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The Use of Transcriptomics to Address Questions in Behaviour: Production of a Suppression Subtractive Hybridisation Library from Dominance Hierarchies of Rainbow Trout*

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ABSTRACT

Microarrays, or gene chips, are transforming the way that gene expression is measured by allowing us to determine the expression of thousands of genes from a sample. This gives immense power to examine gene expression on a global scale within individual animals and between animals. The scope for analysing complex animal functions at the molecular level is within our grasp. Relatively few studies have examined complex behaviours and correlated them with gene expression in the central nervous system. Here, we review the use of microarray technology in the dissection of behaviour and focus specifically on dominance status. A cDNA library using suppression subtraction hybridisation on rainbow trout *Oncorhynchus mykiss* of differing status has been produced to enrich the cDNA library for genes that are differentially expressed between individuals of different dominance status. A preliminary analysis demonstrated that there were 1,165 genes that differed between fish of different dominance status. Therefore, there is the potential of correlating gene expression profile with rank position within dominance hierarchies, thus identifying targets for candidate gene approaches.

Introduction

Microarrays and Behaviour

All behaviours are influenced by the actions of many genes (Robinson 2004), and candidate gene studies that target one or a few genes have had some success (Fitzpatrick et al. 2005). For example, polymorphism in the promoter region of the vasopressin V1a receptor gene has been shown to promote monogamy in prairie voles *Microtus ochragaster* (Insel and Young 2001). The foraging gene (*for*) causes inheritable differences in foraging behaviour of the fruit fly *Drosophila melanogaster*, with active foragers, or “rovers,” who have high expression to “sitters,” who show little foraging activity (Osborne et al. 1997). Shifts in the arginine vasotocin system in the bluehead wrasse *Thalassoma bifasciatum* affect aggression and courtship in males but are dependent on initial social status. Territorial males showed a decrease in aggression whereas nonterritorial males became more aggressive (Semsar et al. 2001). Selection for high- and low-stress responsiveness also affects behavioural output in rainbow trout *Oncorhynchus mykiss*, with low responders being more aggressive and dominant over high responders (Pottinger and Carrick 2001). Gene knockout studies have also been useful but are limited to model organisms where the genome is available, for example, mice, *Drosophila*, and the nematode worm *Caenorhabditis elegans*. Knockout experiments can show a causal relationship between a particular behaviour and a gene. Mouse knockouts of the serotonin gene have shown an increase in aggression and impulsiveness as well as addictive behaviour, and thus these are correlated with low serotonin (Stark and Hen 1999). One problem with knockouts is that one gene may have a plethora of effects, and this can be problematic in interpreting data (Mogil et al. 2000; Wilson and Mogil 2001). Other methods of targeting genes include transgenics or using antisense oligonucleotides that cause acute downregulation of a gene (Mogil and McCarron 2000). Transgenics are relatively difficult to produce in nonmodel organisms (where the genome is not complete or available). Antisense knockouts produce a transient downregulation of a targeted gene, and this has been achieved using antisense oligonucleotides for cell adhesion molecules in zebra fish *Danio rerio* that had a detrimental effect on memory formation (Pradel et al. 2000).

Gene expression in the brain constitutes the first measurable indicator of the interactions of genes and behaviour (Robinson

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2004). New genomic technology allows a more sensitive and efficient means of assessing gene expression and allows the examination of thousands of genes in parallel (Gracey and Cossins 2003; Gracey et al. 2004). The complementary DNA (cDNA) microarray, or gene chip, is essentially a library of cDNA probes or oligonucleotides fixed in a grid on a glass slide or nylon membrane that will bind cDNA from a given sample. So by extracting mRNA, converting it to cDNA by reverse transcription, labelling it with fluorescent dyes (usually Cy3 and Cy5), and hybridising it to the array, it is possible to obtain information on genes upregulated, downregulated, and unchanged at a specific time (see Gracey and Cossins 2003 for a comprehensive review of the technology). The initial application of this technology has been to identify clusters of genes that respond differentially to a treatment or that differ between tissues (Gibson 2002). By correlating gene expression profile to a particular state, behavioural, physiological, and so on, it is possible to identify groups of genes that are related to this state. This has been elegantly pursued in a microarray analysis of gene expression in multiple tissues in the common carp *Cyprinus carpio* subject to physiological stress (Gracey et al. 2004). In this study, carp were subjected to a cooling regime, and several tissues were sampled over the time course after the onset of cooling. A common response shown by all tissues was identified by examining more than 13,000 genes. This expression signature common to all tissues consisted of 252 upregulated genes involved in RNA processing, translation, mitochondrial metabolism, proteasomal function, and modification of lipid membranes and chromosomes. Tissue-specific responses were also identified. For example, the brain responds to cold by upregulating genes involved in fatty acid metabolism and cholesterol, and this may indicate a mechanism to protect the neural tissue from the cold stress. Skeletal muscle responds to cold by upregulating genes involved in structural components of the sarcomere, such as myosin heavy and light chains, tropomyosins, and actins, indicating possible structural remodelling of the muscle tissue. This study illustrates the immense power of microarray analysis that can reveal a level of complexity of response at both the cellular and tissue level (Gracey et al. 2004). Another study on common carp treated with the stress hormone cortisol specifically targeted the gene expression responses of the head kidney and found that many stress-related genes were coregulated in the physiological consequences (Kawano et al. 2003). In other physiological studies, microarrays were successfully used to identify differences in transcripts between male and female zebra finch telencephalon (Wade et al. 2004), and thyroid hormone treatment in male rats identified 11 candidate genes out of a total of 1,224 neural-specific targets that are upregulated in hyperthyroidism (Haas et al. 2004). Much work has been carried out on the effects of endocrine disruptors, which mimic estrogens, on two species of fish, the sheepshead minnow *Cyprinodon variegatus* (Larkin et al. 2002a, 2003) and the largemouth bass *Micropterus sal-*

moides (Larkin et al. 2002b). These chemicals impair reproduction and development, and studies using microarrays aim not only to understand the mechanisms of these detrimental effects but also to produce a signature of exposure so that the gene expression profiles can be used as a diagnostic tool in site analysis. The research has shown that chemicals can be separated by assessing the transcript profile and that some contaminants such as ethylene have different effects on the sexes, with males showing an upregulation of estrogen-responsive genes whereas in females, there is a downregulation of these normally upregulated genes that may account for sterility, reproductive failure, and behavioural responses (Larkin et al. 2002b). Therefore, adopting this microarray approach to understand complex behaviour at the molecular level may reveal the scale and intricacy of the underlying molecular basis of behaviour and provide fresh new insights into the causes and consequences of behavioural variation.

Indeed, microarrays have been successfully used to identify candidate genes related to behaviours. In the honeybee *Apis mellifera*, the transition from hive worker to forager is correlated with changes in more than 1,500 genes in the brain providing a molecular signature that is robustly associated with these behavioural changes (Whitfield et al. 2003). Of the 50 most predictive genes, 17 belong to functional categories and relate to neural and behavioural plasticity. Before the transition to forager, bees had an upregulation of genes involved in axonogenesis and cell adhesion that may reflect brain structure changes that precede the shift to forager. After the transition, forager bees had an upregulation of carbonic anhydrase (similar to CAH1), which plays important roles in synaptic plasticity and cognition in mammals, including spatial learning and memory, and may perform the same function in the bee. Analysis of gene expression in the hippocampus revealed that 27 genes are correlated with learning in mice (Leil et al. 2002). In this study, strains of mice with good learning and memory capabilities were tested against strains with learning and memory deficits. The two good-learning and good-memory strains separated clearly from the two poor-learning and poor-memory strains in the principal components analysis of gene expression profiles, suggesting that these 27 genes are in fact valid candidate genes for learning and memory in mice. It also supports the notion that the different strains of mice share common molecular pathways for learning and memory but that the effectiveness of these mechanisms varies between strains. Of the genes with known functions, some have intriguing prospects for a role in learning and memory. For instance, the mouse homolog of the Wolframin gene, the gene involved in Wolfram syndrome, was detected as being more highly expressed in the strains with deficits. Wolfram syndrome is an autosomal recessive disorder in which homozygotes have severe behavioral defects that often include mental retardation. The evidence from this study suggests that Wolframin may play a role in normal learning and memory. A recently discovered gene, the

transcription factor mbFZB, was also identified in this study. mbFZB has high homology to the *Xenopus laevis* putative Zic3 binding protein, and the mouse gene has been shown to be strongly expressed in the developing brain as well as in the adult mouse hippocampus and piriform cortex. The genetic basis of geotaxis was revealed using microarrays on *Drosophila* brain, where candidate gene approaches previously had been less successful (Toma et al. 2002). The genes that correlated with geotaxis were *Pen*, which encodes importin—homozygous mutants that have these pleiotropic phenotypes have hypertrophied brains—and *cry* and *Pdf*, which mediate pigmentation and circadian locomotor activity, respectively. A causative relationship between these three genes and geotaxis was established by examining the geotactic behaviour of mutant lines with knockdown of these genes. Therefore, there is great potential in the application of microarrays to questions in behaviour and the scrutinisation of the identified candidate genes.

Library Production for Microarrays

There are a variety of methods that are used to produce libraries of genes (Gracey and Cossins 2003), and they are dependent on whether the genome is known. In model organisms, microarrays are available commercially and generally consist of oligonucleotides that can be fabricated by specialised equipment in the laboratory. In “nonmodel” organisms, the absence of extensive sequence data cDNA probes can be generated using amplified inserts of clones isolated from cDNA libraries. These can be identified through end sequencing and homology searching of sequence databases, and repeated clones can be removed by “cherry picking.” Alternatively, the representation of this clone set can be maximised without resorting to expensive sequencing through one of several strategies. Normalisation is a procedure that tends to equalise the amounts of different transcripts by decreasing abundant transcripts while simultaneously increasing the amount of less highly expressed cDNAs (Soares et al. 1994), overcoming the imbalance in the representation of different transcripts. Suppression subtractive hybridisation (SSH) produces a cDNA library enriched for genes that are differentially expressed between mRNA sampled from control and treatment groups (Diatchenko et al. 1996). This produces a clone set whose gene representation is enriched for the problem under investigation, and, therefore, it would be expected that all of the genes on the array are interesting.

Questions in Dominance Behaviour

Functional approaches have been successful in elucidating the role of behaviour as a component of fitness, but it is now evident that a full understanding of how natural selection acts on such behavioural traits requires information about the mechanisms that cause them. Game theory models (Enquist and Leimar 1983) have incorporated assumptions about the

mechanisms that underlie fighting behaviour, and these assumptions need to be tested. These mechanisms may form part of the structure by which fights are resolved, and, therefore, it is necessary to link these two approaches by examining molecular changes and physiology in relation to dominance behaviour in a species in which the fighting behaviour is well studied (Hearn 1987). Behavioural endocrinologists and biochemists have shown rapid changes in circulating levels of hormones and other behaviourally active substances in response to behavioural experience, including encounters with an aggressive rival (Huntingford and Turner 1987; Sneddon et al. 2000; Oliveira et al. 2001). This is likely to be linked to changes in gene and protein expression of key neuroendocrine tissues. Very little is known about the genetic basis of behaviour in fish, although gene knockout studies have shown the genetic basis of certain behaviours in *Drosophila* and mice (e.g., Ferrus and Canal 1994; Wersinger and Rissman 2000), and selective breeding for stress responsiveness in rainbow trout also affects aggressive behaviour (Pottinger and Carrick 2001) as well as other behaviours (Øverli et al. 2002).

New genomic technology, in particular, the cDNA microarray, is revolutionising the rate at which information about changes in gene expression can be collected (Duggan et al. 1999; Lipshutz et al. 1999). Combined with new hierarchical clustering techniques, this offers a particularly efficient means of genome-wide screening to identify correlations of altered transcript expression with experimental treatment. This powerful approach could be used as a means of correlating gene expression profile with rank status. The model independence of the approach will offer fresh new insights into the role of previously uncharacterised genes and processes, such as those responsible for the production of brain monoamines, which correlate with dominance status in rainbow trout (Winberg and Lepage 1998; Øverli et al. 1999). These insights will complement the transcript assessment of more established physiological responses, such as colour change (dominance related; Baker et al. 1995; Francis et al. 1997), growth (growth hormones; Gross et al. 1992), proliferation factors and repressors, stress (cortisol-binding proteins, adrenergic receptors, etc.; Lovejoy and Balment 1999), and resistance to pathogens.

The dominant individual performs the most aggression and has priority or even exclusive access to resources. It has been demonstrated that the second-ranked individual in brown trout hierarchies has the highest plasma cortisol concentrations and is the most stressed within the hierarchy (Sloman et al. 2000). However, when the dominant individual is removed from salmonid hierarchies, the second rank becomes dominant. This is likely to be accompanied by physiological changes more appropriate to the now assumed dominant status. This change in physiology would be evident from, and probably dependent on, changes in the expression genes and their corresponding gene products. Indeed, following the transition from second rank to dominant is a useful experimental tactic for identifying

the genes, their corresponding proteins, and the systems they serve as components of behaviourally dependent physiology. This can be efficiently achieved by screening gene expression over time after rank manipulation using hierarchical clustering techniques.

Changes in gene expression are an important component of adaptation to a new environment (Schulte 2001) and possibly to a new behavioural situation such as an aggressive encounter. One option for an individual to respond to a new predicament, such as environmental change or a confrontation with a competitor, is to alter the expression of individual genes. This suite of changes in gene expression, which integrates with biochemical, physiological, and behavioural alterations occurring in response to the demands of an environmental variable or a new behavioural circumstance, collectively makes up the adaptive response. Research on the shore crab demonstrated that there

are rapid changes in neuromodulators in response to engaging in fights (Sneddon et al. 2000). Behavioural responses are usually the front line of adaptive responses to new circumstances. However, social status has a great impact on the physiological responsiveness of an animal, and so individuals of differing dominance status have been shown to have quite contradictory physiological outputs. Dominant individuals of rainbow trout and Nile tilapia had a better food conversion efficiency for a given food ration than did subordinates and as such had profound metabolic differences promoted by social position (Metcalfe 1986; Fernandes and Volpato 1993). This resulted in dominants having high growth rates whereas subordinates showed negative growth. Thus, dominance-subordinate relationships can have profound consequences for the physiological status and responsiveness of an animal. Therefore, any research that intends to examine the consequences of dominance behaviour

Figure 1. Flowchart of suppression subtractive hybridisation procedure (from Ji et al. 2002).

Figure 2. Flowchart of microarray technique for a reference-based design.

using an integrated approach to obtain novel results on the molecular correlates of behaviour may provide evidence on which genes are upregulated, remain unchanged, or are down-regulated in individual rank members, and this information can potentially be used to explain the mechanisms underlying dominance behaviour.

The aim of this study is to construct a microarray using SSH from a highly tractable and well-studied model system, dominance hierarchies of rainbow trout, by subtracting between dominant (rank 1), subdominant (rank 2), and subordinate (rank 3) fish. We will describe the techniques employed to produce a cDNA library from a brain. A preliminary analysis was carried out to establish whether there were any significant differences between individuals of different dominance status.

Material and Methods

Animal Maintenance and Behaviour

Rainbow trout ($n = 60$; mean $\pm$ SE weight = 50.3 ± 1.0 g; length 15.9 ± 0.2 cm) were obtained from a commercial fish supplier and were held in 2×2 -m tanks in an open system supplied by freshwater, fully aerated, and fed ad lib. with commercial trout pellets. The fish were kept on a 12L : 12D regime and were provided with an opaque cover over half of the tank to provide an area for sheltering. The fish were kept for 2 wk to allow the recovery from the stress of transportation and were used only once feeding had resumed, and thus the fish were unstressed. Fish were individually removed from the stock tank and anaesthetised in benzocaine (0.05 g/L water), measured

using vernier callipers to 0.01 cm, and weighed to 0.01 g. The fish were tagged subcutaneously above the eye on either side using visible implant elastomer (VIE) tags. VIE tags can be applied to a wide variety of small fishes without compromising their growth, survival, or behaviour (Olsen and Vøllestad 2001). Six groups of three size-matched (to within 2%) rainbow trout were placed in glass tanks (90 cm $\times$ 45 cm $\times$ 55 cm) containing a flow of freshwater and were aerated and kept on the same light regime. Each tank was covered in opaque polythene at the sides and rear of the tank along with an opaque screen in front of the tank to minimise visual disturbance. Observations were made through a small opening in the front of each tank and commenced after a settling period of 1 d. The behaviour of each fish within a group was recorded for 15 min twice a day, and food was introduced at a rate of 1.5% body weight during the observations. Behaviours noted were number of attacks, number of retreats, and number of pellets eaten for each of the three fish. Aggressive interactions allowed a daily dominance score for each fish to be calculated (number of attacks – number of retreats), and a linear hierarchy could be determined for each group on each experimental day. Therefore, the fish with the highest score was rank 1, the dominant; a fish with an intermediate score was rank 2, the subdominant; and, finally, the fish with the lowest score was rank 3, the subordinate. Only groups that showed a clear difference in behaviour were sampled, and the hierarchy had to be stable for 7 d. After 7 d, the fish were removed and humanely killed, and the brains were carefully removed and placed in a 12-well plate on dry ice. The tissues were then stored at -80°C before mRNA extraction took place.

Library Production

Total RNA was extracted from the whole brain using the TRIzol method (Sigma Chemical), where the brains were homogenised in TRIzol (1 : 10 vol) in a 15-mL tube. Total RNA was then extracted using chloroform (1 : 5 vol) and then precipitated using isopropanol (1 : 2 vol) followed by 70% ethanol (1 mL). After the addition of each reagent, the tubes were centrifuged at 10,000 g for 15 min at 4°C and kept on ice during the

Table 1: The function of the 71 genes identified that differ significantly between trout of different dominance status

Gene Function	No. Clones
Ribosomal proteins	18
Protein turnover	14
Metabolism	13
Behaviour	11
Stress	9
Unknown	6

procedure. The supernatant was discarded, and the resulting pellet containing the RNA was dissolved in 100–200 μ L DEPC water. The quality of the RNA sample was confirmed by running a northern blot on a 1% agarose gel. mRNA was isolated using the GenElute Direct mRNA Miniprep Kit (Sigma). At this stage, mRNA from each dominant ($n = 6$) was pooled, as was mRNA from the subdominant and subordinate, to give the three dominance states. The following protocols were carried out on these three samples.

First, strand cDNA synthesis was carried out using the Clontech SMART cDNA Library Kit (BD Biosciences). Details of this are available at <http://www.clontech.com/clontech/techinfo/manuals/PDF/PT3000-2.pdf>. When we used the constituents in the kit, the mRNA was reverse transcribed to cDNA and amplified by polymerase chain reaction (PCR). The resulting cDNA samples were digested using Hae III (New England Biolabs) to give cDNA fragments cut at 5' ... GG/CC ... 3' and 3' ... CC/GG ... 5' recognition sites. These were separated by base size using Chromaspin filters, and fragments lower than 300 base pairs were discarded.

From each sample 1 μ L of cDNA (300 ng/ μ L) was diluted in 5 μ L of PCR-grade water. In separate 0.5-mL tubes, the N1 adaptor and N2R adaptor were ligated to each of the three samples using T4 DNA ligase (New England Biolabs). The N1 adaptor was comprised of a long-sequence 5' CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC 3' and a short-sequence 5' ACC TGC CCG G 3', and the N2R adaptors consisted of a long-sequence 5' CTA ATA CGA CTC ACT ATA GGG CAG CGT GGT GCG GGC 3' and a short-sequence 5' ACC TCG GCC G 3' (MWG Biotech). These were incubated overnight at 16°C and inactivated by increasing the temperature to 72°C for 5 min. This provided three tubes for each sample: (1) driver cDNA with no adaptors, (2) cDNA with N1 adaptors, and (3) cDNA with N2R adaptors (see Fig. 1). Eight hybridisations were run for 20 h at 68°C: (A) 2 μ L of dominant cDNA plus 2 μ L of N1 subdominant cDNA, (B) 2 μ L of dominant cDNA plus 2 μ L of N2R subdominant cDNA, (C) 2 μ L of dominant cDNA plus 2 μ L of N1 subordinate cDNA, (D) 2 μ L of dominant cDNA plus 2 μ L of N2R subordinate cDNA, (E) 2 μ L of subdominant cDNA plus 2 μ L of N1 dominant cDNA, (F) 2 μ L of subdominant cDNA plus 2 μ L of N2R dominant cDNA, (G) 2 μ L of subordinate cDNA plus 2 μ L of N1 dominant cDNA, and (H) 2 μ L of subordinate cDNA plus 2 μ L of N2R dominant cDNA. After 20 h, 0.5 μ L of dominant cDNA was combined with A and B into one tube; 0.5 μ L of dominant cDNA was combined with C and D into one tube; 0.5 μ L of subdominant cDNA was combined with E and F into

one tube; and 0.5 μ L of subordinate cDNA was combined with G and H into one tube. The four resulting tubes were incubated at 68°C for 20 h. The tubes were then heated to 72°C for 7 min and stored at –20°C.

From each of the four tubes, 1 μ L of subtracted cDNA was placed in a PCR tube along with a PCR mixture containing PCR primer 1 (5' CTA ATA CGA CTC ACT ATA GGG C 3'; MWG Biotech). After this PCR reaction, 1 μ L of the resulting samples was placed in separate PCR tubes for a second PCR containing the nested PCR primer 1 (5' TCG AGC GGC CGC CCG GGC AGG T 3') and the nested PCR primer 2R (5' AGC GTG GTC GCG GCC GAG GT 3'). After this PCR, the mixture was enriched for differentially expressed cDNAs. In addition, those that varied in abundance in the original mRNA sample should have been in roughly equal proportions. See work by Diatchenko et al. (1996) for a full review of the methodology and concepts behind SSH.

The PCR products were placed in Chromaspin 1000 filters (BD Biosciences) to fractionate the samples and obtain only large cDNAs because fragments above 300 base pairs were suitable for library production. The cDNAs were then ligated into a vector, pGEM-T (Promega), by using T4 ligase from the Clontech SMART cDNA Library Kit. The cDNA clones were then transformed into Electromax *E. coli* cells (Invitrogen) and incubated at 37°C for 1 h in SOC medium (Invitrogen). The cells were then diluted 1 μ L into 9 μ L of sterile water and spread on LB agar plates with ampicillin, IPTG, and X-Gal. This ensured that colonies containing a clone would be white in colour whereas other colonies would be blue. The plates were incubated overnight at 37°C. The plates were kept at 4°C for 2 d, and then the white colonies were carefully picked into 384-well plates containing 50 μ L of LB broth with ampicillin in each well. These were incubated overnight at 37°C and then placed at –80°C for storage.

The clones were amplified by PCR in the presence of SP6 and T7 primers (5' ATT TAG GTG ACA CTA TAG 3' and 5' AAT ACG ACT CAC TAT AGG 3', respectively; Sigma) in 96-well plates. Clones were checked for quality on 1% agarose gel to ensure they were of an acceptable length (>300 base pairs). Purified PCR products were gridded onto poly-L-lysine-coated slides using a BioRobotics MG2 spotting robot. Approximately 3,000–8,000 products were printed per slide, including a set of house-keeping genes and synthetic standards, with genomic and vector DNA as internal standards and controls for normalisation.

Figure 3. Gene expression profiles of dominant, subdominant (*Subdom.*), and subordinate (*Subord.*) rainbow trout ($n = 9$). Lines across the profiles represent one cDNA probe. Those in red show upregulation, those in green show downregulation, and those in yellow are unchanged. On the left are the results of a hierarchical cluster analysis that clusters together coexpressed genes showing a similar profile.

Hybridisation to Microarrays

Three hierarchies were chosen for a preliminary analysis of gene expression; therefore, the brains of the three ranks from three hierarchies had mRNA extracted. Probes were produced from the tissue mRNA by reverse transcription of mRNA in the presence of amino-allyl adducts and were subsequently labelled with Cy3 (green) and Cy5 (red) dyes for the control and treatment groups, respectively. Microarrays were cohybridised with these labelled cDNA probes at 65°C overnight in Genetix hyb boxes. A single-control mRNA was formed by pooling from all sources (see Fig. 2). Fluorescent images from the hybridised microarrays were captured using a Genepix 4000A laser scanning microscope. The fluorescence data were expressed as a normalised ratio of Cy5/Cy3 fluorophores bound to each spot on the slides by using proprietary and public domain software. A global normalisation factor was applied to the data so that the median log fluorescence ratio on each array was 0. Statistical analysis was performed using a hierarchical cluster analysis technique to identify coexpressed genes that are up- or down-regulated in dominant, subdominant, and subordinate individuals. ANOVA was used to test whether these differences were significant at the 5% level using the Benjamini Hochberg multiple correction factor.

Results

One 384-well plate was produced for each of the four subtractions, giving 1,536 brain cDNA clones. Out of these clones, 490 were sequenced, with 307 producing significant hits using BLAST searching and 183 having no hit and thus being unknown. Out of the identifiable clones, there was a high level of redundancy, with only 71 gene identities. For example, one clone had 75 copies within the library. The identities of the genes will be published elsewhere (L. U. Sneddon and A. R. Cossins, unpublished manuscript). However, the biological function of these genes is shown in Table 1. The majority of genes are involved in metabolism followed by protein synthesis and catabolism, ribosomal proteins, genes known to be implicated in behaviour, and stress with a number whose function is unknown.

From the statistical analysis of three dominant individuals, three subdominants, and three subordinates, 1,165 probes on the array showed significant differences ($P < 0.05$) in expression (Fig. 3). Each line across the profile represents one cDNA probe, with probes in red upregulated, probes in green downregulated, and probes in yellow unchanged in expression. A hierarchical clustering analysis that clusters together coexpressed genes was carried out on the significant genes to identify genes with similar expression profiles, and the results can be viewed in Figure 3 and Table 1. Out of this analysis, there are nine major clusters although the analysis does show clusters within these nine primary expression profiles. Genes showing similar expression profiles between the ranks are illustrated in Table 2. The ri-

Table 2: The nine major clusters identified from the hierarchical clustering analysis

No. Clones	Subdominant	Subordinate
478	Down	Up
283	Up	Down
134	Same	Up
92	Up	Same
89	Down	Up
44	Up	Same
14	Up	Same
13	Same	Down
10	Up	Down

Note. Table shows the expression patterns of the subdominant and subordinate relative to the expression profile of the dominant, that is, those genes showing significant downregulation (down) and upregulation (up) and those that were not significantly different (same).

bosomal genes tended to cluster together with an upregulation in the subdominants and downregulation in subordinates relative to the dominant. Protein catabolism and also metabolic genes showed a downregulation in subdominant. Stress-related genes were upregulated in the subdominant (full analysis, L. U. Sneddon and A. R. Cossins, unpublished manuscript).

Discussion

This preliminary analysis has shown that there are profound gene expression differences between rainbow trout of differing dominance status. There were clusters of genes showing particular patterns between the ranks, with some genes showing upregulation in the dominant compared with the other ranks and some genes showing upregulation in the subdominant and/or subordinate compared with the dominant. Therefore, from this study, it is possible to correlate gene expression profile with rank. It is difficult to separate cause and consequence in behaviour, and because these samples were taken after the dominance hierarchy had been stable for 7 d, it is more likely that these are consequences. However, identifying these genes and possibly manipulating them in future studies may provide a means of better understanding the causes and consequences of dominance at the molecular level. Transgenics and knockout technology are currently difficult to study in fish models, but this is possible in mice, so it may be that in the immediate future, one may have to swap models to achieve these aims. Studies have shown that there are differences in physiological parameters before dominance is established, and this could be used to predict dominance, for example, serotonin and dopamine (Sneddon et al. 2000) stress responsiveness in trout (Pottinger and Carrick 2001). This link between rank-dependent behaviour and molecular phenotype is poorly described, but we hypothesise that it is of major importance in understanding how fitness varies between otherwise similar in-

dividuals. Because there are major differences in behaviour, growth rates, and other physiological parameters such as stress hormones (Sloman et al. 2000), it would be intuitive that gene expression is different between animals of different status. Dominant individuals have a higher growth rate because they obtain the greatest proportion of food and have a better growth conversion efficiency (Metcalf 1986); therefore, this will potentially affect protein turnover and metabolism as well as many other factors and may explain the differences in gene expression. The majority of genes were indeed involved in ribosomal processes, protein turnover, and metabolism, which is a similar result shared by other microarray studies (Gracey et al. 2004). However, genes that are related to stress and behaviour were also identified and may act as candidates for future studies to establish a causative link with dominance behaviour (identities; L. U. Sneddon and A. R. Cossins, unpublished manuscript).

Aggression is a major problem in humans, and understanding this complex behaviour has fuelled the nature/nurture debate that attributes aggressiveness to DNA rather than the environment and free will (Robinson 2004). Behavioural variation is affected by genes, the environment, and the interaction between them. This study shows that gene expression is correlated with dominance rank, with animals of different status showing different behaviours. More than 1,000 genes appeared to differ, and they may contribute to the obvious behavioural differences as well as the plethora of physiological measurements made by other studies (e.g., Winberg and Lepage 1998; Øverli et al. 1999; Sloman et al. 2000; Pottinger and Carrick 2001). Genes can affect natural behavioural variation in distinct ways (Ben-Shahar et al. 2002). Allelic variation causes alternative behavioural phenotypes whereas changes in gene expression can influence changes in behaviour at different stages (e.g., the *for* gene in honeybees; Ben-Shahar et al. 2002). It may be the promoter or regulatory elements of these genes that differ between animals rather than the genes per se. Therefore, this may explain the differences in gene regulation seen between the ranks in this study where the dominant genome responds in a distinct way to the social situation, with the two other ranks having a different gene expression profile. Variation in the promoter regions of the serotonin transporter gene have been shown to cause behavioural disorders in hominids and mice rather than any difference in the gene sequence (Lovejoy et al. 2003).

Microarrays have been successfully used in understanding physiological responses to hypoxia in the goby *Gillichthys mirabilis* (Gracey et al. 2001) and zebra fish (Ton et al. 2002); natural variation in populations of killifish *Fundulus heteroclitus* (Olesiak et al. 2002); identification of genes involved in development in the gastrula of zebra fish (Dickmeis et al. 2001); the response of channel catfish *Ictalurus punctatus* (Ji et al. 2002) and common carp *Cyprinus carpio* (Gracey et al. 2004) to cold; and the response of sheepshead minnow (*Cyprinus variegatus variegatus*) and largemouth bass (*Micropterus sal-*

moides) to endocrine disruptors (Larkin et al. 2002a, 2002b, 2003). The SSH strategy used here demonstrated that approximately 76% of the cDNA probes on the array were significantly different between dominance states, thus demonstrating the efficiency of our library although perhaps not surprising. It may be that the remaining 24% of cDNA probes were not significant at the 5% level or that occasionally the robot pin heads do not contain enough PCR product, so some probes are not printed on the array. This was taken into account in the analysis by including only gene spots that appeared in 16 of the 18 arrays. The library, however, had a high level of redundancy, with a number of clones being repeats; therefore, perhaps the SSH did not successfully produce a fully subtracted library. The problem may lie in the fact that the SSH procedure uses digested fragments of DNA; therefore, if the fragments of one gene are not complementary to one another, they will not be removed by the subtractive hybridisation. We recommend full-length cDNA recapture with normalisation and subtraction because this technique has been a success in this laboratory for carp and roach libraries. This study has shown the feasibility of this approach in a traditional behavioural context, and it has the potential to identify multigene systems that correlate with dominance status. This model-independent approach will generate new hypotheses testing the ecological and evolutionary significance of dominance status by manipulation of the candidate genes identified in this study.

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