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Fish & Chips: Functional Genomics of Social Plasticity in an African Cichlid Fish

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Abstract

Behaviour and physiology are regulated by both environment and social context. A central goal in the study of the social control of behaviour is to determine the underlying physiological, cellular and molecular mechanisms in the brain. The African cichlid fish *Astatotilapia burtoni* has long been used as a model system to study how social interactions regulate neural and behavioural plasticity. In this species, males are either socially dominant and reproductively active or subordinate and reproductively suppressed. This phenotypic difference is reversible. Using an integrative approach that combines quantitative behavioural measurements, functional genomics and bioinformatic analyses, we examine neural gene expression in dominant and subordinate males as well as brooding females. We confirm the role of numerous candidate genes that are part of neuroendocrine pathways and show that specific co-regulated gene sets (modules), as well as specific functional Gene Ontology categories, are significantly associated with dominance or reproductive state. Finally, even though the dominant and subordinate phenotypes are robustly defined, we find a surprisingly high degree of individual variation in the transcript levels of the very genes that are differentially regulated between these phenotypes. Our results demonstrate the molecular complexity in the brain underlying social behaviour, identify novel targets for future studies, validate many candidate genes, and exploit individual variation in order to gain biological insights.

Keywords

cichlid; microarray; social behaviour; behavior

INTRODUCTION

Brain and behaviour are sculpted by a dynamic interplay between genotype and environment. Changes in social status mediate changes in a wide range of molecular, neuroanatomical, endocrine and behavioural characteristics (Oliveira et al., 2002; Fernald, 2004; Sapolsky, 2005) at different time scales (Hofmann, 2003). Nervous systems respond by modulating neural properties and neuroendocrine responses. Such rapid, short-term changes will often lead to sustained longer-term changes in brain and behaviour through

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differential gene expression leading to subsequent structural and physiological changes. However, few studies have attempted to determine the molecular underpinnings of socially regulated phenotypes within a comprehensive genomic framework, especially in vertebrates. Here we apply a molecular systems approach in a highly social fish to ask how social context regulates the molecular and physiological substrates that in turn sculpt subsequent behaviour.

The African cichlid fish, *Astatotilapia burtoni* (formerly: *Haplochromis burtoni*) has become an important model system to study the mechanisms underlying socially mediated behavioural change. In this species, 20–30% of males are dominant (**D**), slow growing, brightly coloured and actively defend territories for mating. The remaining subordinate (**S**) males mimic females by schooling and displaying cryptic colouration, while experiencing faster growth (Fernald, 1977; Hofmann et al., 1999a). Subordinate males show little aggression and territoriality, and, importantly, have regressed gonads and thus are not reproductive (Fernald and Hirata, 1977a; 1977b; Francis et al., 1993). These behavioural and physiological characters are plastic and influenced by the immediate social environment, such that an individual male switches between the dominant and subordinate phenotypes several times during its life depending upon its relative ability to obtain and maintain access to a territory through encounters with other males (Hofmann et al., 1999a). Environmental conditions, availability of territorial shelters, relative body size and physiological condition influence the probability of acquiring and maintaining a territory. The phenotypic switch occurs over a timescale of minutes to days to weeks in both the field and the laboratory (White et al., 2002; Hofmann, 2003; Burmeister et al., 2005).

In the laboratory, *A. burtoni* has been the focus of hormonal and molecular studies related to a broad range of phenotypic traits that are affected by the transition between the two male phenotypes (Fig. 1) (for detailed reviews see: Hofmann and Fernald, 2000; Fernald, 2002; 2004; Hofmann, 2003). Variation in several different components of the endocrine system reflects the complexity underlying the corresponding phenotypic switch between these dramatically different male phenotypes. Neuroendocrine pathways regulating, androgen production (Parikh et al., 2006), growth (Hofmann et al., 1999a; 1999b), and stress response (Fox et al., 1997) change in a complex fashion as animals undergo phenotypic change (Fig. 1). Parikh et al (2006) suggested that the higher levels of testosterone (T) and 11-ketotestosterone (11KT) they measured in dominant males might promote aggressive behaviour, as has been shown using androgen manipulation in other fish species. Production and release of neuropeptides and neuromodulators such as gonadotropin releasing hormone (GnRH1) (White et al., 2002) and somatostatin (Hofmann and Fernald, 2000; Trainor and Hofmann, 2006, 2007) are higher in dominant males. According to White et al (2002), GnRH mRNA levels and GSI (a measure of gonadal development) are positively correlated. The higher level of GnRH in dominant males coincides with acquisition of territory and mating opportunity, as such it similar to GnRH changes in seasonally reproducing species, (Amano et al. 1995, Dawson et al. 2001, Nelson 2005; Hofmann 2006). Fluctuations at the molecular level also co-vary with the observed behavioural switch of male phenotype. In the case of GnRH (Au et al., 2006; Parhar et al., 2005) and somatostatin (Trainor and Hofmann, 2006), specific receptor subtypes are regulated according to social status. Furthermore, the membrane properties of GnRH1-expressing neurons, reduced excitability in subordinate males, corresponds with the differences in peptide release between dominant and subordinate males (Greenwood and Fernald, 2004).

Manipulation of the social environment allows experimental control of the phenotypic switch (Francis et al., 1993; White et al., 2002; Burmeister et al., 2005). The ease of experimental manipulation is paired with a wealth of ecological and evolutionary information available for haplochromine cichlids in general (for review, see Kocher, 2004;

Salzburger et al., 2005). For example, there is thought to be a trade-off between reproduction and survival, such that territorial males, the sole reproducers, are also subject to higher mortality rates through predation, likely due to their conspicuous colouration (Fernald and Hirata, 1977a; 1977b; Maan et al., 2008). Furthermore, *A. burtoni* is currently undergoing whole genome sequencing, along with three other African cichlid species (see URL: www.broad.mit.edu/models/tilapia/) and thus offers an unrivaled laboratory-based model system for the genomic analysis of complex and ecologically-relevant phenotypes.

cDNA microarrays, for transcript profiling, have become a powerful tool when applied to species of behavioural, ecological or evolutionary interest (*e.g.* alternative life histories: Aubin-Horth et al., 2005a; cooperative breeding: Aubin-Horth et al., 2007; social behaviour: Grozinger and Robinson, 2002; Robinson et al., 2005; Whitfield et al., 2003; response to heat stress: Buckley et al., 2006; response to environmental estrogens: Martyniuk et al., 2006; physiology of drug addiction: Rhodes and Crabbe, 2005). In the present study, we employ a microarray platform that contains many known candidate genes as well as ~4000 brain-derived cichlid cDNAs (Renn, Aubin-Horth and Hofmann, 2004). Such a combined candidate gene and genomic strategy allows hypothesis-driven and discovery-based experiments on a single platform. The obviously complex nature of behavioural traits – such as this socially regulated, reversible switch between dominant and subordinate phenotypes – requires a discovery-based approach in order to identify the many genes involved.

In the present study, we analyze gene transcript patterns for reproductively active dominant males and reproductively suppressed subordinate males as well as for brooding females. We then compare the expression pattern for each of the three phenotypes in order to identify gene sets associated with reproduction or dominance behaviour, providing insight into the molecular modularity underlying these phenotypes. Next, we annotate the array features according to Gene Ontology (GO) with the goal of identifying gene regulation within molecular categories free of *a priori* expectations or experimenter bias. Finally, we examine variation in gene expression patterns between individual animals within a social phenotype and ask whether any of these variable genes are also those that are differentially expressed between the social phenotypes.

RESULTS AND DISCUSSION

Male Dominance Behaviour and Gene Expression Differences

Individually tagged males were placed into community tanks with females such that naturalistic dominance hierarchies were established among the males according to standard protocol (Hofmann et al., 1999a). Regular observations over the course of five weeks identified males of definitive dominant (D) and subordinate (S) phenotypes. The territory holding males (n = 6) behaved more aggressively than subordinate males (n = 6), as reflected by their significantly higher dominance index (DI; D males: 15.7±1.7; S males: −3.5±0.8, t-test $p < 0.001$) and reproductive index (RI; D males: 2.12±0.57; S males: 0.0±0.0; t-test $p = 0.004$). The gonadosomatic index (GSI) varied continuously across the two groups showing the expected (yet in this study non-significant) trend for higher GSI in dominant males (GSI, D males: 0.85±0.23; S males: 0.56±0.04%; t-test $p = 0.25$). There was no significant difference in growth rate over the course of the experiment (GR; D males: 1.25±0.215; S males: 1.39 ±0.285; t-test $p = 0.69$) or condition factor (residuals) at the time of sacrifice (CF; D males: 0.01±0.053; S males: −0.01 ±0.061; t-test $p = 0.58$). Note, however, that CF differed marginally when the social communities were established at the beginning of the experiment (D males: 0.03±0.051; S males: −0.03±0.068; t-test $p = 0.07$), which could suggest that increased condition may be advantageous when ascending to dominance in the first place. Specific behaviours also varied significantly and predictably between the dominant and subordinate males (Fig. 2): increased presence of an eye-bar in

dominants ($p < 0.001$); increased schooling behaviour of subordinates ($p = 0.0005$); more fleeing behaviour by subordinates ($p = 0.004$); and more frequent chasing behaviour ($p = 0.0003$), border threat behaviour ($p = 0.08$) and sexual behaviour ($p = 0.003$) by dominants. These results are representative of dominant and subordinate males in *A. burtoni* as previously described (*e.g.*, Francis et al., 1993; Hofmann and Fernald, 2000; White et al., 2002).

For the first investigation of our microarray data, we conducted Bayesian analysis of gene expression (Townsend and Hartl, 2002) by treating the six individuals of each phenotype as biological replicates and calculated gene expression differences at the level of phenotype (dominant, subordinate or brooding female). When comparing the different phenotypes, array features representing 3647 cichlid unique sequences passed quality filters in a sufficient number of hybridizations to be considered in the results. Dominant and subordinate phenotypes showed significant differences in gene expression for 171 genes (Bayesian Posterior Probability, BPP ≥ 0.99) (87 up- regulated; 84 down-regulated with dominance; Supplementary tables 1 & 2). Permutation analysis of the data shows that, at this significance threshold of BPP ≥ 0.99, only ~17% of these 171 genes could be expected to show significant differential expression by chance, a reasonable rate of false positives (Table 1). Therefore, almost five percent of the genes studied were differentially regulated in the brain according to male dominance phenotype. This percentage is considerably smaller than that found in typical honey bee colonies (39%) where nurse and forager phenotypes are not only distinguished by their social role, but also differ in their daily behavioural routines and the environment in which they move about (Whitfield et al., 2003). These results also show phenotype specific regulation of a considerably smaller portion of the genome than the 15% observed difference between alternative mating tactics in a study of Atlantic salmon males, which similarly compared sexually mature and immature males (Aubin-Horth et al., 2005a).

Candidate Genes—The microarray was designed to include candidate genes previously studied in the context of social dominance and other behavioural contexts in *A. burtoni* (Hofmann, 2003; Fernald, 2004). The inclusion of known candidate genes allowed us to test multiple hypotheses and also offered validation of the microarray results by comparison with previous studies for some of these genes (summarized in Fig. 1). For instance, peptidergic neurons in the pre-optic area (POA), and other brain regions, express several neurohormones (e.g., arginine vasotocin, GnRH1, galanin) as well as neurohormone receptors and steroid receptors, which have previously been shown in separate studies to play a role in the regulation of social behaviour. Below, we provide for the first time a combined analysis of these neuroendocrine pathways in *A. burtoni*.

As predicted from previous studies using ribonuclease protection assays and *in situ* hybridization, among the three GnRH neuropeptide genes that are expressed in the brain of fish, only GnRH1, the form expressed in the POA (White et al., 1994; White et al., 2002) showed highly significant regulation in the microarray results (BPP = 0.9998) with dominant males having higher levels. Given the small number of cells expressing this neuropeptide (~300 Munz, 1999; Soma et al., 1996), confirmation of GnRH1 regulation by our microarray analysis provides an important cross-validation and confirms the sensitivity of the array even when using whole brain RNA. As predicted from previous studies (White et al., 1994; White et al., 2002), our results also confirmed that the other two forms of GnRH, (midbrain) GnRH2 and GnRH3 (terminal nerve), are not regulated according to male social phenotype (BPP = 0.366; BPP = 0.700 respectively). None of the GnRH receptor subtypes on the array were significantly regulated, although studies have demonstrated their regulation in the pituitary in relationship to sexual maturity and social status (Parhar et al., 2005; Au et al., 2006).

Galanin, a neuropeptide that links metabolic activity and reproduction through regulation of GnRH release (reviewed by Kageyama et al., 2005; Tortorella et al., 2007), was marginally up-regulated in dominant males (BPP = 0.9592). There is considerable evidence from mammals that galanin reduces nociception (Wiesenfeldhallin et al., 1992), increases food intake (Schick et al., 1993) and stress reactivity (Holmes et al., 2002), and that it plays a role in regulation of sexual behaviour, and is itself regulated by GnRH and estrogen (Gabriel, et al., 1993). Specifically, in fish, galanin is thought to play a role in regulation of food intake and is widely distributed in the brain, being localized to the olfactory bulb, telencephalon, hypothalamus, midbrain, and posterior brain (reviewed by Volkoff et al., 2005) as well as the pituitary (Jadhao and Pinelli, 2001) and peripheral tissues (Johnsson et al., 2001). Our results suggest the intriguing possibility that galanin might be up-regulated in dominant males as a response to reduced food intake and constant challenges to their social status by other individuals. Future studies will test these novel hypotheses.

Arginine vasotocin (AVT; represented by multiple clones on the array), the non-mammalian homolog of arginine vasopressin (AVP), was among the most strongly regulated genes in this study, being up regulated in the brains of dominant males (BPP > 0.9999). AVP/AVT has been implicated in the regulation of social behaviour across vertebrates, including aggression and social affiliation (Goodson, 1998; Goodson and Adkins-Regan, 1999; Winslow et al., 1993). In teleost fish, AVT is known to play a role in male mating tactics (peacock blenny: Carneiro et al., 2003; Grober et al., 2002; midshipman: Goodson and Bass, 2001), as well as in the behavioural regulation of sex change and the associated territory acquisition (bluehead wrasse: Semsar and Godwin, 2003; Semsar and Godwin, 2004). AVT is also associated with territorial aggression (damselfish: Santangelo and Bass, 2006) as well as dominant and territorial behaviour in both the male and female of a breeding pair as compared to their subordinate helpers in another cichlid species, the cooperative breeding *Neolamprologus pulcher* (Aubin-Horth et al., 2007). Our data from *A. burtoni* suggest a role for AVT in regulation of dominance and are consistent with an *in situ* hybridization-based study (Greenwood et al., submitted). The AVT V1a receptor, which plays a fundamental role in affiliative behaviors in voles (Lim et al., 2004), was not represented on the array.

The enzyme aromatase, which converts testosterone to estrogen, is important in sex determination (Nakamura and Kobayashi, 2005), sex change in fish (Black et al., 2005; Marsh et al., 2006), and regulation of social behavior (Hallgren et al., 2006). There are two isoforms of aromatase, one localized to the brain and the other to the gonads, and both are represented on the microarray. Here, dominant males showed increased neural expression of the brain form (2 features on the array, BPP = 0.9914; 0.9997), but not the gonad form of aromatase (BPP = 0.3192). This result suggests that the elevated testosterone levels found in dominant males (Parikh et al., 2006) may affect aggression, courtship or dominance through aromatization and subsequent action via estrogen receptors in the brain. In birds, aromatase activity increases during the territorial period and correlates with aggression (e.g. Soma et al., 2003; Silverin et al., 2004). Blocking brain aromatase reduces male courtship in guppies (Hallgren et al., 2006), further suggesting estrogen-mediated neuroendocrine regulation of reproductive behavior for some species. However, studies on gonadal sex change in fish (Black et al., 2005; Marsh et al., 2006) suggest the opposite relationship between brain aromatase and male aggression and thus a more complex mechanism possibly involving differences in receptor expression, binding proteins, or anatomical localization. Estrogen receptors did not show differential regulation on the array, although they may have been expected to, according to Burmeister et al. (2007). The inability to reliably detect differences in receptor gene expression is likely due to small, localized effects that are masked by whole brain gene expression levels.

Novel Genes—We bioinformatically annotated the ESTs obtained from the cichlid cDNA library features represented on the microarray (see methods). Several of the genes thus identified fall into categories that represent candidates likely to play a role in the social regulation of a complex phenotype (Table 2). These genes are considered here as "novel genes", rather than "candidate genes" because the annotation process does not involve rigorous manual curation of genes *a priori* that was employed for the candidate genes discussed above. In addition to many genes involved in cellular metabolism that are differentially regulated between the two social phenotypes, we find genes encoding structural proteins, cell-cycle regulators, specific transcription factors, a plethora of neuropeptides, components of the neurosecretory machinery, and neurotransmitter receptors.

Genes coding for structural proteins such as tubulin and actin, proteins that bind scaffold elements, such as septin 7 and ELF-1a, were more highly expressed in dominant males reflecting the observed differences in soma size between dominant and subordinate for preoptic neurons expressing GnRH1 (Francis et al., 1993) and somatostatin (Hofmann and Fernald, 2000). Furthermore, genes involved in axonal growth, neuromodulin (also known to play a role in modeling of sex-specific brain regions: Simerly, 2002) and neuroserpin (Miranda and Lomas, 2006), also to be up-regulated in dominant males. Taken together, the regulation of this gene set strongly suggests increased neuronal re-wiring in dominant males not previously reported and possibly similar in scale to the massive remodeling of neural circuits seen in seasonal accession to territoriality and mating accompanied by increased testosterone levels in song birds (Devoogd and Nottebohm, 1981; reviewed in Arnold, 1992). It is particularly intriguing that neuroserpins may play a role in anxiety and sexual behaviours. Specifically, neuroserpin-deficient rats showed decreases in exploratory behaviour along with increases in anxiety and neophobia (Madani et al. 2003) and, in swordtail fish, neuroserpin expression increased in the brain of females exposed to an attractive male compared to females exposed to a non-attractive male (Cummings et al., 2008). This association of neuroserpin with social behavior is intriguing in that it may enable dominant males to approach and interact with novel stimuli such as competitors and potential mates.

Similarly, several cell cycle regulators (Table 2) were significantly regulated in dominant and subordinate phenotypes, suggesting that the extent of neurogenesis and subsequent cell death may also differ between these phenotypes, a hypothesis consistent with the finding that cell proliferation in the brain is correlated with high social status in rainbow trout (Sorensen et al., 2007). While there is currently no other evidence for plasticity of this kind in neuroanatomical structures outside the pre-optic area (POA) in *A. burtoni*, gross neuroanatomical differences that correspond to species typical reproductive strategies have been identified in other cichlid species (Pollen et al., 2007).

Genes encoding neuropeptides and protein hormones that have not been previously studied in this system were perhaps the most striking, though not unexpected, class of genes regulated according to social status. In addition to the neuropeptides GnRH1, AVT and galanin discussed above, we found somatotropin/growth hormone (GH), prolactin, somatolactin (all members of the GH family of genes), as well as proopiomelanocortin (POMC) to be up-regulated in dominant males. Interestingly, a similar pattern of endocrine gene regulation (GH, prolactin, somatolactin, POMC) is observed in Atlantic salmon, such that the expression profile for the early maturing "sneaker" male compared to for reproductive dominant males, suggesting conserved function of these pathways (Aubin-Horth et al., 2005a). We found cholecystokinin (CCK) and natriuretic peptide to be up-regulated in subordinate males.

Since the activity of pituitary somatotrophes is associated with testis maturity and is stimulated by high levels of GnRH in several fish species (reviewed by Legac et al., 1993; Yu and Peter, 1991), we suggest that the increased expression of growth-related genes in dominant males is likely related to gonad maturation. Somatolactin, which thus far has been found only in teleost fish, is involved in both growth (Forsyth and Wallis, 2002) and colour change (Fukamachi and Sugimoto, 2004): two plastic traits associated with social dominance in *A. burtoni*. Importantly, the observed increase in expression of GH is consistent with the previous finding that circulating GH levels are higher in dominant males (Hofmann et al, 1999b). Additionally, the Growth Hormone-Releasing Hormone (GHRH)/GH axis facilitates territorial behaviour in *A. burtoni* (Hofmann et al, 1999b; Trainor and Hofmann, 2006; note that GHRH was not represented on the array). Finally, antagonists of the neuropeptide somatostatin (which inhibits GH production and release in the pituitary; this gene was not represented on the microarray) inhibited aggressive behaviour in *A. burtoni* males without affecting sexual behaviour (Trainor and Hofmann, 2006).

In addition to neuropeptide genes, we found many genes involved in production, maturation, release and reception of neuropeptides and neurotransmitters to be differentially expressed between social phenotypes. For example, secretory granule proteins, such as a homolog of synaptophysin as well as members of the granin family of acidic proteins, were up-regulated in dominant males. These are notably found in a wide variety of endocrine and neuroendocrine cells (for reviews see Gerst, 1999; Helle, 2004) and the regulation pattern found here may simply be a consequence of increased neuroendocrine activity in dominant males.

While not many neurotransmitter receptors were present on the array, there is one very intriguing result to report: A GABA-(A) receptor was up-regulated in dominant males whereas at least two subunits of the kainate-type glutamate receptor were up-regulated in subordinate males. Clearly, these differences were not predicted nor do we have, at this point, a clear understanding of their specific roles in regulating social dominance. However, the fact that these receptors are affected by (or affect) social phenotypes likely reflects the interconnected nature of neurotransmitter systems and suggests that regulation at all levels of neural circuits may underlie the transition from one social phenotype to another. Interestingly, an antagonistic relationship between GABA and kainate signaling has been suggested in the regulation of reproductive physiology in other systems (e.g., Sagrillo et al., 1996; Chu and Moenter, 2005; Clarkson and Herbison, 2006; Liu et al., 2006; see discussion in Molecular Modules below).

Molecular Functions, Biological Processes and Cellular Locations

The Gene Ontology annotation scheme applied to the cichlid microarray allows rigorous statistical analysis for over- and under-representation of particular molecular functions, biological process, and cellular locations in the genes that are differentially expressed in each male type. Despite the biased nature of the GO terms due to their origin and application in model organisms and directed research, this tool offers a mechanism for statistical analysis of microarray results according to function (Shaw et al., 1999). These terms, unlike specific gene names, avoid experimenter bias and cross-referencing between experiments and even between species, to relate experimental results between organisms and platforms. Of the 171 features regulated according to male social phenotype, GO terms could be applied to 85. Analysis at all GO levels revealed 22 categories that are statistically over- or under-represented among the genes that are regulated by social phenotype ($p \leq 0.05$; compared to their representation among all genes above threshold) (Fig. 3). Using permutation analysis, we determined that only five GO terms were expected to show significant over- or under-representation by chance alone (hyper geometric test $p \leq 0.05$, only l GO term at $p \leq 0.01$). Importantly, this unbiased statistical approach confirms our observation discussed earlier that cytoskeleton/structural molecules as well as hormone

signaling are up-regulated in dominant males (Table 3). Furthermore, cation/potassium transport pathways appear to be important building blocks for each male phenotype. In dominant males, the biological processes of GTP binding, iron ion binding and motor activity were significantly enriched, whereas in subordinate males potassium ion transport, regulation of cellular cation transport and ligand-gated ion channel function were activated. Though difficult to interpret directly, this bioinformatic approach results in a considerable data reduction, facilitates comparisons across species and platforms, and provides a framework of hypotheses for future studies on the molecular underpinnings of socially regulated brain function.

Molecular Modules Underlying Dominance and Reproduction

The notion that biological entities (*e.g.,* cognitive tasks, developmental programs, neural circuits, metabolic pathways) operate as functional and discrete (*i.e.,* largely non-overlapping) units, or modules, is not new (Fodor, 1983; Redies and Puelles, 2001; Schlosser and Wagner, 2004; Op de Beeck et al., 2008). In molecular systems biology, a module can simply be defined as a set of co-regulated genes or proteins (Segal et al., 2004). Many such modules may serve as building blocks for assembly of more complex processes. To date, most studies in this area have primarily been concerned with molecular and cellular networks and pathways in simple unicellular systems (Hartwell et al., 1999; Wolf and Arkin, 2003). However, the ultimate challenge in the biology of complex systems is the integration across many levels of biological organization, from molecules to whole organisms in an ever-changing environment. In the following, we make an initial attempt at such an integration of genomic data with physiological and behavioural phenotypes to provide a comprehensive conceptual framework for understanding phenotypically plastic traits. Specifically, we examine male dominance phenotypes in terms of molecular modules of socially controlled traits such as aggression, territoriality, reproduction and growth.

Because we also obtained neural expression profiles of brooding females, we examined variation in transcript levels in relation to sex. 571 genes were regulated according to sex (318 male up-regulated, 253 female up-regulated; Table 1), a number that is far greater than that for dominance phenotypes within males (171 genes or ca. 5%) and suggests that while the switch between dominance phenotypes is multifaceted (see Fig. 1), the difference in brain gene expression profiles between males and females is even more dramatic, affecting ca. 16% of the genes on the array. Interestingly, this proportion is comparable to the 15% observed difference between alternative mating tactics in males of Atlantic salmon (Aubin-Horth et al., 2005a). A considerable fraction of the 171 genes associated with male social phenotypes were also regulated according to sex (Supp. Table 1 and 2). However, among the social status-regulated genes, there were similar proportions of female-enriched and male-enriched genes (Fig. 4). Among the 87 dominant up-regulated genes, were 11 female-enriched and 20 male-enriched genes (hypergeometric test; $p = 0.09$), and among the 84 subordinate up-regulated genes there were 16 female-enriched genes, and 21 male-enriched genes (hypergeometric test; $p = 0.13$). In other words, subordinate males do not appear to be molecularly feminized nor are dominant fish simply “super-males”. This result makes sense in light of the reproductive state of these animals. Although female behaviour is, in many ways, similar to that of subordinate males, both brooding females and dominant males are reproductively active. Furthermore, just as the metabolic demands of maintaining a territory are associated with reduced growth in dominant males (Hofmann et al., 1999a), mouth-brooding females starve while incubating their offspring and exhibit a marked reduction in body mass (Mrowk, 1984). Subordinate males, on the other hand, do not reproduce and metabolic energy is directed toward growth (Hofmann et al., 1999a). Thus, the eleven genes (including synaptophysin, neuroserpin and GABA-receptor) up-regulated in both dominant

males and brooding females may be part of a module facilitating reproduction and/or reducing growth, and may not necessarily be involved in dominance behaviour *per se*.

Twenty of the 318 male-enriched genes were even more highly expressed in dominant compared to subordinate males. We suggest that these genes, which include several structural proteins (actin, tubulin, ELF-1) and hormones (GnRH-I, AVT, somatolactin, POMC) (Sup. Table 1) are part of a "super-male" module, which likely plays a role in the aggressive and/or sexual behaviours typical of the dominant male (Fig. 4). Even though we know from previous studies using other approaches that the absolute levels of GnRH1 (Francis et al., 1993; White et al., 2002) and GH (Hofmann et al., 1999b) are higher in dominant males, it is important to note that cDNA microarrays measure only relative transcript levels. Future studies will have to determine whether these genes must be activated in order to produce a dominant phenotype or whether their expression needs to be suppressed for the subordinate phenotype to occur. Following a similar rationale, we suggest that the 21 male-enriched genes (including NeuroD and Kainate receptor) that were further up-regulated in subordinate males relative to dominant males represent a masculinizing module in some sense. In this case, reduced expression would be critical for the manifestation of behaviours linked to aspects of social dominance and reproduction. Consequently, the 21 genes in this masculinizing module could be considered to be complementary to the "super-male" module. Finally, since brooding females and subordinate males all display submissive behaviors and very little aggression, it is tempting to suggest that the 16 genes (including natriuretic peptide) expressed in a similar pattern in these two (Fig. 4). phenotypes

The opposing pattern of regulation for receptor expression in two classic neurotransmitter systems that we observed between male social phenotypes (see above) is also maintained in relation to sex. The gene encoding a GABA-(A) receptor was up-regulated in dominant males and also in females, whereas the kainate-type glutamate receptor was up-regulated in males in general and particularly so in subordinate males. There is a wealth of research that ties the GABA-(A) receptor to the regulation of hypothalamic-pituitary-gonadal axis via integration of steroid feedback to GnRH neurons (for review see Sagrillo et al., 1996). In mammals, GABA has mixed inhibitory and excitatory effects on the release of GnRH due in part to a developmental switch from GABA-(A) receptor depolarization to hyperpolarization (Clarkson and Herbison, 2006). In fishes, depending on species, GABA has either excitatory or inhibitory effects on GnRH release (Trudeau et al., 2000). Interestingly, glutamate-controlled GABA release has been implicated in GnRH regulation (Chu and Moenter, 2005; Clarkson and Herbison, 2006). The kainate system has also been proposed to underlie observed sex differences in the mechanisms of the neural glucocorticoid/stress response. Female mice show less atrophy of hippocampal neurons in response to elevated glucocorticoid levels possibly due to the increased expression of NMDA, AMPA, and kainate glutamate receptor subtypes (Liu et al., 2006). In *A. burtoni,* the increased kainate receptor expression seen in subordinate males could similarly provide a neuroprotective effect against the elevated cortisol levels seen in subordinate males during specific social situations (Fox et al., 1997). Future pharmacological and neurohistochemical experiments will elucidate the mechanistic interactions of these neurotransmitter systems in relation to sex and social behaviour.

Taken together, our molecular systems analysis supports the notion that transcript patterns may indeed be organized in a modular fashion and can be strongly associated with behavioural and/or physiological traits associated with social phenotypes or sex in either concordant or contrasting ways. Additionally, we can exploit expression variation between phenotypes for tentatively annotating gene function and predicting functional roles of these genes.

Individual Variation in Gene Expression

To appreciate the importance of variation in gene expression for phenotypic plasticity, we need to evaluate the expression differences between individuals. To determine the extent to which individuals of the same phenotype differ in their expression profiles, we estimated transcript levels for each individual separately. Individual profiles were then clustered for similarity according to the estimated transcript levels using the gene list that had been identified as significantly regulated between any two phenotypes (BPP ≥ 0.99) (Euclidian distance matrix based on resampling for bootstrap confidence levels) (Fig. 5).

Similarities in expression profiles of individuals—Sex was a strong factor in the clustering of individual expression profiles, as five of the six females clustered separately from all males. While males of same social phenotype tended to have more similar brain expression profiles, two dominant individuals clustered with the subordinate males. The analysis of behavioural data of these males did not suggest any obvious difference from other dominant males (see Fig. 2). It is unlikely (since we controlled for age and size and based on our observations) that these two dominant males were experiencing dominance for their first time. Also, at the level of behaviour, these two dominant males were not similar to each other. While male 5 did show the fewest chases and the most border threats (Fig. 1) of all dominant males, the behaviors of male 4 were not in any way different from those of the other dominant males. While it is possible that there is more than one molecular substrate for constructing and maintaining a dominant male phenotype, the expression profiles of these two dominant males are no more similar to each other than to subordinate males.

When individual profiles were clustered according to estimated transcript levels using all genes on the array that passed filtering for every fish, rather than only those genes that showed significant regulation, similar results were obtained (data not shown). While the bootstrapped confidence values were lower, the same four dominant males formed a cluster suggesting that gene regulation according to sex and according to social status account for the greatest variation among individuals in this study. Principal component analysis corroborated this conclusion (Supplementary Fig. 1).

Variation among individuals within and between male phenotypes—While overall the clustering resulted in a robust separation of the three phenotypes according to sex and social status, the individual variation, already apparent at this level, prompted further inquiry into expression variation between individuals of the same phenotype. In voles, expression patterns of oxytocin receptor for females and vasopressin receptor for males is correlated with individual variation in social and anxiety-related behaviours (Olazabal and Young, 2006). Similarly, male mice show individual variation in estrogen receptor distribution that correlates with aggressive behaviour (Trainor et al., 2006). Previous studies in *A. burtoni* and other cichlid species have also reported strong covariation patterns within a social phenotype, between the expression of candidate genes and specific phenotype measures (somatostatin correlated with aggression: Trainor and Hofmann, 2006; AVT correlated with hormone titers: Aubin-Horth et al., 2007). We therefore asked whether significant differences in gene expression existed between individuals of the same phenotype. Furthermore, we tested whether significant differences in gene expression between individuals of the same phenotype could be found among those genes that are differentially regulated between phenotypes.

In order to investigate the degree of individual variation in gene expression, we determined the number of genes that varied significantly in expression between individuals of the same phenotype and compared it to the number that varied between individuals of different phenotypes. Although statistical power was lower because we had only four technical

replicates per individual as opposed to six biological replicates in the analyses above (Clark and Townsend, 2007), we were able to measure the average number of genes significantly regulated (BPP ≥ 0.99) in each possible pairwise comparison of two individuals within and between phenotypes. For intra-phenotype variations among males, an average of 82.8 (s.e. ± 4.5; 2.3% of all genes analyzed) genes varied between any two dominant males while a mean of 92.3 (s.e. ± 4.3; 2.6%) genes varied between any two subordinate males. The identity of these genes was substantially different for each pairwise comparison, such that 38% of all the array features varied in at least one intra-phenotype comparison. Interestingly, for inter-phenotype variation between individual males an average of 132 (s.e. ± 8.4; 3.6%) genes varied between any two males of differing social phenotype, which was not significantly different from, the intra-phenotype variation (t-test $p = 0.13$). Although it is difficult to set an equivalent threshold for significant variation between individuals and between phenotypes, this high degree of individual variation is consistent with other studies that have examined genome-scale individual differences in gene expression to study the molecular basis of natural variation. Whitehead and Crawford (2006) found that 69% of the metabolic pathway genes showed significant variation between individuals within a population, while only 12% were significantly regulated between populations adapted to different temperatures. Similarly, in yeast, up to 50% of the expressed genes show significantly different levels of expression among individual strains (Brem et al., 2002). Not only do we find a similar number of genes to be regulated between individuals of the same or different phenotypes, we find no significant difference in coefficient of variation of expression level for sets of these individually regulated genes. This result indicates that absolute gene expression levels vary between individuals of the same phenotype as much as between phenotypes.

While one might expect low variation within a phenotype for those genes that define that phenotype, an alternative hypothesis posits that those genes that define the social phenotype vary across individuals displaying that phenotype in a manner associated with variation in physiological and behavioural traits (for examples see Trainor and Hofmann, 2006; Aubin-Horth et al., 2007; Cummings et al., 2008). In support of this notion, we found a statistically significant over-representation of intra-phenotype regulated genes among those that were regulated by social status (Table 4). About 72% of the 87 genes up-regulated in the dominant phenotype were also significantly regulated among individuals within phenotype (41 among dominant and 39 among subordinate, 24 of which are shared). Similarly, 64% of the 84 genes that were up-regulated in the subordinate phenotype were also significantly regulated among individuals (47 among dominant and 52 among subordinate, 38 of which are shared). This variation cannot be explained by technical variation in array hybridizations: for a given animal, an array feature must show consistent results across four dye-reversed hybridizations before it can be identified as regulated across individuals according to our statistical analysis. Rather, our results show that even considerable and potentially important within-phenotype variation in gene expression can give rise to reliable and readily identifiable between-phenotype differences. Future integrative studies will help determine whether the observed variation between individuals is caused by or causes subtle phenotypic differences, or represents a dramatic, alternative molecular mechanisms for constructing the same phenotype.

Summary

By including candidate genes on the microarray, we have validated the discovery-based approach that identifies the gene expression patterns for reproductively active dominant males and reproductively suppressed subordinate males as well as for brooding females. While the regulation of neuroendocrine genes was predicted from previous research, the unexpected and novel discovery of opposing roles for two classic neurotransmitter systems

opens exciting new avenues for future research. Furthermore, the increased neuronal remodeling activity in dominant males suggests that the known neuroanatomical changes observed in preoptic GnRH and somatostatin neurons may extend to other cell types and/or additional brain regions. Using the Gene Ontology (GO) framework to interrogate the data for statistical significance reinforces the importance of hormonal regulation and highlights the hitherto underappreciated roles of cytoskeletal components in addition to neurotransmitter pathways. Interestingly, expression profiles vary across individuals within a male phenotype for roughly the same number of genes and with similar magnitudes of transcript abundance as seen between male phenotypes. Specifically, there is a surprisingly high level of variation among those genes that molecularly define the very phenotypes in the first place. Taken together, our genome-scale analysis of molecular systems in the brain has identified complex patterns of gene expression that are associated with a socially regulated switch in behavioural phenotype.

Methods

Animals

Fish derived from a wild-caught stock population were kept in aquaria under conditions mimicking their natural environment (Fernald and Hirata, 1977b): pH 8.0, 28 C water temperature and 12:12 light: dark cycle. Gravel substrate and terra cotta shelters allowed the establishment and maintenance of territories necessary for reproduction (Fernald and Hirata, 1977a). Fish were fed every morning with cichlid pellets and flakes. All work was carried out in compliance with Animal Care and Use Guidelines.

Behavioural experiments

Males—For the behavioural experiments males were marked (coloured bead combinations attached near the dorsal fin). Nine groups of 2 – 3 males with 2 – 3 gravid females were established in one half of a 30 gallon aquarium. Each group was visually isolated from neighboring fish. Ten-minute behavioural observations were made approximately twice every week for five weeks by direct observation of chasing, threat, display, border threat, courtship, flee, and schooling (as described by Fernald, 1977). Behavioural measures were used to calculate a Dominance Index (DI) as the sum of all aggressive behaviours (threat, chasing, border threat and carousel) minus the number of submissive behaviour (flee) and Reproductive Index (RI) as the total of all reproductive behaviours (court, dig, spawn). On the final day of the experiment, males of each status were taken for gene expression profiling only if they had continuously expressed either dominant or subordinate phenotype for the past 28 days. Six mouth-brooding females were also selected for expression profiling. The eighteen fish used in for gene expression analysis were obtained from a total of seven different groups on the basis of stable phenotype.

For the purpose of hybridization design the animals were treated as equivalent within phenotype, though comparisons between each can be made indirectly (see below). Close inspection of the behavioral and microarray data did not reveal any co-variation between animals derived from the same social group. Standard length, body mass, and gonad mass were measured for each fish to calculate the gonadosomatic index (GSI) as gonad mass/body mass. Condition factor (CF), was calculated based on the residuals from the regression of body mass on standard length for before and after the experiment ($r^2 = 0.95$; $p = 2.79 \times 10^{-6}$), and growth rate (GR) was calculated as the relative change in standard length over the course of the experiment. Whole brains were dissected and stored in RNAlater (Ambion) within 5 minutes of initial tank disturbance.

The array used in this study has numerous redundant features, i.e. two or more features represent the same gene. We exploited this property of our array for quality control purposes and to assess the sensitivity of the approach. For example, the array includes four independent features that represent the neuropeptide GnRH1 (two previously cloned cDNAs and two obtained independently from the cDNA library) known to be expressed in only ~300 neurons and up-regulated in dominant males (Davis and Fernald, 1990; White et al., 2002). All four features were similarly significantly differentially expressed, being up-regulated with dominance and thus serve as both a biological and a technical control demonstrating the sensitivity even with whole brain RNA.

Microarray Analysis

RNA extraction—Brains were homogenized (Tissue tearor, Biospec products) and total RNA was extracted according to standard Trizol (Invitrogen) and phase lock gel (Eppendorf) protocols. RNA integrity was determined on the Bioanalyzer (Agilent) and spectrophotometer (Agilent) prior to indirect RNA labeling protocol, starting with 2 μg of total RNA according to Renn et al. (2004). Briefly, each RNA sample was labeled twice, once with Cy3 and once with Cy5. After purification from unincorporated label, each was divided in half, combined with the appropriate samples and every individual was compared to two individuals from each of the other two phenotypes in a balanced loop design incorporating a dye-reversal (see annotation of submitted data at GEO for details). Targets were hybridized to the brain-specific cDNA array from *Astatotilapia burtoni* (Renn et al., 2004) at 65 degrees Celsius for 12 – 16 hours. Arrays were scanned on an Axon 4000B arrays scanner (Genepix 4.0; Molecular Devices). The loop design allows for the direct comparison of samples of interest thus offering greater statistical power (Churchill, 2002) with fewer replicates.

After filtering for bad feature morphology, hybridization artifacts and low intensity (< 2 standard deviation above local background), raw data was imported into R software (v1.0 R-Development team, 2004) and normalized using the *Linear Models for Microarray Data* package (LIMMA v1.6.6; Smyth et al., 2004). Background-subtracted mean intensities were normalized using a within array printtip-lowess normalization and used to calculate ratios for a Bayesian analysis of gene expression levels (BAGEL v3.6; Townsend and Hartl, 2002). BAGEL takes advantage of additional information obtained from transitive comparisons of individuals in loop designs experiments (Townsend and Hartl, 2002; Churchill, 2002) Genes represented by more than one feature on the array were only counted as significant if at least one representative passed significance threshold and the full complement survived Fisher's test of combining probabilities from multiple tests of significance (Sokal and Rohlf, 1995, pg 794). All raw and processed data are available at NCBI's GEO database (www.ncbi.nlm.nih.gov/projects/geo/) sample numbers GSM267785 - 819 of series GSE10624.

Functional Annotation

The clone templates for PCR amplification were end sequenced (Salzburger et al., 2008) resulting in 4258 expressed sequence tags (EST), 3670 of which were deemed to be of high quality and have been submitted to GenBank (accession number CN472211 - CN468542) and are maintained at the Dana-Farber Cancer Institute as a GeneIndex (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=a_burtoni) such that 1280 clones combine into 399 tentative contigs (TC) leaving 2381 singleton sequences for a total of 2780 unique sequences. Half (49.5%) of the sequences (1408 out of 2842) could be annotated with a "best hit" at a threshold of e −12 or better according to BLAST alignment to uniprot (version 5.2). Gene Ontology terms were applied to 869 unique cichlid genes by transitive annotation, meaning the GO annotations for a cichlid gene's best hit were collected and used

here for further analysis. In order to avoid species bias we collected GO terms from all genes with the same name as the best hit annotation. GO annotations also include confidence codes. The less reliable annotations derive from"inferred sequence otation similarity (ISS)" (IEA)" (The Gene Ontology those annotations when excluded Consortium, 2000), therefore, we transitively applying GO terms to the ESTs represented on the *A. burtoni* microarray. The resulting gene ontology (GO) graphs (referred to as directed acyclic graphs DAGs) were then "slimmed" to 183 terms and a total of 4,102 total annotations. For the "slimming" process, the leaf-most nodes that were selected to contain a minimum of 10 annotated cichlid sequences and parent nodes were retained only if an additional 10 cichlid sequences were annotated at that level.

Over- and under-representation of GO terms for a regulated set of genes was determined in Cytoscape (Shannon et al., 2003) using the Biological Network Gene Ontology tool, BiNGO (Maere et al., 2005), which relies upon hypergeometric statistical significance. As GO categories are highly non-independent, the statistical treatment of these terms is still under discussion (Ge et al., 2003). Also, due to the small number of genes for each ontology term and the relatively small number of genes that are regulated, there is less statistical power to identify significantly under-represented GO terms. For these reasons, we use GO analysis as a hypothesis-generating tool and report only uncorrected hypergeometric p-values.

Clustering

Prior to clustering, features representing replicate ESTs were collapsed by combining probabilities from multiple tests of significance (Sokal and Rohlf, 1995; p 794) and the average expression level was determined for each set of features. A hierarchical clustering analysis was applied to the list of genes that were significantly regulated according to dominant, subordinate and brooding female phenotypes. The estimated gene expression levels were used to obtain the dissimilarity matrix applying Euclidian distance measure, which integrates effects of amplitude of ratios as well as direction (correlation) in patterns. Clustering analysis of gene expression patterns of each individual was performed using the *hclust* function in R software v2.0.1. Clustering was based on dissimilarity measures obtained using the *dist* functions in the *stats* package. The consensus tree and bootstrap confidence values for each tree node were obtained with the *consensus* function in the *maanova* package (Wu et al., 2002). The consensus tree and confidence values were calculated as the proportion of trees obtained with bootstrapped datasets that agreed with the original tree. Each bootstrapped tree was based on the Euclidian distance matrix calculated for each of 1000 permutated gene expression profile datasets obtained by resampling with replacement. Alternate clustering methods, and different measures of distance are available and are similarly appropriate for gene expression analysis. Hierarchical clustering, based upon all features on the full array rather than only regulated genes, provided a similar tree with reduced confidence values at each node (not shown). The *heatmap* function and colour options in the package *gplots* were used to visualize clusters of gene expression, the z-transformed expression ratios were grouped by k-means function in the stats package of R and ordered as such while the samples were ordered according to the consensus hierarchical cluster. Gene Ontology terms provide a means to address the possible functional relationship of a cluster of genes that are coordinately regulated. However, no statistically significant over- or under representation of Gene Ontology terms was seen for any of the gene groups identified by the k-means clusters according to a hypergeometric test (not shown).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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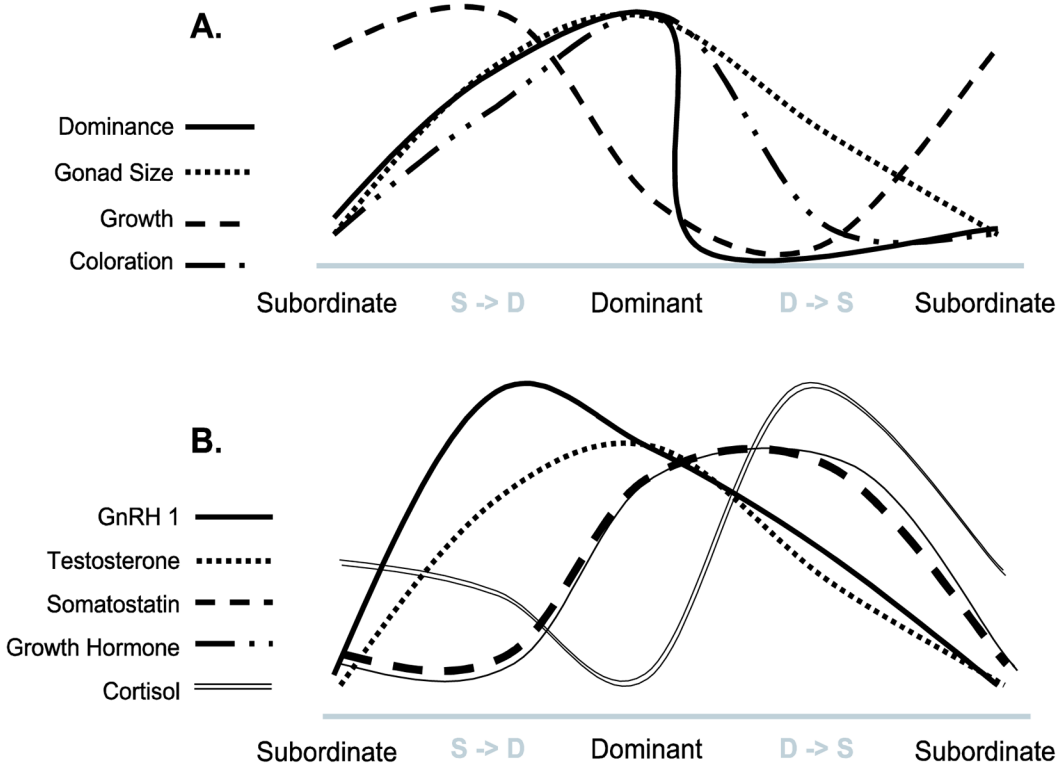

Figure 1.
Schematic representation of A) phenotypic and B) physiological characteristics associated with Dominant and Subordinate male phenotypes in *A. burtoni*. The graphs are based on the following studies: gonad size: Hofmann and Fernald 2000; growth: Hofmann et al., 1999a; GnRH1: White et al., 2002; testosterone: Francis et al., 1993; somatostatin: Hofmann and Fernald 2000; growth hormone: Hofmann et al 1999b; cortisol: Fox et al., 1997.

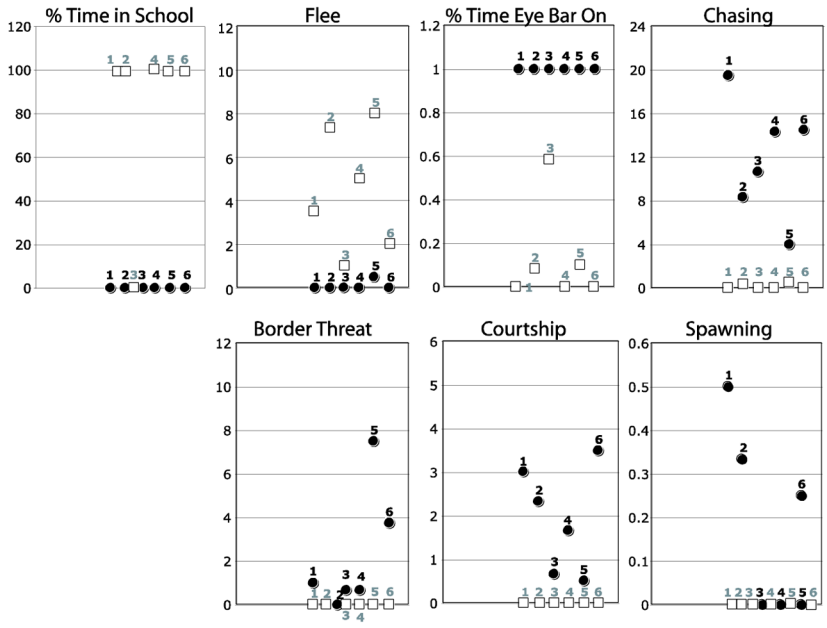

Figure 2.

Social behaviour varied significantly and predictably between the dominant and subordinate males. Y-axis represents (A, C) the percent time or (B, D, E, F) the average number of observed events per 10 minute focal observation. Identified squares, dominants, and circles, subordinates are consistent with figure 5.

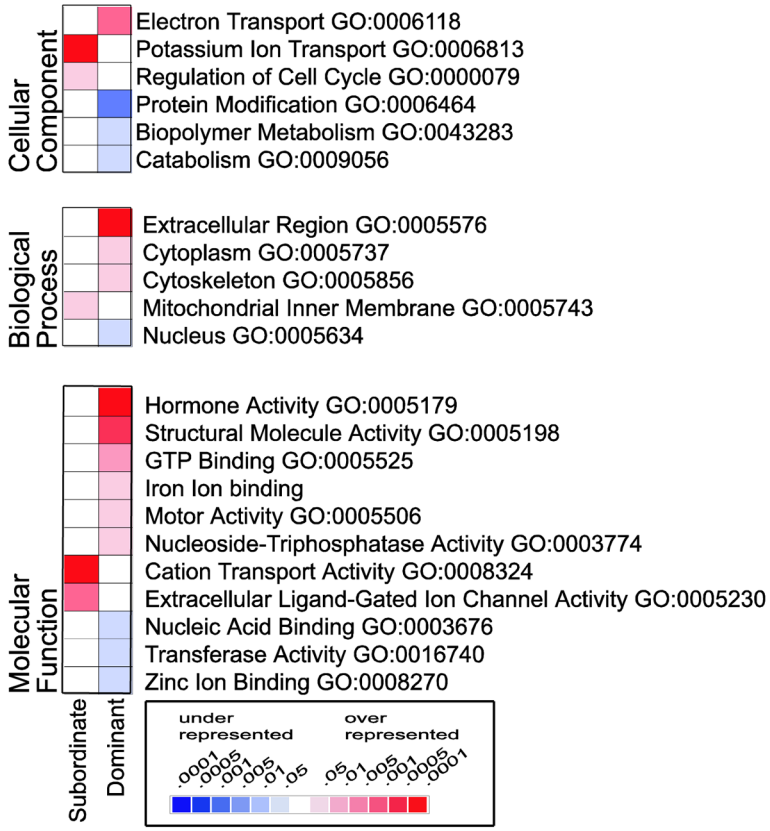

Figure 3.

Analysis, at all Gene Ontology (GO) levels, revealed 22 categories that were statistically over- (red) or under- (blue) represented. The three separate GO vocabularies (molecular function, biological process and cellular location) provide overlapping information, as many of the genes are annotated according to each. P-values represent uncorrected results for the hypergeometric test. GO number and name are according to 200605 releases.

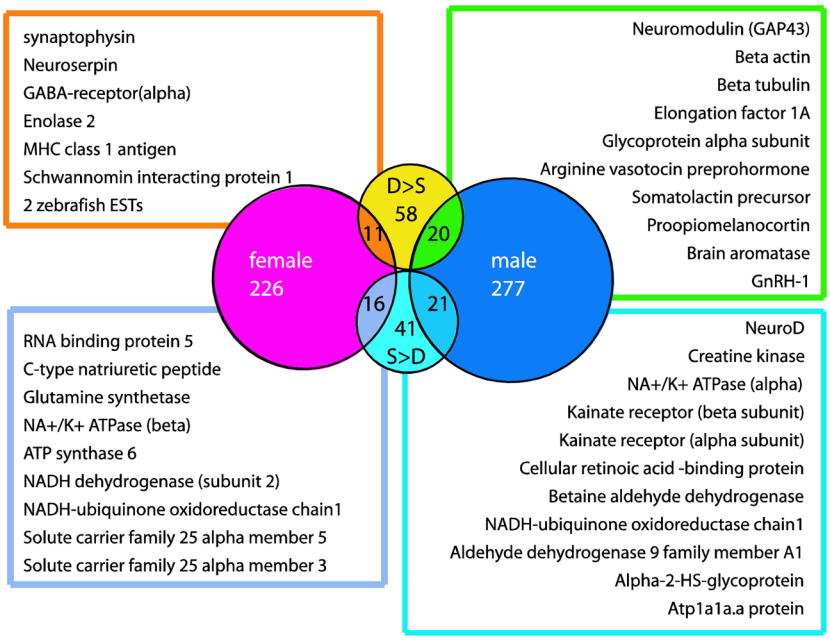

Figure 4.

Venn diagram depicting the relationship of sexually regulated and socially regulated genes. These relationships subdivide the gene classes to indicate modules of gene expression that potentially underlie reproduction (orange), submissive behaviour (lavender), and super-male dominance (green) and opposing super-male dominance (teal). Numbers indicate total unique sequences, and unsequenced array features. Gene names given represent best hit blast annotation for available sequences. The Venn diagram indicates regulation at a BPP of ≥0.99, (the specific BPP, down to 0.80, for regulation is indicated in supplementary tables 1 and 2)

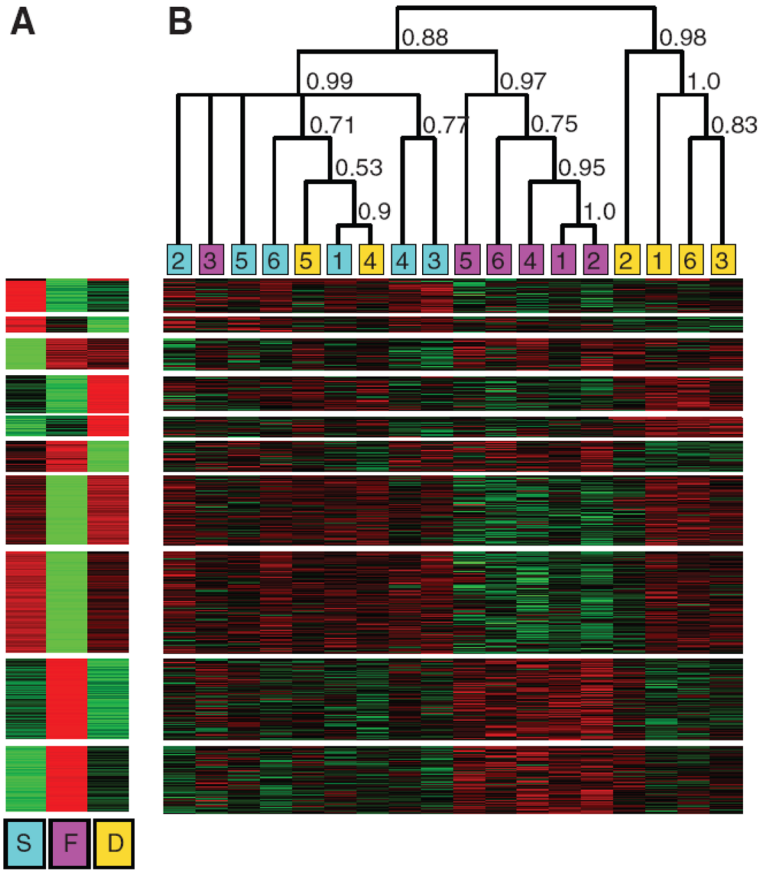

Figure 5.

Hierarchical clustering of (left) phenotypes and (right) individuals (based on expression profiles for the genes regulated at level of phenotype). The genes have been ordered according to K-means clustering. The heatmaps (red – up-regulated, green down-regulated) show estimated gene expression levels. Heat values are relative only within, not across genes. The numbers identify individual males consistent with figure 2. Confidence values at the nodes were obtained by bootstrap analysis (1000 permutations with resampling).

Table 1

False Discovery Rate estimated by randomization of hybridization ratios both between genes and between arrays using permutation with replacement and resampling for analyses conducted at the level of phenotype and sex. The number of features identified by analysis of the permutated dataset as significant for each threshold is reported as the average (n=5) percentage of the number of features identified at that threshold by analysis of the original dataset.

		Bayesian Probability Threshold			
		>=0.99	**>=0.995**	**>=0.999**	**>=0.9995**
phenotypes compared	Male Dominant > Male Subordinate	89	61	41	37
	Male Dominant > female	273	218	148	127
	Male Subordinate > Male Dominant	84	61	26	23
	Male Subordinate > female	277	220	136	116
	Female > Dominant male	205	154	96	78
	Female > Subordinate Male	219	164	95	83
	Males > Female	318	269	174	147
	Female > Males	253	211	119	103
	FDR %	17.08	10.76	3	2.07
	Sterr	0.61	0.57	0.39	0.39

Table 2

Best hits for dominant and subordinate regulated genes grouped according to presumed functional category (see Methods for details). For array feature identification and BPP for differential regulation see supplementary tables 1 & 2.

	Dominant greater than Subordinate	Subordinate great than Dominant
Peptides Neurotransmitters and growth hormones	Prolactin 1 Prolactin 2 Somatotropin Somatolactin Proopiomelanocortin AVT GABA - receptor beta subunit Tilapia growth hormone. Glycoprotein alpha GNRH-1 Brain aromatase	Orphan nuclear hormone receptor Kainate Receptor Cholecystokinin C-type natriuretic peptide
Granins	Chromogranin Neurogranin Secretogranin II	
Cell Structure	Beta actin Beta tubulin Septin 7 Elongation Factor 1a	Clasp2 -regulation of microtubule dynamics
Synaptic Vesicle	similar to Synaptophysin Q5RCZ2_PONPY - secretory vesicle electron transport	
Transcription factor	ETS family efr1	bHLH HLH
Axonal Growth	Neuromodulin Neuroserpin	
Cell Cycle regulation	Schip-1 Pdcd4	Rbm5 protein putative tumor suppressor
cellular metabolic enzymes	Enolase 1 Enolase 2 Uridine kinase Lactate dehydrogenase	many ATPase's, NADH dehybdrogenase and oxidoreductase Glutathion S-transferase Glutamine synthetase Betaine aldehyde dehydrogenase Leucine carboxyl methyltransferase

Table 3

Gene Ontology terms that were significantly over- or under-represented among the genes regulated according to male phenotype. Each of the three independent ontologies is given. Significance is determined to be $p<0.05$ for the uncorrected hypergeometric p-value. TC number refers to "tentative contig" number assigned in release 1.0 *A. burtoni* cichlid gene index (compbio.dfci.harvard.edu).

BIOLOGICAL PROCESS			
GO: 0006118 Electron Transport D>S overrepresented p=1.01E-03 8 of 55			
TC400	0.9991	Q5RCZ2_PONPY	similar to secretory vesicle-specific electron transport protein
HH_NB_Harvardcol_000005749	0.9914	cloned fragment	Brain Aromatase
HH_AB_BRAIN2000_000003171	0.9999	Q803D7_BRARE	Sb:cb825 protein (Fragment).
HH_AB_BRAIN2000_000001582	0.9997	Q9DEZ3_ORENI	Brain aromatase (Fragment).
HH_AB_BRAIN2000_000001970	0.9925	Q6GLW8_XENLA	Hypothetical protein.
TC59	0.9988	Q94SU5_9TELE	Cytochrome c oxidase polypeptide II
TC2	>0.9999	COX1_GADMO	NADH dehydrogenase 1
TC9	>0.9999	CYB_TROMR	cytochrome b family
GO:0006813 Potassium Ion Transport S>D overrepresented p=4.83E-04 5 of 25			
TC15	>0.9999	Q90279_CARAU	Kainate receptor alpha subunit.
TC34	0.9972	Q7ZU25_BRARE	Atp1a1a.1 protein.
TC16	0.998	Q90278_CARAU	Kainate receptor beta subunit.
TC17	>0.9999	Q90278_CARAU	Kainate receptor beta subunit.
TC139	0.9909	Q9DGL2_BRARE	Na+/K+ ATPase beta subunit
G0:0000079 Regulation of Cell Cycle S>D overrepresented p=4.76E-02 3 of 30			
TC109	0.9971	SERF2_HUMAN	Small EDRK-rich factor 2
HH_AB_BRAIN2000_000001151	0.9919	RAS_CARAU	Ras-like protein
HH_AB_BRAIN2000_000005191	0.9965	RBM5_HUMAN	RNA-binding protein 5
GO:0006464 Protein Modification D>S underrepresented p=1.7E-02 none of 148			
GO:0043283 Biopolymer metabolism D>S underrepresented p=3.47E-02 only 2 of 161			
TC42	0.9999	PAB1_HUMAN	Polyadenylate-binding protein 1
HH_AB_BRAIN2000_000004166	0.9992	Q6NX86_BRARE	High mobility group box 1.
GO:0009056 Catabolism D>S underrepresented p=3.38E-02 none of 81			
CELLULAR COMPONENT			
GO:0005737 Cytoplasm D>S overrepresented p=1.27E-02 23 annotated genes out of 383 analyzed			
TC40	0.9997	Q8AVI3_XENLA	Arbp-prov protein.
TC236	>0.9999	LDHB_FUNHE	L-lactate dehydrogenase B chain
HH_AB_BRAIN2000_000001815	0.993	Q9I921_CARAU	Beta actin.
TC2	>0.9999	COX1_GADMO	NADH dehydrogenase 1
TC28	>0.9999	Q9DFT6_9PERC	Beta tubulin.
TC32	0.9998	Q6PC12_BRARE	Enolase 1 (Alpha).
HH_AB_BRAIN2000_000001970	0.9925	Q6GLW8_XENLA	Hypothetical protein.
TC348	>0.9999	RB6A_HUMAN	Ras-related protein Rab-6A

BIOLOGICAL PROCESS			
TC118	0.9998	Q9DG82_BRARE	Prosaposin.
TC291	0.9987	ACT2_FUGRU	Actin, cytoplasmic 2 (Beta-actin 2).
HH_AB_BRAIN2000_000002804	0.9995	Q6P4P6_BRARE	Zgc:73142 protein (HMP19 protein).
TC18	>0.9999	Q8UW60_ORENI	Elongation factor 1a.
TC42	0.9999	PAB1_HUMAN	Polyadenylate-binding protein 1
TC399	0.9999	Q802G7_BRARE	Selenoprotein M.
HH_AB_BRAIN2000_000003171	0.9999	Q803D7_BRARE	Sb:cb825 protein (Fragment).
TC37	0.9944	Q6GQM9_BRARE	Enolase 2.
HH_AB_BRAIN2000_000003205	0.9947	Q6GNE7_XENLA	MGC82852 protein ribosome receptor activity
HH_AB_BRAIN2000_000003279	0.9905	Q9YHD1_ANGAN	Vacuolar-type H+ transporting ATPase B2 subunit.
TC59	0.9988	Q94SU5_9TELE	Cytochrome c oxidase polypeptide II
TC48	0.9934	Q9DFT6_9PERC	Beta tubulin.
TC9	>0.9999	CYB_TROMR	cytochrome b family
HH_AB_BRAIN2000_000003055	0.9989	Q5U4S4_XENLA	LOC495448 protein.
GO:0005856 Cytoskeleton D>S overrepresented p= 2.85E-02 7 of 77			
TC291	0.9987	ACT2_FUGRU	Actin, cytoplasmic 2 (Beta-actin 2).
HH_AB_BRAIN2000_000001469	0.9967	SEPT7_MOUSE	Septin 7
TC28	>0.9999	Q9DFT6_9PERC	Beta tubulin.
TC19	>0.9999	TBA6_HUMAN	Alpha-tubulin 6
TC12	>0.9999	ACT1_FUGRU	cytoplasmic 1 (Beta-actin 1).
HH_AB_BRAIN2000_000001815	0.993	Q9I921_CARAU	Beta actin.
TC48	0.9934	Q9DFT6_9PERC	Beta tubulin.
GO:0005576 Extracellular Region D>S overrepresented p=5.25E-06 15 of 106 analyzed			
HH_AB_BRAIN2000_000004166	0.9992	Q6NX86_BRARE	High mobility group box 1.
TC49	>0.9999	SOMA_ORENI	Somatotropin precursor
HH_AB_BRAIN2000_000003361	0.99	O70534_RAT	ZOG protein.
TC124	0.9998	Q6ZM74_BRARE	Similar to synaptophysin
HH_AB_BRAIN2000_000005345	>0.9999	Q9W7F7_OREMO	Proopiomelanocortin.
HH_AB_Stanfordcol_000005677	0.9998	cloned fragment	Arginine vasotocin
TC290	0.9986	GBRA5_HUMAN	GABA receptor alpha-5 subunit
TC118	0.9998	Q9DG82_BRARE	Prosaposin.
TC194	>0.9999	NEUS_CHICK	Neuroserpin precursor
HH_AB_BRAIN2000_000001923	>0.9999	PRL2_OREMO	Prolactin-2 precursor
TC325	>0.9999	PRL1_OREMO	Prolactin-1 precursor
TC331	>0.9999	SOML_PAROL	Somatolactin precursor
TC310	0.9987	Q8JHD7_HAPBU	Arginine vasotocin
HH_AB_Stanfordcol_000005722	>0.9999	cloned fragment	Arginine vasotocin
TC134	>0.9999	Q9DEI5_OREMO	Glycoprotein alpha subunit
GO:0005743 Mitochondrial Inner Membrane S>D overrepresented p=3.07E-02 4 of 44			

BIOLOGICAL PROCESS			
TC36	0.9929	Q8JHI0_BRARE	Solute carrier family 25 member 5protein
TC13	>0.9999	Q8M522_HAPBU	NADH dehydrogenase
TC21	0.9998	Q8HLY8_9SMEG	NADH-ubiquinone oxidoreductase
TC4	>0.9999	Q7YDM6_9LABR	ATP synthase 6.
GO:0005634 Nucleus D>S underrepresented p=2.3E-02 only 5 of 275			
TC12	>0.9999	ACT1_FUGRU	cytoplasmic 1 (Beta-actin 1).
TC32	0.9998	Q6PC12_BRARE	Enolase 1 (Alpha).
HH_AB_BRAIN2000_000001906	0.9910	O42448_ONCMY	Id2 protein.
HH_AB_BRAIN2000_000003955	0.9919	Q5MBG6_BRARE	ETS family transcription factor efr1.
HH_AB_BRAIN2000_000004166	0.9992	Q6NX86_BRARE	High mobility group box 1.
MOLECULAR FUNCTION			
GO:0005525 GTP Binding D>S overrepresented p=5.23E-03 7 out of 56			
HH_AB_BRAIN2000_000001469	0.9967	SEPT7_MOUSE	Septin 7
TC18	>0.9999	Q8UW60_ORENI	Elongation factor 1a.
TC19	>0.9999	TBA6_HUMAN	Alpha-tubulin 6
HH_AB_BRAIN2000_000005599	0.9959	Q6PBW0_BRARE	Hypothetical protein zgc:73218.
TC28	>0.9999	Q9DFT6_9PERC	Beta tubulin.
TC48	0.9934	Q9DFT6_9PERC	Beta tubulin.
TC348	>0.9999	RB6A_HUMAN	Ras-related protein Rab-6A
GO:0005179 Hormone Activity D>S overrepresented p=1.08E-09 9 of 16			
TC49	>0.9999	SOMA_ORENI	Somatotropin precursor
HH_AB_BRAIN2000_000001923	>0.9999	PRL2_OREMO	Prolactin-2 precursor
TC325	>0.9999	PRL1_OREMO	Prolactin-1 precursor
HH_AB_BRAIN2000_000005345	>0.9999	Q9W7F7_OREMO	Proopiomelanocortin.
TC331	>0.9999	SOML_PAROL	Somatolactin precursor
TC310	0.9987	Q8JHD7_HAPBU	Arginine vasotocin
TC134	>0.9999	Q9DEI5_OREMO	Glycoprotein alpha subunit
HH_AB_Stanfordcol_000005722	>0.9999	cloned fragment	Arginine vasotocin
HH_AB_Stanfordcol_000005677	0.9998	cloned fragment	Arginine vasotocin
GO:0005506 iron ion binding D>S overrepresented p=1.05E-02 5 of 35			
TC400	0.9991	Q5RCZ2_PONPY	similar to secretory vesicle-specific electron transport protein
HH_NB_Harvardcol_000005749	0.9914	cloned fragment	Brain Aromatase
HH_AB_BRAIN2000_000001582	0.9997	Q9DEZ3_ORENI	Brain aromatase (Fragment).
TC2	>0.9999	COX1_GADMO	NADH dehydrogenase 1
TC9	>0.9999	CYB_TROMR	cytochrome b family
GO:0003374 Motor Activity D>S overrepresented p=1.91E-02 3 of 15			
TC291	0.9987	ACT2_FUGRU	Actin, cytoplasmic 2 (Beta-actin 2).
TC12	>0.9999	ACT1_FUGRU	cytoplasmic 1 (Beta-actin 1).
HH_AB_BRAIN2000_000001815	0.993	Q9I921_CARAU	Beta actin.

BIOLOGICAL PROCESS			
GO:001711 Nucleoside-Triphosphatase Activity D>S overrepresented p=2.50E-02 7 of 75			
TC18	>0.9999	Q8UW60_ORENI	Elongation factor 1a.
TC19	>0.9999	TBA6_HUMAN	Alpha-tubulin 6
HH_AB_BRAIN2000_000005599	0.9959	Q6PBW0_BRARE	Hypothetical protein zgc:73218.
TC28	>0.9999	Q9DFT6_9PERC	Beta tubulin.
HH_AB_BRAIN2000_000003279	0.9905	Q9YHD1_ANGAN	Vacuolar-type H+ transporting ATPase B2 subunit.
TC48	0.9934	Q9DFT6_9PERC	Beta tubulin.
TC348	>0.9999	RB6A_HUMAN	Ras-related protein Rab-6A
GO:0005198 Structural Molecule Activity D>S overrepresented p=6.53E-04 11 of 93			
TC40	0.9997	Q8AVI3_XENLA	Arbp-prov protein.
TC236	>0.9999	LDHB_FUNHE	L-lactate dehydrogenase B chain
TC291	0.9987	ACT2_FUGRU	Actin, cytoplasmic 2 (Beta-actin 2).
HH_AB_BRAIN2000_000001469	0.9967	SEPT7_MOUSE	Septin 7
TC19	>0.9999	TBA6_HUMAN	Alpha-tubulin 6
TC12	>0.9999	ACT1_FUGRU	cytoplasmic 1 (Beta-actin 1).
HH_AB_BRAIN2000_000005599	0.9959	Q6PBW0_BRARE	Hypothetical protein zgc:73218.
TC28	>0.9999	Q9DFT6_9PERC	Beta tubulin.
HH_AB_BRAIN2000_000001815	0.993	Q9I921_CARAU	Beta actin.
HH_AB_BRAIN2000_000003205	0.9947	Q6GNE7_XENLA	MGC82852 protein ribosome receptor activity
TC48	0.9934	Q9DFT6_9PERC	Beta tubulin.
GO:0008324 Cation Transporter Activity S>D overrepresented p=2.84E-04 9 of 84			
TC13	>0.9999	Q8M522_HAPBU	NADH dehydrogenase
TC15	>0.9999	Q90279_CARAU	Kainate receptor alpha subunit.
TC1	>0.9999	NU1M_BRARE	NADH-ubiquinone oxidoreductase chain 1
TC21	0.9998	Q8HLY8_9SMEG	NADH-ubiquinone oxidoreductase
TC4	>0.9999	Q7YDM6_9LABR	ATP synthase 6.
TC34	0.9972	Q7ZU25_BRARE	Atp1a1a.1 protein.
TC16	0.998	Q90278_CARAU	Kainate receptor beta subunit.
TC17	>0.9999	Q90278_CARAU	Kainate receptor beta subunit.
TC139	0.9909	Q9DGL2_BRARE	Na+/K+ ATPase beta subunit
GO:0005230 Extracellular Ligand-Gated Ion Channel activity S>D overrepresented p=2.78E-03 3 of 11			
TC15	>0.9999	Q90279_CARAU	Kainate receptor alpha subunit.
TC16	0.998	Q90278_CARAU	Kainate receptor beta subunit.
TC17	>0.9999	Q90278_CARAU	Kainate receptor beta subunit.
GO:0003676 Nucleic Acid Binding D>S underrepresented p=3.47E-02 only 6 of 280			
TC18	>0.9999	Q8UW60_ORENI	Elongation factor 1a.
TC32	0.9998	Q6PC12_BRARE	Enolase 1 (Alpha).
TC40	0.9997	Q8AVI3_XENLA	Arbp-prov protein.
TC42	0.9999	PAB1_HUMAN	Polyadenylate-binding protein 1

BIOLOGICAL PROCESS			
HH_AB_BRAIN2000_000003955	0.9919	Q5MBG6_BRARE	ETS family transcription factor efr1.
HH_AB_BRAIN2000_000004166	0.9992	Q6NX86_BRARE	High mobility group box 1.
GO:0016740 Transferase Activity D>S underrepresented p=1.25E-02 only 1 of 150			
HH_AB_BRAIN2000_000004040	0.9925	UCKL1_HUMAN	Uridine/cytidine kinase-like 1.
GO:0008270 Zinc Ion Binding D>S underrepresented p=1.13E-02 none of 106			

Table 4

Enrichment of intra-phenotype gene expression variation is significant for genes that are both up-regulated and down-regulated for that phenotype in comparison to the other phenotype. Hypergeometric test.

		Regulated by Phenotype	
		Dominant	**Subordinate**
Regulated by Individual		87	84
w/in dominant	824	41 (p=2.9E-07)	47 (p=2.76E-11)
w/in subordinate	925	39 (p=4.3E-05)	52 (p=1.2E-12)

(3598 total genes analyzed)