Sex, Sex-roles, and Dominance: A Functional Genomics Approach

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Abstract

How do environment and genome interact to create a phenotype? I have addressed this question by profiling gene expression in the brain of the African cichlid fish *Astatotilapia burtoni*. Male *A. burtoni* reversibly switch between two behavioral phenotypes, dominant and subordinate, a change that is controlled by social cues. Previous work has defined neural gene expression profiles for these two phenotypes. In the absence of males, females can change their sex-role and exhibit male-typical phenotypes without changing sex. I compared neural expression profiles of dominant and subordinate females with those of males, thus controlling for the effects of sex and reproductive status, which can confound the identification of the molecular basis of social dominance. Many of the genes identified encode plausible candidates such as neurohormones, transcription factors, and structural (synaptic) proteins. Interestingly, brain expression profiles of sex-role reversed dominant females share a significant number of genes that were previously identified as "male-typical" (regardless of social status). Examples include centractin, Y-box binding protein, and elongation factor 1a. Even more striking was my finding that genes such as gonadotropin, AVT, and aromatase are up-regulated in the brains of dominant fish, regardless of sex. These results suggest that the brains of sex-role reversed females do become masculinized to some extent; however, despite the astonishing behavioral similarities, these two phenotypes are not identical at the molecular level. Microarrays are more than a mere gene discovery device. By exploiting cichlid fish plasticity and diversity, we can use expression profiles to identify the molecular building blocks of complex phenotypes behavioral phenotypes.

Introduction

Behavior and Social Environment

A fundamental question in biology is how an organism's social environment affects its behavioral phenotype. In particular, many researchers are attempting to elucidate the mechanisms behind social dominance and aggression, in the hope of applying this research to human behavior. Researchers are taking many different approaches to the problem and use a variety of model organisms: Edwards *et al* (2003) identify specific neural circuits in the crayfish; Carlson *et al* correlate hormone levels and dominance in meerkats; and Broom and Cannings (2002) model hierarchies using game theory, to mention just a few. Crayfish have a relatively simple hierarchical social system (Edwards *et al*, 2003). Meerkats have a complex society in which almost all breeding is done by a single dominant pair, but subordinate individuals contribute to offspring care (Carlson *et al*). The work of Broom and Cannings (2002), unlike the previous two, is entirely theoretical. These examples illustrate the breadth of approaches and systems used to study the same question. The molecular mechanisms underlying behavior are one of the most difficult topics to study, in part because a behavioral phenotype can be incredibly complex and in part because research suggests that genes and environment interact to create a phenotype, and therefore their effects cannot be studied separately (Hofmann, 2003).

Sex Roles and Dominance Hierarchies

Some of the most interesting behavioral paradigms under inquiry are those relating to sex roles and social dominance. Many species demonstrate social systems in which some

animals are dominant over others, with the dominant animals often attacking subordinate animals. Often one sex is can be considered dominant over the other, usually the male. In some species, however, the female is the more aggressive. This phenomenon is linked to sex-role reversal, in which females compete more vigorously for mates than males. Sex-role reversal occurs in varying degrees in many groups of vertebrates; though rare, examples have been found in fish (e.g. pipefishes), amphibians (e.g. green dart-poison frog), and birds (e.g. spotted sandpiper), but not in mammals (Eens and Pinxten, 2000). Hierarchies among males are also common. One model for a dominance hierarchy is the crayfish, which forms transitive linear hierarchies that determine access to food sources (Edwards *et al*., 2003). A very different system is found in some fish, including salmon. In salmon, some males leave the place of spawning, mature in the ocean, and return very large to the site of spawning, where they compete for mates and territory. Others, known as "sneakers" mature quickly at a smaller size and intrude on matings between females and large males to fertilize some eggs (Aubin-Horth and Dodson, 2004). This dichotomy exists in other species as well, such as the plainfin midshipman, *Porichthys notatus* (Foran and Bass 1998). Relative size usually plays a significant role in determining status; in the crayfish, larger individuals are usually dominant over smaller individuals (Edwards *et al*., 2003), while in the salmon, males smaller at emergence mature earlier and become sneakers, while smaller males wait and eventually become the large anadromous males (Aubin-Horth and Dodson, 2004).

Hormonal/Neurological Bases for Dominance-related Traits

Traditional approaches to the genetic basis of complex traits are less applicable to dominance because dominance is a function not only of the animals involved but also of their interaction (Moore *et al*., 2002). However, many genetic, hormonal, and neurological studies have identified important contributors to dominant behavior.

Both testosterone and estrogen have been strongly linked to aggression and male territorial behavior. Many of these studies have been carried out in songbirds, which show increases in testosterone in males during the breeding season, when males engage in extensive territorial behavior and aggression toward intruders (Soma *et al*., 2003). Aromatase is the name of several related enzymes that convert androgens into estrogen. Silverin *et al*. (2000) identified a correlation between plasma testosterone and brain aromatase in songbirds. However, the fact that testosterone levels did not change during aggressive behavior in pied flycatchers (*Ficedula hypoleuca*) suggested that brain aromatase levels are the limiting factor in controlling territorial behavior (Silverin *et al*., 2004). Evidence that aromatase inhibition decreases aggression in song sparrows during the non-breeding season supports a role for estrogen in aggressive behavior (Soma *et al*., 2000). In mice, too, males lacking the aromatase gene showed disrupted male-specific behaviors (Matsumoto *et al*., 2003).

Arginine vasotocin (AVT) (and its mammalian homolog arginine vasopressin, or AVP) is a neuropeptide that has been associated with a variety of reproductive and social behaviors. Vasotocin generally increases male reproductive behaviors in a variety of

vertebrates, including fish, amphibians, and birds (Salek *et al*., 2002; Moore and Miller, 1983; Jurkevich and Grossman, 2003). In the plainfin midshipman, AVT immunoreactive cells in the preoptic area of the brain are larger in Type I (large, nesting) males and females and smaller in juveniles and Type II (sneaker) males (Foran and Bass, 1998). However, AVT/AVP also appears to influence aggression in a manner that is dependent on the social system of the species and the status of the individual involved. A particularly striking example is the vole experiment by Young *et al*. (1999) in which transgenic mice (normally polygamous) expressing the AVP receptor from monogamous voles increased affiliative behavior in response to AVP. The receptors from the two species have different expression pattern, and the transgenic mice displayed a pattern similar to that of the monogamous voles. In the bluehead wrasse, AVT appears to increase aggression and other territorial-like behavior in non-territorial males, but increase mating behavior while decreasing aggression in territorial males, showing that effects of the molecule vary by individual social status as well as by species (Semsar *et al*, 2001). Semsar *et al* (2001) interpret this adjustability of response as evidence that AVT acts through general motivation rather than directly affecting specific behaviors.

African Cichlids

One very useful model system for studying dominance and aggression is the African cichlid fish of Lakes Victoria, Tanganyika, and Malawi, many of which are popular as pets because of their intense aggression and complex social structures. The African cichlids are a group of extremely closely related species (Verheyen et al., 2003) that are nevertheless very diverse in morphology and behavior due to rapid recent and repeated

radiation to fill dramatically different ecological niches through specialization in feeding, habitat use, and social behavior (Barlow, 2000; Kocher, 2004). They show a wide range of social and reproductive systems that are useful for studying both the evolution of behavior and the effects of environment on behavior (Barlow, 2000).

Astatotilapia burtoni

The Lake Tanganyika cichlid *Astatotilapia burtoni* is a useful model for studying aggression and dominance as well as behavioral plasticity because the fish show significant social regulation of behavior (Hofmann & Fernald, 2001). In the wild as well as in standard tank setups, males take on one of two very different phenotypes, territorial (T) and non-territorial (NT). T males are socially dominant and very aggressive. They can be recognized by their bright yellow or blue coloring and dark eyebar (Figure 1A). They defend a three-dimensional territory with border threats (charging with opercula and pelvic fins spread) against other T males, chase NT males and females, dig a spawning pit in their territory, and court and spawn with females. NT males are subordinate to T males. In contrast to brightly colored T males, NT males show dull coloring with no eyebar (Figure 1B), and their behavior is mostly limited to fleeing from aggressive attacks and feeding (Fernald 1977). Unlike "sneaker" males in other social systems, NT *A. burtoni* males have immature gonads and are completely reproductively inactive. Furthermore, these phenotypes are not fixed. Males usually switch between territorial and non-territorial status several times within their lifetimes. Generally, size is a strong predictor of dominance, and NT males grow significantly faster than T males,

which leads to a frequent exchange of positions. The average male holds a territory for 9.5 weeks (Hofmann *et al*., 1999).

Different aspects of status change at different rates during a male's transformation from one status to another. A territorial male can become completely non-territorial in approximately three weeks (as far as reproductive physiology is concerned). A non-territorial male can complete changes in reproduction in only a week, but growth rates decline over two weeks. Moreover, descending males lose territorial behavior instantaneously, while ascending males gain it gradually (Hofmann 2003). Changes in coloration begin to happen within seconds in both cases.

Hormonal/Neurological Bases for *A. burtoni* Territorial Behavior

Earlier work, using the candidate gene approach, has shown that the size of neurons in the preoptic area producing gonadotropin-releasing hormone (GnRH) and that of neurons producing somatostatin (which inhibits growth hormone release) is increased in territorial males (Francis et al., 1993; Hofmann and Fernald, 2000; Hofmann and Fernald, 2001) providing initial evidence that this behavioral switch is orchestrated through gene expression. GnRH neurons also change size in females, shrinking while a female is brooding (White and Fernald, 1993). GnRH induces the pituitary to release gonadotropic hormones, which act on the gonads and regulate sexual maturity in both sexes. As in mammals, these hormones vary with the reproductive cycle in female fish (Mateos *et al*., 2003; Kumar and Trant, 2004). Gonadal steroids in turn regulate gonadotropin levels, forming a feedback loop (Mateos *et al*., 2002). Somatostatin-containing neurons in the hypothalamus have up to three times higher volume in territorial males than in non-

territorial males (Hofmann and Fernald, 2000). If this correlates with increased somatostatin output, it may account for changes in growth rates.

Microarrays

In order to go beyond the single gene candidate approach, the Hofmann lab has created a cDNA microarray from an *A. burtoni* brain cDNA library. In spite of the valuable work that has been done, traditional approaches to finding molecular mechanisms behind behavior have their limits. Genes with large effects can be found with analysis of mutations in model organisms (de Belle, 2002; Bolivar *et al*., 2000), and quantitative trait locus analysis has been used successfully to identify genes whose sequence differences affect behavior (Flint, 2003), but is less useful for non-traditional model organisms. However, neither of these approaches is particularly useful for identifying subtle changes of expression that probably underlie much variation in behavior (Hofmann, 2003). A candidate-gene approach, the standard method in the 1980s and 1990s is laborious and depends on the researcher having hypotheses about what genes might make could candidates. Because of this, discovery of new genes is slow. The cutting-edge technology of microarrays allows a researcher to spread a much wider net for molecular causes of complex phenotypes. The extensive use of microarrays in yeast biology and other model genetic organisms is providing great insight into cellular physiology, development, disease, and other complex processes (DeRisi *et al,* Miki *et al,* Xiang et al). Only now are these tools beginning to be applied to the complex phenotype of behavior (e.g. honeybee: Whitfield et al., 2003). Results of a microarray comparison can be used as a less biased candidate gene list that may include genes hitherto completely unknown,

or one can take a bioinformatics approach, looking for modules of expression. The bioinformatics approach to microarrays also yields valuable information about the genes because this data can serve as functional annotation for novel genes that have never been studied before in any context or in the organism in question.

Gene Expression in *A. burtoni*

The Hofmann lab *A. burtoni* array has 4570 spots that represent up to 3000 genes (Renn *et al*., 2004). Using this array, a comparison was made between territorial males, non-territorial males, and brooding females (Renn et al., in prep.). Though some regulation may be post-transcriptional, the comparison demonstrated sustained and measurable changes in gene expression associated with the behavioral phenotypes. Genes identified as territorial specific included some that make sense in the context of reproduction/behavior such as AVT, gonadotropin, and aromatase, some with other potentially interesting functions such as ion channels, transcription factors, and neurotransmitter receptors, and many novel genes whose functions are completely unknown (Renn *et al,* in preparation). However, territorial and non-territorial males differ in more respects than dominance alone. Many differentially expressed genes are likely to be related to growth, male reproduction, or locomotor activity rather than dominance.

Removal of Confounds Using Induced Female Dominance

To remove some of these confounds, I use female dominance to separate dominance-related changes in gene expression from sex-related changes. Under normal conditions,

female *A*. *burtoni* behave much like non-territorial males. They swim in schools with non-territorial males, feed, and spawn with territorial males. After spawning, females brood the eggs in their mouths for approximately two weeks before releasing the offspring. However, when all males are removed from a tank, some females show coloration and behavior typical of territorial males. They acquire dark eyebars, defend a territory, chase other females, and court and spawn with other females. Unlike some species of teleost fish, *A*. *burtoni* do not change sex, only behavior (Rhodes, 1995). It is likely that similar mechanisms underlie male and female dominance, so a comparison between gene expression profiles in territorial males and gene expression profiles in territorial females should identify common aspects that relate to territorial behavior independent of sex. Careful behavioral observations and physiological measures are necessary to ensure appropriate control of confounds.

Materials and Methods

Housing of Fish

Fish were kept in 100 L aquaria with gravel bottoms. Water was kept at pH 8.5, 28°C and light was full spectrum on a 12hr light/12 hr dark cycle with full-spectrum illumination. Lights came on at 8:00 a.m. after a 10 minute dawn period and went off at 8 p.m. followed by a 10 minute dusk period. Fish were fed every morning with TetraCichlid cichlid flakes (Tetra). Tanks had terracotta flowerpots to simulate natural caves in rock used for territories. Each tank (or side of a divided tank) had on pot in each corner and a smaller piece of one near the center.

All experiments involved fish that were sharing a tank with others in the experiment, leading to non-independence, particularly in behavioral measures. Single-sex tanks for females used in microarrays were split by clear dividers with 5-6 fish per side. When tanks were set up, means of standard lengths ranged from 3.18 g to 3.54 g with standard deviations 0.49-0.63, and means of lengths ranged from 4.88 cm to 5.08 cm with standard deviations 0.10-0.33. Fish were replaced after each round of sacrifice, and for the last round, remaining females (10) were combined in a single tank. Mixed tanks contained 5-6 males and 5-6 females. Males were not measured at setup but were approximately the same age as the females, and therefore much larger. When tanks were set up, means of female masses were 4.03 g and 4.33 g with standard deviations of 0.68 g and 0.72 g respectively. Lengths averaged 5.11 cm and 5.17 cm with standard deviations of 0.17 cm and 0.14 cm respectively. For long-term behavior analysis, 9-10 females were kept in

each tank. At setup, means of masses were 4.90-7.48 g with standard deviations 0.58-1.03 and means of lengths were 5.59-6.36 cm with standard deviations 0.18-0.39.

Behavioral Observations

I observed each fish in person 2-3 times per week between 8 and 10 a.m. (0-2 hours after lights came on). Fish were given 5-10 minutes to adjust to the presence of an observer. Observations were three-minute focal observations of each fish, based on a systematic description of behavior previously developed for territorial and non-territorial *A. burtoni* males (Fernald, 1977). Aggressive behaviors included chasing or biting, threatening, and engaging in border threats. Mating behaviors consisted of digging, courting, and spawning. Submissive behavior was fleeing from an attacker. I counted how many times each fish engaged in each behavior, as well as noting coloring, reproductive status (i.e. brooding/gravid), how long the individual spent as part of the school, and where its territory was located, if any.

Data for Statistical Comparisons

Data from the 18 females that were sacrificed for expression profiling and six additional males were used in statistical analysis of behavior across phenotypes. The fish included six T-like females and six NT-like females from the three all-female tanks and six control females from two regular (mixed male and female) tanks. Behavior data came from the four weeks before sacrifice and included 4-10 observations per fish (mean = 7.94). All six males came from a single mixed tank, with six observations per fish over the same four weeks.

Statistical Analysis

I used SPSS for Windows (SPSS Inc.) to perform a one-way ANOVA for each behavior, with a Games-Howell post-hoc test to identify significant differences between pairs of phenotypes, since for most behaviors homogeneity of variances could not be assumed. Because of the high significance in the ANOVA, I did not correct for multiple hypothesis testing.

Growth

Every 2-4 weeks, I removed all fish from their tank to be weighed and measured. Standard length is measured from the mouth to the base of the tail. Growth data were used from all females in the experiment. Growth data were grouped by individual and by status during the period between measurements (so while a single measurement contributed to only one status categories, a single individual might contribute to multiple status categories if that animal changed status over the course of the experiment), and they were normalized to percent increase in standard length per week. A one-way ANOVA was performed in SPSS.

Spawning

I assessed reproductive status visually during focal observations. I counted a spawn each time it appeared that a fish was brooding that had not been brooding during the previous observation. For calculating frequency of spawning, I divided the number of spawns that took place while the fish was in a particular status category by the number of days that

individual held that status, using only those individuals that held the status for over 70 days to avoid outliers due to individuals spending only a short time as T-like or NT-like. I compared overall growth and spawn rates in the 29 females from long-term tanks and 10 females from mixed tanks using the Bivariate Correlation function (calculating Pearson's correlation coefficient) in SPSS to determine whether spawning rate affects growth.

Hormone Measurement

Blood (ca. 40ul per animal) was obtained from the dorsal aorta using heparinized butterfly infusion sets (25 gauge, Surflo). The plasma was then separated from the serum using a tabletop centrifuge (5000rpm for 10min) and stored at -20° C. Circulating testosterone levels were measured by another lab member with a specific radioimmunoassay (Diagnostic Systems Laboratories) after determining a standard curve from a pool of plasma.

Dissection

Animals were sacrificed by decapitation according to an animal experimental protocol issued by the Harvard University/Faculty of Arts and Sciences (HU/FAS) Standing Committee on the Use of Animals in Research and Teaching, Assurance of Compliance Number 22-22, and brains were dissected out and stored in pieces in 1 mL RNALater (Ambion). Brains were stored at 4°C for 48 hours to allow RNALater to permeate the tissue, and then stored at -20°C. I weighed ovaries and divided by body mass to calculate

gonadosomatic index (GSI). A One-way ANOVA with Games-Howell post-hoc test was used to compare GSI across phenotypes. I sacrificed 18 females, 6 from each phenotype.

Extraction of RNA

I homogenized brain tissue with the Tissue Tearor (Biospec) and extracted RNA using the PhaseLock Gel Heavy (Eppendorf) with a standard Trizol protocol (Invitrogen), using 1 mL Trizol per brain. I measured RNA concentration with a NanoDrop ND-1000 spectrophotometer and used an Agilent Bioanalyzer 2100 to confirm RNA quality based on the ratio of ribosomal RNA bands.

Microarrays: Experimental Design

The most common design for a microarray experiment is to compare every sample to a reference sample. All comparisons between experimental samples are thus the same number of steps away. However, this leads to a less efficient experiment by using comparisons to a sample of no interest and increasing technical variation (Churchill, 2002). A one-way loop can be more efficient as long as it is small, but larger loops make it difficult to compare all samples and loss of a single array can jeopardize the whole experiment. A series of interlocking loops can increase both efficiency and robustness and does not require a reference sample, but additional samples cannot be easily added late in the experiment as they can when using a reference (Churchill, 2002). To maximize efficiency of the experiment, I used a loop with 15 females, 5 from each phenotype, in which each individual was compared to four others, two of each of the other two phenotypes (Figure 2). To correct for potential dye bias, dyes were swapped

between comparisons with two individuals of the same phenotype (e.g. if a sample from a T-like female was labeled with Cy3 when compared with a sample from one NT-like female, it was labeled with Cy5 when compared to a sample from another NT-like female – see Figure 2 arrows).

Microarray

The array used is a spotted cDNA array constructed in the Hofmann lab from a cDNA library. It contains 4570 good spots (i.e. with one and only one clone represented), about 94% of which are detectable in a whole-brain competitive hybridization experiment. This array represents approximately 3000 genes (Renn *et al,* 2004).

Reverse Transcription and Labeling of cDNA

I used a standard indirect labeling protocol for amino-allyl coupling. I labeled two µg of total RNA plus positive control spike RNA for each sample by first annealing primer in a 15 µL reaction with 1 µL of 5 µg/µL poly dT 12–18 primer solution. I prepared reverse transcription reactions on ice: 5 µL 5X 1st strand buffer (Invitrogen); 2 µL 0.1 M DTT; 0.6 µL 50 × amino-allyl-dUTP/dNTP mix (2.5 mM each dATP, dCTP, dGTP, 1.5 mM dTTP (Invitrogen) and 10 mM amino-allyl dUTP (Sigma)); and 2 µL (200 U/µL) SuperScript II (Invitrogen), and then incubated at 42°C for 2 hours. RNA was hydrolyzed by adding 10 µL of 1 M NAOH, and the enzyme was inhibited with 10 µL of 0.5 M EDTA and placed at 65°C for 7 min. The reaction was neutralized with 25 µL of 1 M HEPES pH 7.5 (GIBCO BRL). I then rinsed and concentrated the cDNA on a YM-30 filter (Millipore). The dye-coupling reaction required adding 1.5 µL of 1 M sodium

bicarbonate pH 9.0 and the appropriate Cy3 or Cy5 CyDye Post-labeling reactive Dye Pack (Amersham) and placing it for 1 hour at room temperature in the dark.

Hybridization to array

I purified the labeled cDNA using a Qiagen QIAquick column, pooled with the appropriate sample for competitive hybridization and concentrated to 50 µL on a YM 30 filter. The appropriate hybridization buffer conditions were achieved by adding 6 µL 20X SSC (Gibco), 3 µL poly (dA) poly(dT) (Sigma), 0.96 µL 1 M HEPES, and 0.9 µL 10% SDS to each combined labeled probe. The probe was heated to 100°C to denature and 20 µL placed on each array. (Each slide thus contained two replicates.) Arrays were hybridized overnight in the dark at 65°C in a humidified chamber (Telechem) submerged in a water bath. I removed excess probe rinsing slides in SSC at room temperature and centrifuged to dry. The first rinse was 0.6X SSC with 0.025 % SDS and 0.001M DTT and the second was 0.05X SSC with 0.001M DTT and no SDS.

Scanning and Normalization of Microarray Data

I scanned arrays using an Axon GenePix 4000B confocal laser scanner with GenePix software. After using the software to find spots on the array, I adjusted the gridding manually. I imported raw data from GenePix into R and used the LIMMA library (Linear Models for Microarray Data, Smyth *et al*, 2003) to filter out spots already marked as bad and those that were below two standard deviations above local background and for within-array loess normalization of intensities.

Microarray Analysis

Rather than use a fold-change cutoff to reduce false positives as many microarray users do, I used Bayesian analysis (using B.A.G.E.L. software; Townsend and Hartl, 2002) on the delogged normalized data. This statistical method gives a posterior probability as a significance value and identifies small but significant changes in expression A fold-change cutoff can only identify large expression changes and therefore, in reducing false positives increases false negatives. A Bayesian analysis offers the greatest statistical power and is robust to missing data that arise due to microarray imperfections. In addition to B.A.G.E.L. analysis of female arrays with respect to phenotype and individual, a computer meta-analysis was done using my data and data from the earlier array experiment, which was an experiment similar in design to the current study in which T males, NT males, and brooding females were compared in a loop design (Renn *et al,* in preparation). GenePix data were normalized from each loop separately using within-array print-tip loess normalization of intensities, and a B.A.G.E.L. analysis was done on all the data combined, treating all control females as belonging to the same group. In this meta-analysis, all comparisons between T-like and NT-like females and males were indirect.

Clustering

With the heatmap function of the Stats package in Bioconductor, I did hierarchical clustering of B.A.G.E.L. data using Euclidean distance.

qPCR Primers

To verify microarray results, I performed quantitative real time PCR on the same RNA samples I used in the microarray. For hh_Ab_Brain2000_000001582, I also used RNA from three T males and three NT males that had not been used in any arrays. I designed primers from the clone sequence using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to obtain a product 150-200 bp. Primers were ordered from Qiagen, resuspended, and tested on 20 ng pooled cDNA with 2.5 μL 10X Buffer with MgCl, 0.5 μL 20mM dNTP, 0.25 μL 5U/μL FastStart Taq polymerase (Roche Diagnostics), and 2.5 μL 10μM forward and reverse primers in a 25 μL reaction which was run on a thermocycler at 94°C for 1 min.; 35 repetitions of 94°C for 30s, 55°C for 30 s, 72°C for 30 sec; followed by 72°C for 10 min. and 4°C forever.

DNAse Treatment and RNA Quantification

I removed DNA contamination from RNA samples by treating 1 μg RNA (based on NanoDrop reading) with DNAse I (Invitrogen). I used 1 μL 10X DNAse I Reaction Buffer and 1 μL 1U/ μL DNAse I with 1 μg RNA and water to bring the volume to 10 μL and incubated 15 min. at room temperature before stopping the reaction by adding 1 μL 25 mM EDTA and heating samples for 10 minutes at 65ºC. I quantified RNA concentrations using RiboGreen High range assay (Invitrogen).

Reverse Transcription

I annealed primer in a 12 µL reaction with 1 µL of 500 ng/µL oligo-dT/ 100 ng/ µL random hexamers primer solution, 1 µL 10mM dNTP mix, and 100 ng RNA. I added 4 µL 5X 1st strand buffer (Invitrogen) and 2 µL 0.1 M DTT and incubated 2 min. at 42ºC, then added 2 µL (200 U/µL) SuperScript II (Invitrogen) and incubated at 42°C for 1 hour. Finally, I inactivated the reaction by incubating it at 70°C for 15 min.

Quantitative Real-Time PCR

Because "housekeeping" genes have been shown to change expression and are therefore unreliable as controls for quantitative PCR (e.g. Thellin *et al,* 1999; Eleaume and Jabbouri, 2004), I used precise quantitation of total RNA to ensure that differences in C(T) values come from differences in the expression of the gene of interest. I created a standard curve from PCR product to verify that C(T) values are linear with respect to log(quantity) in the range being measured. I performed qPCR in a 96-well plate. Each reaction was 25 µL: 1 µL 10 µM primer (forward and reverse), 12.5 µL SYBR Green RTPCR Mix, and 5ng cDNA (or 1 µL standard curve and 10.5 µL water). I spun the plates down at 1000 rpm for 1 min. and then ran the reaction in the MJ Opticon2 with the following cycle: 15 min. at 95ºC followed by 40 repetitions of: 15 sec. at 94ºC, 30 sec. at 55ºC, 30sec. at 72ºC. At the end, I ran a melting curve from 50ºC to 95ºC. For hh_Ab_Brain2000_000000598 and hh_Ab_Brain2000_000001508, PCR testing of the primers indicated that they worked better with an annealing temperature of 50ºC, so I changed the Opticon protocol for those genes to include an annealing temperature of 50ºC and began the melting curve at 45ºC.

qPCR Analysis

SYBER green intercalates double stranded DNA; fluorescence therefore increases as product accumulates. The cycle threshold (C(T)) is the cycle at which the fluorescence crosses a threshold. In a perfectly efficient reaction, an increase in C(T) of 1 is equivalent to halving the amount of template (i.e. quantity = $k * 2^{-C(T)}$ for some constant k). I set the C(T) threshold manually for each experiment in the Opticon 2 software and used a one-way ANOVA on SPSS to identify significant changes in C(T) values. If the Homogeneity of Variances test gave $p>0.05$, I used a Tukey post-hoc test; otherwise I used Dunnett's T3 post-hoc test.

Results

T-like Female Behavior

Within two weeks females in each of the three all-female tanks could be divided into two groups based on behavior (Figure 3). 2-3 of the 5-6 females in each tank began to show territorial behavior similar to that of T males (Table 1). These "T-like" females (n=6) showed increased male-like aggression, with more chasing compared to "NT-like" females (n=6) ($p=0.004$) $\mu \pm$ s.e.m. events per 3 minute observation: T-like 11.13 ± 1.43, NT-like 0.32 ± 0.11, Figure 4A). There was a trend toward increased threat displays and border threats, but differences were not significant ($p=0.136$, $p=0.189$) (Threat display: T-like 1.52 ± 0.48, NT-like 0.07 ± 0.07, Figure 4B; Border threat: T-like 0.42 ± 0.16, NT-like 0 ± 0, Figure 4C). T-like females displayed male-like mating behavior such as courting and digging, but differences between T-like females and NT-like females were not significant ($p=0.167$, $p=0.467$) (Court: T-like 0.40 ± 0.14, NT-like 0 ± 0, Figure 4D; Dig: T-like 0.45 ± 0.21, NT-like 0.08 ± 0.04, Figure 4E). The increase in aggressive behaviors corresponded to a decrease in submissive behavior. T-like females fled significantly less than NT-like females ($p=0.004$) (T-like 0.28 ± 0.0, NT-like 5.93 ± 0.80, Figure 4F). NT-like females closely resembled control females (those kept in tanks with males, n=6), with no significant differences in any behaviors, while T-like female showed significantly increased chasing ($p=0.004$), a non-significant trend towards increases in other aggressive and mating-related behaviors, and a significant decrease in fleeing ($p=0.001$, Table 1, Table 2).

I assessed aggression in T-like females in all-female tanks with regard to levels of aggression in *A. burtoni* males under normal social conditions. The only significant difference in any behavior between T-like females and T males was that T males (n=3) showed much higher levels of digging ($p=0.002$) (T-like male 4.28 ± 0.29, T-like female 0.45 ± 0.21, Figure 4E). Otherwise, behavior of T-like females was very similar to that of T males (Figure 3, Figure 4). NT-like females, on the other hand, showed less chasing than T males ($p=0.004$), and both NT-like and control females showed less threat displays and digging and more fleeing. Differences in border threats and courting were not significant; however, NT-like and control females never showed these behaviors. T-like females showed no significant differences in behavior from NT males, and neither did NT-like females or control females (Table 2). However, with such a small sample size, there was high variability in male behavior.

T-like Females Resemble T Males

T-like females displayed black eyebars approximately 90% of the time, while NT-like females showed an eyebar 10% of the time and control females showed it 2-4% of the time. (For each observation, I estimated the percentage of time that the individual displayed an eyebar.) T males always showed an eyebar, and NT males showed one about 33% of the time. T-like females also showed other T-specific coloration; however, it was less bright than in T males (Figure 5A). Though 16 T-like females showed some yellow coloring at least part of the time and 3 T-like females became slightly blue, no females showed the red humeral patch typical of T males or male-like egg spots on the anal fin. NT-like females looked like normal females, with dull coloring (Figure 5B).

Maintenance of Territories

In the long term behavior study, 14 out of 29 individuals in 3 tanks were territorial for at least some portion of the experiment. The three tanks averaged 2.85, 4.13, and 3.13 T-females respectively on any given day. (Tanks held 10, 9, and 10 fish respectively). The first tank was the only tank watched from initial establishment; if data from before the tank stabilized (10 days) are removed, the average number of T females becomes 2.95. Of these 14 females who were T-like at some point, 11 lost territoriality at least once (3 lost it twice and 1 lost it 3 times), but only 2 were non-territorial at the end of the experiment (Figure 6). Of the 16 incidents of territory loss, 10 (68%) occurred shortly after spawning; of these, 5 (50%) regained territoriality within one week. (Also, in one case the experiment ended less than one week after territory loss.) Median time spent territorial was 3.6 weeks (25^{th} percentile = 1.1 weeks, 75^{th} percentile = 10.1 weeks) and median time non-territorial was 3.4 weeks (25^{th} percentile = 0.6 weeks, 75^{th} percentile = 12.1 weeks). If short (less than one week) periods of non-territoriality after brooding were ignored, median time spent territorial was 3.9 weeks (25^{th} percentile = 2.0 weeks, 75^{th} percentile = 11.7 weeks) and median time non-territorial was 4.9 weeks (25^{th} percentile = 1.0 weeks, 75^{th} percentile = 12.1 weeks) (Figure 7). Removal of fish for weighing and measuring did not disrupt hierarchies; behavior after each measurement was similar to behavior directly before, and T-like females returned to the same territories.

Growth

Cichlids, like most teleost fish, exhibit indeterminate growth throughout life. In my study, females averaged 1.08%/week increase in standard length. There were no significant differences in growth rates with respect to social status (One-way ANOVA, $F_{4,82} = 1.807$, $p = 0.135$), though there was a trend toward lower growth rates in control females and socially descending females (Figure 8). When T-like, NT-like, and control females were sacrificed for gene expression profiling, gonadosomatic index (GSI), the ovary mass divided by body mass, was recorded. While differences across phenotypes were significant (One-way ANOVA: $F_{2,13} = 6.748$, $p = 0.010$), post-hoc tests failed to identify significant differences between pairs of phenotypes ($\mu \pm$ S.E.M.: Control females 0.936% ± 0.161%, NT-like females 5.196% ± 1.405%, T-like females 7.540% ± 2.046%).

Spawning

No fish changed gonadal sex during the experiment. All females continued to produce eggs and spawn, although the eggs were not fertilized. All control females spawned at least once. 11 of the 14 fish that spent some time territorial spawned while territorial; 13 of the 25 fish that spent some time non-territorial (including 9 of the 15 that spent the entire time non-territorial) spawned while non-territorial. 8 (out of 29) females were never observed brooding eggs. T-like females averaged 0.41 spawns per 30 days, or about once every 73 days, NT-like females averaged 0.38 spawns per 30 days, or about once every 78 days, and control females 0.62 spawns per 30 days, or about once every 48

days (Figure 9). These differences were not significant (One-way ANOVA $F_{2,46} = 1.908$, $p = 0.165$).

While brooding, T-like females became less aggressive and sometimes lost territories. T-like females showed significant differences in both chasing and fleeing with respect to reproductive status (One-way ANOVA $F_{2,65} = 9.520$, $p<0.001$ for chase/bite, $F_{2,65} = 6.259$, $p = 0.003$ for flee. However, post-hoc tests only identified significant differences in chasing, with brooding females showing a significant decrease in chasing compared to gravid or eggless females ($p = 0.001$, Figure 10). There also seems to be a trend to increased fleeing while brooding in T-like females. Spawning was negatively correlated with growth (Pearson correlation = -0.434, $p = 0.006$, Figure 11). Over the course of the experiment, 9 T-like females (out of 24 that were T-like for some period of time) had male-like spawning pits at least partially dug.

Hormones

Blood samples were taken from all females in the three long-term tanks and from all males and females in a single mixed tank as well as from seven additional males (Control female n=6, NT-like female n = 15, T-like female n=10, NT male n=7, T male n=6) for testosterone measurement. All fish had held their status at least three weeks before blood was drawn (3 females had lost or gained territoriality within the past 3 weeks and were not included). Testosterone levels varied significantly between phenotypes (One-way ANOVA $F_{4,42} = 17.353$, $p<0.001$). Testosterone was significantly higher in territorial males than in all females ($p<0.03$), and T-like females had higher testosterone than NT-

like females ($p = 0.038$, Figure 12). Mean ± S.E.M. for T males was 99.78 ± 18.09 ng/mL, for T-like females was 12.78 ± 2.00 ng/mL, for NT males was 54.81 ± 20.75 ng/mL, for NT-like females was 5.36 ± 0.86 ng/mL, and for control females was 7.31 ± 0.97 ng/mL. There was high variability in males, particularly in NT males.

Microarrays

Microarray results are being prepared for public access at the Gene Expression Omnibus (SERIES ID = GSE975, available online). 4571 spots passed the filtering threshold, with 1479 regulated between any two female phenotypes with significance $p \leq 0.05$; 473 were regulated with significance $p \leq 0.01$ (Figure 13). Differentially expressed genes included several that had been identified in the earlier microarray experiment with males (Table 3). Genes that were upregulated in T-like females included arginine vasotocin (AVT), p450 aromatase, and the gonadotropin alpha subunit, all of which have previously been linked to reproductive and social behaviors in many species and were upregulated in T males, as well as structural (synaptic) proteins, transcription factors, and ion channels. Over half the spots that were regulated between female phenotypes represented unidentified genes. Hierarchical clustering of individual transcription profiles using data from all spots regulated between any two female phenotypes ($p \leq 0.01$) demonstrated that expression data from these genes are good predictors of status (Figure 14). Four out of five T-like females were grouped together, while NT-like and control females were mostly indistinguishable. 165 spots had been identified in the earlier male microarray experiment (Renn *et al* in preparation) to be specifically regulated according to male social phenotype (T male and NT male). When hierarchical clustering of the female

microarray data was performed using only these male phenotype specific spots, the individual females failed to cluster by social phenotype (Figure 15). A meta-analysis was performed in B.A.G.E.L. n order to compare the relative expression levels of male and female social phenotypes. Hierarchical clustering of values from the male-female meta-analysis for genes that were up- or down-regulated in T-like females relative to NT-like females or control females clustered T-like females closer to T males than to other females (Figure 16).

Quantitative Real-Time PCR

I chose five genes to validate with quantitative PCR (Table 4). To test gene expression corresponding to clones hh_Ab_Brain2000_000000598, hh_Ab_Brain2000_000001789, hh_Ab_Brain2000_000001508 (SCHIP1), and hh_Ab_Brain2000_000003435, I used the same RNA from the females as was used for the microarray experiment. I added RNA from 3 T males and 3 NT males (not used in the male array experiment) for qPCR of hh_Ab_Brain2000_000001582 (Aromatase). According to the female array experiment, hh_Ab_Brain2000_000000598 was higher in control females than in NT-like females with a trend toward higher expression in T-like females than NT-like females ($p<0.07$), but qPCR suggested that the gene was expressed more in T-like females than in control females, with no difference between control females and NT-like females. In fact, there appeared to be a trend toward higher expression in NT-like females than in control females. (Figure 17A). Similarly, the arrays showed hh_Ab_Brain2000_000001789 upregulated in T-like females and higher in control females than in NT-like females; however, qPCR showed no differences between female phenotypes ($F_{2,10} = 2.44$, p =

0.137, Figure 17B). Arrays showed SCHIP1 higher in T-like females than in control females and higher in control females than NT-like females. Again, qPCR did not detect any differences in expression between female phenotypes (Figure 17C). For hh_Ab_Brain2000_000003435, qPCR results confirmed microarray results, with no significant differences in expression between any phenotypes (Figure 17D). Based on the array data from this study, aromatase was higher in T-like females than NT-like females and higher in NT-like females than control females. Similarly, the previous study found aromatase higher in T males than in NT males and higher in NT males than in control females. qPCR was only able to confirm that control females had lower levels of aromatase expression than any other phenotype; no other differences were detected (Figure 17E).

Discussion

T-like Females are a Good Control for Sex, Sexual Maturation, and Growth

Behavior data on territorial females suggests that they are a good control for three major confounding factors (sex, reproductive maturity, and growth) when finding genes that are regulated in social dominance behavior in *A. burtoni*. T-like females displayed many of the aggressive and territorial behaviors normally associated with T-males without changing sex. However, T-like females and NT-like females continued spawning (Figure 9), meaning that the female ovaries are still cycling and producing eggs in both phenotypes. Growth rates also showed no differences between phenotypes (Figure 8), and these two traits may be linked. It is not clear what the primary source of the difference in male growth rates is; however, it may be related to the fact that in males only T males spend energy on mature gonads, spawning, a difference not present in females. T males also have increased motor activity, which consumes additional energy. The most likely reason for the trend toward slower growth in control females is that more time is probably spent brooding (since eggs are fertilized), during which time they eat much less (since they brood the eggs in their mouths). Females are known to grow more slowly when brooding (Hofmann, personal communication), and this is supported by the negative relationship between spawning and growth (Figure 11).

The similarity in growth rates between T-like and NT-like females would suggest that T-like females would retain territories longer than males. Since dominance is mostly determined by relative size in the group, T males lose territoriality when NT males, growing faster, surpass them in size. Males generally retain territoriality for a median of

9.5 weeks (Hofmann *et al,* 1999). However, females retained territories only 40% as long (Figure 7). This may be because females had an additional source of instability: brooding reduced aggression and sometimes induced loss of territoriality. It is interesting to note that more than half the fish remained non-territorial the entire time, which is reflected in the high mean time spent non-territorial. This suggests that the similarity in growth rates is affecting the stability of the tanks in ways not immediately obvious. Moreover, 8 of the remaining 14 never lost territoriality once they had gained it except while brooding (for less than a week) (Figure 6). Therefore, much of the perceived instability of the hierarchy comes from less than a fifth of the individuals. It appears that females do not maintain territories as long as males do; however, the primary sources of instability (spawning versus growth) differ between the sexes.

Remaining Confounds: Motor Activity and Female Reproduction

Although I was able to control for growth, sex, and male reproduction by using females, there still remains the confound of motor activity. I have no measure of motor activity in females, so it is not clear whether it differs between T-like and NT-like females, although it is likely. In order to make useful comparisons between this study and the previous study using males (Renn *et al,* in preparation), it was important to keep the control group consistent between the two studies. Because of this, I have necessarily introduced another confound: female reproduction. As in the earlier study, all control females were brooding when sacrificed. However, since brooding decreases aggression (Figure 10), all T-like females used in the microarray and qPCR experiment were gravid when sacrificed. NT-like females varied. As a result, differences in gene expression between female

phenotypes may be due to reproduction. However, by using a meta-analysis as well as direct comparison to the male experiment, I was able to identify genes that were more likely to be related to social dominance. A microarray experiment comparing normal brooding and non-brooding females would control for this confound. Moreover, it would be interesting in its own right as a study of maternal care and of the relationship between brooding and aggression.

Molecular Mechanisms of Dominance: Expression Modules

Once we have observed that the behavior of T-like females is externally similar to that of T males, the next question is whether this behavior is mediated by the same molecular and neurological mechanisms. There are two possible hypotheses. One is that the mechanism for social dominance in females is completely different from that in males. In this case, the genes expressed in a T-like female brain would include female-specific genes and T-like female genes, while a T male would express male-specific genes and T male genes (Figure 18A). However, given the similarity in behavior between T males and T-like females, it is more likely that there is at least some overlap between T-like female genes and T male genes. Figure 18B depicts a scenario in which T-like females use exactly the same mechanisms for social dominance. The failure of expression data for genes differentially regulated between T and NT males to cluster females by phenotype (see Figure 15) could be seen as evidence for the first hypothesis. However, it is more likely that this is a result of other traits, such as growth rates and the presence of mature gonads, which distinguish T males from NT males but do not differ between T-like females and NT-like females. If genes involved in male growth and reproduction are

regulated differentially between social male phenotypes but not in females, it could add significant noise to the effort to cluster individual females.

Using the meta-analysis to cluster all phenotypes by their expression of genes that were differentially regulated in T-like females demonstrates that the genes can also be clustered into modules specific to different phenotypes. These include not only male-specific, female-specific, and territorial-specific clusters, but also clusters of genes differentially regulated in T-like females compared to all other phenotypes (Figure 19). This implies that the actual mechanism was something intermediate between the two hypotheses (Figure 20). In order to acquire T-like behavior, females use some of the same regulatory modules used by T males, but they also use modules whose expression pattern is shared with T and NT males and modules whose expression pattern is unique to T-like females.

Aromatase

One exciting outcome of my study is that brain aromatase cytochrome P450 appears to be upregulated in T-like females and T males. Most work on aromatase has been done in rodents (Lephart, 1996). Aromatase is expressed in specific regions of the brain, where it converts androgens to generate estrogen. This local production of estrogen drives the sexual differentiation of brain regions during a perinatal critical period, for example the sexual dimorphic nucleus (SDN) in the preoptic region, which is larger in males than in females (Lephart, 1996). *In situ* hybridization on brain sections from T males, NT males, T-like females, NT-like females and normal females using probes for aromatase could

identify similarly sexually dimorphic regions in the *A. burtoni* brain. It would be interesting to know whether these regions differ between territorial and non-territorial animals of either sex and to identify the extent of "masculinization" in T-like female brains.

The main developmental effects of aromatase seem to be to establish the gonadotropin secretion profile, which is steady in males and cyclical in females, and to regulate sexual behavior (Lephart, 1996) The effects of aromatase on gonadotropin suggest that higher aromatase may be the cause of the increased gonadotropin in territorial *A. burtoni*. The regulation of sexual behavior by aromatase can apparently be affected long past the critical period, since testosterone or estrogen restores mating behavior to castrated adult rats (Lephart, 1996). Aromatase also affects territorial aggression in sparrows, even during the non-breeding season when territoriality is not affected by gonadal testosterone (Soma *et al,* 2000). In zebra finches, aromatase in the brain also seems to be responsible for circulating estrogen in males (Schlinger and Arnold, 1993).

The estrogen produced by aromatase affects synaptic and dendritic organization, even post-natally (Lephart, 1996). Teleost fish have high levels of brain aromatase activity which may be related to their continuous neurogenesis, and in the plainfish midshipman, it is primarily present in radial glia, especially in the telencephalon (Forlano *et al,* 2001). Many functions have been proposed for these radial glia, including stems cells that could give rise to neurons as well as glia (Forlano *et al,* 2001). This suggests that higher aromatase levels in T-like females and T males might be involved in reorganizing

neurons and masculinizing the brain on a physical and cellular level as well as a molecular level. Oberlander *et al* (2004) demonstrated that aromatization of testosterone is necessary for spatial memory in zebra finches. Aromatase, therefore, may be important in memory and learning in *A. burtoni* claiming territories. Since temporary removal from the tank disrupted neither the hierarchy nor the spatial arrangement of territories, T-like females must have a spatial memory of the tank layout and their own territory in it. Hofmann *et al* demonstrated that changes in the layout of the tank disrupt hierarchies. An adaptation of that experiment could be used to quantify spatial memory abilities in *A. burtoni*. Interestingly, aromatase activity appears to be necessary for sex change in the protandrous black porgy (Lee *et al,* 2001). This includes changes in sex-specific behavior, so some of the pathways used in this change may be used when female *A. burtoni* become behaviorally masculinized even though they do not change gonadal sex. If female *A. burtoni* are using some subset of the regulatory modules involved in black porgy sex change to acquire T-like behavior, that also suggests that the process of sex change can be divided into two co-regulated but at least partially independent processes: changes in behavior and changes in physiology. Godwin *et al* (1996) have provided some support for this by discovering that under conditions that would normally induce sex-change, gonadectomized females were capable of acquiring the full range of TP-male behaviors but did not acquire permanent coloration changes.

Pharmacological manipulations of a molecule of interest are useful for identifying the functions of the molecule, particularly when genetic tools would be difficult to use. To investigate the role of aromatase in territorial behavior, fadrozole or another inhibitor of

aromatase would be a useful tool. A similar experiment in the ring dove, in which courting behavior was measured before and after a fadrozole-releasing osmotic pump was implanted, demonstrated that aromatase is necessary for nest soliciting, but not for other mating behaviors (Fusani *et al,* 2001). Behavior comparisons between treated and untreated animals would confirm whether aromatase is an inducer of territorial behavior, while a microarray comparison between brain RNA of treated and untreated animals would help identify what parts of the dominance pathway are downstream of aromatase. Since the effects of aromatase are caused by the conversion of androgens to estrogens and T-like females had higher testosterone than NT-like females, it would also be useful to see whether injections of testosterone or dihydrotestosterone (non-aromatizable) have the same effects on behavior as up-regulation or down-regulation of aromatase.

AVT

Arginine vasotocin (AVT) has been repeatedly linked to many forms of social behavior, generally increasing mating-related behavior. Semsar and Godwin (2003) showed that the presence of gonads did not affect AVT mRNA levels in sex-changing bluehead wrasses, suggesting that the AVT system represents an independent pathway for control of social behavior. Up-regulated AVT in T-like females may be inducing male-like courtship as well as territorial aggression. As with aromatase, injecting AVT or an AVT antagonist (Semsar *et al,* 2001, used Manning compound, an AVT receptor antagonist, to manipulate the AVT system in the bluehead wrasse) and then observing behavior and brain gene expression would help identify what behaviors AVT is regulating in T-like females. Identifying regions of the brain that express AVT and its receptors through *in*

situ hybridization with AVT- or AVT-specific probes is one step toward understanding the mechanisms by which AVT regulates dominance and sexual behavior. This has been done in males by Greenwood *et al* (in preparation). Lewis *et al* (2004) have developed a method for visualizing the effects of AVT on cells: an AVT conjugate that fluoresces while remaining fully functional. While AVT might directly control specific behaviors, its appearance as a general factor for species- and status-specific social behavior suggests, as Semsar *et al* (2001) pointed out, that AVT plays a more general role as a motivator for sexual behavior.

Brain areas expressing genes known to be of interest in dominance behavior can also be dissected out of the whole brain and used in more focused microarray experiments using amplified cDNA. These experiments would be likely to have less noise from irrelevant brain regions, so genes that change expression in localized areas would appear as significant when they might be overwhelmed by RNA from other regions in a whole-brain experiment.

qPCR Validation of Array Data

Out of five genes examined, one confirmed the array results completely and one partially. I am in the process of attempting to confirm array results for more genes and determine whether this reflects a problem with the microarray data, a problem with the qPCR, or an expected number of false positives and false negatives. If, when many more genes have been analyzed with qPCR, around 95% of the total confirm microarray results, I can conclude that the first group of genes, by chance, represented a small number of array false positives that are to be expected from a technique that is essentially over 4000

competitive hybridization experiments on one slide. On the other hand, if future testing fails to confirm microarray results, it will imply a serious problem with either the microarray experiment or the qPCR experiment, and a more in-depth analysis will be necessary. Some sample sizes were smaller in the qPCR experiment (3 control females for hh_Ab_Brain2000_000000598 and hh_Ab_Brain2000_000001789 and 4 for hh_Ab_Brain2000_000001508, hh_Ab_Brain2000_000003435, and aromatase compared to 5 control females in the array experiment; 4 T females for hh_Ab_Brain2000_000001508, hh_Ab_Brain2000_000003435, and aromatase compared to 5 in the array experiment; 3 T males and 3 NT males compared to 6 of each in the array experiment). This may have exacerbated the effects of individual variability.

Genetic approaches

In choosing genes to confirm with qPCR, I am currently focusing primarily on genes which are interesting either because the gene name and function is known or because the gene was highly regulated. This will generate a list of candidate genes to be studied using more traditional approaches. Since *A. burtoni* is not a traditional model species for genetics, it will be necessary to devise new genetic strategies or tools to determine the functions of these genes. The lack of a sequenced genome makes it difficult but not impossible to use targeted knockdown strategies such as RNAi or morpholinos (because specified genes and genomic regions would have to be cloned and sequenced for interference design and because it is impossible to test for cross-reactivity without comparing the proposed morpholino sequence to all expressed sequences) (for information on using morpholinos in zebrafish, see Nasevicius, A. and Ekker, 2000).

Observing the appearance and behavior of individuals with decreased expression of the genes of interest would help pinpoint the effects of these genes and assist in constructing regulatory networks that can explain the social control of social dominance behavior in *A. burtoni* and perhaps in other species as well.

Evolution of Female Dominance

It is not clear why *A. burtoni* would have evolved this ability for females to take on male behavior without changing sex. There are many fish species that change sex at some point in their life cycle either from male to female or from female to male, depending on how fertility and size are related in that species (Allsop and West, 2003). There are even fish such as *Lythrypnus dalli* (Black *et al,* 2004) which can change sex based on the sex ratios in the surrounding population to become the rarer sex, obviously advantageous in competition for mates. However, T-like *A. burtoni* females are not only more aggressive but also attempt to court and mate with NT-like females, which is unlikely to be beneficial. However, since males are generally much larger than females of the same age and *A. burtoni* generally live in large groups (Fernald and Hirata, 1977), it is likely that a situation such as I created in the lab never occurs in the wild. This may explain why T-like females need to up- or down-regulate genes in a pattern that is very different from other male and female phenotypes. If T-like female behavior is never or rarely seen in the wild, it may be that while males and females possess similar behavioral plasticity, selection only acts on its presence in males. It has been suggested that female dominance behavior may be evidence of a protandrous ancestor (Rhodes, 1995). However, there is no other evidence of a sex-changing ancestor, since neither *A. burtoni* nor any other

cichlid changes sex (Barlow, 2000). On the other hand, genes such as aromatase and gonadotropin are relevant in sex determination. It is possible that sex-determination is socially regulated in *A. burtoni* (Rhodes, 1995) even if gonadal sex cannot change once it is established. In this case, male-specific genes would be socially repressed in females. When this repression is removed, some degree of male sex-determination is activated.

It is possible to speculate that if this plasticity existed in an ancestor, it could have allowed the evolution of sex-role reversed species such as *Julidochromis marlieri* (another Lake Tanganyika cichlid) and possibly other *Julidochromis* species. Lande *et al* (2001) have proposed a model for certain groups of haplochromine cichlids in which sex-determination genes play a crucial role in sexual selection and eventual speciation.

Social regulation of aggressive behavior or dominance occurs in many species. I am interested in the molecular mechanisms of this behavior, which are often confounded by simultaneous changes at many levels. *A. burtoni* is a good model to address this problem because I can use the artificial lab manipulation of female behavior to remove some of the confounds that exist when looking at the natural behavioral switch in males. This strategy has successfully identified molecular modules of behavior through microarray analysis of gene expression profiles in the brain.

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Figures and Tables

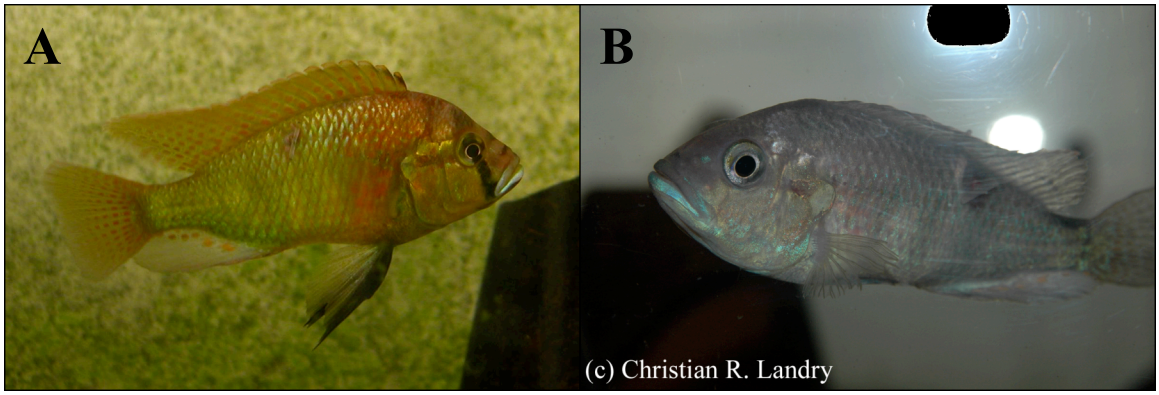

Figure 1: (A) A territorial male displays bright colors, including a characteristic black eyebar. (B) A non-territorial male is gray and has no eyebar.

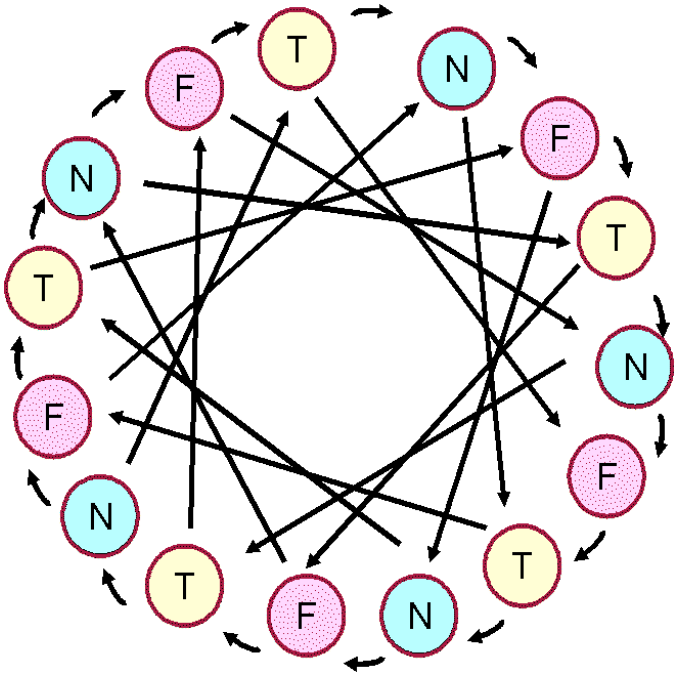

Figure 2: A loop design was chosen for the microarray experiment for greater efficiency and statistical power. Each circle denotes an individual fish. Arrows represent microarray comparisons, with the direction of the arrow going from the sample labeled with Cy3 to the sample labeled with Cy5. T: T-like female; N: NT-like female; C: Control female.

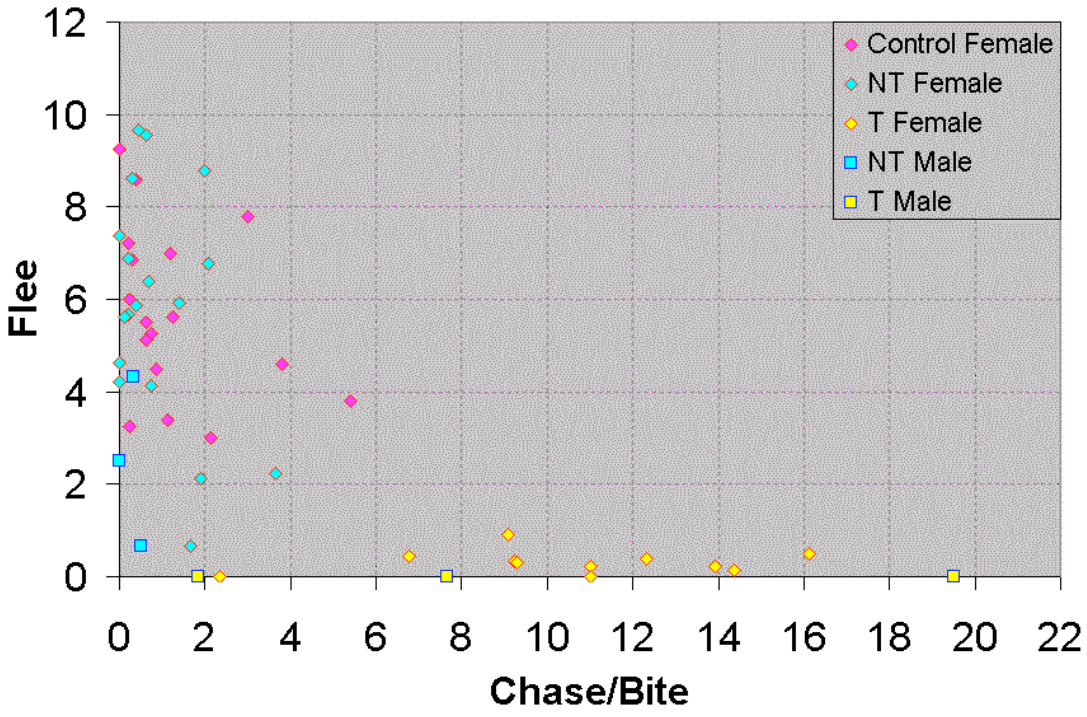

Figure 3: In all-female tanks, some females (yellow diamonds with red outlines) show chase and flee behavior similar to that of territorial males (yellow squares with blue outlines), while others (blue diamonds with red outlines) show chase and flee behavior similar to that of non-territorial males (blue squares with blue outlines) and normal females (pink diamonds with red outlines). Numbers represent events/three minute focal observations, averaged over a month of observations.

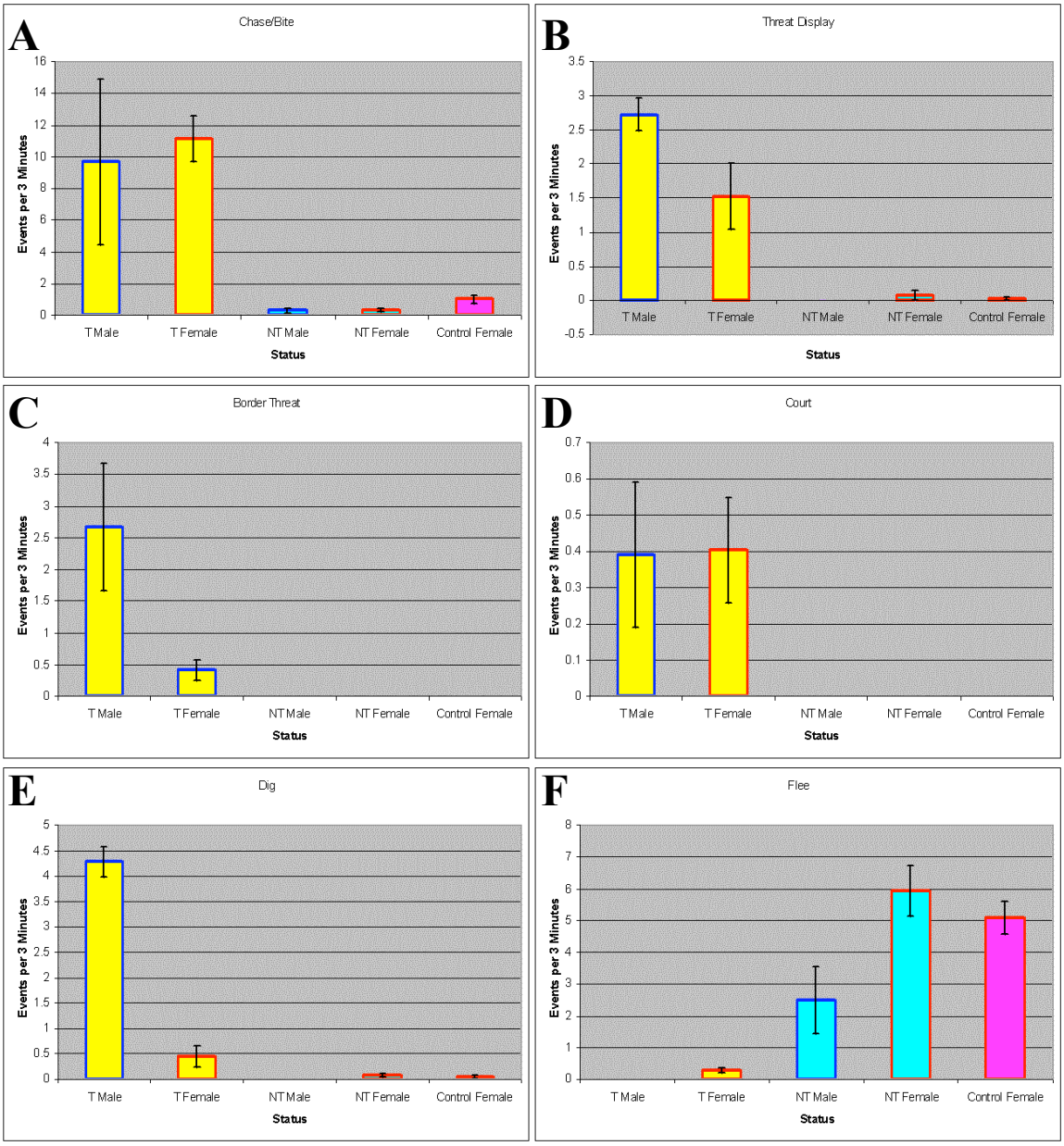

Figure 4: T-like females show behavior similar to territorial males. Error bars represent S.E.M. Females were the 18 (6 of each phenotype) that were sacrificed; behavior data is from the month before death. Data for males comes from one month of observations of 6 males (3 and 3) in a single mixed tank. See Table 2 for significances.

Behavior	Mean ± S.E.M.					One-way ANOVA	
	T Male (n=3)	T-like Female (n=6)	NT Male (n=3)	NT-like Female (n=6)	Control Female (n=6)	F(4,19)	p
Chase/ Bite	9.67 ± 5.20	11.13 ± 1.43	0.28 ± 0.15	0.32 ± 0.11	1.01 ± 0.26	12.092	<.001
Threat display	2.72 ± 0.24	1.52 ± 0.48	0.00 ± 0.00	0.07 ± 0.07	0.02 ± 0.02	14.497	<.001
Border threat	2.67 ± 1.00	0.42 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.445	<.001
Court	0.39 ± 0.20	0.40 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.916	0.007
Dig	4.28 ± 0.29	0.45 ± 0.21	0.00 ± 0.00	0.08 ± 0.04	0.04 ± 0.03	114.164	<.001
Flee	0.00 ± 0.00	0.28 ± 0.08	2.50 ± 1.06	5.93 ± 0.80	5.08 ± 0.52	20.723	<.001

Table 1: Means, standard errors, and ANOVA data for all behaviors from fish in Figure 4. See Table 2 for pairwise significances.

Comparisons		Games-Howell p-values					
Group 1	Group 2	Chase/Bite	Threat display	Border threat	Court	Dig	Flee
T Male	T-like Female	0.070	0.270	0.412	1.000	0.002	0.070
T Male	NT Male	0.384	0.024	0.325	0.493	0.014	0.384
T Male	NT-like Female	0.004	0.016	0.325	0.493	0.014	0.004
T Male	Control Female	0.583	0.023	0.325	0.493	0.014	0.001
T-like Female	NT Male	0.450	0.116	0.189	0.167	0.315	0.450
T-like Female	NT-like Female	0.004	0.136	0.189	0.167	0.467	0.004
T-like Female	Control Female	0.004	0.121	0.189	0.167	0.395	0.001
NT Male	NT-like Female	0.224	0.846	*	*	0.355	0.224
NT Male	Control Female	0.205	0.846	*	*	0.565	0.371
NT-like Female	Control Female	0.207	0.951	*	*	0.947	0.893

Table 2: p-values given by Games-Howell post-hoc test for differences in aggressive, mating-related, and submissive behavior. Yellow: p≤0.05; orange: p≤0.01. *This behavior was never shown in these groups during this time period.

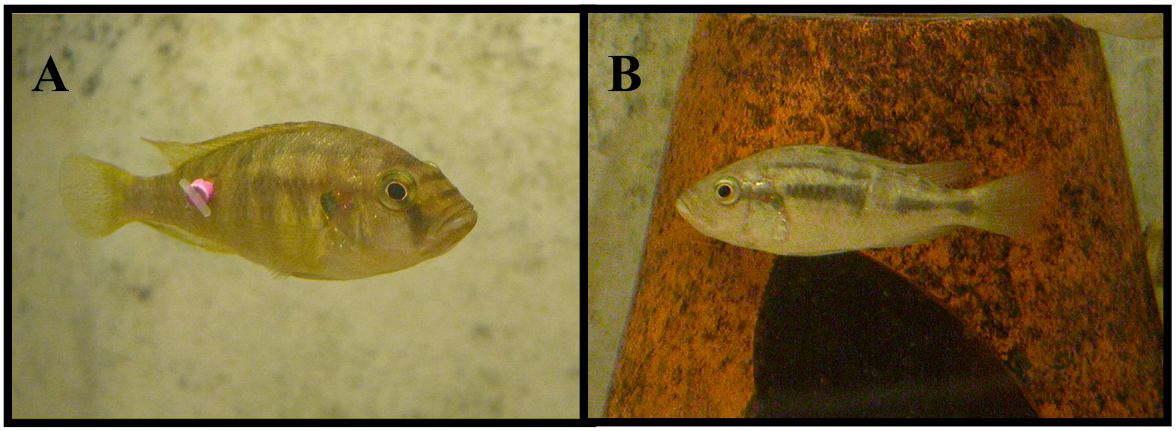

Figure 5: (A) A T-like displays shows brighter colors, including a black eyebar. (B) A non-territorial female is gray and has no eyebar, like a normal female or non-territorial male.

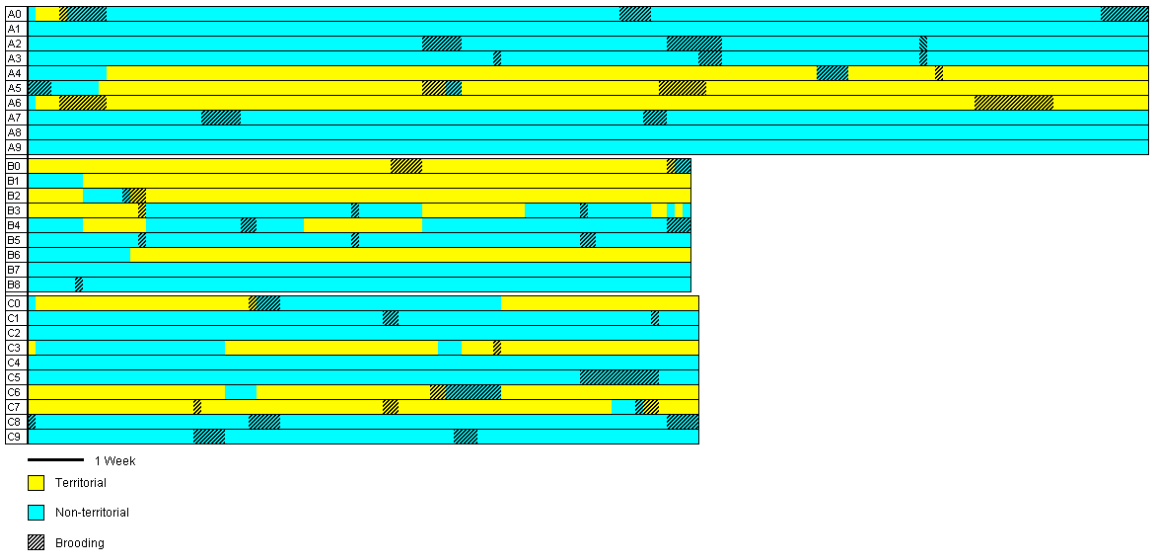

Figure 6: T-like females retained territoriality for long periods of time, but often lost it temporarily while brooding. Yellow: territorial; blue: non-territorial; hatched: brooding. 29 females from 3 long-term tanks are represented. Tank A: 10 females, 142 days; Tank B: 9 females, 84 days; Tank C: 10 females, 85 days.

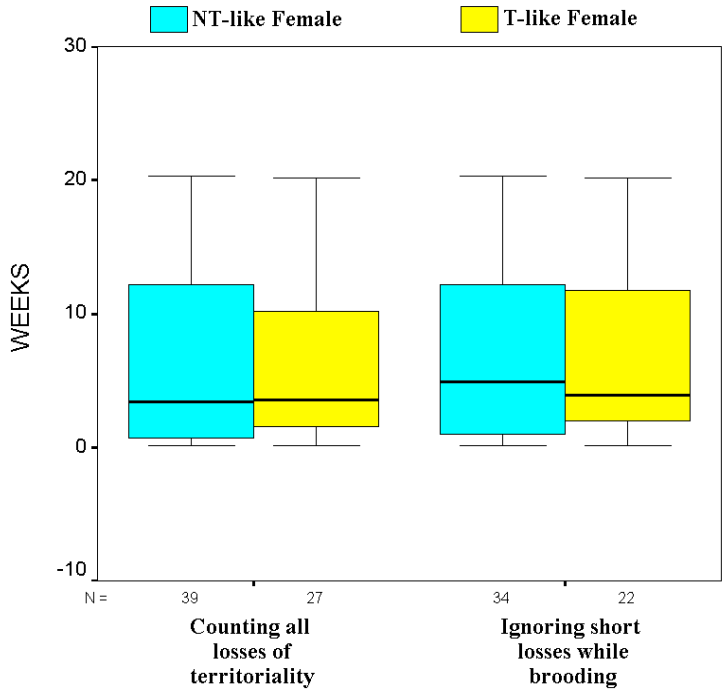

Figure 7: In the three long-term all-female tanks, the median time spent territorial was 3.6 weeks (25^{th} percentile = 1.1 weeks, 75^{th} percentile = 10.1 weeks) and median time non-territorial was 3.4 weeks (25^{th} percentile = 0.6 weeks, 75^{th} percentile = 12.1 weeks). If short (less than one week) periods of non-territoriality after brooding were ignored, median time spent territorial was 3.9 weeks (25^{th} percentile = 2.0 weeks, 75^{th} percentile = 11.7 weeks) and median time non-territorial was 4.9 weeks (25^{th} percentile = 1.0 weeks, 75^{th} percentile = 12.1 weeks).

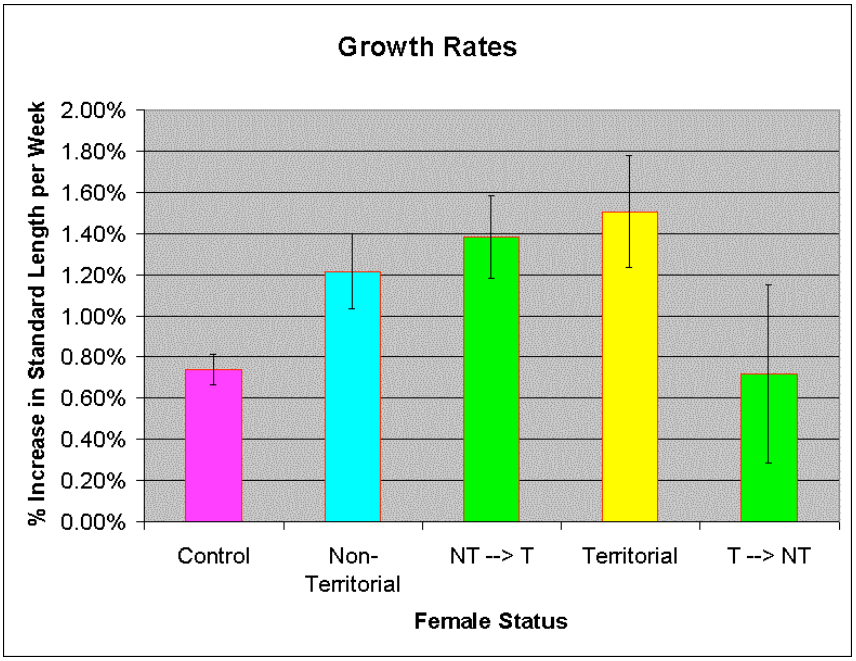

Figure 8: Non-territorial females did not decrease growth rates. N → T: Ascending from NT-like to T-like, T → NT: Descending from T-like to NT-like. Error bars represent S.E.M. and differences are not significant between phenotypes.

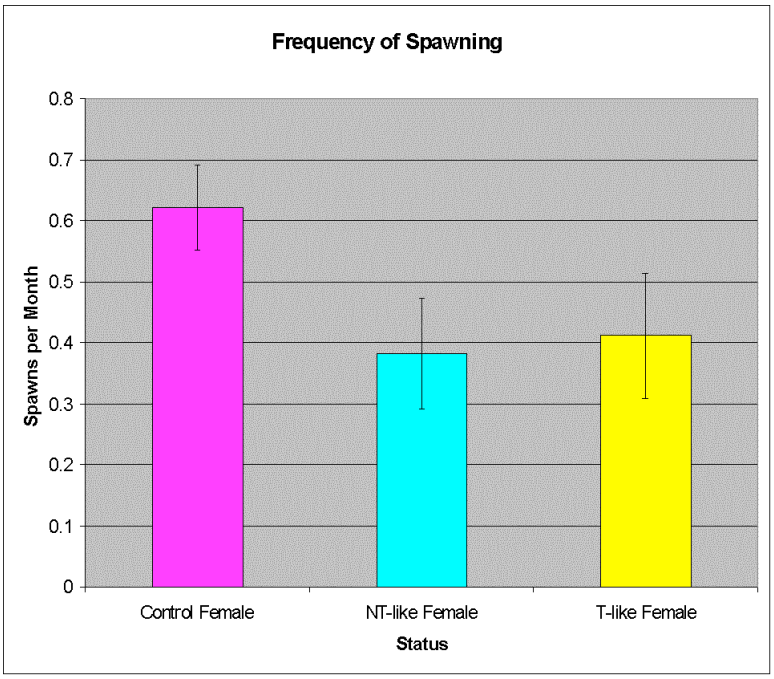

Figure 9: Spawns per 30 days: T-like females and NT-like females continue to spawn at comparable rates to each other and to control females. Differences are not significant (p>0.15).

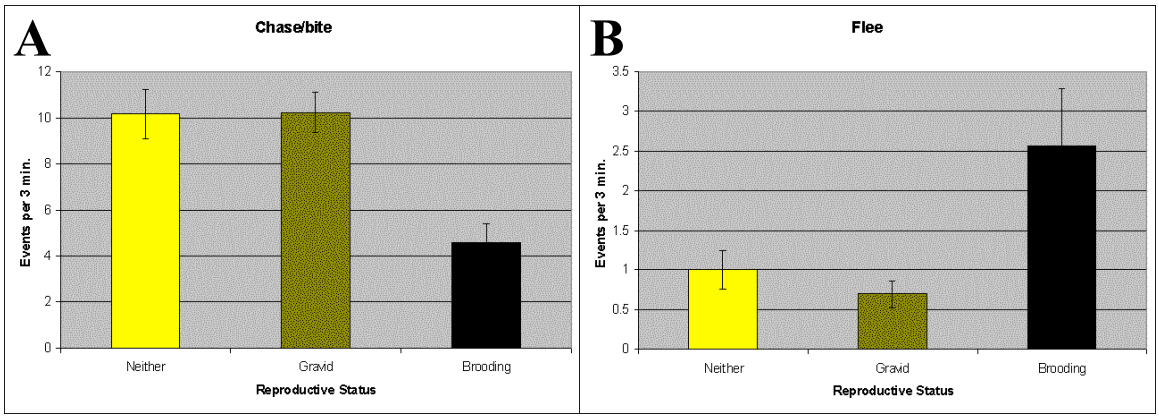

Figure 10: Brooding decreases aggression in T-like females. Error bars represent S.E.M. (A) Chasing and biting is significantly reduced in T-like females while brooding (p=0.001). (B) There is a trend towards an increase in fleeing while brooding, but differences are not significant.

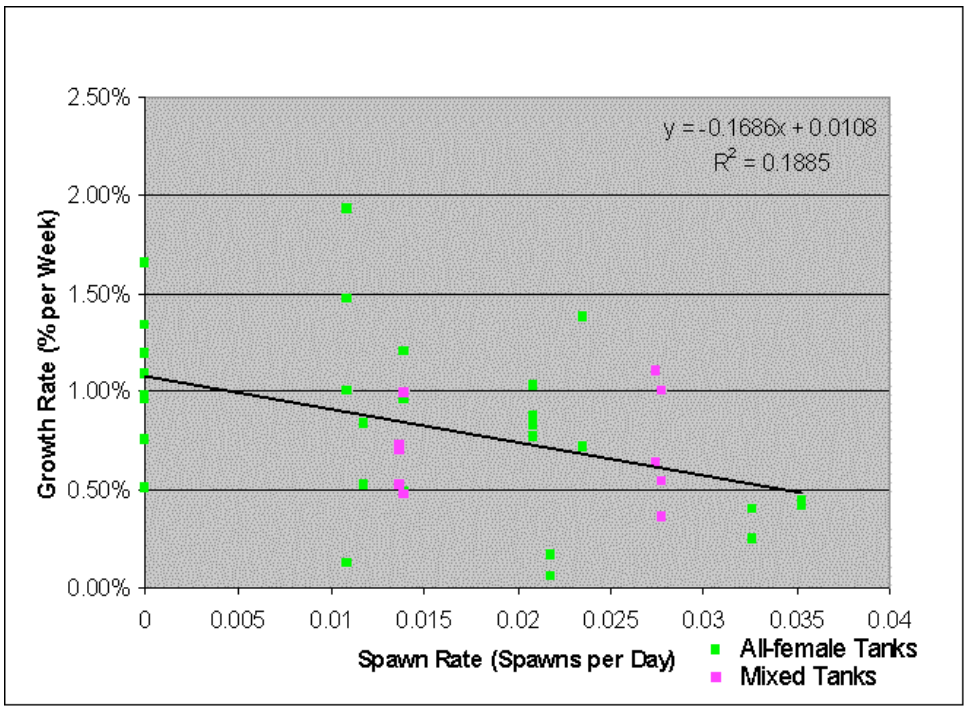

Figure 11: Growth was negatively correlated with spawn rate (Pearson correlation = -0.434, p=0.006). Data from 3 All-female tanks (29 fish) and 2 mixed tanks (10 fish) was combined.

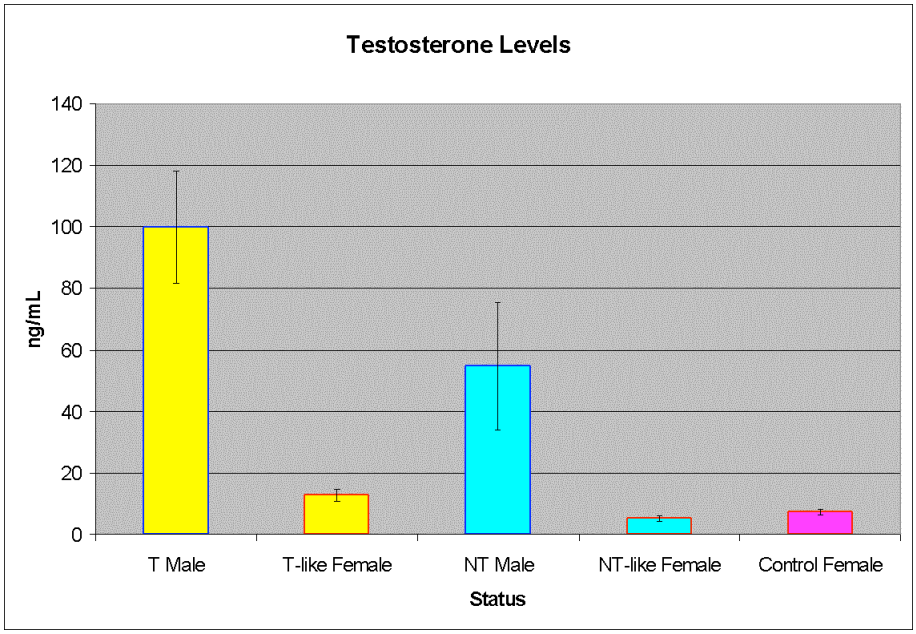

Figure 12: Testosterone levels varied significantly between phenotypes (One-way ANOVA $F_{4,42}$ = 18.509, $p<0.001$). Testosterone was significantly higher in territorial males than in all females ($p<0.03$), and T-like females had higher testosterone than NT-like females ($p = 0.015$)

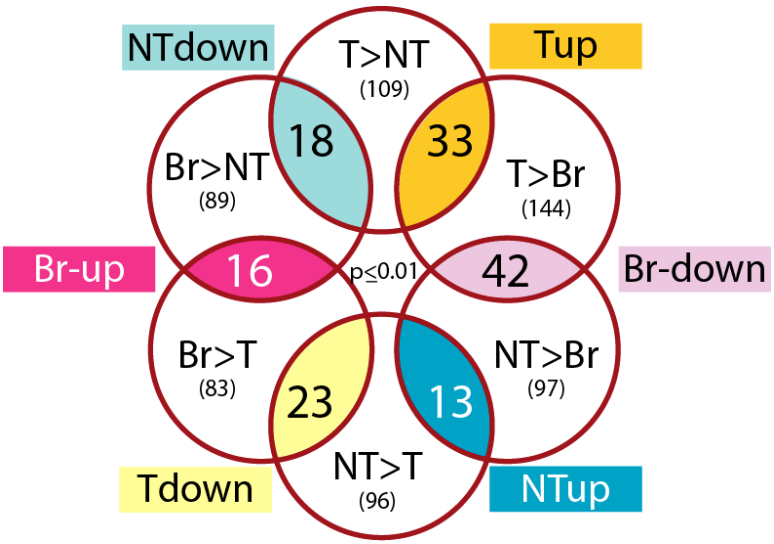

Figure 13: Many genes were differentially expressed between pairs of phenotypes. Numbers represent number of array spots that showed a difference with p≤0.01.

Up-regulated in T Males and T-like Females			
Clone Name	Gene Name	T-like Female Up	T Male Up
hh_Ab_Brain2000_000001582	Aromatase	p<0.01	p<0.01
hh_Nb_HarvardCol_000005749	Aromatase	p<0.01	p<0.01
hh_Ab_Brain2000_000004330	AVT	p<0.01	p<0.01
hh_Ab_StanfordCol_000005672	AVT	p<0.01	p<0.01
hh_Ab_StanfordCol_000005673	AVT	p<0.01	p<0.01
hh_Ab_StanfordCol_000005722	AVT	p<0.05	p<0.01
hh_Ab_Brain2000_000000835	Gonadotropin	p<0.05	p<0.01
hh_Ab_Brain2000_000000931	Gonadotropin	p<0.01	p<0.05
hh_Ab_Brain2000_000001102	Gonadotropin	p<0.01	p<0.05
hh_Ab_Brain2000_000000825	Tubulin	p<0.05	p<0.05
hh_Ab_Brain2000_000001026	Unknown	p<0.05	p<0.01
hh_Ab_Brain2000_000005498	Unknown	p<0.05	p<0.05
Down-regulated in T Males and T-like Females			
Clone Name	Gene Name	T-like Female Down	T Male Down
hh_Ab_Brain2000_000003500	Unknown	p<0.05	p<0.05
hh_Ab_Brain2000_000004550	Unknown	p<0.05	p<0.05

Table 3: Many spots on showed similar regulation in T males and T-like females. p-values for females are from this experiment; p-values for males are from the male loop (Renn *et al*, in preparation).

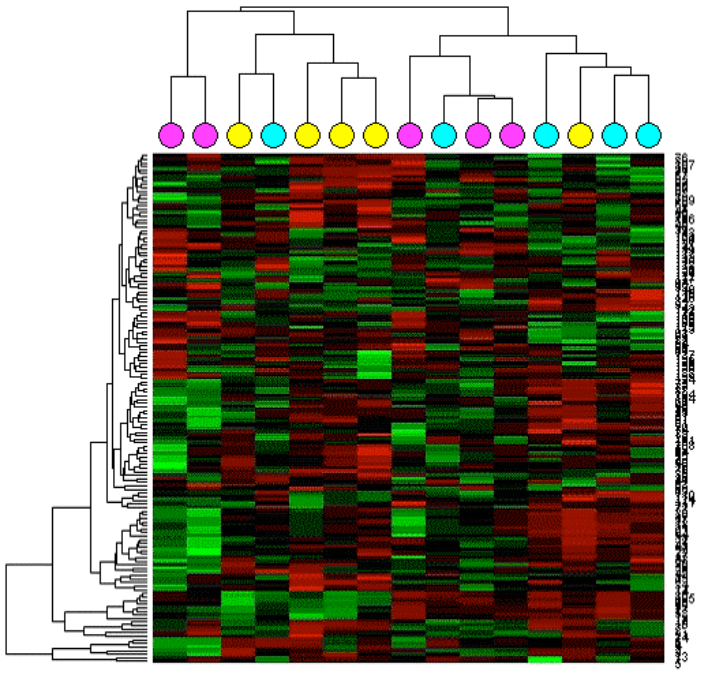

Figure 14: Individual females cluster by phenotype based on transcriptional profiles. Each circle represents an individual. Yellow: T-like; Blue: NT-like; Pink: Control. Spots whose array data were used in the cluster were those that were differentially expressed between any two female phenotypes with $p \leq 0.01$. Hierarchical clustering was done using Euclidean distance.

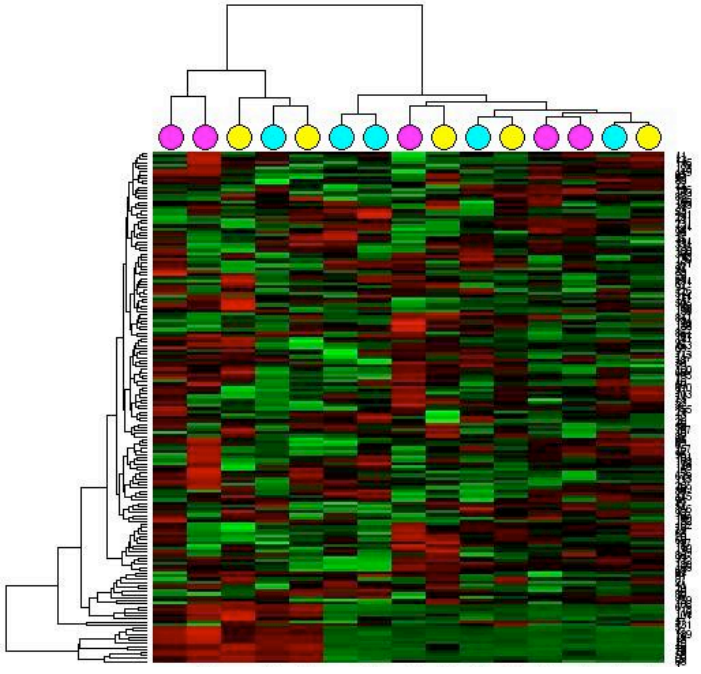

Figure 15: Expression data from genes that are differentially expressed between territorial males and non-territorial males (from Renn *et al*, in preparation) are not sufficient to cluster. Hierarchical clustering was done using Euclidean distance.

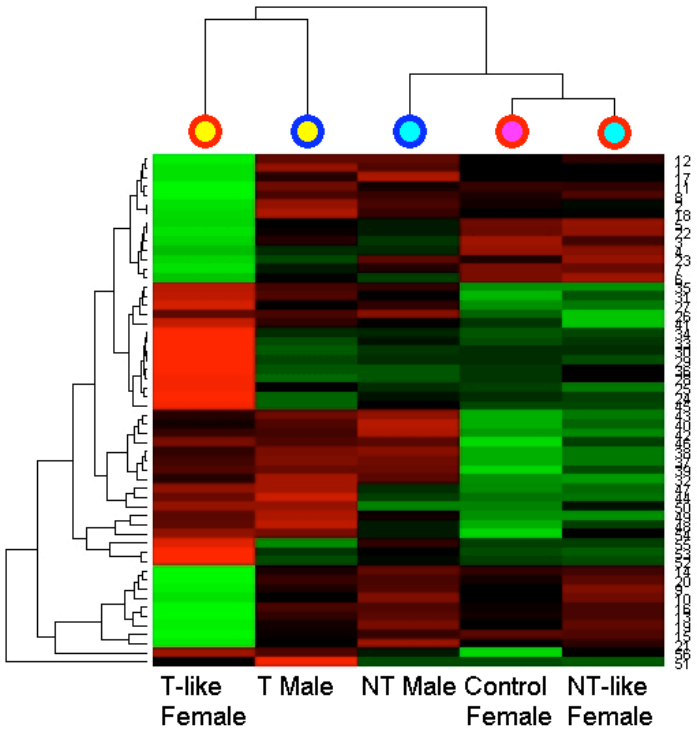

Figure 16: Genes that are up-regulated or down-regulated in T-like females relative to other females ($p \leq 0.01$) cluster T-like females and T-males together in a computer meta-analysis. Hierarchical clustering was done using Euclidean distance.

Clone	Name	Female array results	Male array results	qPCR results
hh_Ab_Brain2000_000000598	No match	Control Female > NT-like Female ($p \leq 0.01$)	Control Female > NT Male ($p \leq 0.01$)	T-like Female > Control Female ($p \leq 0.05$)
hh_Ab_Brain2000_000001789	No match	T-like Female > NT-like Female ($p \leq 0.05$) Control Female > NT-like Female ($p \leq 0.01$)	T Male > NT Male ($p \leq 0.01$) Control Female > NT Male ($p \leq 0.01$)	No Difference ($p=0.137$)
hh_Ab_Brain2000_000001508	Schwannomin Interacting Protein 1 (SCHIP1)	T-like Female > NT-like Female ($p \leq 0.05$) Control Female > NT-like Female ($p \leq 0.05$)	Control Female > T Male ($p \leq 0.01$) Control Female > NT Male ($p \leq 0.01$) T Male > NT Male ($p \leq 0.05$)	No Difference ($p=0.582$)
hh_Ab_Brain2000_000003435	No match	No Difference ($p>0.24$)	No Difference ($p>0.19$)	No Difference ($p=0.103$)
hh_Ab_Brain2000_000001582	Aromatase	T-like Female > NT-like Female ($p \leq 0.01$) T-like Female > Control Female ($p \leq 0.01$) NT-like Female > Control Female ($p \leq 0.01$)	T Male > Control Female ($p \leq 0.01$) NT-like Female > Control Female ($p \leq 0.01$) T Male > NT Male ($p \leq 0.01$)	T Male > Control Female ($p \leq 0.01$) NT Male > Control Female ($p \leq 0.01$) T-like Female > Control Female ($p \leq 0.01$) NT-like Female > Control Female ($p \leq 0.01$)

Table 4: Results of qPCR confirmation of microarrays. Many differences identified by the microarray experiments could not be confirmed by qPCR (though there were no direct contradictions). Observations marked in orange appeared in both microarray data and qPCR data.

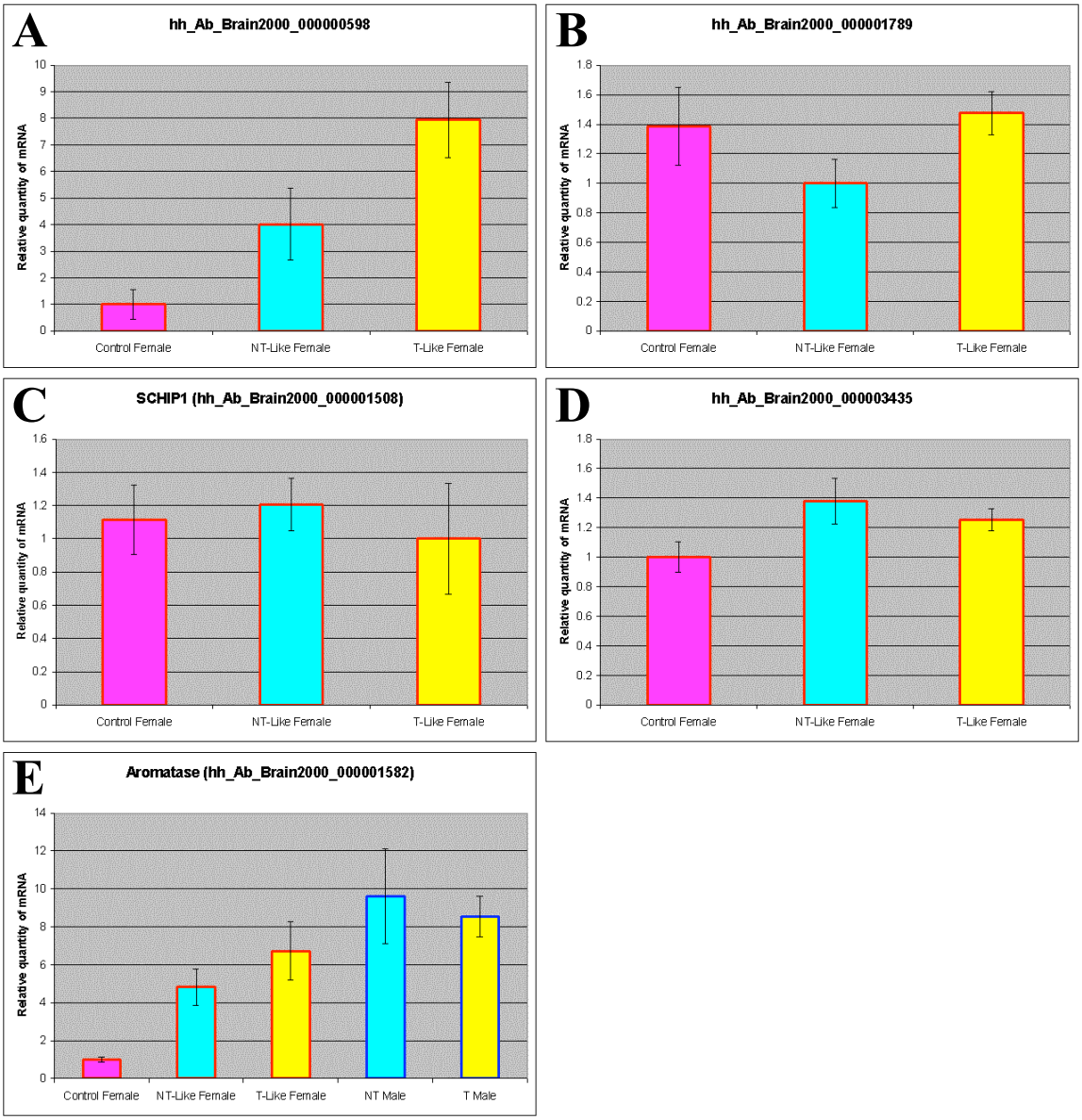

Figure 17: qPCR confirmation of microarray results. (A) Control Female < T-like female (p=0.018); other differences are not significant. (B-D) Differences are not significant. (E) Control females are lower than all other groups (p<0.002); differences between other groups are not significant.

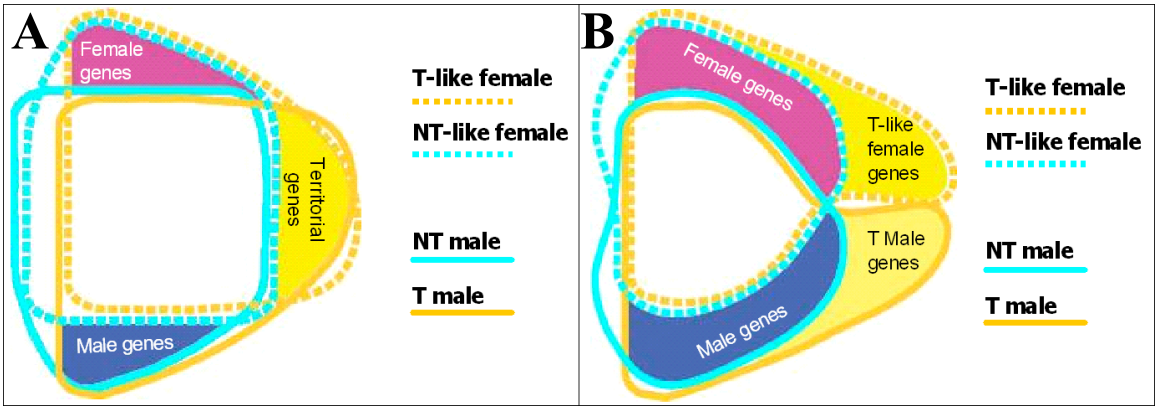

Figure 18: Two hypotheses for how territorial behavior is controlled in males and females. (A) Both males and females might use the same mechanisms. In this module, the genes whose expression differs between T-like and NT-like females would overlap with the genes whose expression differs between T and NT males. (B) Males and females might use completely different mechanisms to regulate territoriality. In this model, the module used for dominance in T-like females does not overlap with the module used by T males.

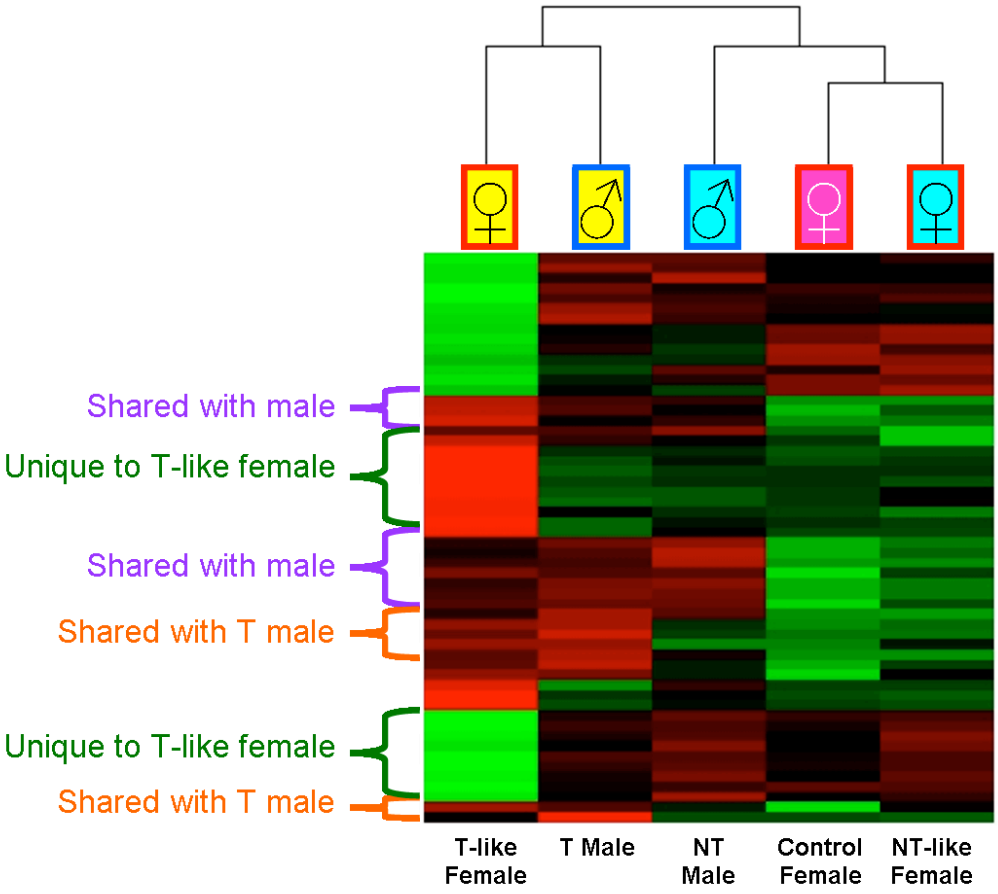

Figure 19: Clustering all phenotypes by expression data from the 56 genes that were up- or down-regulated in T-like females, we can identify modules of expression. There are some genes for which T-like females share an expression pattern with T males, some shared with all males, and some whose expression pattern is different in T-males from any other phenotype.

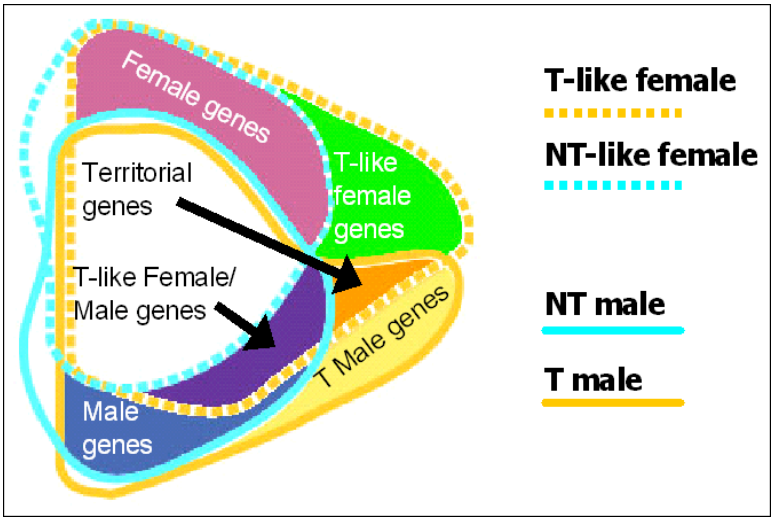

Figure 20: The genes that change expression when females become territorial include some genes that change the same way that they do in T males (territorial genes), but they also include genes that whose expression pattern is shared with all males (T-like female/ male genes) and some genes that are expressed differently in T-like females than in any other phenotype (T-like female genes).