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Alternative life histories shape brain gene expression profiles in males of the same population

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Atlantic salmon (*Salmo salar*) undergo spectacular marine migrations before homing to spawn in natal rivers. However, males that grow fastest early in life can adopt an alternative 'sneaker' tactic by maturing earlier at greatly reduced size without leaving freshwater. While the ultimate evolutionary causes have been well studied, virtually nothing is known about the molecular bases of this developmental plasticity. We investigate the nature and extent of coordinated molecular changes that accompany such a fundamental transformation by comparing the brain transcription profiles of wild mature sneaker males to age-matched immature males (future large anadromous males) and immature females. Of the *ca.* 3000 genes surveyed, 15% are differentially expressed in the brains of the two male types. These genes are involved in a wide range of processes, including growth, reproduction and neural plasticity. Interestingly, despite the potential for wide variation in gene expression profiles among individuals sampled in nature, consistent patterns of gene expression were found for individuals of the same reproductive tactic. Notably, gene expression patterns in immature males were different both from immature females and sneakers, indicating that delayed maturation and sea migration by immature males, the 'default' life cycle, may actually result from an active inhibition of development into a sneaker.

Keywords: microarray; gene expression; plasticity; behaviour; reproduction; brain

1. INTRODUCTION

In many species, differences in environmental conditions can result in striking divergences in life history. Atlantic salmon, *Salmo salar*, exhibit spectacular male alternative reproductive life histories that are the result of developmental plasticity. Males either attain sexual maturity precociously as parr (first reproduction at 1–3 years) to become *sneakers* (stealing mating) that are 10 times smaller than their migratory conspecifics or migrate out to sea and return at the age of 3–7 years as large *anadromous* animals (Fleming 1998). The quantitative genetic, behavioural and physiological differences between sneakers and immature males have been studied extensively (figure 1; Hutchings & Myers 1994; Aubin-Horth & Dodson 2004). However, little is known regarding the underlying molecular mechanisms of this developmental plasticity. It is generally assumed that the regulation of gene activity is an important component of phenotypic plasticity (West-Eberhard 2003). The advances in functional genomics, particularly the simultaneous monitoring of thousands of genes in individual tissues, can now facilitate a synthesis between mechanistic insights at the molecular, cellular, and physiological levels and an organismal understanding of plasticity within an evolutionary framework.

Here we present the first systematic analysis of the activity of thousands of genes in the brains of these wild animals, the location where environmental demands are integrated with physiological status to sculpt the phenotype (Hofmann 2003; Whitfield *et al.* 2003). Analysing brain gene expression profiles allows us to identify (i) how many genes vary in expression between sneaker and immature male brains; (ii) what biological processes are implicated and how they relate to known physiological, behavioural and morphological differences between tactics as well as to life-history theory predictions; (iii) genes previously not implicated in the sneaker tactic; and (iv) determine if expression patterns can classify individuals according to their tactic in wild animals. We measured brain gene expression profiles in four sneaker males and eight immature juveniles (four males and four females), collected at spawning time in October 2002 in a tributary of the Connecticut River, Massachusetts, USA, using a microarray constructed from salmonid cDNA libraries (Rise *et al.* 2004), which allows the monitoring of the transcriptional level of thousands of genes simultaneously. We focused on differences between individuals of the same age (freshwater life before migration), but with different phenotypes, and therefore did not include large anadromous males and females, who are several years older and are protected. Our results illustrate how hypotheses regarding life-history evolution can be addressed using a genomics approach (Stearns & Magwene 2003).

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2. METHODS

(a) *Sampling*

We collected immature females, immature males and mature sneaker males of Atlantic salmon (*Salmo salar*) of age 1 in the Sawmill River (42° 30′ N, 74° 30′ W), a tributary of the Connecticut River in Western Massachusetts, USA, in October 2002. All fishes used were collected between 1100 and 1400 of the same day. Electrofishing was used to collect fishes individually. Each fish was immediately euthanized in buffered MS-222 (100 mg l^{-1}). Length and mass were then measured, and the brain was dissected within 5 min after death and transferred to RNAlater storage solution (Ambion), which stops RNAse activity. The interval between collecting and tissue storage was always less than 11 min. Sex was then determined by dissection.

(b) *Microarray hybridization*

Four fishes (biological replicates) were assayed separately per phenotype. Total RNA was extracted from brains according to a standard Trizol protocol (Invitrogen), following tissue homogenization (Tissue Tearor, Biospec Products). All RNA samples were kept separate in subsequent analyses. Four independent reverse transcriptions were performed on total RNA extracted from each brain sample according to a standard 3DNA array 350 protocol (Genisphere). These cDNA samples from one individual were then pooled and aliquoted. cDNA from each fish was used four times (technical replicates, including two dye-swaps) in a loop design comparison, such that an individual of a given group (sneaker male, immature male, female) was directly compared with two individuals of each of the other groups (figure 2), without the need for a reference sample (Churchill 2002; Townsend 2003). Using such a loop design with biological and technical replicates and including dye-swaps for each individual has been shown to substantially increase reliability of microarray results and to reduce the probability of dye bias by means of transitive comparisons (Liang *et al.* 2003; Townsend 2003). The microarray used was constructed from 18 high complexity salmonid cDNA libraries (described in Rise *et al.* 2004). We purchased this microarray, called the '3700 array' from the Genomic Research on Atlantic Salmon Project (GRASP) consortium. Physical platform description is available at NCBI GEO GPL966. Clone sequences, library of origin, and annotation are available from the GRASP Web site (http://web.uvic.ca/cbr/grasp/). We used 24 microarrays to compare 48 independent labelling reactions (figure 2). cDNA samples were competitively hybridized on a microarray slide according to a standard 3DNA Array 350 protocol (Genisphere). Arrays were scanned with an Axon 4000B scanner (Axon Instruments) using GENEPIX 4.0 software (Axon Instruments). Spots were examined individually and flagged as 'bad' if irregularities occurred.

(c) *Transcription analysis*

Raw data (after flags filtering) were imported into R software (v. 1.9; R Development Core Team 2004) and normalized using the Linear Models for Microarray Data package (Limma v. 1.6.5; Smyth *et al.* 2003). Background-subtracted mean intensities were normalized using a within array loess normalization. These intensities were used in a Bayesian analysis of gene expression levels (BAGEL v. 3.6 (Townsend & Hartl 2002)). Out of the 7356 spots containing salmonid cDNA, 659 spots could not be reliably analysed because of low hybridization quality in most replicates, such that 6697 spots were used in the BAGEL analysis. We determined that this corresponded to 2917 genes, using the TIGR gene indices for ATLANTIC SALMON v. 1.0 (Quackenbush *et al.* 2000). This method estimates gene expression level and error variances for each gene by Markov chain Monte Carlo integration of the likelihood function of observed gene expression ratios, and incorporates a prior distribution for the parameters. A non-informative uniform prior distribution is used by default by this method. BAGEL takes advantage of additional information obtained from transitive comparisons of individuals in loop designs experiments (Churchill 2002; Townsend & Hartl 2002; Townsend 2003). For each gene, we obtain a relative gene expression level for each group, 95% credible intervals for gene expression level and a Bayesian posterior probability (p) of significant differences between two groups. Genes found to be significantly highly expressed in sneaker male brains relative to immature male brains ($p<0.01$) were labelled 'sneaker biased' while genes found significantly highly expressed in immature males compared with sneaker males ($p<0.01$) were labelled 'immature male biased'.

(d) *Functional annotation*

Each significantly differentially regulated gene between sneaker and immature males, as determined by the Bayesian analysis, was assigned to a biological process category following its annotation by the GRASP EST database (http://web.uvic.ca/cbr/grasp/) and TIGR gene indices. Genes with no sequence, that had a BLAST hit to unannotated sequence or that were of unknown function in the brain were removed from analysis. Seventeen biological processes were associated to differences in the brain of sneaker and immature males. Each gene found to be significantly upregulated in one male type compared with the other male type had an associated biological process. Therefore, genes related to a biological process can be sneaker biased, while other genes classified in that same biological process can be immature male biased. We hypothesize that if a biological process is generally independent of the alternative phenotypes examined here, genes associated with that process would probably be represented at random (equally) in both sneaker-biased and immature male-biased gene lists. If non-random distributions of genes that are linked to a biological process are found between the sneaker-biased gene list and the immature male-biased gene list, then we hypothesized that this biological process is statistically associated with, and is probably important for, the development of the phenotype. For each biological process category, the proportion of sneaker-biased and immature male-biased genes was calculated. An exact binomial test (R software v. 1.9) was performed to detect statistically significant over-representation towards one reproductive tactic in the number of genes in this category.

(e) *Individual variation in transcription profiles*

We asked whether the overall gene expression profiles are shared among representatives of the same phenotype despite the individual variation probably present in animals collected in the natural environment. To this end, we used unsupervised hierarchical clustering to investigate how expression profiles of individual fish group. Using the Bayesian analysis of gene expression levels (BAGEL), we first estimated gene expression levels for each of the twelve individuals separately.

In the cluster analysis, we then used the expression levels for each individual of the 1017 genes that had shown significant differential expression in the first Bayesian analysis comparing phenotypes. We created 1000 gene expression datasets of the same size as the original by re-sampling gene expression levels with replacement in the individual gene expression dataset (bootstrap; Sokal & Rohlf 1995). Pearson correlation coefficients (r) were calculated between $\log_2$-transformed brain gene expressions of all individuals for each of these 1000 datasets and the resulting dissimilarity indices (1-r) were used in a clustering analysis of individuals (successive agglomerative clustering using an average-linkage method, UPGMA method, Neighbour package, PHYLIP v. 3.6 (Felsenstein 2004)). The 1000 clusters obtained were used to construct a consensus tree (Consense package, PHYLIP v. 3.6), which was visualized using TREEVIEW (v. 1.6.6; Page 1996).

(f) *Quantitative real-time PCR*

The same individuals used in the microarrays were used in the quantitative real-time PCR validation experiments except for one individual (immature male) for which there was no RNA left. RNA samples were first treated with DNAse I following manufacturer protocol (Invitrogen). An aliquot of these RNA samples was used in a Ribogreen assay (Invitrogen) to quantify RNA concentration of each sample based on fluorescence. This allows using exactly the same amount of starting RNA for each sample in later reverse transcription reactions and alleviates the need for inclusion of 'house keeping genes' (Hashimoto *et al.* 2004). Reverse transcription was performed in duplicate according to standard superscript II protocol (Invitrogen) using a primer solution containing poly dT and random hexamers (Invitrogen). cDNA from two replicates was then pooled. 5 ng of cDNA was used in a quantitative real-time PCR assay using a standard SYBR-GREEN RTPCR manufacturer's protocol (Qiagen) in an Opticon thermocycler (MJ research). Each fish was tested in three replicates for a given gene. Primers were designed using PRIMER3 and AMPLIFY (1.0) software. Primer sequences were: Prolactin: 5-CGCCCACTCTACCACACC-3 and 5-AGCAGGACATGAGGAAGTGG-3; Pro-opiomelanocortin (POMC): 5-GAGCAGTGGTTTCTGACTGC-3 and 5-GGAGGCTGGGACTGCGGA-3; and Somatostatin: 5-AGCCAAGGAGCTGCCTCG-3 and 5-ACACGCAGG TCCTCTTTGG-3.

3. RESULTS AND DISCUSSION

Of the 2917 genes included in the analysis, 432 genes or 15% were significantly differentially expressed in the brain of mature sneaker males compared with immature males ($p<0.01$; Electronic Appendix, supplementary table S1). There is no other study to date that has examined brain gene expression profiles of plastic phenotypes in a vertebrate, although it has been found that even higher proportions of the genome (up to 40%) are differentially regulated in two honeybee behavioural phenotypes (Whitfield *et al.* 2003). For simplicity, genes found to be significantly upregulated in sneaker male brains relative to immature male brains are called 'sneaker biased', while genes found significantly upregulated in immature males compared with sneaker males are called 'immature male biased'. As the genes represented on the array were randomly selected with regard to the tactics studied, we infer that roughly 15% of the genome varies in gene expression in the brain between the two male tactics. Of course, it is probable that some differences in genes expressed only in a few cells and/or at very low levels may be masked by examining whole brains. This would result in false negatives (e.g. a gene is found to be not differentially expressed between the two male types when in fact it is). For most of the differentially expressed genes, the differences between the two male types were very small (less than twofold) but highly significant ($p<0.01$); 85% of sneaker-biased genes and 94% of immature male-biased genes. Therefore, large changes in phenotype were accompanied mostly by subtle changes in transcription levels when considering the whole brain, demonstrating that significant but subtle variation and/or localized to specific brain regions could be captured using our approach.

According to life-history theory, trade-offs between reproduction and growth are expected to result in differential allocation of resources in divergent phenotypes (Stearns 1992). However, the mechanisms underlying trade-offs have rarely been examined and are usually treated as a black box (Stearns & Magwene 2003). We now know that developmental switches are largely determined by changes in gene expression (Carroll *et al.* 2001; Abouheif & Wray 2002). We therefore expect developmental trade-offs to be detectable at the transcriptional level. We hypothesize that this reproduction/growth trade-off should be reflected at the molecular level within specific biological processes. We test this prediction by classifying genes according to their biological function. We grouped the differentially expressed genes into 17 biological process categories following functional annotations (Rise *et al.* 2004) and tested whether these processes were preferentially activated in one phenotype relative to the other (Electronic Appendix, supplementary table S2; figure 3). Here, we find that the diverse molecular and cellular processes that are differentially activated in the two phenotypes are consistent with trade-offs between reproduction and growth. We find a significant disparity in the number of sneaker-biased and immature male-biased genes for many processes (exact binomial test; table 1): genes relevant in reproduction ($p=0.01$), feeding, reproduction and associated processes ($p=0.01$), growth hormone related, and neural signalling and neural plasticity ($p<0.001$), were sneaker biased significantly more often. Conversely, genes involved in transcription regulation, protein synthesis ($p<0.0001$), protein folding and maturation (chaperones), and neurodegeneration ($p=0.03$), were mostly upregulated in immature males, consistent with resource allocation towards somatic growth. In contrast, genes relating to blood, immune function, protein degradation, cellular metabolism and development were sneaker biased and immature biased in comparable proportions (table 1).

Finding *reproduction*-associated genes to be almost exclusively sneaker biased (table 1), although not extremely surprising, is a validation of our approach. For example, gonadotropins have previously been shown to be upregulated in sneaker males (Antonopoulou *et al.* 1999). Other biological processes were also in broad agreement with earlier findings that focused on candidate genes and hormones in Atlantic salmon and other species; growth hormone-related genes were found upregulated in sneaker males, which is consistent with findings that growth

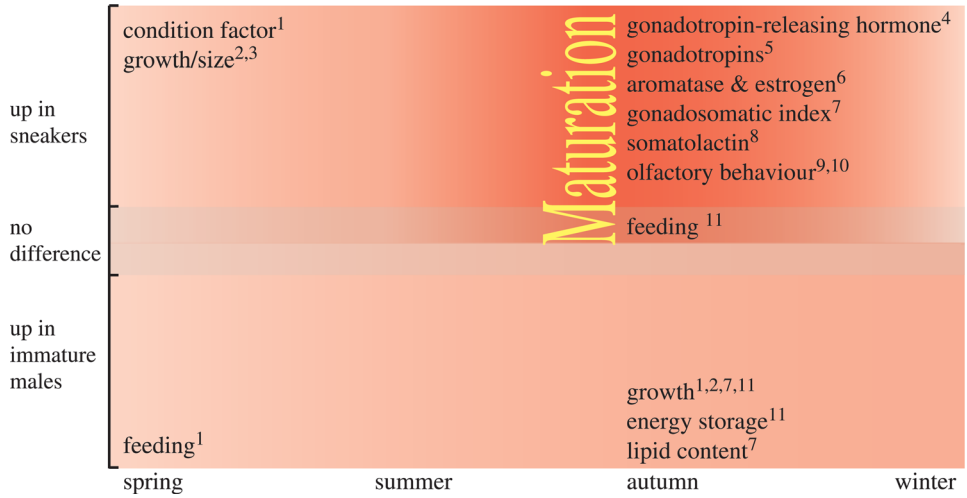

Figure 1. Physiological and life-history differences between early maturing sneakers and immature males of the same age. Juveniles that exhibit increased growth/size and condition during spring mature into sneakers during the summer/early autumn ('maturation' label), in time for spawning of large anadromous individuals. References: 1 (Rowe & Thorpe 1990), 2 (Whalen & Parrish 1999), 3 (Aubin-Horth & Dodson 2004), 4 (Amano *et al.* 1995), 5 (Antonopoulou *et al.* 1999), 6 (Mayer *et al.* 1991), 7 (Saunders *et al.* 1982), 8 (Mayer *et al.* 1998), 9 (Moore & Scott 1991), 10 (Waring *et al.* 1996), 11 (Arndt 2000).

hormone, notwithstanding its name, is elevated in plasma during sexual maturation of anadromous individuals of this species (Bjornsson *et al.* 1994). The *circadian clock* gene Bmal1, a basic helix-loop-helix transcription factor, was upregulated in sneakers, which is intriguing since in salmonids photoperiod and maturation are linked (Amano *et al.* 1995).

Other biological processes yielded some surprising insights, as many genes were in categories not previously implicated in this type of developmental divergence, such as *neural plasticity* and *neural signalling*. For example, genes involved in synaptic function and plasticity like neuronal pentraxin (a secreted immediate early gene product induced by synaptic activity (Reti *et al.* 2002)), synaptotagmin (triggers calcium-induced fusion of pre-synaptic vesicles with the plasma membrane (Zamponi 2003)), MHC class I proteins (activity-dependent synaptic remodelling (Huh *et al.* 2000)) and ependymin (hormonally induced glycoprotein involved in learning and memory formation in fishes (Schmidt 1995)) were all highly expressed in sneaker males compared with immature males. In addition, several genes involved in the synthesis of the gaseous neurotransmitter nitric oxide were upregulated in sneakers, which may be indicative of its role in the regulation of neuropeptides (Cioni *et al.* 2002). These differences in gene expression may reflect the cognitive demands of sneaking behaviour. Clearly, behavioural and neurobiological studies that explore some of these genes in the context of learning and cognition are needed.

Among genes relevant for *feeding, reproduction and associated processes*, somatolactin was sneaker biased, which confirms previous work (figure 1). The same was found for another pituitary hormone, prolactin, which has been implicated in salmon reproductive function and osmoregulation (Onuma *et al.* 2003). Another intriguing gene upregulated in sneaker males codes for POMC, a master regulator of feeding, reproduction, and a variety of other physiological processes (Raffin-Sanson *et al.* 2003). These microarray results have been validated using quantitative real-time PCR (figure 4). The *growth* category

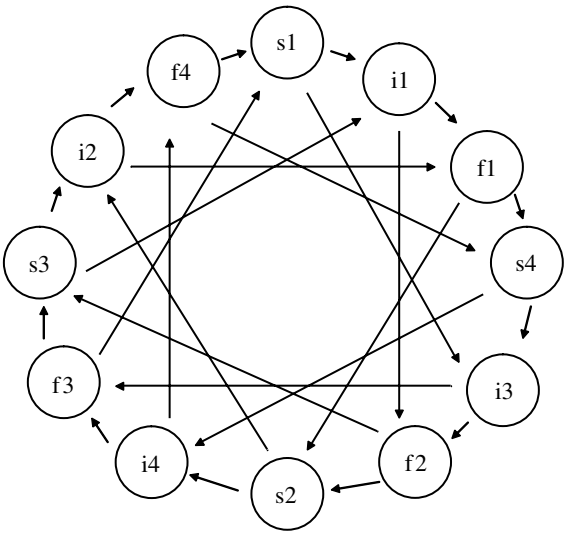

Figure 2. Microarray hybridization design. RNA extracted from the brains of immature females (F), immature males (I) and mature sneaker males (S) Atlantic salmon individuals 1–4. Arrow-heads represent cy5-dye labelling and arrow tails cy3-dye labelling.

included insulin-like growth factor 2 receptor (IGF-2-R) upregulated in sneakers, which facilitates neuronal proliferation and differentiation and has been implicated in neuroendocrine regulation (Hawkes & Kar 2004). Genes involved in *neurodegeneration* were over-represented in immature males. Examples include β-1 integrin, which is involved in neural proliferation, differentiation, and apoptosis (Van der Flier & Sonnenberg 2001) and Brp44l, an apoptosis-regulating basic protein. Many immature male-typical genes are involved in *transcription regulation* (including histone H3), *protein synthesis* (ribosomal proteins), *protein folding and maturation* (chaperones) and *protein degradation* (proteasome-related genes), possibly indicating the increased somatic (and neural) growth in those animals compared with sneakers in the fall at the time of capture (figure 1).

Because considerable variation may exist (based on studies in other species and tissues (Oleksiak *et al.* 2002)),

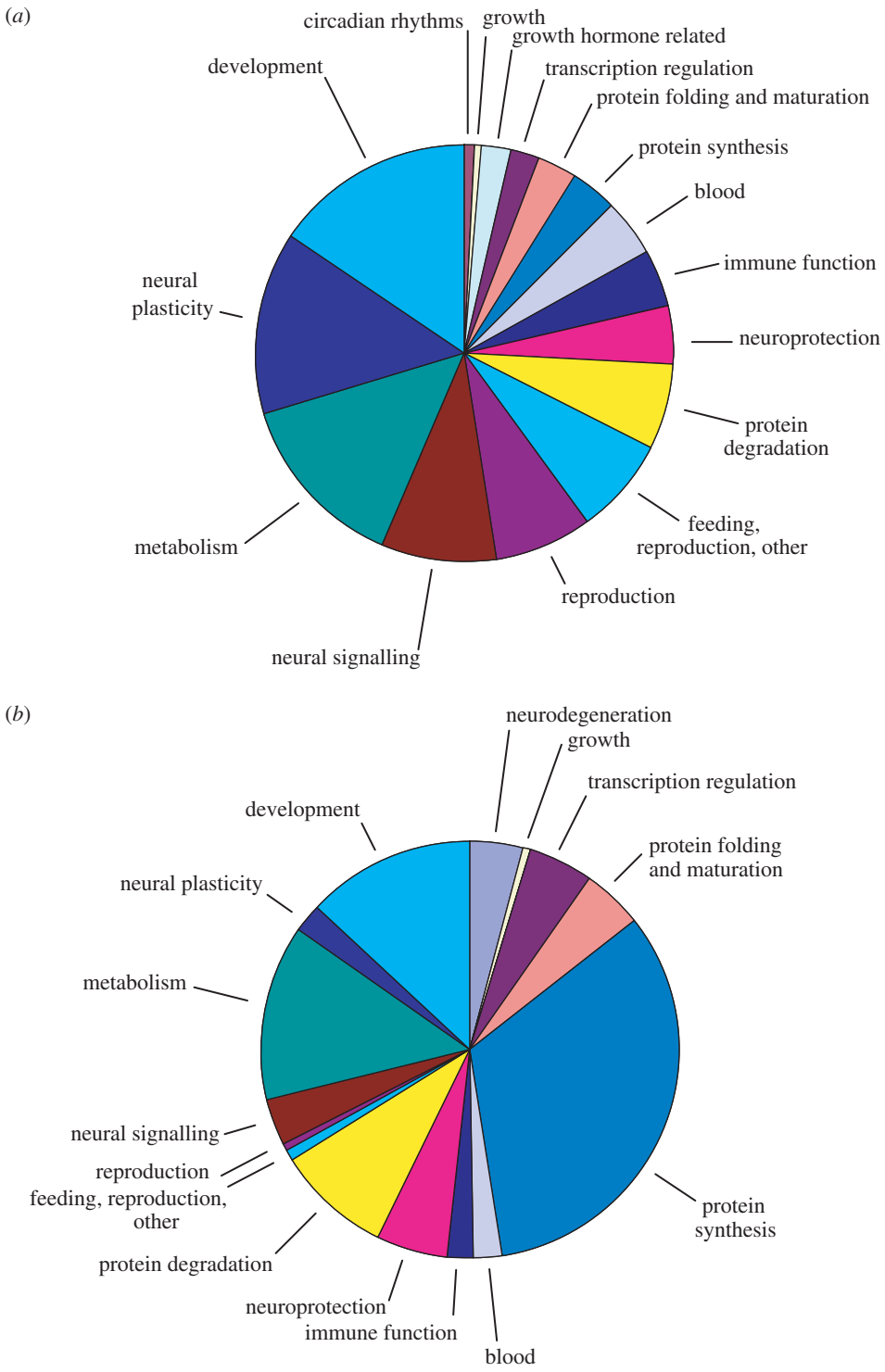

Figure 3. Proportion of genes from 17 biological processes up-regulated in (*a*) sneaker males and (*b*) immature males. A biological process was assigned to 135 of 235 genes upregulated in sneakers and to 145 of 197 genes upregulated in immature males, after removing spots without sequence information (sneaker males $n=6$, immature males $n=5$), no annotated hits (sneaker males $n=38$, immature males $n=25$), and unknown function (sneaker males $n=56$, immature males $n=22$). Note that some processes are unique to one tactic and that size of pie section representing a given biological process cannot be compared between sneaker and immature male figures, as this size is dependent on other categories found for this tactic.

we determined whether the broad range of environmental and genetic factors involved in an individual's developmental trajectory, particularly in wild animals, result in variable gene expression profiles among individuals of the same phenotype. If individuals would cluster independently of phenotype, then this would reflect the null hypothesis of no correlation between expression profiles at the individual level. However, in this study, individual expression profiles clustered mostly according to their macroscopic phenotypes (e.g. sneaker males, immature males, immature females) in a correlation-based consensus tree, which groups individual expression profiles according to their similarity (figure 5). This indicates that despite the potential for large environmental effects in natural habitats, specific cellular and physiological processes are consistently activated in a phenotype. In the face

Table 1. Number of genes significantly upregulated in sneaker male brains and immature male brains for 17 biological process categories and significance level of exact binomial statistical test.
(The five biological process categories with significant bias towards one male type in the number of genes represented in that category (in bold) were still all significantly biased after applying the Dunn-Sidak method of sequential correction of alpha level of significance (Sokal & Rohlf 1995).)

biological process	sneaker males	immature males	*p*-value
circadian rhythm	1	0	1
growth hormone related	3	0	0.25
feeding/reproduction/ others	10	1	0.01
reproduction	10	1	0.01
neural plasticity	19	3	0.0009
neural signalling	12	5	0.14
blood	6	3	0.51
immune function	6	3	0.51
development	21	19	0.87
growth	1	1	1
metabolism and energy production	19	20	1
neuroprotection	6	8	0.79
protein degradation	9	13	0.52
protein folding and maturation	4	7	0.55
transcription regulation	3	7	0.34
protein synthesis	5	48	<0.0001
neurodegeneration	0	6	0.03

of the continuous variation found in many internal and external traits relevant to this kind of developmental plasticity (Roff 1996; Nijhout 2003; West-Eberhard 2003), the developmental trajectories at both the molecular and physiological levels nonetheless produce two discrete and canalized alternative phenotypes instead of a continuous spectrum.

Surprisingly, the cluster analysis showed that brain gene expression profiles of sneaker males were more similar to those of immature females than immature males. Although evidence at the molecular and cellular level is limited, this suggests that early maturation as a sneaker could be the default developmental pathway in salmon, with males that fail to reach the threshold size to develop into sneakers, the immature males of this study, being the ones adopting an alternative tactic by actively repressing maturation (Thorpe *et al.* 1998). Indeed, a high percentage of the genes that were upregulated in sneaker males compared with immature males were not significantly different when compared with female brains. In fact, the percentage of 'sneaker-specific genes' (e.g. those found significantly highly expressed in sneaker male brains compared both to immature males and females) was only 17% ($n=39$, Electronic Appendix, supplementary table S2) of the genes upregulated in sneaker male brains compared with immature male brains only (no difference between sneaker males and immature females). In contrast, out of the 197 genes that were upregulated in immature males compared with sneaker males, 41% ($n=81$) were upregulated in immature males compared with both sneaker males and immature females and were therefore 'immature male-specific'. Therefore, because at

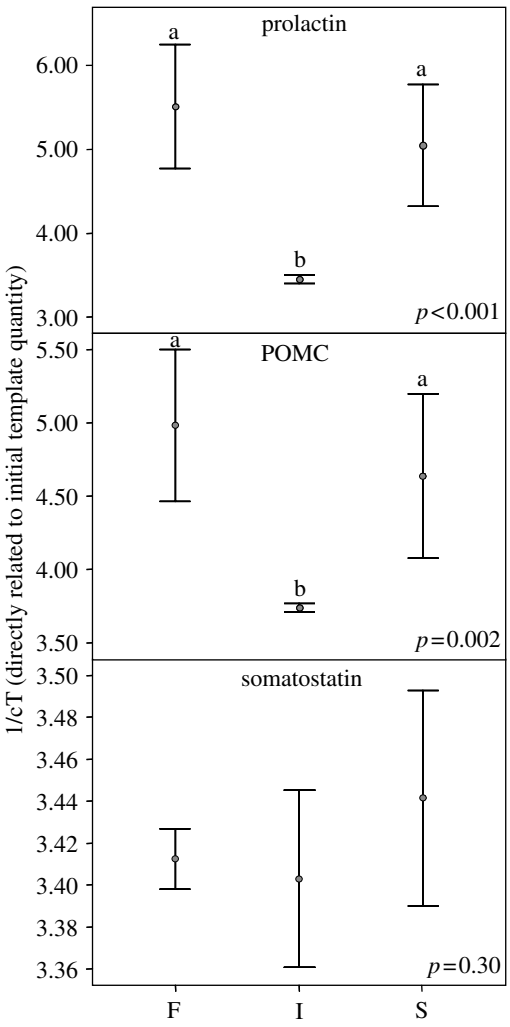

Figure 4. Quantitative real-time PCR comparison of mean gene expression in the brains of immature females (F), immature males (I) and mature sneaker males (S). 1/cT (cycle threshold) value is directly proportional to initial amount of gene product. Vertical bars represent the 95% confidence interval and letters results of *a posteriori* tests, with same letter indicating no difference between groups at $p=0.05$. Both microarray and Q-RT-PCR showed prolactin and pro-opiomelanocortin (POMC A) to be upregulated in sneaker male brains (see Electronic Appendix, supplementary table S1). Additionally, Q-RT-PCR gave consistent results for genes such as somatostatin (see Supplementary table S1), which according to the microarray, were expressed at similar levels in the brains of all three phenotypes.

the level of brain transcription, immature males are different not only from sneakers, but also from immature females, the expression differences between sneakers and immature males cannot solely be explained by the sexual maturation component of the divergence between these two male types. Delayed maturation and future sea migration, as exhibited by immature males, is often seen as the 'default' life-history tactic. However, our study suggests it may actually result from actively inhibiting the developmental trajectory towards becoming a sneaker male.

The goal of our study was also to lay the foundation for future expression profiling studies to determine whether the same molecular networks (or 'modules') are used across species in males showing the (plastic or genetically determined) sneaker tactic (Hartwell *et al.* 1999). Fishes

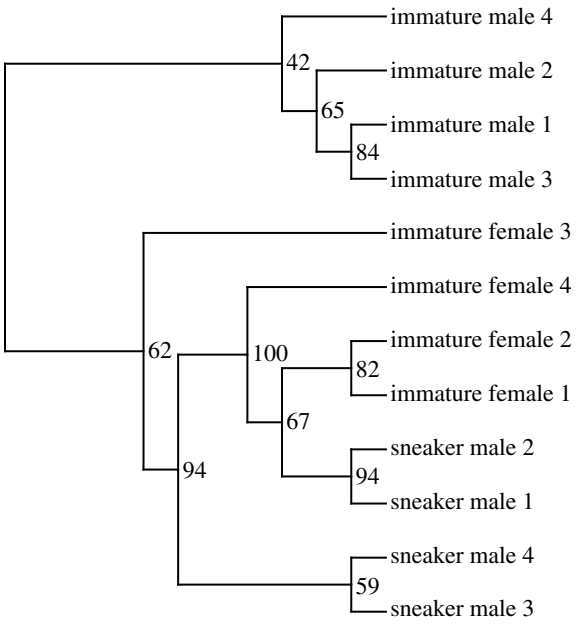

Figure 5. Cluster analysis of similarity of individual gene expression profiles of mature sneaker males 1–4, immature males 1–4 and immature females 1–4. Distance matrix based on Pearson correlation. Numbers at branch nodes in the consensus tree represent the percentage of trees that showed this clustering configuration of individuals when those trees were built using 1000 bootstrapped datasets (see §2).

are ideally suited for this comparative approach, as numerous species are known to exhibit such alternative male reproductive tactics (Taborsky 2001; Knapp 2003). Additionally, it has recently been shown that the same microarray platform can be used to study even relatively distantly related teleost species (Renn *et al.* 2004; Aubin-Horth *et al.* 2005). Combined with the existing research into the ultimate causes of phenotypic plasticity and behavioural ecology, functional genomics paves the way for similar analyses in a wide variety of organisms in order to uncover the molecular and cellular determinants and constraints underlying evolutionary trajectories of these complex plastic traits.

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