

ORIGINAL ARTICLE

Ecotype differences in aggression, neural activity and behaviorally relevant gene expression in cichlid fish

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Abstract

In Lake Malawi, two ecologically distinct lineages of cichlid fishes (rock- vs sand-dwelling ecotypes, each comprised of over 200 species) evolved within the last million years. The rock-dwelling species (*Mbuna*) are aggressively territorial year-round and males court and spawn with females over rocky substrate. In contrast, males of sand-dwelling species are not territorial and instead aggregate on seasonal breeding leks in which males construct courtship “bowers” in the sand. However, little is known about how phenotypic variation in aggression is produced by the genome. In this study, we first quantify and compare behavior in seven cichlid species, demonstrating substantial ecotype and species differences in unconditioned mirror-elicited aggression. Second, we compare neural activity in mirror-elicited aggression in two representative species, *Mchenga conophoros* (sand-dwelling) and *Petrotilapia chitimba* (rock-dwelling). Finally, we compare gene expression patterns between these two species, specifically within neurons activated during mirror aggression. We identified a large number of genes showing differential expression in mirror-elicited aggression, as well as many genes that differ between ecotypes. These genes, which may underlie species differences in behavior, include several neuropeptides, genes involved in the synthesis of steroid hormones and neurotransmitter activity. This work lays the foundation for future experiments using this emerging genetic model system to investigate the genomic basis of evolved species differences in both brain and behavior.

KEYWORDS

aggression, cichlid fish, evolution, gene expression, neural induction, PhosphoTRAP, pS6, RNA-seq, social behavior, species difference

1 | INTRODUCTION

Animals exhibit aggressive behavior for many reasons, including acquiring and defending limited resources, subduing prey, protecting vulnerable offspring, and competing for mates. However, the specific forms of agonistic behavior vary widely across species and contexts. Aggression, overt behavior with the intention of inflicting harm on another individual, is a polygenic trait with strong environmental components. In both humans and nonhuman animals, aggression is highly

heritable, with approximately 50% of variance in aggression being associated with genotype.^{1–9} Unsurprisingly, the number of genes and genetic variants that have been associated with variation in aggressive phenotypes using genome-wide association studies is very large.^{10–12}

The evolution of aggressive behavior and its complex genetic architecture has been the subject of study since the early days of ethology.¹³ Animal studies have implicated several conserved and well-studied neural and physiological pathways involved in aggression, including sex steroid hormones (androgens and estrogens), serotonin

(5-HT), nonapeptides (arginine vasopressin, AVP and oxytocin, OT), γ -aminobutyric-acid (GABA) and dopamine (DA).^{14,15} Although these neural and molecular systems have been well-characterized, we still have a very poor understanding of how phenotypic variation in aggression is produced by the genome. A better understanding of the genetic basis of aggression would provide insight into the mechanisms of behavioral diversity, as well as point us to genetic and neural networks that may be therapeutic targets in the treatment of behavioral disorders.

Unbiased data-driven approaches in tractable animal systems—such as transcriptomic profiling or quantitative trait loci (QTL) mapping—have the potential to uncover novel genetic mechanisms underlying behavioral variability. Several studies have successfully mapped QTL in inbred lab strains of mice or *Drosophila* which vary in aggressive behavior.^{16,17} Additionally, transcriptomic analyses have uncovered many differentially expressed genes in rats, canines and Silver foxes artificially selected for either aggression or tameness.^{18–20} Unbiased methods such as these are critical for discovering genetic variants and relevant molecular pathways, but these studies also have important drawbacks: the limited generalizability of genetic variants found in the context of a lab-adapted, inbred, or artificially selected organisms.

In the current study, we take advantage of a naturally occurring species difference in aggressive behavior in a genetically tractable animal system, Lake Malawi cichlid fish. In Lake Malawi, two ecologically distinct groups of cichlid fish species (rock- vs sand-dwelling ecotypes, each comprising over 200 species) have evolved within the last million years.²¹ The adaptive radiation of Malawi cichlids has led to remarkable phenotypic diversity in both brain and behavior, yet Malawi cichlid species possess remarkably similar genomes and share polymorphism because of both periodic hybridization and retention of ancestral variation.^{22–24} The rock-dwelling species (also known as *Mbuna*) are aggressively territorial year-round and males court and spawn with females over the rocky substrate. Border contests between territorial *Mbuna* males involve overt physical aggression, including face-to-face lunges and jaw locking.²⁵ In contrast, males of the sand-dwelling species are not territorial and instead aggregate on seasonal breeding leks in which each male constructs a courtship “bower” in the sand where he displays to females.²⁶ Sand males exhibit a wide range of agonistic behaviors to defend their bowers from rival males, although it has been hypothesized that bowers and their size, as well as a number of display behaviors, serve to reduce physical aggression among sand species.^{27–29}

Although ecotype differences in aggression have been reported in field studies in Lake Malawi,^{25,30–33} little is known about the neural and genetic basis of this difference. To quantify species differences in aggressive behavior in males under controlled conditions, we employed a classic mirror test assay.³⁴ African cichlids, like other fish, do not recognize themselves in the mirror and reliably respond aggressively toward their own reflection.^{35–37} Mirror tests are conceptually similar to resident-intruder tests, very reliably elicit aggressive behaviors, and have been used extensively to measure unconditioned agonistic behavior in fish. A number of researchers have criticized

whether both behavior and neural responses in the mirror test are ecologically valid.^{37–41} However, mirror tests have the advantage of eliminating the variance introduced by the opponent and minimizing the risk of injury associated with real-life aggressive encounters. Furthermore, behavior in mirror tests has repeatedly been found to positively correlate with aggression during live agonistic trials.^{37,42,43}

Here, we quantify and compare behavior during the mirror test in seven species of Lake Malawi cichlid (three sand- and four rock-dwelling species) and show substantial ecotype and species differences in unconditioned mirror-elicited aggression. Second, we compare neural activity in mirror-elicited aggression in two representative species, *Mchenga conophoros* (MC, sand) and *Petrotilapia chitimba* (PC, rock). Finally, we compare gene expression patterns between these two species specifically within neurons activated during mirror aggression using PhosphoTRAP.^{44,45} This method uses antibodies to phosphorylated ribosomal protein S6 (pS6) to enrich for transcripts bound to phosphorylated ribosomes. In neurons, this phosphorylation occurs downstream of the binding of neurotransmitters. Thus, pS6 antibodies are increasingly being used to label neurons activated by a stimulus, similar to immediate early genes (IEGs) like *c-fos* or *egr-1*, especially in nontraditional or nonmammalian systems.^{46–50} Using whole brain samples, we use this unbiased RNA-sequencing approach and identify a large number of genes associated with species differences in aggressive behaviors. Several of these candidate genes are in peptide and hormonal signaling pathways. In addition, we consider the analytical challenges of PhosphoTRAP data sets and suggest next steps for future analyses.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult Malawi cichlids were housed in a recirculating aquarium system at 28°C. Water quality monitoring, feedings of spirulina flakes (Pentair ZSF25, 2× daily), and husbandry were provided by the Georgia Tech Physiological Research Laboratory staff. All animal experiments were approved by the Georgia Tech Institutional Animal Care and Use Committee (Protocol # A100028).

2.2 | Mirror test assay and behavior scoring

The following protocol was used for all mirror assays. Mature, mirror-naïve males were removed from mixed-sex social tanks and were housed alone in a 95-L tank (0.44 m × 0.44 m × 0.41 m) for 6 days. Each experimental housing tank was equipped with a single PVC pipe on a randomly selected side to serve as a shelter and facilitate territory establishment. Each tank was surrounded on three sides with white plastic, to prevent visual interaction between adjacent tanks.

On the seventh day, a 0.3 m × 0.3 m square mirror was introduced to the tank against the wall of same side of the tank as the PVC pipe shelter (Figure 1A). Behavior was filmed using a GoPro 4.0 HD

video camera for 15 minutes, after which the mirror was removed from the tank. In the control condition, the same mirror was introduced backward, with the opaque gray backing of the mirror facing the tank. This resulted in a similar disturbance, but there was no reflective surface with which the fish could interact. All mirror tests were conducted between 11:00 and 16:00.

Behavior was analyzed by a trained observer using Noldus Observer XT. We recorded the amount of time subjects spent in proximity to the mirror (within 5 cm of the mirror surface), performing lateral displays (body oriented parallel to the mirror with fins extended), and with their nuptial colors "on." In addition, we scored the occurrence of frontal attacks (ie, bites and/or open mouth contact with the mirror). Although other aggressive behaviors such as body quivering and operculum flaring displays were occasionally observed, these occurred at very low frequencies in a small number of individuals and were thus excluded from the analyses.

2.3 | Behavioral experiments

To test the hypothesis that males of rock species and sand species differ in aggressive behavior, we tested seven species of Lake Malawi cichlid (three sand and four rock species) in the mirror test assay:

Copadichromis virginalis (CV, sand, $n = 8$), *Tramitichromis intermedius* (TI, sand, $n = 8$), *Mchenga conophoros* (MC, sand, $n = 15$), *Petrotilapia chitimba* (PC, rock, $n = 17$), *Cynotilapia afra cobue* (CA, rock, $n = 8$), *Labeotropheus fuelleborni* (LF, rock, $n = 9$) and *Metriaclima zebra* (MZ, rock, $n = 9$). In this behavioral comparison, all subjects were in the mirror condition.

In order to identify the brain regions which exhibited differential activation during mirror-elicited aggression, we compared pS6 immunostaining between two representative species, MC and PC, in either the mirror test (MC, $n = 7$; PC, $n = 6$) or in the control condition (MC, $n = 6$; PC, $n = 6$). These species were chosen because they are representative of the ecotype difference (particularly in terms of lateral display behavior), well-studied, and breed readily in the lab. In the pS6 RNA-immunoprecipitation experiment, we compared behaviorally relevant gene expression in the same two species (MC and PC) in either the mirror test or in the control condition, with two males per group ($n = 8$ total).

2.4 | Tissue collection

Ninety-minutes after the introduction of the mirror or control stimulus (75 minutes after the end of the stimulus), the subjects were sacrificed by rapid decapitation. For tissue processed for

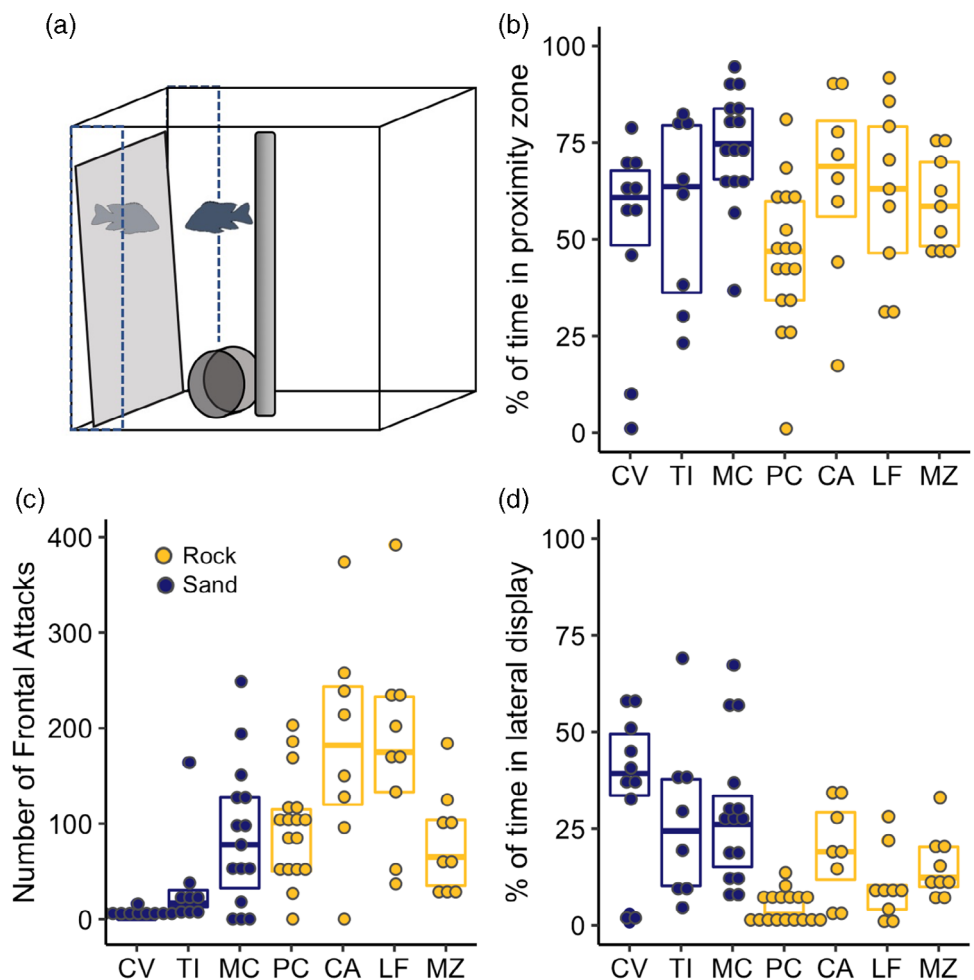


FIGURE 1 A, Diagram of mirror test aggression assay. Dotted lines delineate the zone of proximity within 5 cm of the mirror surface. B, Boxplot of % of 15 minutes assay spent (within 5 cm of the mirror surface) [Rock vs sand, $Z = -1.98$, $P = .047$]. C, Number of frontal attacks ($Z = 4.20$, $P < .0001$) d) Time (s) performing lateral displays during ($Z = 4.67$, $P < .0001$). Sand species are shown in blue; rock species are in yellow. Species: *C virginalis*, CV ($n = 10$); *T. intermedius*, TI ($n = 8$); *M conophoros*, MC ($n = 15$); *P chitimba*, PC ($n = 17$); *C afra*, CA ($n = 8$); *L fuelleborni*, LF ($n = 9$); *M zebra*, MZ ($n = 9$)

immunohistochemistry, the top of the skull was removed and the whole head was immersed overnight in 4% paraformaldehyde (PFA) in 1× PBS, pH 7.4 at 4°C. After fixation, the tissue was washed in 1× PBS and carefully removed from the skull. The brain was then cryoprotected in 30% sucrose in 1× PBS at 4°C overnight and frozen in embedding medium and stored at −80°C until sectioning. The brain was sectioned in the coronal plane at 20 µm in four series, thaw-mounted onto positively-charged slides, and stored at −80°C until immunohistochemistry. For tissue used for pS6-immunoprecipitation, the brain was fully extracted from the skull in under 3 minutes and flash frozen in cold 2-methylbutane and stored at −80°C until immunoprecipitation.

2.5 | Immunohistochemistry

Sectioned brains were labeled with an antibody to neural activation marker phospho-S6-ribosomal protein (pS6). Slides were air-dried at room temperature for 40 minutes. The tissue was fixed a second time on the slides for 5 minutes in chilled 4% PFA and then rinsed in 1× PBS. To inactivate endogenous peroxidases, the slides were incubated in 1% hydrogen peroxide in 1× PBS for 20 minutes and then rinsed in 1× PBS 3 times for 5 minutes each. To block nonspecific binding, the slides were incubated for 1 hour in a 5% blocking solution (5% normal goat serum and 0.3% Triton X-100 in 1× PBS) followed by 1× PBS for 2 minutes. The sections were then incubated in the same pS6 primary antibody used in the pS6-immunoprecipitation (1:300, Cell Signal #2215 pS6 ribosomal protein S240/244 antibody, 2% NGS, 0.3% Triton X-100 in 1× PBS) overnight at room temperature. Slides were then rinsed in 1× PBS three times for 5 minutes each. Next, sections were stained using the Vectastain ABC Anti-Rabbit IgG Kit (VectorLabs PK-6101, Burlingame, California) per manufacturer's instructions and visualized by reaction with DAB for ~15 minutes. Slides were rinsed in distilled water and mounted with an aqueous mounting medium (Vector Labs H-5501). Tissue was processed in two batches.

Slides were analyzed using a Zeiss AxioObserver Z1 brightfield microscope. We quantified the number of cells in five brain regions previously implicated in aggressive behavior in fish: the granular zone of lateral zone of the dorsal telencephalon, DI-g; ventral part of the lateral zone of the dorsal telencephalon, subdivision 2, DI-v2; medial part of the dorsal telencephalon, subdivision 1, Dm-1; medial part of the dorsal telencephalon, subdivision 3, Dm-3; and the anterior parvocellular preoptic nucleus, which is homologous in part to the preoptic area (POA) in other vertebrates.⁵¹ Images were captured from 1 to 6 sections per side, depending on structure size and tissue quality. Cells were quantified by hand, using the Image J Cell Counter plugin to keep track of cell counts within fixed counting frames which varied depending on the shape of the structure: 180 × 360 µm counting frame for Dm-1 (oval) and POA (rectangle), a 360 µm circle for DI-g, and a 360 × 360 µm square for both Dm-3 and DI-v2.

2.6 | pS6 RNA-immunoprecipitation (ie, PhosphoTRAP)

The following methods are adapted from References 44,45. Prior to the immunoprecipitation (IP), magnetic Protein A Dynabeads (Life Technologies 10001D) were loaded with pS6 antibody (Cell Signal #2215). Note that the same pS6 antibody was used for both the IHC and the pS6-immunoprecipitation. For each IP, we rinsed 100 µL of dynabeads in 0.15 M KCl wash buffer (10 mM HEPES, 150 mM KCl, 5 mM MgCl₂ and 1% NP40 in RNase-free water prepared in 500 mL sterile disposable filter units). Beads were then resuspended in 250 µL of 0.15 M KCl buffer for each IP. Then 20 µL of antibody and 30 µL of BSA was added per IP. The antibody-bound beads were mixed end-over-end overnight at 4°C.

The following homogenization steps were performed on ice in the fume hood. All procedures in the homogenization and immunoprecipitation steps were performed with low-bind microcentrifuge tubes. Samples were processed in two batches, each representing a balanced experimental block. Each batch was processed on a separate day. Whole brains were homogenized in 2 mL of homogenization buffer [10 mM HEPES, 150 mM KCl, 5 mM MgCl₂ in RNase-free water prepared in advance in 500 mL sterile disposable filter units with the following supplements added per 2 mL aliquot within 1 hour: 5 µL of 1 M DTT, 1/2 EDTA-free protease inhibitor tablet (Roche 11836170001), 5 µL RNasin (Promega N2611), 5 µL cycloheximide (100 mg/mL stock in DMSO), 10 µL phosphatase inhibitor cocktail (ThermoScientific 78440) and 2 µL of Calyculin A (1 mg/mL in ethanol stock)].

Immediately after removal from the −80°C, the frozen brain was homogenized in 1.4 mL homogenization buffer on ice using a RNase away-treated glass pestle and tube. The supernatant was then transferred to two microcentrifuge tubes (~700 µL each) and each were topped with 300 µL of additional homogenization buffer to ~1 mL. The homogenized lysate was then centrifuged at 2000g for 10 minutes at 4°C. The supernatant from each tube was transferred to new tubes and 70 µL of 10% NP40 and 70 µL DHPC (300 mM stock in chloroform) was added to each ~1 mL tube. The tubes were mixed briefly and then incubated for 2 minutes on ice. The tubes were then spun at Rmax for 10 minutes at 4°C. The supernatant was transferred again to two new tubes. For the input RNA sample, 25 µL from each tube (50 µL total) of this supernatant was added to 350 µL of buffer RLT (Qiagen RNeasy Micro Kit, Venlo, Netherlands) and stored on ice until purification.

Next, we rinsed the pS6-loaded magnetic beads 2× in 0.15 M KCl buffer and distributed the beads into two microcentrifuge tubes for each IP. After the second wash, the 0.15 M KCl buffer was removed and then ~1 mL of clarified supernatant was added to each tube and mixed to resuspend the beads. The tubes were incubated with end-over-end mixing for 10 minutes at 4°C, which allows for antibody binding. After a brief spin, the tubes were placed on the magnet, and the supernatant was removed. The beads were then rinsed 4× in 0.9 mL of cold 0.35 M KCl buffer (10 mM HEPES, 350 mM KCl, 5 mM MgCl₂, 1% NP40, plus the following supplements added per 4 mL:

5 μ L 1 M DTT, 10 μ L RNasin, 5 μ L cycloheximide and 2 μ L Calyculin A). After the third wash, the samples incubated at room temperature for 5 minutes, which allows for the dissociation of some of the non-specific RNA. After the fourth wash, the supernatant was removed, and the beads were resuspended in 350 μ L of buffer RLT and incubated on ice for 5 minutes.

RNA from both the input and IP samples was purified using the Qiagen RNeasy Micro Kit (Qiagen 74004), with two minor modifications to the protocol. First, we added 350 μ L of 70% ethanol to the IP RNA in RLT and 375 μ L of 70% ethanol to the input RNA in RLT. Second, in order to maximize yield, we used 14 μ L of RNase-free water which had been heated to 65°C during the final elution step. RNA samples were analyzed using the Agilent RNA 6000 Pico Kit, Santa Clara, California. Input RNA had RNA integrity number (RIN) scores ranged 7.1 to 8.6 and concentrations of 658 to 4571 pg/ μ L. IP RNA had RIN scores of 6.8 to 8.8 and concentrations of 22 to 100 pg/ μ L.

cDNA synthesis was performed using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio 634 888, Mountain View, California). We used 2 μ L of input RNA and the 10.5 μ L of the remaining IP RNA for cDNA synthesis and the maximum number of PCR cycles (18) during the cDNA amplification step. We analyzed cDNA samples using the Agilent DNA High Sensitivity Kit, Santa Clara, California. cDNA samples had concentrations of 10 to 75 ng/ μ L for input samples and 0.340 to 1.5 ng/ μ L for IP samples as measured by DeNovix Micro-volume Spectrophotometer.

2.7 | Library preparation and sequencing

Library preparation was performed by the Molecular Evolution core facility at GT using NEBNext Ultra II FS DNA library Prep Kit for Illumina (NEB E7805S, Ipswich, Massachusetts). Either 1 ng (IP samples), or 5 ng (input samples) of cDNA were used as a starting material for the library preparation. The enzymatic fragmentation was performed by adding 7 μ L of Ultra II FS Buffer and 2 μ L Ultra II FS Enzyme mix to each sample. The samples were then incubated at 37°C for 15 minutes and 65°C for 30 minutes in a thermocycler. Adaptor ligation was performed in accordance to the manual for NEB Ultra II FS DNA library Prep Kit. The adaptors were diluted 25-fold for IP cDNA samples and 10-fold for all other samples. A mix of 30 μ L of NEBNext Ultra II Ligation Master Mix, 1 μ L NEBNext Ligation Enhancer and 2.5 μ L NEBNext diluted Adaptor for Illumina was added to the FS reaction mixture and incubated at 20°C for 15 minutes in a thermocycler. 3 μ L of USER Enzyme were added to the ligation mixture and incubated at 37°C for 15 minutes with the heated lid set to \geq 47°C.

Adaptor clean-up was performed without size selection because of the low initial DNA quantity. NEBNext Sample purification beads were used in this process by adding 57 μ L (0.8 \times) of the beads to the adaptor ligation reaction. The samples were incubated at room temperature for 5 minutes, then placed on magnetic stand for 5 minutes to separate the beads from the supernatant. Supernatant was

discarded. The beads were washed by adding 200 μ L freshly prepared 80% ethanol to the tubes while still on the magnetic plate; incubated for 30 seconds, and then discarded. This step was repeated once. The beads then were air dried for 5 minutes while still standing on the magnetic plate. The DNA was then eluted by adding 17 μ L of 0.1 \times TE buffer directly to the beads. After mixing the beads with TE buffer, the samples were incubated at room temperature for 2 minutes, then placed back on the magnetic stand until the supernatant was cleared.

PCR enrichment of adaptor-ligated DNA was performed with the use of 15 μ L of eluted DNA for each sample. A mix of the following components was added to each 15 μ L: 25 μ L NEBNext Ultra II Q5 Master Mix, 5 μ L Index Primer/i7 Primer and 5 μ L Universal PCR Primer/i5 Primer (total reaction volume 50 μ L). The samples were pooled by equimolar normalization and sequenced on together in a single run on an Illumina NextSeq 500 instrument with 76 cycles (single-end).

2.8 | RNA-sequencing analyses

Raw FASTQ files were processed using *cutadapt* to (a) trim sequences from the SmartSeq Kit, Illumina TruSeq Indexed adapter, and both poly-A and poly-T contamination and (b) exclude sequences shorter than 25 bp. The remaining sequence reads were then aligned to the *Metriaclima zebra* (Malawi cichlid) reference genome UMD2A^{23,52} using HiSat2.⁵³ Input samples had a slightly higher alignment rates on average than IP samples (85.5% vs 70.5%). Although PC samples had a higher alignment rate than MC samples (85.2% vs 70.7%), this was not driven by sequence divergence between the species and the MZ reference genome, but rather by sample quality. There is no statistically significant difference between the alignment rate between the two species to the *M. zebra* (reference) genome vs "consensus" genome sequences generated for those species ($\chi^2[2] = 2.09$, $P = .351$; Streelman lab, unpublished data). Transcripts were quantified using StringTie⁵⁴ and differential expression analyses were performed using the Bioconductor package DESeq2.⁵⁵

2.9 | Statistical analysis

All statistical analyses were performed with R software (Version 3.6.1, R Development Core Team 2007). To analyze behavioral data, which were not normally distributed, we used nonparametric tests to separately evaluate the effects of ecotype (Wilcoxon signed rank test) and of species (Kruskal-Wallis test followed by posthoc tests using Dunn's test for multiple comparisons).

To analyze cell count data in the pS6-immunohistochemistry, we used generalized linear mixed models (GLMMs) with a Poisson link function using raw cell counts from each brain region using the R package *lme4*. Condition (mirror test vs control), species (MC vs PC), and their interactions were included as main effects predicting the number of pS6+ cells. Individual ID nested within IHC-processing batch was included as a random effect, allowing us to control for

batch effects and variability in staining across individual slides. In addition, we tested whether any of the measured behaviors (time in proximity to the mirror, duration of lateral displays, and the number of frontal attacks) were predictive of the average number of pS6+ cells within any brain region using simple linear models. In these models, the average number of pS6+ cells within a brain region was the dependent variable and the behavior was the independent variable, using a Bonferroni correction to adjust the *P*-value to take into account multiple testing. To perform model comparisons for both the linear models and the GLMM models, we used likelihood ratio tests to compare the full model to a reduced null model with the factor of interest removed using the ANOVA function to perform a chi-square test.

For the RNA-seq analyses in the pS6-RNA immunoprecipitation experiment, we used the DESeq2 package to compare transcript abundance in whole-brain input RNA to pS6-IP RNA from two species (PC, rock and MC, sand) exposed to either a mirror or control stimulus (Table 1). DESeq2 uses a negative binomial generalized linear modeling approach, followed by likelihood ratio tests to compare a full model to a reduced model with the variable of interest removed.⁵⁵ We examined three separate models, each testing separate hypotheses. We first aimed to identify genes that were differentially expressed between input and IP samples, controlling for batch, species, and condition (model 1). For models 2 and 3, we consider the interaction terms using likelihood ratio tests to identify genes for which the effects of condition (model 2) and of the effects of both species and condition (model 3) differ between input and IP samples. The R code and input files, have been made publicly available at: <https://github.com/nmbaran/CichlidAggression>.

Data using the PhosphoTRAP approach differ from traditional RNA-seq data in the both (a) the lower detection rate of many genes (ie, a larger number of zeroes, especially in IP samples) and (b) some genes with very high read counts (ie, high variance). These issues, which have also emerged in the analysis of single-cell RNA-sequencing data and in 16S microbial abundance data, present special challenges for statistical analysis.⁵⁶⁻⁵⁹ It is important to note, however, that the lower detection rate (ie, the average proportion of nonzero counts per gene for each sample, 54.1% for input and 12.6% for IP

samples) and high read count variance are in this case the result of both technical and biological factors. The higher number of “dropouts” may be because of either low concentration RNA samples or because those genes were not expressed in the neurons activated during aggression. Similarly, high read counts could be a function of PCR amplification bias introduced during the cDNA synthesis step or because those genes are in fact highly expressed in activated neurons. Of interest here is the differential enrichment of genes in pS6 immunoprecipitate across species and conditions, where the ratio of fold-enrichment value for each gene is the IP sample read count divided by the input sample read count for each subject (IP/input).

We explored a number of options for using an unbiased differential expression analysis for this “zero-inflated” data. DESeq2’s default normalization procedure is based on the geometric means of counts, where any gene for which any samples have a zero read count are excluded from normalization. This is problematic when there are a large number of zeroes and when the frequency of zeroes vary systematically by sample, as is the case with PhosphoTRAP data. It is worth noting that the DESeq2 vignette recommendation for single-cell analysis using the *zinbwave* package does not apply well to our data set either.⁵⁶ PhosphoTRAP data in fact have a lower number of “samples” (ie, 16 samples in our case vs the several 1000 cells now typical of single-cell sequencing data sets). In addition, we have a larger number of genes expressed per sample than are typically uncovered per cell in single cell sequencing experiments.

After evaluating multiple options, we chose to use the DESeq2 default settings with a pseudocount of 1 added to all reads to statistically test for differential expression. This is in fact a standard approach for analyzing 16S microbial abundance data, although some have criticized that such approaches may lead to a high false discovery rate.⁶⁰⁻⁶⁴ Adding a pseudocount of 1 to our raw data also facilitated the calculation of fold-enrichment for each gene in each sample. Although reporting fold-enrichment values and calculating differential fold enrichment has been standard in previous PhosphoTRAP studies,^{44-46,65,66} the fold-enrichment value is not itself amenable to unbiased statistical testing. Thus, all analyses were performed with the read count (with an added pseudocount of 1) as the dependent variable and *SampleType* (input vs IP) as an independent variable. For

TABLE 1 Numbers of statistically significant genes (adjusted *P*-value <.05) found using each model, assessed using the likelihood ratio test to compare the full model to a reduced model without the bolded variable

Model	PosFC	NegFC	Total	PosFC (with HGNC)	NegFC (with HGNC)	Total (with HGNC)
Input vs IP Only, LRT	1443	14 786	16 229	476	9661	10 137
~ Batch + Species + Condition + SampleType						
IP × MT Interaction, LRT	307	475	782	222	359	581
~ Batch + SampleType + Condition + Species + SampleType × Condition						
IP × MT × Species Interaction, LRT	392	601	993	307	479	786
~ Batch + SampleType + Condition + Species + SampleType × Species + Condition × Species + SampleType × Condition + SampleType × Condition × Species						

Note: Lists the number of differentially expressed genes with positive fold-change (FC), negative fold change and total, with the number of each set successfully matched to a human homolog (with HGNC).

the purposes of visualization and as a sanity-check, the results in the figures are presented in terms of the calculated fold-enrichment values, as well as the \log_{10} (Fold Change) and P -values standard for differential expression analyses.

To identify gene ontology categories and KEGG pathway enrichment based on lists of differentially expressed genes, we used ToppFun using the human homolog HGNC gene names.⁶⁷⁻⁶⁹ We present Bonferroni corrected P -values, which accounts for multiple testing.

3 | RESULTS

3.1 | Species comparison of aggressive behavior in the mirror test assay

Sand species spent a slightly higher proportion of time within 5 cm of the mirror surface than rock species (0.66 [IQR 0.57-0.79] vs 0.59 [IQR 0.43-0.70], $Z = -1.98$, $P = .047$, Figure 1B). This effect was largely driven by the difference between two species, MC & PC (Kruskal-Wallis test of effect of species, $\chi^2[6] = 17.63$, $P = .007$ followed by Dunn Test: $Z = 4.01$, $P_{adj} = .001$ for PC-MC comparison). No other species difference in time in proximity to the mirror was significant.

Rock species performed more frontal attacks than sand species (107 [IQR 53-170] vs 17 [IQR 1-78], $Z = -4.20$, $P < .0001$, Figure 1C). Rock species also had a shorter attack latency, as well (51 seconds [IQR 26-86 seconds] vs 287 seconds [IQR 136-696 seconds], $Z = 4.82$, $P < .0001$). Once approaching the mirror, rock species escalate to a frontal attack in a median of 14 seconds (IQR 3-35 seconds) whereas sand species took substantially longer (115 seconds [IQR 51-179 seconds], $Z = 5.696$, $P < .0001$). In contrast, sand species spent a greater proportion of time performing lateral displays than rock species (0.40 [IQR 0.30-0.69] vs 0.15 [IQR 0.08-0.35], $Z = 4.68$, $P < .0001$, Figure 1D).

In each of these two aggressive behaviors, there is substantial variability across species, as well. CA and LF performed the most frontal attacks whereas CV and TI perform the fewest (Kruskal-Wallis $\chi^2[6] = 33.12$, $P < .0001$). PC perform the fewest lateral displays closely followed by LF, whereas the sand species all perform a similarly high number of lateral displays (Kruskal-Wallis $\chi^2[6] = 33.15$, $P < .0001$). MC and PC exhibit a similar number of frontal attacks (Kruskal-Wallis $\chi^2[6] = -0.57$, $P = .66$), but MC perform a substantially higher number of lateral displays (Kruskal-Wallis $\chi^2[6] = 4.53$, $P < .0001$).

3.2 | pS6 immunohistochemistry

To determine whether any brain regions exhibited differential activation during mirror-elicited aggression, we examined pS6 staining in several regions previously implicated in aggressive behavior (Figure 2). In the DI-g, we found a higher number of pS6+ cells per

counting frame in sand species ($\chi^2[1] = 8.00$, $P = .0047$), but there was no effect of condition on the number of pS6+ cells ($\chi^2[1] = 1.065$, $P = .30$) and no interaction between condition and species ($\chi^2[1] = 0.60$, $P = .44$). In the DI-v2, we found a higher number of pS6+ cells per counting frame in sand species ($\chi^2[1] = 6.96$, $P = .0083$), but similarly no effect of condition alone ($\chi^2[1] = 0.091$, $P = .76$) and no interaction between condition and species ($\chi^2[2] = .78$, $P = .68$). In the Dm-1, there is a nearly significant effect of both species ($\chi^2[1] = 3.7889$, $P = .052$) and condition ($\chi^2[1] = 3.48$, $P = .062$) on the number of pS6+ cells. However, there was no statistically significant interaction between species and condition on the number of pS6+ cells in the Dm-1 ($\chi^2[1] = 0.018$, $P = .89$). In the Dm-3, there was no significant effect of either species ($\chi^2[1] = 1.14$, $P = .29$) or condition ($\chi^2[1] = 0.03$, $P = .86$) on the number of pS6+ cells. There was also not a significant interaction between species and condition ($\chi^2[1] = 0.22$, $P = .64$). In the parvocellular POA, there was no effect of either species ($\chi^2[1] = 1.14$, $P = .29$) or condition ($\chi^2[1] = 0.031$, $P = .86$) on the number of pS6+ cells, and no effect of interaction between the two ($\chi^2[1] = 0.22$, $P = .64$). The number of pS6+ cells in the DI-v2 and in Dm-3 was positively predicted by lateral display behavior independent of species (DI-v2: $\chi^2[1] = 2343$, $P_{adj} = .027$ and Dm-3: $\chi^2[1] = 1074$, $P_{adj} = .046$). All associations between behavior and pS6-labeling are shown in Figure S1.

3.3 | RNA-immunoprecipitation quality control and analysis

The average RNA concentration for input samples was 1812.5 pg/ μ L and only 56.4 pg/ μ L for IP samples, resulting in an average yield of 0.15% from the immunoprecipitation. The average RNA quality between input and IP samples was comparable: 7.9 RIN score for input samples vs 7.6 RIN score for IP samples. RNA-sequencing

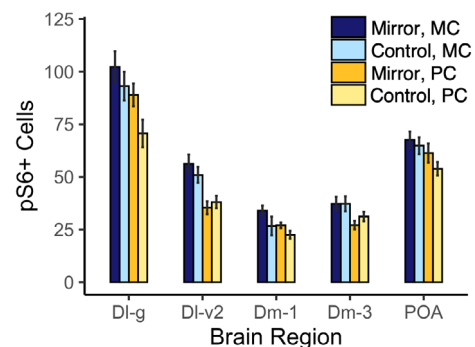


FIGURE 2 Mean \pm within-subject SE of cells immunopositive for pS6 in *M. conophoros* (sand) vs *P. chitimba* (rock). Granular zone of lateral zone of the dorsal telencephalon, DI-g; ventral part of the lateral zone of the dorsal telencephalon, subdivision 2, DI-v2; medial part of the dorsal telencephalon, subdivision 1, Dm-1; medial part of the dorsal telencephalon, subdivision 3, Dm-3; and preoptic area, POA

yielded an average of 16 million reads per sample (21 million for input and 11 million for IP samples). The average alignment rate was 85.5% for input samples and 70.5% for IP samples. Detailed quality control information can be found in Supporting Information.

3.4 | Differential expression in input vs IP samples

PhosphoTRAP RNA-seq analyses from eight individuals showed a large number of genes which showed differential expression between input and IP samples, controlling for differences in batch, species, and condition (Table 1, model 1). We used *DESeq2* to evaluate the following model: *SampleType* + *Batch* + *Condition* + *Species*. We then used a likelihood ratio test to evaluate the effect of *SampleType*, an independent variable for whether the sample was input or IP. We found 16 229 genes (10 137 with human homologs, that is, HUGO Gene Nomenclature Committee (HGNC) names) which were differentially expressed between input and IP samples (Figure 3), accounting for 45.1% of annotated genes in the genome. Only 1443 (8.9%) were more highly expressed in IP samples. Genes involved in the MAPK ($P = 1.312 \times 10^{-8}$), PI3K/Akt ($P = 9.418 \times 10^{-3}$), cAMP ($P = 9.368 \times 10^{-5}$) and mTOR ($P = 5.855 \times 10^{-5}$) pathways were overrepresented in the list of differentially expressed genes (S6 is phosphorylated downstream of these four pathways).

3.5 | Differential expression in IP samples in the mirror test

Next, we sought to identify the genes that were differentially expressed in IP samples during mirror aggression independent of

species. To do this, we used the following model: *Batch* + *SampleType* + *Condition* + *Species* + *SampleType* × *Condition*, using a likelihood ratio test to evaluate the effect of the interaction term (Table 1, model 2). This approach identifies either (a) the genes that are expressed in the population of cells that are activated during mirror aggression in both species or, alternatively, (b) the genes whose expression is altered within the population of cells that are activated during mirror aggression.

We found 782 genes (581 with HGNC names) that exhibited this pattern of differential expression: 307 with positive fold-change (FC) (ie, up in mirror test condition and in IP samples) and 475 with negative FC. For all genes that were found to be statistically significant, the interaction term effect is at least 16 FC (ie, $\log_2 FC > 4$). Results from this analysis are shown on the scatterplot as the average fold-enrichment value (IP/input) for control vs mirror test samples for each gene (Figure 4). Genes that emerged as significant are clustered away from the identity line. Genes with a positive FC are also clustered primarily in Q2 and, to a lesser extent in Q3. Thus, genes with a significant positive FC have a higher fold-enrichment value in mirror test samples relative to the control samples. The top three most statistically significant gene ontology molecular function categories for positive FC genes are “DNA-binding transcription factor binding” ($P = 2.51 \times 10^{-3}$), as well as the related sub-categories “RNA polymerase II-specific DNA-binding transcription factor binding” ($P = 1.10 \times 10^{-2}$) and “RNA polymerase II repressing transcription factor binding” ($P = 3.34 \times 10^{-2}$). Among the genes exhibiting this pattern of expression are several less investigated genes involved in the hypothalamic-pituitary-gonadal axis, including HSD17B (hydroxysteroid 17- β dehydrogenase 8) and GNRH2 (gonadotropin-releasing hormone 2). A complete list of differentially expressed genes can be found in Supporting Information.

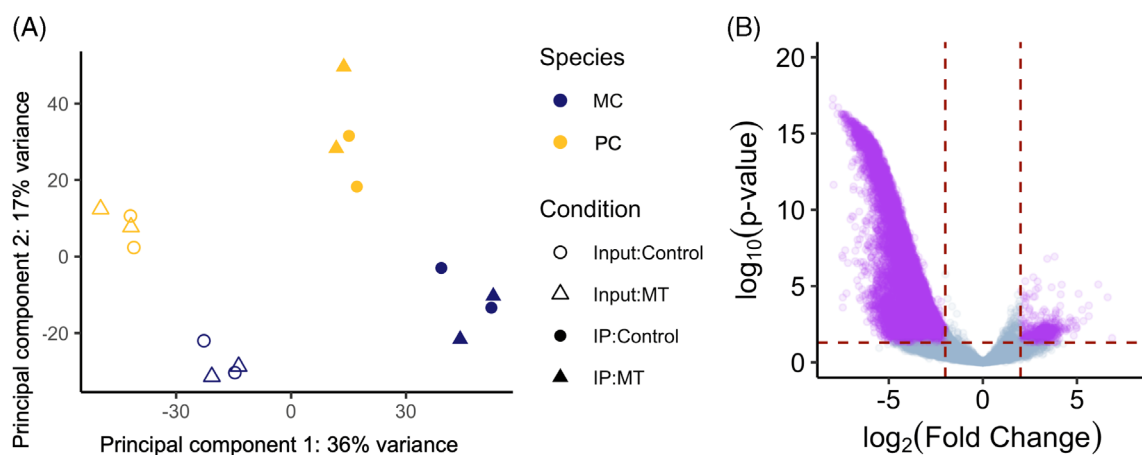


FIGURE 3 A, Scatterplot of principal component 1 vs principal component 2 shows clustering of overall expression profile by sample type and species, but not by condition. Principal component 1 accounts for 36% of all gene expression variation and, by inspection, separates the Input (open circles and triangles) and IP samples (closed circles and triangles). Principal component 2 accounts for 17% of the variation in gene expression and varies significantly with species. B, Volcano plot showing gene expression fold-difference and significance between input and IP samples (model 1). We found that 1443 genes are up-regulated in the IP samples while 14 786 genes are up-regulated in the input samples. Genes for which the adjusted P -value $< .05$ and where the \log_2 Fold Change > 2 are shown in purple

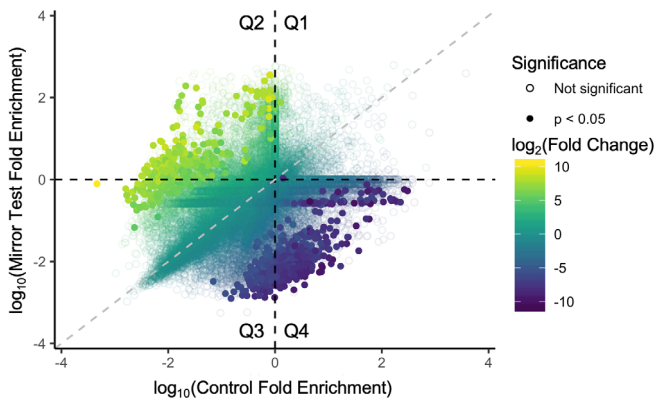


FIGURE 4 Log₁₀ average fold-enrichment values of each gene for Control vs Mirror Test samples. Color is the Log₂ Fold Change for IP and mirror test samples (model 2). Solid circles have an adjusted *P*-value < .05. Gray dashed line is the identity (1:1) line

3.6 | Differential expression in IP samples in the mirror test by species

Finally, we aimed to identify the genes that showed a pattern of differential expression in activated neurons during mirror aggression and which varied by species. To do this, we used the following three-way interaction model: *Batch + SampleType + Condition + Species + SampleType × Condition + Sampletype × Condition × Species* (Table 1, model 3). These are genes for which their expression in activated cells during mirror-elicited aggression varies by species and, thus, candidate genes that underlie species differences in mirror-elicited aggression.

We found 993 genes (786 genes with HGNC names) that exhibited this pattern of expression, 392 (307) with positive FC and 601 (479) with negative FC. The three significant gene ontology biological function categories for all differentially expressed genes were “response to peptide hormone” ($P = 8.347 \times 10^{-4}$), “response to peptide” ($P = 1.146 \times 10^{-2}$), and “response to insulin” ($P = 3.161 \times 10^{-2}$). Two of these categories, “response to peptide hormone” and “response to insulin” were significant when considering just negative FC genes.

Three-way interactions can be difficult to interpret in gene expression analyses, thus we performed t-distributed stochastic neighbor embedding (t-SNE) using the R package *Rtsne* to identify genes that exhibited similar patterns of gene expression to each other. t-SNE is nonlinear technique for dimensionality reduction that is used for the visualization of high-dimensional datasets. In this case, the input for the t-SNE algorithm was the raw count reads for the 993 significant genes identified by *DESeq2*. This analysis results in a plot in low-dimensional space which we found retains the features of the two major outputs of the *DESeq2* analysis: log₂FoldChange and base mean (the mean of normalized read count of all samples; Figure 5A, B). In this case, genes with a positive FC in the three-way interaction, in general, have higher fold-enrichment values in individuals of the rock species in the mirror test condition compared with rock individuals in the control condition (Figure 5A,C). In contrast, genes with a

negative FC are genes that have higher fold-enrichment values in sand individuals in the mirror test condition compared with controls (Figure 5A,D).

Among the genes that exhibit a positive log₂FoldChange are neuropeptide Y (NPY), insulin receptor A (INSR), and steroid 5 alpha-reductase 3 (SRD5A3). Among the genes that have a negative log₂FoldChange are G-protein coupled estrogen receptor 1 (GPER1), gamma-aminobutyric acid (GABA) A receptor, theta (GABRA6), gamma-aminobutyric acid (GABA) A receptor, alpha 6 (GABRQ) and glutamate receptor, metabotropic 7 (GRM7).

Finally, we compared the list of genes that are differentially expressed across MC and PC in the mirror test (model 3) to the genes that either contain or are near genomic variants that differ between rock and sand species (Streelman lab, unpublished data). We found that 49 of the 778 differentially expressed genes with a human homolog (~6.3%) contain some kind of alternately-fixed variant between rock and sand genomes within 10kb of the gene, either a single-nucleotide polymorphism (SNP) (38/778), an alternately-fixed insertion-deletion (InDel, 11/778), or both (9/778) (see Supporting Information for gene list).

4 | DISCUSSION

Our results confirmed that ecotype differences in aggression reported in field studies can be reliably assayed in the lab. We found that males of rock-dwelling species were faster to attack the mirror and attacked a greater number of times, whereas males of sand-dwelling species performed more lateral displays. This observation is consistent with ecological pressures experienced by these species in the wild. *Mbuna* males must aggressively defend their rocky territories against intruders, as it is both where they feed and their spawning site.^{30,70} Border contests between territorial males are described as including face-to-face lunges and jaw locking.²⁵ In contrast, the sand species are not territorial, although they will defend their bower against rival males. However, there is support for the hypothesis that sand species evolved bower building to reduce the likelihood of agonistic encounters, because the complex extended phenotype allows males to more accurately evaluate their rival's size, quality and species.^{27,29} The higher time spent performing lateral displays and increased latency to frontal attack in sand species is consistent with this hypothesis.

By comparing neural activity in two closely related species which differ in aggressive phenotype, we hoped to identify brain regions that were differentially engaged in agonistic interactions across species. We found that sand species had more pS6+ cells than did the rock species in two brain regions in the dorsolateral telencephalon (DI-g and DI-v2), which together comprise the putative teleost homolog of the mammalian hippocampus.⁷¹ Contrary to our expectations, we did not observe any differences in pS6+ staining in across conditions or any Condition × Species interactions. It is possible that species differences in neural induction during mirror-elicited aggression are subtle and that our sample size was not large enough to detect small changes. Alternatively, it is possible that the differential activity is

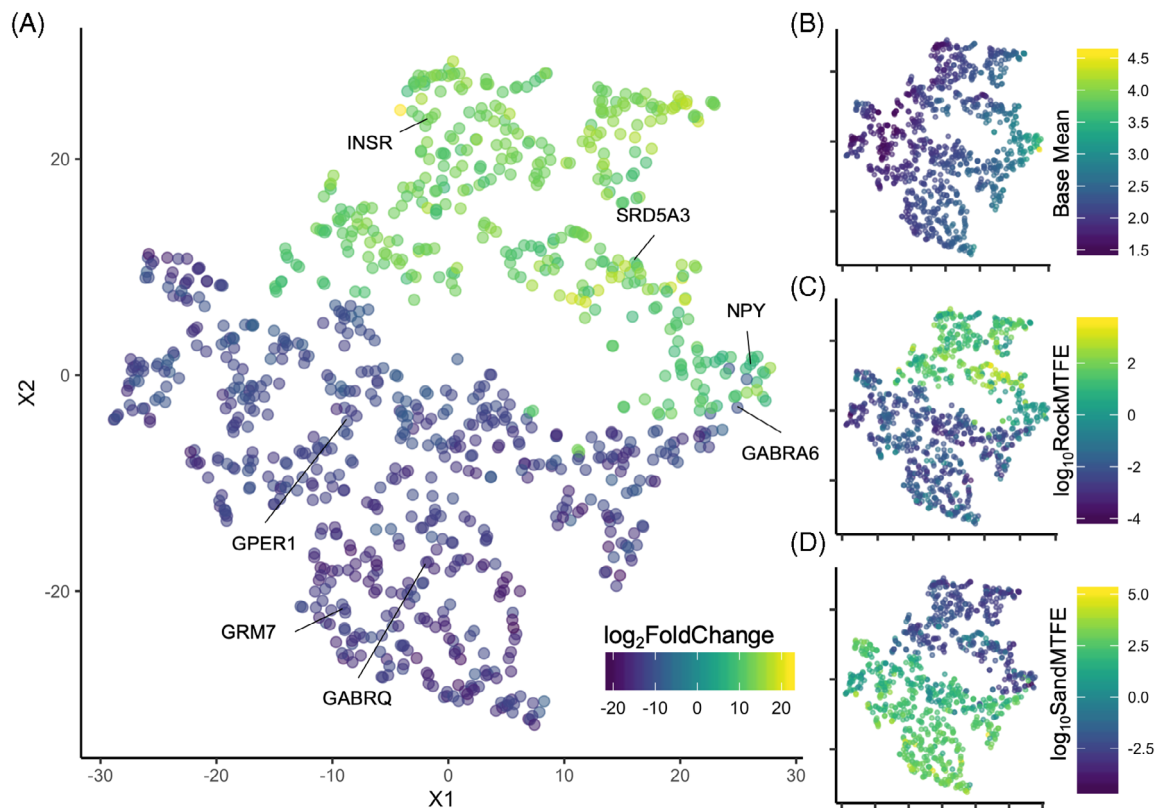


FIGURE 5 t-SNE plot showing the overall relationship between the 993 genes significant in the three-way interaction with eight immunoprecipitated and eight input samples per gene. Genes that are clustered closer together exhibit a similar pattern of expression across samples. A, Color scale is the \log_2 FoldChange for each gene in the three-way interaction. B, Color scale depicts the base mean, that is, the mean of normalized counts of all samples, normalizing for sequencing depth. C, Color scale depicts the \log_{10} average fold-enrichment values for the rock species (PC) in the mirror test relative to rock species in the control condition. This pattern is highly similar to the \log_2 FoldChange. D, Color scale depicts the \log_{10} average fold-enrichment values for the sand species (MC) in the mirror test relative to sand species in the control condition

occurring within different sub-populations of cells within the same region or different brain regions altogether.

Previous studies in other closely-related cichlid species have reported a “dissociation between the behavioral and the physiological responses toward mirror images.”^{35,36} This has led a number of researchers to be skeptical about the validity and reliability of mirror-elicited aggression and whether the mirror tests should be used to investigate the physiological and neural correlates of aggression in the brain.^{37,38,40} The mirror image is a highly unusual stimulus, and mirror fights lack the sensory richness of real-life encounters, which include auditory, olfactory, and tactile cues. Additionally, cichlids encounter reversed image of their rival in the mirror. Normally, cichlids will perform lateral displays to each other nose-to-tail and aggressive display behaviors are highly lateralized, as they are in many species.^{39,41,43,72-74} Mangrove rivulus fish males (*Kryptolebias marmoratus*) were found to be more aggressive in their behavior when the experimenters used a nonreversing mirror and the fish's behavior in this apparatus was more predictive of real-life contests than a normal mirror.⁷⁵ Thus, we may have failed to find a difference across species in our p56+ staining because of the use of a mirror induced a lesser or distorted behavioral response compared with real-life aggressive behaviors.

However, we nevertheless find substantial differences in gene expression in response to mirror-elicited aggression, suggesting that there is indeed a robust transcriptional response to the stimulus. The pattern of differentially expressed genes we observed in both MC and PC, suggests that mirror aggression results in widespread changes to the regulation of gene expression at the level of transcription factor binding. In addition, we noted several interesting genes in the hormone pathways that may play under-explored roles in fish aggression. The gene HSD17B (hydroxysteroid [17-beta] dehydrogenase 8, 17 β -HSD) encodes a steroidogenic enzyme responsible for the last step in the synthesis of 11-ketotestosterone, the primary fish androgen. Surprisingly little is known about the role of 17 β -HSD in the brains of fish, but 17 β -HSD type 8 is ubiquitously expressed in the related fish species Nile tilapia, *Oreochromis niloticus*.^{76,77} Another gene of interest is gonadotropin-releasing hormone 2 (GnRH-2). GnRH-2 is less well-understood than GnRH-1. Across vertebrates, GnRH-2 is expressed exclusively in the midbrain tegmentum and is thought to play roles in the control of reproductive motor behavior and feeding.⁷⁸⁻⁸⁴ However, it has not previously been linked to aggression and two previous studies failed to find an association between aggressive behavior and GnRH-2 in fish.^{85,86}

Finally, we identified a large number of genes that show differential patterns of activation across species during mirror-elicited aggression, many of which are specifically involved in peptide hormone signaling pathways. Several of the genes that emerged from the analysis also play important roles in feeding and metabolism, including a number of genes in the insulin pathway and neuropeptide Y. There is, in fact, a large literature linking metabolic syndromes and insulin resistance to aggression.^{87–89} For example, diabetic rats show a loss of aggression and an increase in submissive displays.⁸⁷ Several researchers have theorized that the overlap between the same signaling molecules that are associated with insulin resistance are also associated with reduced aggression, likely because of the evolutionary origins of aggression in the service of acquiring food or resources, as well as the metabolic changes needed to fight or recover from agonistic encounters.^{88,89} Neuropeptide Y, an abundantly expressed peptide with well-known roles in regulating food intake, growth, and reproduction, is also similarly linked to aggression.⁹⁰ For example, the knockout of Y1 receptors for NPY was found to substantially increase in aggression in mice.⁹¹ NPY has also been associated with aggression in zebrafish (*Danio rerio*)⁸⁵ and NPY mRNA levels were found to be substantially higher in the POA of socially subordinate vs dominant rainbow trout (*Oncorhynchus mykiss*).⁹² In the Lake Malawi cichlids, the gene for NPY is near a highly divergent region between rock and sand species, which may also account for the observed pattern of differential expression.

Intriguingly, we found that two different 5 α -reductase genes were differentially expressed in the two species: SRD5A3 was more highly expressed in PC during mirror-elicited aggression, whereas SRD5A1 was more highly expressed in MC. The inferred evolutionary history for the SRD5 α family of genes suggests that SRD5A1 and SRD5A3 diverged early in the evolution of eukaryotes.⁹³ The SRD5A1 gene encodes an enzyme that catalyzes the reduction of testosterone, progesterone, corticosterone, and estrone into the more active 5 α -reduced derivatives, including the conversion of testosterone (T) to 5 α -dihydrotestosterone (5 α -DHT), a nonaromatizable, potent androgen.⁹³ In mice, 5 α -reductase type 1 was found to mediate testosterone enhancement of aggression during a resident-intruder test.⁹⁴ 5 α -DHT has been found to induce territorial aggression in both mice and songbirds,^{95–97} as well as male-typical social signals in weakly electric fish.⁹⁸ It was only recently discovered that SRD5A3 is likely not a steroidogenic enzyme, but it is instead involved in glycosylation.^{99,100} Humans with mutations of the SRD5A3 gene typically present with severe ocular issues, cerebellar malformations, and cognitive impairment.¹⁰¹ SRD5A3 is known to be widely expressed in fish brains in a region-specific manner,¹⁰² but it remains unclear whether it plays a role in aggression.^{103,104}

Another interesting candidate gene for further exploration is GPER1, which encodes the gene for the G-protein coupled estrogen receptor. A number of studies have shown rapid, nongenomic effects of estradiol on aggression across taxa, but the specific role of GPER1 remains poorly understood.¹⁰⁵ In fish, rapid effects of estrogens have been reported in Plainfin midshipman fish¹⁰⁶ and the GPER1 gene was implicated in the pacemaker region of weakly electric fish.¹⁰⁷ This

suggests that evolution of the G-protein coupled estrogen receptor may underly differences in behavioral aggression in fish, especially in the production of male-typical social signals. Furthermore, G-protein coupled receptors are hypothesized to be particularly evolvable neuromodulatory systems, as a result of their compartmentalization and subfunctionalization within the brain.¹⁰⁸

We also observed differences in several neurotransmitter receptors. In rodents, aggressive behavior is thought to arise as a result of an imbalance between glutamatergic excitation and GABA-ergic inhibition.¹⁰⁹ GABA_A receptors have previously been linked to aggressive behaviors (see^{110,111} for review). In addition, we also identified differential expression of metabotropic glutamate receptor subtype 7 (mGluR7). mGluR7 in the medial bed nucleus of the hypothalamus has been shown to be essential for male-male aggression in mice¹¹² and agonists to mGluR7 significantly reduce both threat and attack behaviors.¹¹³ Both pharmacological and genetic studies have shown that almost all subtypes of glutamate (NMDA, AMPA, kainate receptors, and mGluRs) and GABA (GABA_A and GABA_B) receptors are involved in aggression in some capacity. However, the role of each of the receptors may vary depending on the composition of receptor subunits, their localization, and the type of aggressive behavior studied, all of which can vary across species.

We were surprised that we did not observe any differences in expression in either the gene for arginine vasotocin (homolog of arginine vasopressin, AVP) or in the vasopressin-1a receptor (V1aR), both of which have previously been linked to aggression in other species, including dominance behaviors in the African cichlid *Astatotilapia burtoni*.^{114–116} V1aR contains an alternately fixed 11-base pair deletion in sand species, which is predicted to alter the C-terminal domain of the G-protein coupled receptor via a frameshift, removing multiple palmitoylation sites.¹¹⁷ However, this mutation is predicted to alter protein functions like the trafficking of the receptor to the membrane, which may not be observable at the level of gene expression and further exploration of the consequences of this variant is warranted. We were also surprised to not observe any genes related to serotonin (5-HT) or in DA emerge from these analyses, as both have been frequently linked to aggressive behaviors.

In our analyses, we identified a large number of genes that exhibited patterns of expression consistent with differential involvement in aggressive behaviors across two closely related cichlid species. In general, there are two ways to interpret our PhosphoTRAP data. First, the data may show specific neuronal cell types activated during mirror-elicited aggression, in the cases in which the differentially expressed genes have been identified as cell-type specific markers, such as NPY. Alternatively, PhosphoTRAP can show stimulus- or behavior-driven changes in gene expression within the population of activated cells. However, it is impossible to distinguish between these possibilities with PhosphoTRAP data alone. Additionally, one drawback of using whole-brain samples vs punches of tissue from a single brain region pooled across multiple samples for PhosphoTRAP (as is more common), is that different populations of cells of the same type may show opposite patterns of activation. Although

our goal was to examine differences in gene expression throughout the brain, this approach may lead us to fail to identify region-specific patterns of differential expression, which are undoubtedly important in the context of complex social phenotypes. Future studies using single-cell RNA sequencing technologies, coupled with activation-based cell sorting, would enable us to parse these two different interpretations of our data and provide more detailed information about cell types.^{118,119}

Here we used the immunoprecipitation of translational ribosomes via phosphorylated ribosomal pS6 to produce a rich set of candidate genes involved in aggression in two species of Lake Malawi cichlid fish which differ in aggressive behaviors. To our knowledge, this study is the first time that PhosphoTRAP has been performed on whole-brain tissue samples, rather than brain-region specific punches pooled from multiple individuals (see References 44,46,65,66. We used an unbiased approach, with the aim of discovering novel genes and pathways involved in a complex social behavior to provide a foundation for further exploration. Future studies can interrogate the causal relationship between specific genomic variants and aggressive behavior using genetic approaches such as genetic mapping or CRISPR-Cas9 genome editing.¹²⁰⁻¹²⁴ This work thus lays the foundation for future experiments using this emerging genetic model system to investigate the genomic basis of evolved species differences in both brain and behavior.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the Gene Expression Omnibus (GEO) database (accession number GSE140690) at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140690>. In addition, the R code and input files, have been made publicly available at: <https://github.com/nmbaran/CichlidAggression>.

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SUPPORTING INFORMATION

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