 Estimates of gene expression between cross type from the June samples. S6.6
763 estimates of gene expression between resident and smolt samples from the June time
764 point. S6.7 TPM values for February R x R samples. S6.8 TPM values for February A x
765 A samples. S6.9 TPM values for June R x R samples. S6.10 TPM values for June A x A
766 samples.
767 Supplementary file 7: All biofunctions that were enriched between R x R and A x A
768 samples. Positive values for Z scores indicate more genes within the pathway were
769 upregulated in R x R samples, a negative Z scores indicate more genes are upregulated in
770 the A x A samples.
771 Supplementary file 8: All biofunctions that were enriched between residents and smolts.
772 Positive values for Z scores indicate more upregulated genes in the residents, negative Z
773 scores indicate more upregulated genes in the smolts.
774 Supplementary file 9: Altered canonical pathways between cross type.
775 Supplementary file 10: Altered canonical pathways between residents and smolts.
776 Supplementary file 11: Differentially expressed genes that mapped to the *O. mykiss*
777 genome (Berthelot et al., 2014). Provided are the genes, chromosome, percentage

778 sequence similarity, number of mismatches, e-values, and annotation information (where
779 available).
780
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793
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1103

1104 Table 1. Top altered canonical pathways between cross type in the February samples.
1105 Pathways above the line were altered in the female samples, pathways below the line
1106 were altered in the male samples. The differentially expressed genes within the pathway
1107 are listed as abbreviations, for the complete name see Supplementary File 6 S6.3.

Altered Canonical Pathways	p-value	Differentially expressed genes in pathway
Phototransduction Pathway	0.001	PDE6A,PDC,GUCA1B,GNAT2,RCVRN
G Protein Signaling Mediated by Tubby	0.001	LCK,GNG5,TUB,JAK2
NF-ŒʃB Signaling	0.008	LCK,CD40,TLR1,PIK3R5,MALT1,TNFRSF11B, JAK2
Role of JAK2 in Hormone-like Cytokine Signaling	0.011	
Alanine Biosynthesis III	0.013	NFS1
Role of Tissue Factor in Cancer	0.018	LCK,ARRB1,PIK3R5,F7,JAK2
Dermatan Sulfate Biosynthesis	0.021	CHST2,SULT1C4,NDST1
VDR/RXR Activation	0.022	LRP5,COL13A1,IL1RL1,KLF4
RhoA Signaling	0.022	NRP2,SEPT4,PFN2,DLC1,MSN
MSP-RON Signaling Pathway	0.023	ITGB2,PIK3R5,JAK2
Cellular Effects of Sildenafil	0.000	PRKG1,CACNA1E,GNAS,MPRIP,CACNA1D,GUCY1A3,ADCY5,ITPR2 ,MYLPF,MYH9,MYLK,PRKG2,PLCD4
Synaptic Long Term Depression	0.000	PRKG1,GNAS,GUCY1A3,ITPR2,NPR1,RYR3,GNAQ,PLA2G3,RYR1,PP P2R2C,PRKG2,PLCD4,GRIA3
Dopamine-DARPP32 Feedback in cAMP Signaling	0.000	GRIN2B,CACNA1D,GNAS,CSNK1G1,GUCY1A3,ITPR2,GNAQ,PRKG2 ,ATP2A2,CACNA1E,PRKG1,ADCY5,PPP2R2C,PLCD4
Glutamate Receptor Signaling	0.001	SLC17A8,GRIN2B,GRIK5,HOMER2,SLC17A6,SLC1A7,GRIA3
Corticotropin Releasing Hormone Signaling	0.002	GNAS,JUN,GUCY1A3,ITPR2,ADCY5,NPR1,GNAQ,MAPK11,PTCH2
Sperm Motility	0.003	PRKG1,GNAS,GUCY1A3,ITPR2,NPR1,PLA2G3,PRKG2,PLCD4, CNGA3
Gap Junction Signaling	0.006	PRKG1,GNAS,CSNK1G1,GUCY1A3,ITPR2,ADCY5,NPR1,GNAQ,PRK G2,PLCD4
GŒ±s Signaling	0.007	GNAS,ADCY5,HTR7,RYR3,PTH1R,RYR1,RAPGEF4,CNGA3
Inhibition of Angiogenesis by TSP1	0.010	JUN,GUCY1A3,MAPK11,MMP9
Phototransduction Pathway	0.010	PRKG1,CACNA1E,GNAS,MPRIP,CACNA1D,GUCY1A3,ADCY5,ITPR2 ,MYLPF,MYH9,MYLK,PRKG2,PLCD4

1108

1109 Table 2. Top altered canonical pathways between cross type in the June samples.
1110 Pathways above the line were altered in the female samples, pathways below the line
1111 were altered in the male samples. The differentially expressed genes within the pathway
1112 are listed as abbreviations, for the complete name see Supplementary File 6 S6.3.

Altered Canonical Pathways	p-value	Differentially expressed genes in pathway
Phototransduction Pathway	0.000	PDE6G,ARR3,PDE6A,RHO,PDC,RGR,PRKAG2,GUCA1B,PDE6C ,CNGB3 ,CNGA1,PDE6B,RCVRN
The Visual Cycle	0.000	RLBP1,RBP3,RDH8,RPE65
Retinoate Biosynthesis I	0.000	ALDH1A3,RDH16,DHRS7C,RDH8
tRNA Splicing	0.001	PDE6G,PDE6A,PDE6C,PDE6B
RAR Activation	0.001	ALDH1A3,RDH16,PRKAG2,DHRS7C,JAK2,RBP3,RDH8,RBP4
Atherosclerosis Signaling	0.003	COL1A1,LPL,COL2A1,PLA2G3,COL11A2,RBP4
Intrinsic Prothrombin Activation Pathway	0.004	COL1A1,COL2A1,COL11A2
Protein Kinase A Signaling	0.005	PDE6G,PDE6A,RHO,PTPN9,PDE6C,PRKAG2,CNGB3,CNGA1,P DE6B,NFATC1,GRK7
T Cell Receptor Signaling	0.005	CD28,VAV1,MALT1,LCP2,NFATC1
Serotonin Receptor Signaling	0.006	GCH1,TPH1,SLC6A4
Phototransduction Pathway	0.000	PDE6G,PDC,PDE6C,GUCA1B,PRKAG2,CNGB3,GNGT2,CNGA1, RCVRN
Coagulation System	0.001	F8,PROS1,F7,TFPI,A2M
Intrinsic Prothrombin Activation Pathway	0.003	COL1A1,F8,PROS1,COL2A1
Extrinsic Prothrombin Activation Pathway	0.005	PROS1,F7,TFPI
Serotonin and Melatonin Biosynthesis	0.007	TPH1,DDC
tRNA Splicing	0.007	PDE6G,PDE6C,PDE4D,PDE1C
Epithelial Adherens Junction Signaling	0.015	CDH1,MYH6,AKT1,ACTB,PVRL3,TUBB4A,MAGI2,CLIP1
Remodeling of Epithelial Adherens Junctions	0.017	CDH1,ACTB,RAB7A,TUBB4A,CLIP1
cAMP-mediated signaling	0.023	OPRD1,PDE6G,NPR3,DUSP1,OPRM1,PDE6C,CNGB3,PDE4D,C NGA1,PDE1C
HER-2 Signaling in Breast Cancer	0.026	CCNE1,AKT1,CDKN1A,MMP2,ITGB7

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1116 Table 3. Top altered canonical pathways between smolts and residents in the R x R samples.
1117 Pathways above the line were altered in the female samples, pathways below the line were
1118 altered in the male samples. The differentially expressed genes within the pathway are listed
1119 as abbreviations, for the complete name see Supplementary File 6 S6.3.

Altered Canonical Pathways	p-value	Differentially expressed genes in pathway
Phototransduction Pathway	0.000	PDE6G,PDE6C,GUCA1B,CNGA1,CNGA3,PDE6A,ARR3,RHO,PDC, GUCY2F,SAG,CNGB3,GNGT2,PDE6B,RCVRN
The Visual Cycle	0.000	RLBP1,RBP3,RDH8,RPE65
tRNA Splicing	0.000	PDE6G,PDE6A,PDE6C,PDE6B
cAMP-mediated signaling	0.001	PDE6G,PDE6A,VIPR2,CREBBP,PDE6C,CNGB3,CNGA1,PDE6B,CNGA3
Protein Kinase A Signaling	0.001	PDE6G,PDE6A,PTPN11,RHO,CREBBP,PDE6C,CNGB3,CNGA1,EYA1, PDE6B,CNGA3,GRK7
Stearate Biosynthesis I	0.006	FASN,SLC27A6,ACSBG2
GŒ±s Signaling	0.007	VIPR2,CREBBP,CNGB3,CNGA1,CNGA3
Fatty Acid Activation	0.008	SLC27A6,ACSBG2
RAR Activation	0.011	NR2F1,RDH16,CREBBP,RBP3,RDH8,RBP4
eNOS Signaling	0.013	AQP1,CNGB3,AQP8,CNGA1,CNGA3
Phototransduction Pathway	0.000	PDC,GUCA1B,CNGB3,GNGT2,CNGA1,RCVRN
Atherosclerosis Signaling	0.010	COL2A1,PLA2G3,COL18A1,TNFRSF14,RBP4
Ethanol Degradation IV	0.016	TYRP1,ALDH1A3
Endoplasmic Reticulum Stress Pathway	0.016	ERN1,ATF6
Aryl Hydrocarbon Receptor Signaling	0.018	AHRR,CCND2,TP73,ALDH1A3,CDKN1A
Nicotine Degradation III	0.019	CYP2F1,UGT2A1,CYP2U1
GADD45 Signaling	0.019	CCND2,CDKN1A
Melatonin Degradation I	0.021	CYP2F1,UGT2A1,CYP2U1
Superpathway of Melatonin Degradation	0.027	CYP2F1,UGT2A1,CYP2U1
Nicotine Degradation II	0.028	CYP2F1,UGT2A1,CYP2U1

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1123 Table 4. Top altered canonical pathways between smolts and residents in the A x A
1124 samples. Pathways above the line were altered in the female samples, pathways below the
1125 line were altered in the male samples. The differentially expressed genes within the
1126 pathway are listed as abbreviations, for the complete name see Supplementary File 6
1127 S6.3.

Altered Canonical Pathways	p-value	Differentially expressed genes in pathway
Acute Phase Response Signaling	0.000	ALB,HPX,C3,APOA1,MYD88,AHSG,SERPINA1,C5,FGB,FGG
LXR/RXR Activation	0.000	ALB,HPX,APOB,C3,APOA1,AHSG,SERPINA1
Complement System	0.002	C3,C5,CFH
Atherosclerosis Signaling	0.002	ALB,APOB,APOA1,SERPINA1,TNFRSF14
Coagulation System	0.002	SERPINA1,FGB,FGG
Clathrin-mediated Endocytosis Signaling	0.003	ALB,APOB,ARPC1A,APOA1,SERPINA1,LDLRAP1
IL-12 Signaling and Production in Macrophages	0.004	ALB,APOB,APOA1,MYD88,SERPINA1
Extrinsic Prothrombin Activation Pathway	0.006	FGB,FGG
Melatonin Degradation III	0.008	MPO
Phototransduction Pathway	0.008	PRKAG2,CNGB3,GNGT2
Serotonin Receptor Signaling	0.000	GCH1,DDC,SLC6A4,SLC18A3,SLC18A2
Corticotropin Releasing Hormone Signaling	0.000	VEGFA,UCN3,POMC,PTGS2,CREB5,PRKD3,GUCY1B3
Nitric Oxide Signaling in the Cardiovascular System	0.001	VEGFA,PRKG1,ATP2A3,PRKD3,GUCY1B3,CACNA1A
Cardiac Œ≤-adrenergic Signaling	0.006	GNB3,PDE9A,PDE10A,ATP2A3,AKAP9,CACNA1A
Relaxin Signaling	0.006	VEGFA,GNB3,PDE9A,PDE10A,RLN3,GUCY1B3
L-dopachrome Biosynthesis	0.012	TYR
Dopamine Receptor Signaling	0.013	GCH1,DDC,SLC18A3,SLC18A2
Dermatan Sulfate Degradation	0.013	FGFRL1,HYAL4
Sperm Motility	0.014	PRKG1,NPPC,PLA2G3,PRKD3,GUCY1B3
Dopamine-DARPP32 Feedback in cAMP Signaling	0.014	PRKG1,ATP2A3,CREB5,PRKD3,GUCY1B3,CACNA1A

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Figure legends:

Figure 1. Proportion of offspring per family classified as smolts, matures, residents, and indeterminate (Inde) at 24 months after fertilization. A x A samples (black bars) were produced by crossing anadromous parents, R x R samples (gray bars) were produced by crossing resident parents.

Figure 2. Cluster analyses conducted from transcripts per million for all differentially expressed genes (annotation information is included where known). Plot A shows clustering of differentially expressed genes between cross-type in the February samples. Plot B shows clustering of differentially expressed genes between phenotype in the June R x R samples. Plot C shows clustering of differentially expressed genes between phenotype in the June A x A samples. In all heatmaps white indicates high expression and red low expression.

Figure 3. The number of differentially expressed genes between R x R and A x A samples. Gray bars include the number of genes that were upregulated in the R x R samples and black bars indicate the number upregulated in the A x A samples.

Figure 4. The number of differentially expressed genes between resident and smolt samples for the June time point. The gray bar represents the number of differentially expressed genes that are upregulated in smolts, the black bar in residents (A x A F = A x A females, A x A M = A x A males, R x R F = R x R females, and R x R M = R x R males).

Figure 5. Scatter plots showing relationship between estimates of gene expression from RT-PCR and RNA-seq data. A) Samples within the February time point, R x R divided by A x A, values greater than 1 indicate upregulation in the R x R samples, values less than 1 indicate upregulation in the A x A samples. B) Samples within the June A x A cross, values greater than 1 indicate upregulation in the resident samples, values less than 1 indicate upregulation in the smolt samples. C) Samples within the June R x R cross, values greater than 1 indicate upregulation in the resident samples, values less than 1 indicate upregulation in the smolt samples.

Figure 6. Gene network drawn from Ingenuity pathway analysis software (Qiagen) generated from 24 molecules involved in the conical pathways phototransduction and visual cycle in the R x R female samples from the June time point. This network illustrates the complex nature of visual changes between smolts and residents. Molecules with an asterisk signify that molecule was differentially expressed between AMTs. Molecules in green are upregulated in smolts, molecules in gray are missing from the dataset.

1174 Figure 1
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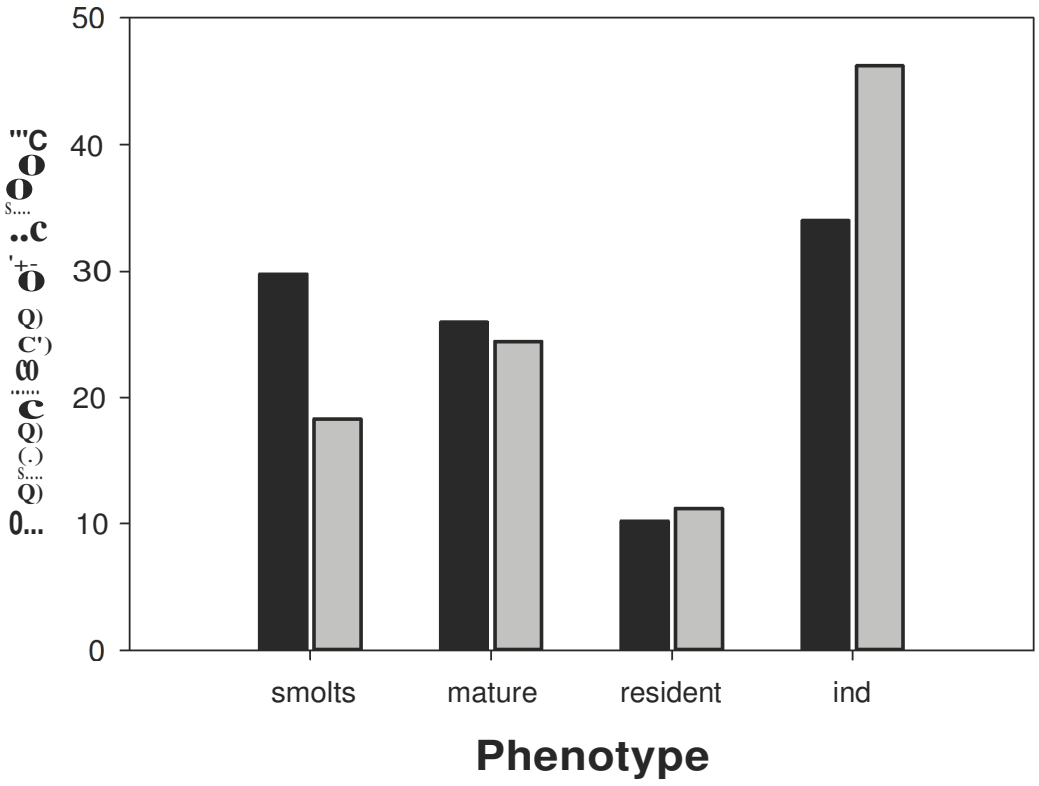

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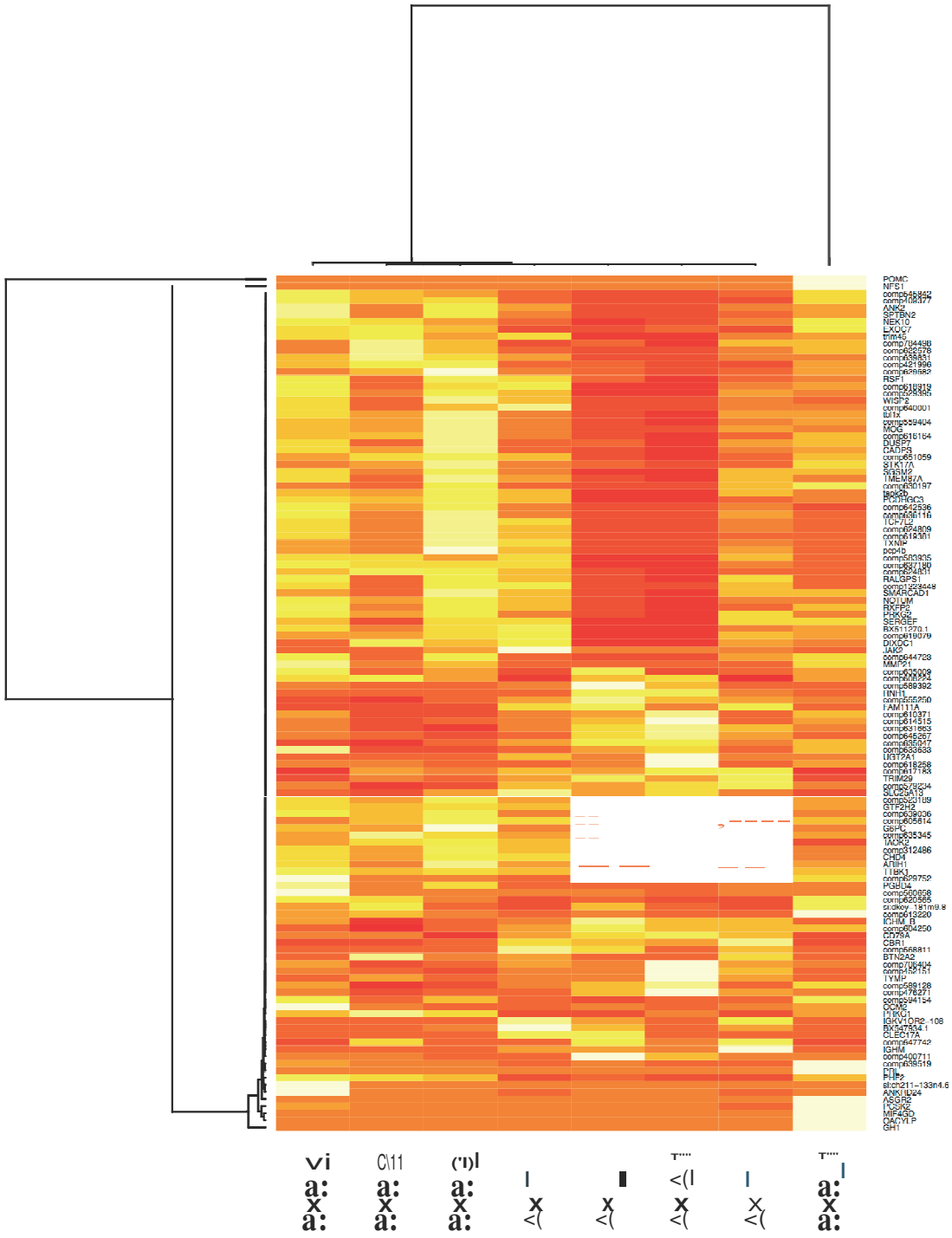
Figure 2 a.



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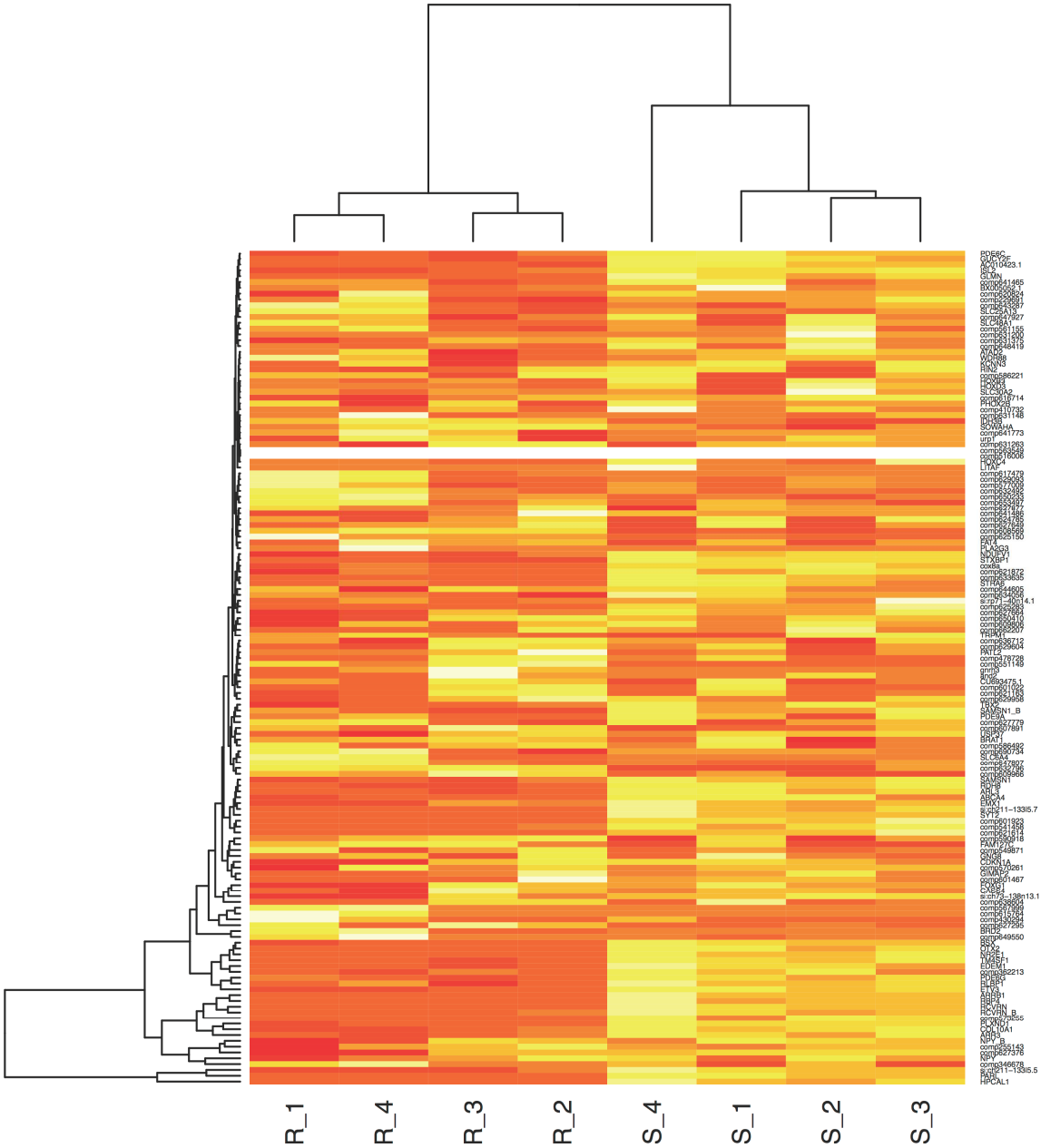

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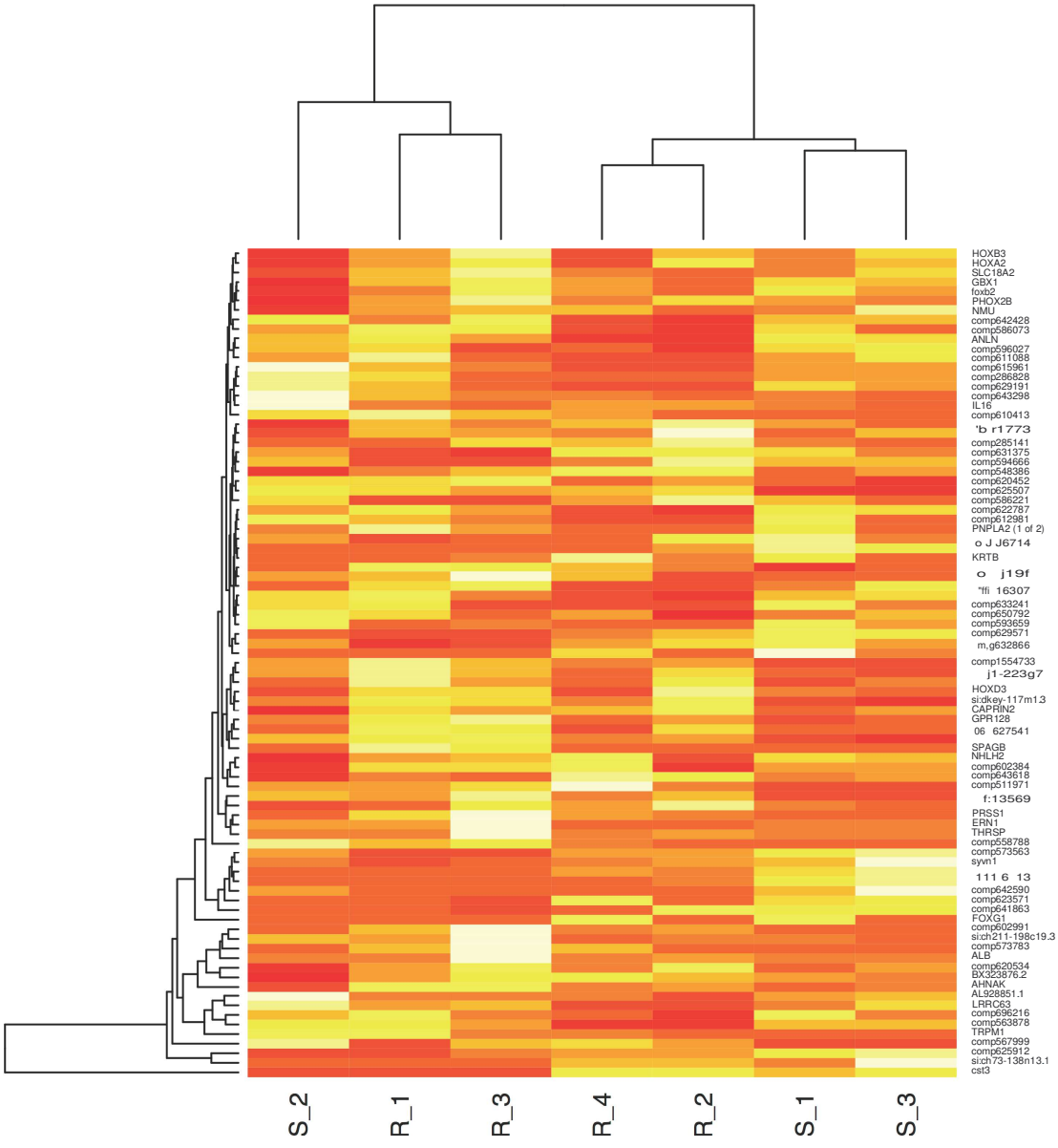
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foxb2
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comp286828
comp629191
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IL16
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o j19f
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comp625912
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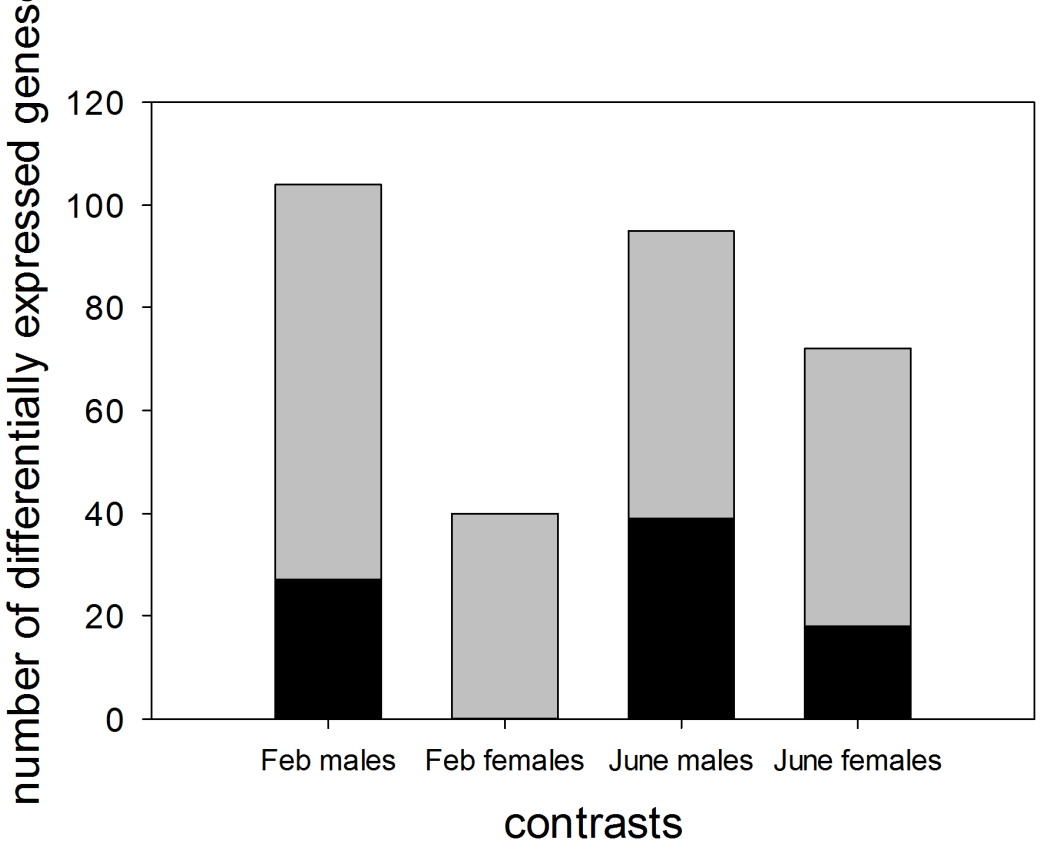
S_2
R_1
R_3
R_4
R_2
S_1
S_3

1198 Figure 3



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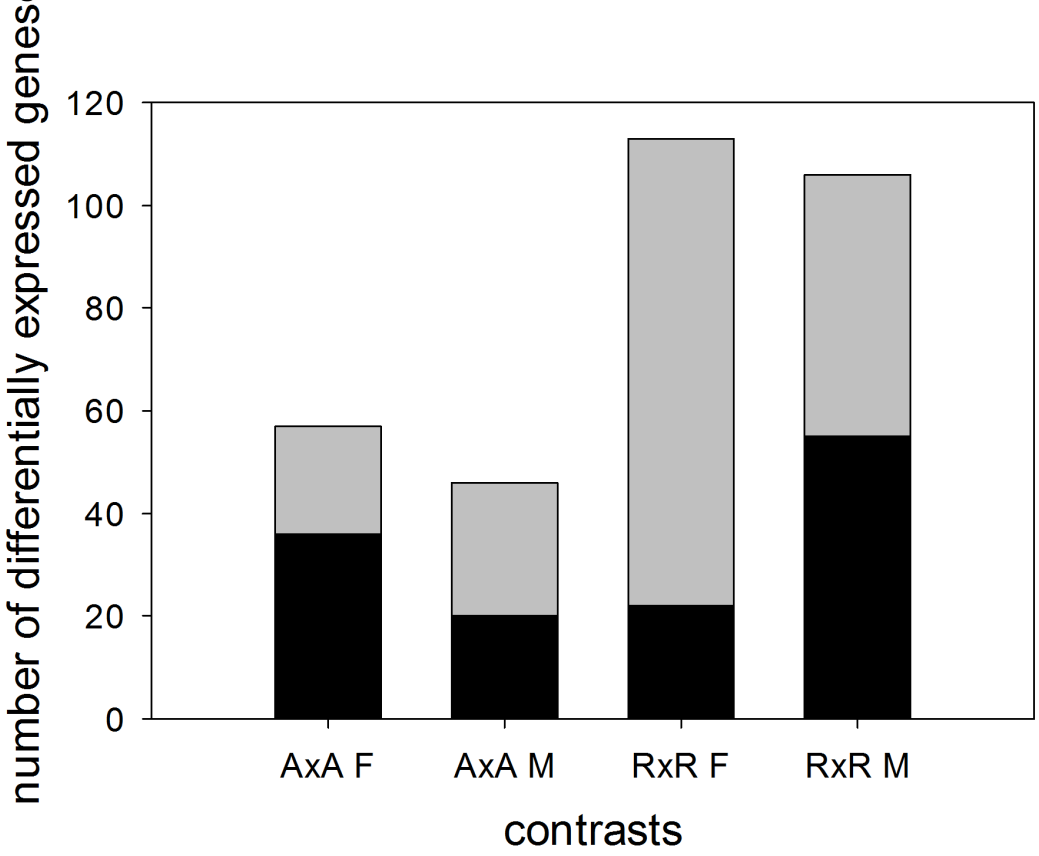
1201 Figure 4



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1204 Figure 5 a.
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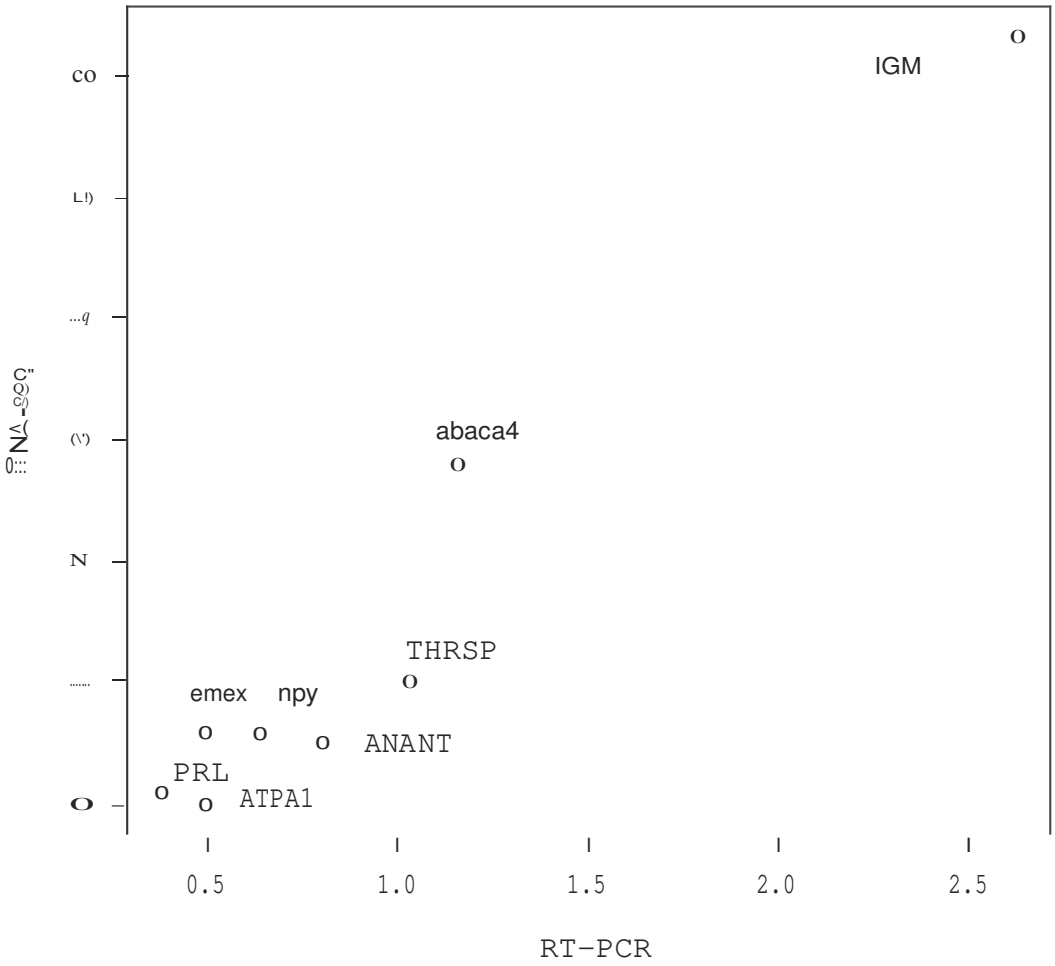

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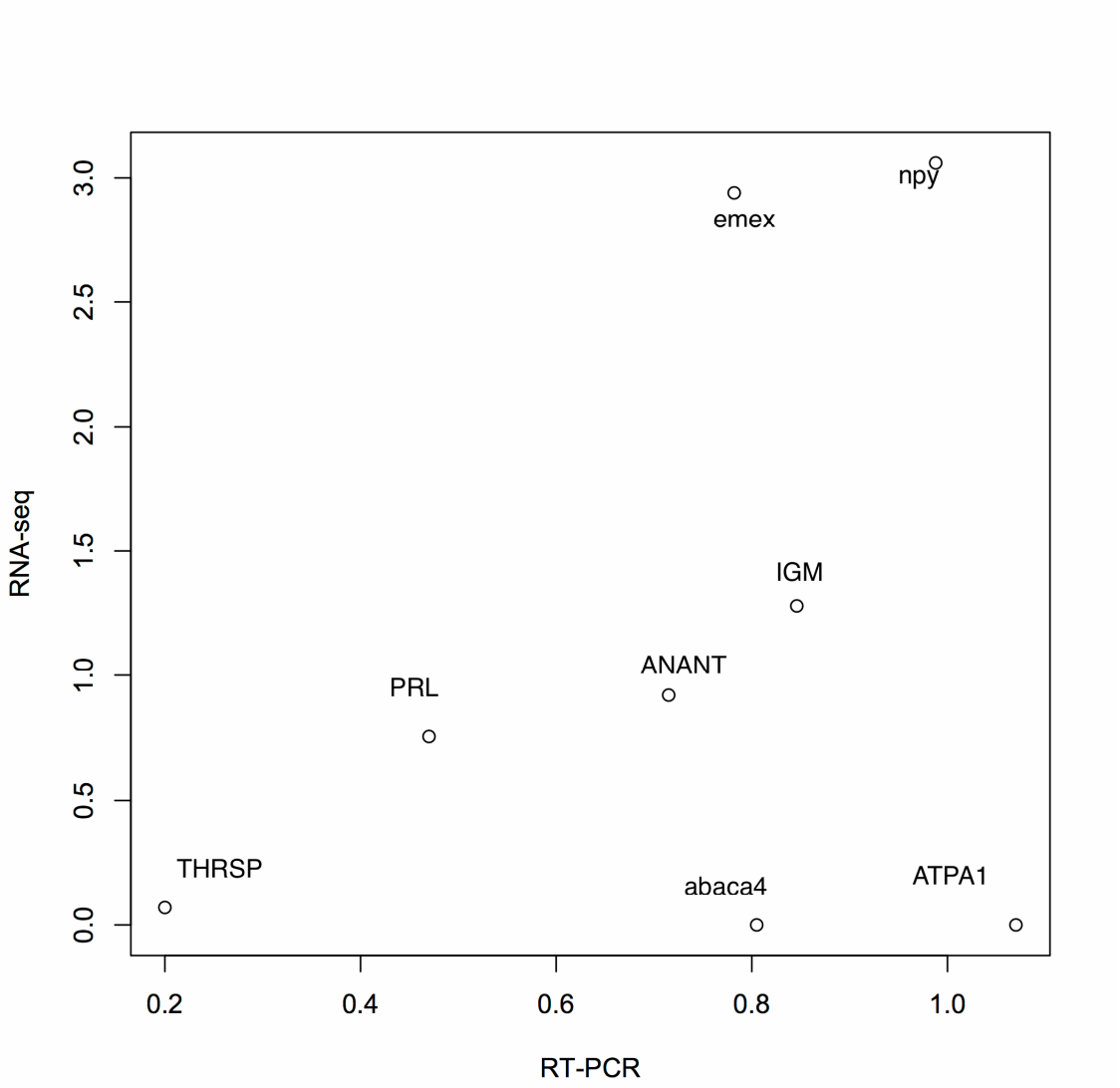

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1211 c.
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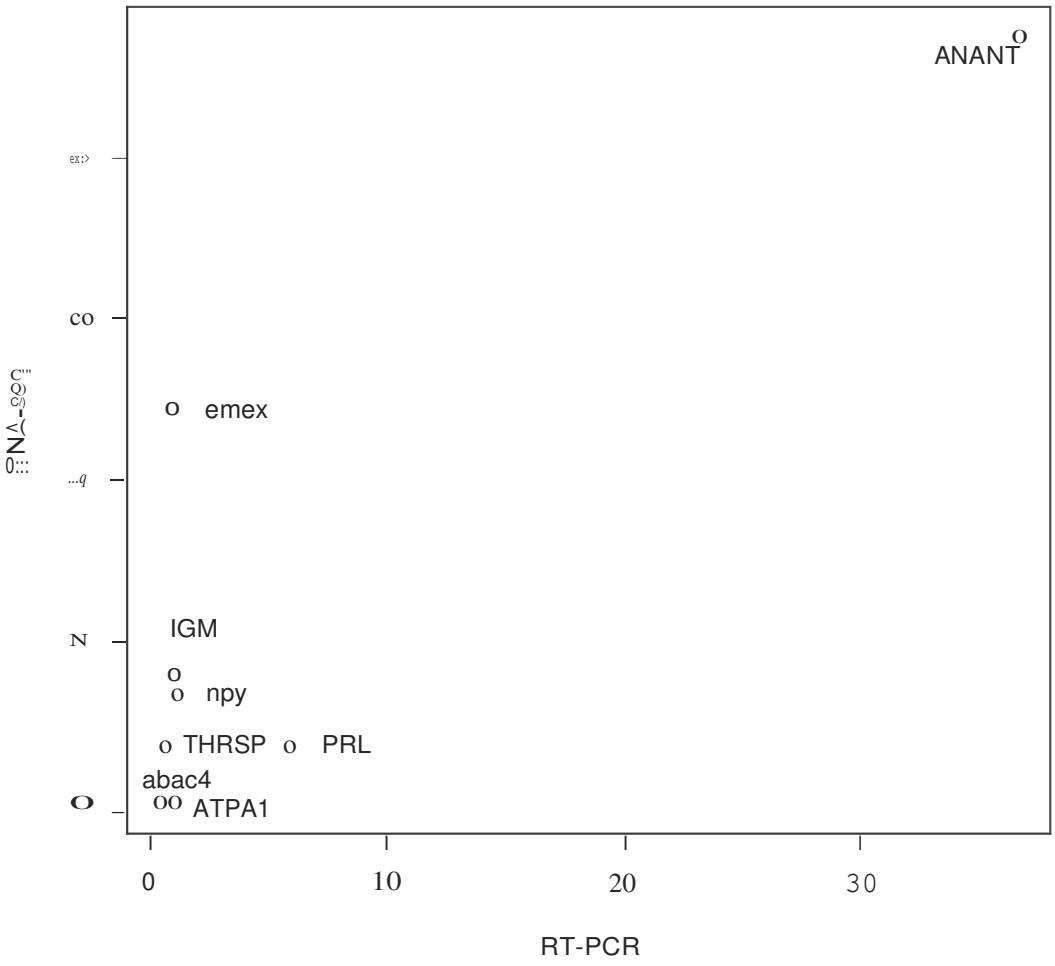

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1214 Figure 6