A MULTIPRONGED APPROACH TO UNDERSTANDING HOW GENES CONTRIBUTE TO SOCIAL BEHAVIOR IN THREESPINE STICKLEBACK

BY

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DISSERTATION

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ABSTRACT

Understanding how genes contribute to behavior requires a two-pronged approach – identifying what genes underlie the behavior and characterizing their molecular mechanisms. Naturally, the behavior under study must be heritable. It should ideally be reliably assayable, a difficult provision to satisfy for ecologically important social behaviors which have large but consistent individual differences. Non-traditional model systems offer a limited molecular toolkit, often constraining behavioral genetics to mainly correlative methods rather than direct manipulation. Therefore, to enable establishing a causal relationship between genes and social behavior in the emerging model system threespine stickleback (*G. aculeatus*), we needed to develop molecular techniques and simplify the already robust behavioral assays.

Threespine sticklebacks are a classic system for the study of behavior, ecology, and evolution. A growing number of quantitative trait loci (QTL) and gene expression studies are identifying genes related to ecologically-important social behaviors in sticklebacks, such as parenting and aggression. In order to visualize the expression of these candidate genes, we developed a fluorescence *in situ* hybridization (FISH) protocol. The FISH protocol resulted in specific labeling under all combinations of dissection (fresh vs. frozen) and embedding (paraffin vs. cryo) conditions. Paraffin embedding preserved morphology better than cryo-embedding. We provide representative results showing the expression of three genes related to social behavior – glial fibrillary acidic protein (*GFAP*), oxytocin receptor (*OXTR*) and tyrosine hydroxylase (*TH*) in the brain.

To enable direct manipulation of genes for social behavior, we focused on aggression. Male sticklebacks demonstrate stereotypical aggressive behaviors during an easily-induced territorial defense. Previous studies in stickleback have shown that aggression is heritable, and that hundreds of genes are differentially expressed in the brain following territorial intrusion.

However, the traditionally ethology focused territorial-intrusion assay is typically synchronized to the reproductive cycle rather than being yoked in time. Given the seasonality and high dropout rate of non-nesters, this methodology would require a prohibitively large sample size during molecular characterization. Therefore, we first sought to evaluate to what degree territorial aggression is moderated by nesting. Neither nest presence, timing of construction, nor nesting outcome were associated with differences in behavioral measures of territorial aggression. Assessed behaviors were robust, repeatable, and intercorrelated. We conclude territorial aggression is neither predictive of nor altered by nesting in threespine stickleback fish and could therefore synchronize aggression assays based on timing rather than nesting state.

Finally, we developed a method for viral-mediated transgenesis in the brain to directly test the effects of increased expression of candidate genes, monoamine oxidase (*MAOA*) and arginine vasopressin (*AVP*), on territorial aggression in the sticklebacks. This method is flexible, fast, and amenable to statistically powerful within-subject experimental designs, making it practical for use in natural populations. Fish transfected with either *AVP* or *MAOA* constructs were more aggressive in response to a territorial intruder, unlike control animals transfected with a fluorescent protein. Our success demonstrates that widely available mammalian plasmids work with this method, lowering the barrier of entry to the technique. It further enhances the growing molecular toolkit in threespine stickleback, a classic ethological system, and is the first step toward using chemogenetics and optogenetics.

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INTRODUCTION

"It begins to be difficult, and even in some cases impossible, to say where ethology stops and neurophysiology begins." - Tinbergen 1963

Threespine stickleback have long been a foundational organism in ethology (reviewed in Huntingford & Ruiz-Gomez 2009). Nobel Laureate Nikolaas Tinbergen used these small fish in developing his four questions on the study of behavior – causation (mechanism), development (ontogeny), function (adaptation) and evolution (phylogeny). As the stickleback molecular toolkit continues to expand, so does their popularity in in other fields including evolution, physiology, comparative genomics, and neuroscience (Fang, Merilä, Ribeiro, Alexandre, & Momigliano, 2018; Norton & Gutiérrez, 2019). In order to address how genes contribute to behavior within this system, a multipronged approach using a variety of techniques is necessary.

First, we need to identify behaviors with underlying genetic components, normally accomplished with quantitative genetics. Most behaviors fall on a continuous scale rather than into discrete categories, somewhat complicating this step. For an accessible conceptual and methodological introduction see Conner & Hartl's *A Primer of Ecological Genetics*. Once a heritable basis is established for a behavior, the genetic mechanism can be explored both directly and indirectly. To detect these mechanisms, it is important to select the right tools for the system as each method has its own assumptions and required extant capabilities within the system (Bengston et al., 2018).

Direct DNA sequence differences giving rise to different phenotypes can be identified with techniques such as quantitative trait locus (QTL) mapping and genome-wide linkage/association studies (GWAS). These techniques yield candidate genes from within regions where variation in genetic markers (RFLPs, RAPD, AFLP, microsatellites, etc.) is

significantly associated with the phenotypic variation. With additional information and detailed computational analysis, a causal relationship can be inferred – although it is suspectable, especially in natural populations, to hidden structure or sampling bias (Civelek & Lusis, 2014; Li, Tesson, Churchill, & Jansen, 2010). Charney's 2017 behavioral genetics review lays out the underlying assumptions for many common linkage study designs. While DNA sequence changes were historically thought to rely on changes in protein efficacy, they can occur in promoter or enhancer regions resulting in changes in expression (Xie et al., 2019).

Changes in gene expression are frequently lumped together under the category of epigenetics which rely on changes outside the DNA sequence such as methylation or histone modifications. These regulatory changes can only alter expression. For a discussion of epigenetics and the conserved underlying mechanism in teleost fish, see Best *et al.* (2018). Methods for detecting epigenetic effects include ChIP-seq, fluorescence *in situ* hybridization (FISH), and bisulfite sequencing. In chapter 1, I present my brain-optimized method for fluorescence *in situ* hybridization, the first FISH protocol in stickleback, which allows for subcellular localization of gene expression.

RNA-Seq, currently one of the most popular methods for identifying candidate genes, detects both sequence-based (mutations, SNPs, etc.) and other (alternative splicing, post-transcriptional modifications, etc.) genetic effects. For sticklebacks RNA-Seq and linkage studies have already yielded numerous candidate genes related to hormones (Kitano et al., 2010) and behavior (Abbey-Lee et al., 2018; Aubin-Horth, Deschênes, & Cloutier, 2012; S. A. Bukhari et al., 2019; Syed Abbas Bukhari et al., 2017; Greenwood, Wark, Yoshida, & Peichel, 2013; Sanogo, Band, Blatti, Sinha, & Bell, 2012). However, the stickleback system lacked methods to establish a causal link between genes and behavior or to investigate the underlying proximate mechanisms. Thus, I developed a new minimally invasive brain injection method in stickleback to enable pharmaceutical and transgenic studies.

In developing direct brain injection, I needed to refine the anesthetization process to allow for a longer procedure. Anesthetization remains one of the most challenging aspects of surgery because proper anesthetizations timing must be determined on an individual basis. To allow me to perform precise brain injections we built a custom surgical rig as there are no commercially-available, water compatible, tiny stereotaxis tables. A custom peristaltic pump allowed fine adjustment for the delivery of maintenance anesthetic throughout the approximately 10 minute out-of-water procedure. With this equipment I was able to improve precision of injections as well as raise survival rates to 90%. I additionally characterized the post-surgical period to determine both the typical pattern of recovery and early indications of failure to recuperate. Recognizing these warning signs allows intervention when necessary, also contributing to the high survival rate.

Pharmacological manipulation can mimic the results of altered gene expression. In chapter 3, I show that systemic administration of vasotocin signaling drugs that pass the blood-brain barrier have similar effects as direct injection into the brain, suggesting that the recovery period from the brain injection does not mask the pharmaceutical effects. This is also true for behavior. However, pharmaceutical manipulation is more useful in examining the signaling mechanisms at the protein level, while directly altering gene expression would be preferable for establishing a causal relationship between genes and behavior.

Viral-mediated transgenesis is a method to increase a gene's expression in a specific location or time. We can think about this like a self-driving car whose destination is the cells we want to influence. Our vehicle is something that can easily deliver its DNA passenger into those cells – a virus such as herpes. However, we replace the harmful Herpes passenger with our gene of interest. The viral vehicle still goes to its normal destination. By adding a fluorescent gene as well, we can visually confirm transfection. Different promotors in the viral payload can change

the timing or location of expression. The three promoters I piloted, discussed in chapter 3, did not alter survival or recovery compared to saline-injected controls.

Viral-mediated transgenesis can be used to increase or decrease gene expression levels, using either a direct gene payload or a CRISPR cassette, respectively (Braasch et al., 2014; Ingusci, Verlengia, Soukupova, Zucchini, & Simonato, 2019). This method of transgenesis is fast and flexible. We can measure the same animal before and after we alter the genes, allowing us to show a causal relationship, as I do in chapter 4. By using a repeated measure within-subject design, each animal acts as its own control which eliminates variation between individuals. This is a major benefit when dealing with behavior, which has high inter-individual variation. When combined with pharmacological manipulation, DREDDs (designer receptors exclusively activated by designer drugs, reviewed in Roth, 2016) can target neural signaling with extremely precise timing. This method is already being used to identify neural circuits via chemical silencing or activation of receptors including those that are serotoninergic (Ingusci et al., 2019). Finally, viral-mediated transgenesis also lays the groundwork for optogenetics, although there are still engineering challenges to design light, tether-free setups that do not interfere with complex behaviors in fish that weigh less than 2 grams.

In order to assess genetic influences on behavior, I considered how to best quantify heritable behaviors and examined how specific changes in gene expression alter those behaviors. For many of the classic behavioral models, the required techniques have yet to be actualized,

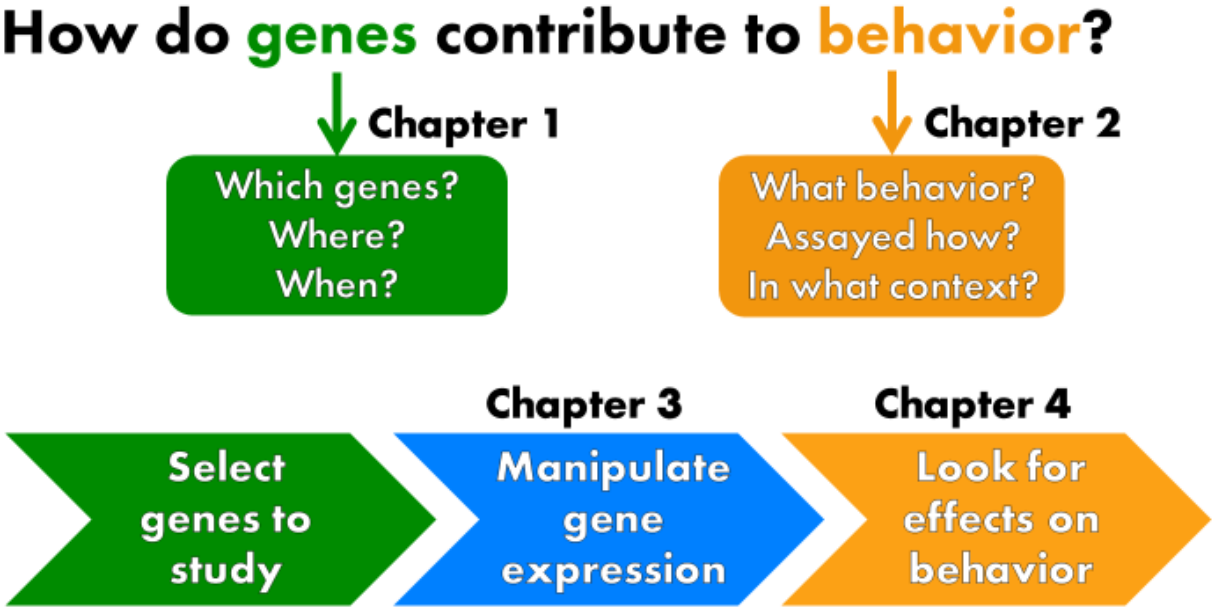

Figure 1 Roadmap to my multipronged approach.

resulting in an uncomfortable choice between working in a genetic model system on developing behavioral assays or working with ecologically verified behaviors but developing genetic methods. Here, I have chosen to do the latter; bringing numerous genetic tools to the classic ethology system threespine stickleback fish, enabling us to address how genes contribute to behavior.

CHAPTER 1: A FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) PROTOCOL FOR STICKLEBACK TISSUE

Threespine sticklebacks are renowned for their phenotypic variation. An increasing number of studies are identifying genes related to morphological (Colosimo et al., 2005; Liu et al., 2014; Miller et al., 2007; Shapiro et al., 2004), hormonal (Kitano et al., 2010), and behavioral (Greenwood et al., 2013) differences between stickleback populations. Behavioral studies are also identifying genes whose expression is influenced by the social environment in sticklebacks (Greenwood & Peichel, 2015; Rittschof et al., 2014; Sanogo et al., 2012; Sanogo, Hankison, Band, Obregon, & Bell, 2011).

Linking genes to traits requires additional techniques to determine both how and where gene expression changes within a tissue. Subtle changes in the location of gene expression can have major consequences for trait development. For instance, pelvic reduction in freshwater sticklebacks is due to the loss of *Pitx1* expression specifically in the developing pelvic girdle but not in other tissues (Chan et al., 2010; Shapiro et al., 2004). qPCR is useful for validating how expression levels differ, but its resolution is limited to the precision of the dissection. Alternatively, antibody staining can be used for localization of proteins, but developing new antibodies can be expensive and time consuming.

For fine localization of gene expression, a visualization technique (Tautz & Pfeifle, 1989) such as fluorescence *in situ* hybridization (FISH) is necessary. In this technique, a labeled RNA probe binds its complementary mRNA, expressed from the targeted gene. FISH is not limited to mRNA; it can be used to label any type of expressed RNA including microRNAs and long

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James, N., Liu, X., & Bell, A. (2016). A fluorescence in situ hybridization (FISH) protocol for stickleback tissue. *Evolutionary Ecology Research*, *17*(4), 603–617.

noncoding RNA (lncRNA). FISH has superb resolution, allowing for subcellular localization (Zimmerman, Peters, Altaras, & Berg, 2013) and the possibility of detecting the expression of multiple genes simultaneously using different fluorescent labels (Barroso-Chinea et al., 2007). FISH can also be used to distinguish qualitative differences in expression between upregulation in the same cells and new expression in previously silent cells. Therefore, we optimized a protocol for FISH on sectioned stickleback tissue. Though we established the protocol for brain tissue, we note key steps where it can be optimized for other tissues.

Protocol

Animals used in the development of this protocol were wild-caught adult threespine stickleback (male and female) collected from Putah Creek, CA. All of the procedures were approved by the University of Illinois at Urbana-Champaign IACUC (protocol #15077). Product ordering information as well as specific primers for our probes are listed in their entirety in the *Supply Information* section following this protocol. All steps are carried out at room temperature unless otherwise specified.

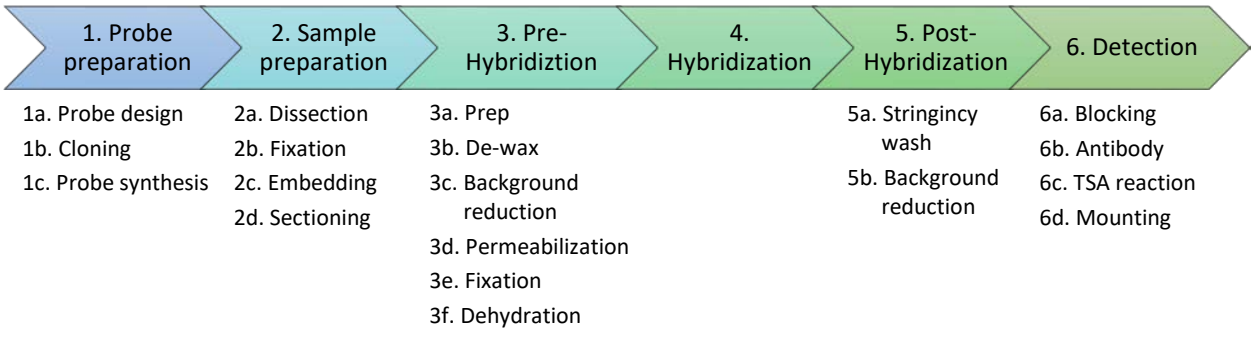

Figure 2 Workflow for FISH broken into the major phases of the protocol. Note that Phase 2 takes a minimum of two days, while Phases 3 and 4 should be done on the same day.

The FISH protocol comprises six distinct phases outlined in Figure 2. The general strategy is to label the target mRNA, which requires a specific probe. As tissue penetration

(Kühn & Köster, 2010) can hamper deep tissue labeling, thin sections are necessary. We compare FISH on two dissection techniques, flash-frozen tissue versus fresh tissue, and on two embedding techniques, cryo- or paraffin embedding. We also show the expression of Glial Fibrillary Acidic Protein (GFAP), Oxytocin receptor (OXTR) and Tyrosine Hydroxylase (TH) in the brain as representative results.

Important: Use RNase-free solutions and tools unless otherwise noted. Degradation of either the RNA probe or the mRNA target will result in weak or no signal.

1. Probe preparation

1a. Probe Design

Design a probe that recognizes more than one exon, preferably with 50% GC content. Spanning an intron with the probe reduces the background that can occur from genomic labeling. Alternatively, probes can be designed to the 3'UTR (Thisse & Thisse, 2008) to reduce cross-reactivity via a unique sequence. The anti-sense strand of your target sequence will be used as the probe. For a negative control, use the sense strand, a scramble sequence, or a gene not expressed in the relevant tissue. This negative control will be used to check for non-specific labeling. Always ensure the uniqueness of a probe by comparing potential target sequences to the stickleback genome assembly (on the UCSC Genome Browser) using the BLAT feature.

1b. Cloning

Using whole brain (or tissue of interest) cDNA collected from a non-experimental fish, do a nested PCR (in which the inner primer set amplifies a region within the product of the outer primers) reaction to amplify the gene's target sequence. The presence of a single band at the expected size by gel electrophoresis should confirm specificity of primers. Transform the

checked PCR product into a vector (pCRII) with both T7 and SP6 promoters using a TOPO TA Cloning Kit. Sequence the inserts to confirm the identity of the probe and to determine the correct sense transcription start site. Alternatively, SP6 and T7 promoter sites can be added to the inner PCR primers to avoid the need for vector transformation. We used both methods of probe design during the testing of this protocol.

1c. Probe Synthesis

Template DNA should be cleaned, concentrated, and resuspended in RNase-free water. It can be generated from either nested PCR or amplified from a long-term storage clone/library so long as it has appropriate RNA Polymerase start sites. The probe synthesis reactions follow the Roche DIG RNA Labeling Kit (SP6/T7). After a 15-30 minute DNase I treatment, ethanol precipitate the probe with lithium chloride for at least 1 hour at -80° and resuspend in aliquots of $50^{ng}/_{\mu l}$ in RNase-free water for storage at -20°.

2. Sample Preparation

2a. Dissection

Decapitate an experimental fish. For the flash-freezing protocol, immediately immerse the head in a dry ice-ethanol bath for 5 minutes until the eyes turn cloudy white. Next, for either protocol, in a room temperature environment, remove the brain from the skull using RNase-free (cleaned with RNaseZap) tools.

We compared FISH on flash-frozen or fresh brain tissue and did not observe an effect on either morphology (Figure 3a) or FISH signal (Figure 3b) regardless of embedding method. Flash-freezing the head can ease dissection by preventing complications from accidental damage to surrounding tissue (e.g. eye punctures), but makes the brain more liable to chipping from the Micro-Rongeurs during extraction. Ultimately, both techniques are viable.

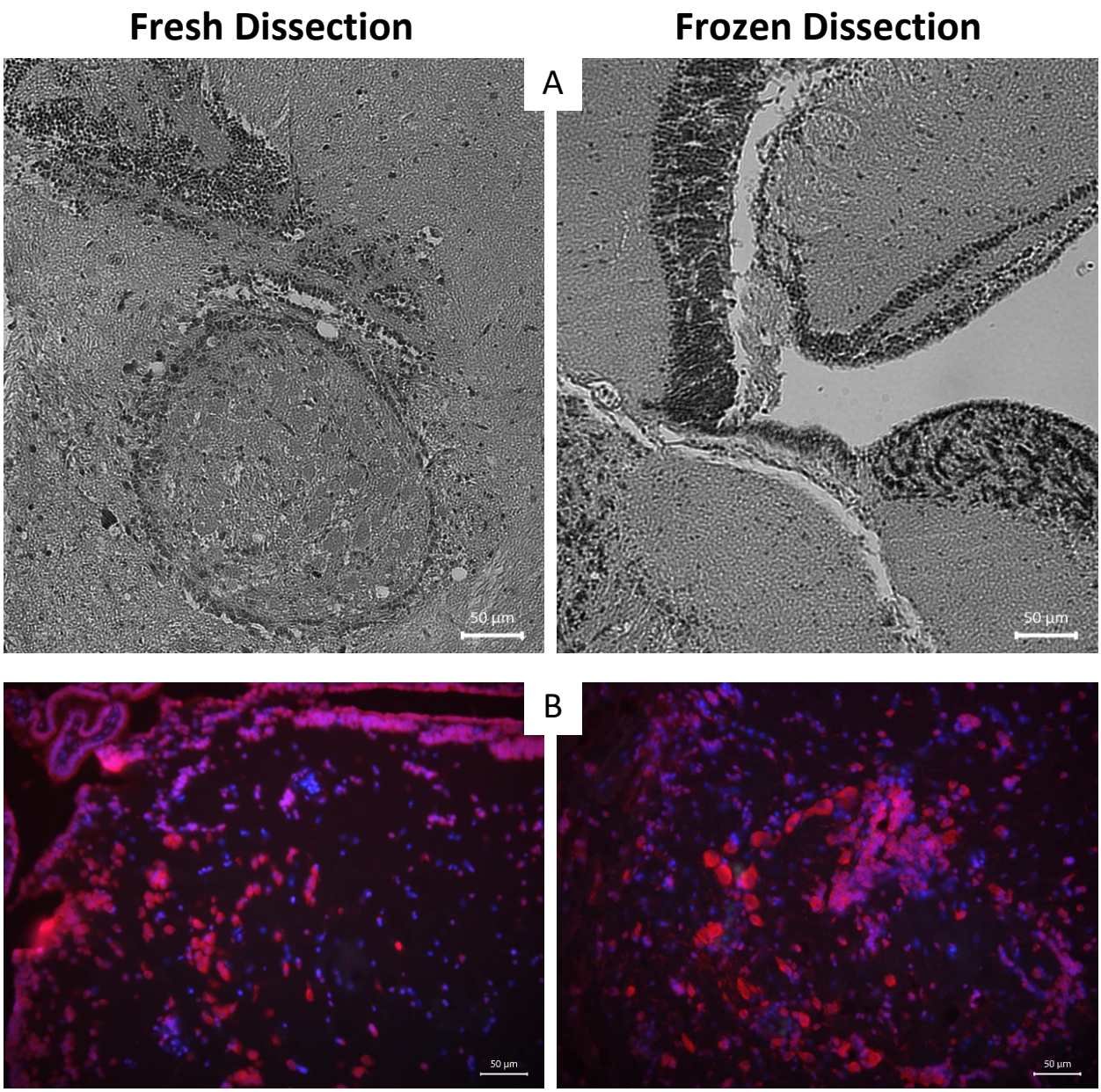

Figure 3 Following decapitation the brain was either immediately placed in fixative or the head was flash-frozen in ethanol on dry ice prior to brain extraction. There was no notable difference in either A) morphology preservation or B) *in situ* hybridization signal quality. Tissue was treated simultaneously and identically following dissection. Paraffin embedded transverse sections of the A) diencephalon with H&E staining and B) posterior telencephalon with the probe *PAC1b-R* (red) and Hoechst nuclear dye (blue). The images were taken sequentially with no light adjustment between the images.

2b. Fixation

Fix the brains immediately following dissection for 12-24 hours at 4° in RNase-free 4% paraformaldehyde (PFA). The 4% PFA (pH 7.4 in PBS) should be < 2 months old and stored in individual aliquots to avoid freeze/thawing. For paraffin embedding, a standard overnight fixation (12-18 hours) is sufficient. However, we found that a longer fixation of 20-24 hours was necessary for cryo-embedding.

2c. Embedding

We compared FISH on cryo- and paraffin embedded tissue. While both produced specific labeling (Figure 4b), paraffin better preserved the tissue morphology (Figure 4a). Cryo-sectioning has less processing time and lower initial equipment costs. However, paraffin embedding reliably allows thinner (<10μm) sectioning, resulting in more sections from a given brain. With paraffin embedding, the concerns are that heat and harsh (toluene) chemicals might damage mRNAs. However, we did not observe signal degradation with our probes. RNA was stable in both paraffin blocks (stored at -20°) and slide-mounted sections (at room temperature for testing; we recommend storage at -20°) for more than 1 month. Cryo-embedded samples were stored in blocks (at -20°) until ready to section and immediately process.

Paraffin embedding: Immediately following fixation, transfer tissue to RNase-free 70% ethanol (EtOH). To begin the embedding process, move the sample to an appropriately-sized mesh tissue cassette, e.g. for brain, we used a Micromesh Biopsy cassette. Run the tissue, either automatically using a Tissue-Tek VIP or by hand, through the following series of washes.

i. Equilibrate in EtOH gradient of 70%, 80%, 95%, 95%, 100%, 100%, 100% for 40 minutes each
ii. Wash in 50% Toluene/EtOH for 40 minutes
iii. Wash 2x in 100% Toluene for 1 hour each
iv. Wash 3x in paraffin at 60° for 40 minutes each

Hold the samples in final paraffin wash at 60° before embedding in a paraffin block using an embedding station. The Tissue-Tek base mold 7 x 7 x 5 mm fit adult brains best, minimizing excess paraffin.

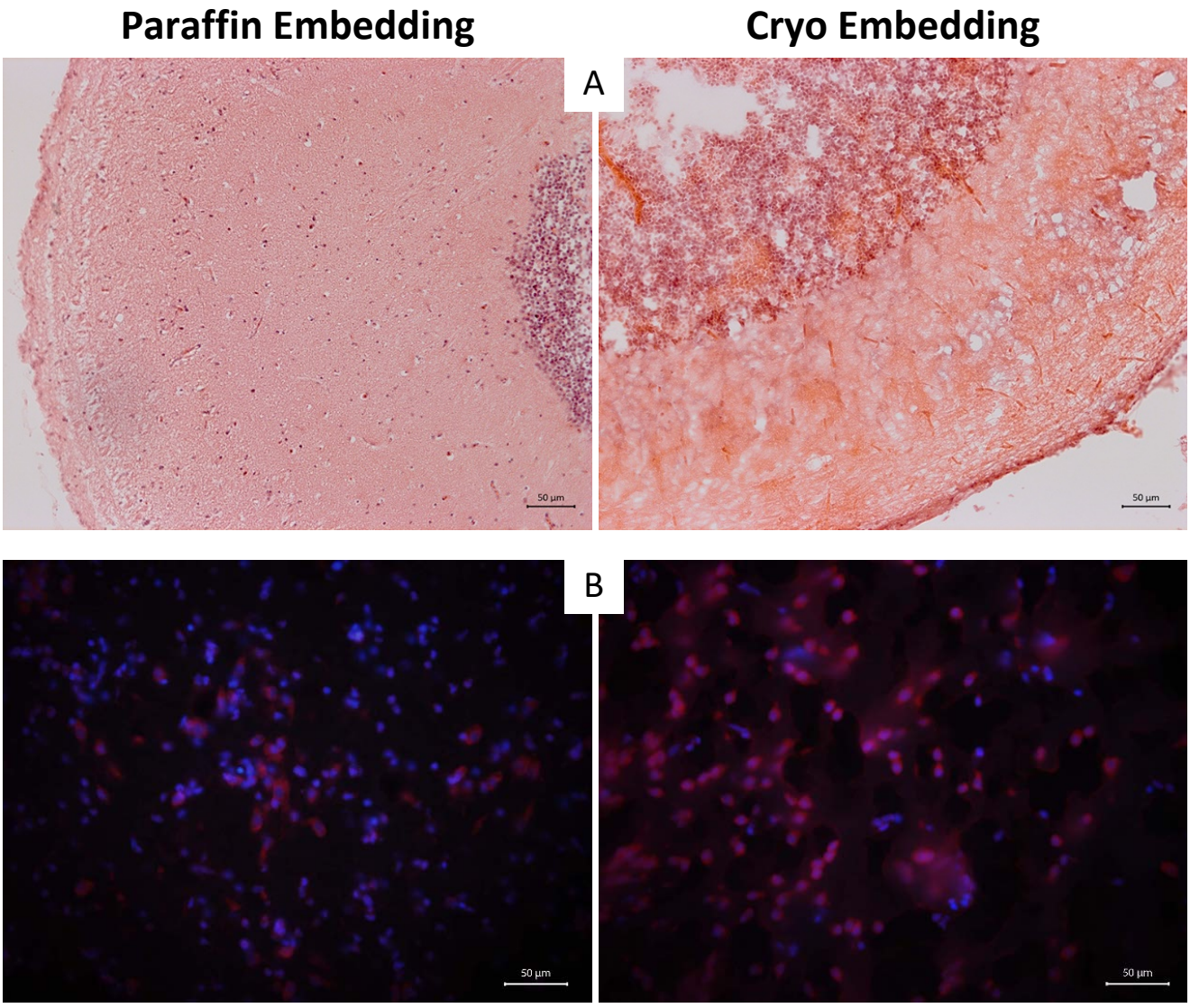

Figure 4 We compared FISH on paraffin and cryo-embedded tissue. A) Paraffin embedding resulted in better morphology preservation. B) Signal quality was consistent between embedding methods. Tissue was fresh dissected and processed for FISH simultaneously. Sections show the A) tectum opticum with H&E staining and B) mid telencephalon with the probe *PAC1b-R* (red) and Hoechst nuclear dye (blue).

Cryo-embedding: Fill cryo-mold with room temperature tissue freezing media. We tested use of a sucrose gradient prior to media immersion but found no improvement to tissue preservation, so the brain may be transferred directly from the fixative. Add the fixed tissue and push to the bottom, oriented such that the desired sectioning plane (sagittal, transverse or coronal) is parallel to the bottom. Place the mold on dry ice and allow the block to freeze completely. The media turns white when fully frozen.

2d. Sectioning

Tissue damage is most likely to occur during sectioning. Uneven or torn sections indicate that the blade was not sharp enough. For cryo-embedded samples torn tissue can also indicate sectioning was done at an improper temperature or humidity for the tissue type. Wrinkling and folding of the tissue arises when the embedding material curls. We apportioned serial sections across 8 to 16 slides for adult brain tissue, resulting in a series of sections at a variety of depths on each slide.

Paraffin: Section (8-10 μm) on a microtome. Immediately float sections on 15% ethanol and then on 42° water before affixing them to a slide. These 30 second to 5 minute floats smooth wrinkles in the tissue and help the section bind to the slide. Dry slides on slide heater for >24 hours before proceeding with labeling.

Cryo: Section (10-25 μm) on a cryostat (Leica CM1850) within 24 hours of embedding. Allow sections to thaw for 5 minutes at room temperature, adhering them to the slide before either storing at -20° or proceeding with labeling.

3. Pre-Hybridization

3a. Prep

3b.1. Calculate temperatures for hybridization (T_{hyb}) and stringency washes (T_{wash}). Generally, probes with similar T_{hyb} can be processed in batches to minimize the need for multiple hybridization ovens or runs. If, for example, there are 3 genes at T_{hyb} of 57/58/59 it is usually sufficient to hybridize them all at 58°C.

$$T_{hyb} = T_m - 25 \qquad T_{wash} = T_m - 14$$

The T_m is calculated by Wilkinson's formula for RNA:RNA in solution:

$$T_m = 79.8 + 18.5 \log[Na] + 58.4(GC\ fraction) + 11.8(GC\ fraction)^2 - \frac{820}{Probe\ length\ (bp)} - 0.35(formamid\%)$$

Calculate T_m using 60% formamide, 5x Saline Sodium Citrate (SSC) buffer (Na = 0.825)

3b.2. Pour 1X Target Retrieval Solution into plastic Coplin jars (glass jars will break) and heat in 95-100°C bath. This solution will be used in 3c.3 (Background Reduction).

Note: For cryo-sections, rinse slides in RNase-free H_2O for 3 minutes, then skip to 3c Background Reduction.

3b. De-wax

3b.1. Preheat slide heater to 55-60°C.

3b.2. Melt wax from tissues in slide heater for 15 minutes and immediately proceed, in a Coplin jar, with washes.

3b.3. Wash 3x in Clearene for 5 minutes each

3b.4. Wash 3x in 100% EtOH for 5 minutes each

3b.5. Wash in 90% EtOH for 5 minutes

3b.6. Wash in 70% EtOH for 5 minutes

3b.7. Wash in RNase-free H_2O for 5 minutes

3b.8. Remove any remnant Clearene or alcohol in the water. This is typically only a problem if slides were placed back to back in Coplin jars.

3c. Background Reduction

Acetylation with triethylamine (TEA) & acetic anhydride prevents non-specific binding of the probe, thereby reducing background signal. This chemical reaction substitutes an amine group (-NH-CO-CH_3) in place of the reactive amine group (NH_3^+). Antigen retrieval combats the deleterious effects of formaldehyde fixation and paraffin embedding. Antigen retrieval is especially important for genes that are expressed at a low level because it increases the signal to noise ratio. TSA-based detection (covered in step 6c) requires quenching of endogenous peroxidase activity, which we accomplish through incubation in hydrogen peroxide (H_2O_2) with methanol.

3c.1. Move slides to TEA solution (2 mL 2M TEA per 40 mL RNase-free H_2O) in a Coplin jar and add 100 µl of acetic anhydride per 40 mL of TEA solution. Cap, invert to mix, and place on shaker for 10 minutes.

3c.2 Rinse in RNase-free H_2O for 5 minutes.

3c.3. Transfer slides to the preheated (95-100°C) Target Retrieval Solution. Heat for 15 minutes with the cap slightly loosened.

3c.4 Take Coplin jar out of water bath and allow it to cool for 25-30 minutes on a shaker.

3c.5. After jar cools, add RNase-free 1x PBS and let sit for 5 minutes.

3c.6. Switch slides to 3% H_2O_2 (4 mL of 30% H_2O_2 diluted in 36 mL Methanol to make 40 mL, make fresh from < 2 month old stock) for 30 minutes up to 2.5 hours.

3c.7. Move to fresh Coplin jar with RNase-free 1X PBS and let shake for 5 minutes. If the slides are placed back-to-back, transfer one-by-one so that remnant methanol is rinsed off the slides. Pull out Proteinase K to thaw upon starting 5 minute shake.

3c.8. Rinse once more with RNase-free 1X PBS for 5 minutes.

3d. Permeabilization

Proteinase K treatment allows the probe better access to the target by partially eliminating proteins, especially those associated with nucleic acids. This is a critical step of the ISH protocol according to the DIG Application Manual for In Situ Hybridization (Eisel, Seth, Grünewald-Janho, & Kruchen, 2008) that will need to be optimized for different tissues. Both time and concentration can be varied to achieve optimum probe penetration with the least tissue damage.

3d.1. Make 5 μg/mL Proteinase K solution in new RNase-free 1X PBS in a Coplin jar. Add slides to the solution and let sit for 10 minutes.

3e. Fixation

Additional on-slide fixation is important for stabilizing tissue and nucleic acids after permeabilization. It can also improve the adhesion of sections to slides, reducing sample damage or loss.

3e.1. Fix in RNase-free 4% paraformaldehyde (made from 13 mL 12% stock diluted in 39 mL RNase-free 1X PBS) for 10 minutes.

3e.2. Wash in RNase-free 1X PBS and let shake for 5 min. If slides are back-to-back, separate and return to jar so that remnant PFA is washed out.

3e.3. Wash again in RNase-free 1X PBS for 5 minutes.

3e.4. Wash in Glycine (made fresh, 3.7 g in 40 mL of RNase-free 1X PBS) for 20 minutes. After starting the wash, preheat hybridization oven and solution to T_{hyb} calculated in step 3a.1.

3e.5. Wash in RNase-free H_2O for 5 minutes.

3f. Dehydration

Dehydrating the sections prior to hybridization prevents dilution of the probe and hybridization buffer and helps affix tissues to the slide. However, this can also result in increased non-specific labeling especially on the edges of tissues. Dilute all solutions with RNase-free water and use repeatedly for up to 2 months.

3f.1. Rinse in 50% EtOH for 2 minutes

3f.2. Rinse in 70% EtOH for 2 minutes

3f.3. Rinse in 90% EtOH for 2 minutes

3f.4. Rinse in 100% EtOH for 2 minutes

3f.5. Rinse in 100% EtOH for 2 minutes

3f.6. Allow slides to dry completely on a clean kimwipe for 5-10 min. While slides are drying, place 6-20 µl of RNA probe (225-375ng) in 250 µl of hybridization solution and heat 5 minutes in a heat block at 80°C.

4. Hybridization

The recommended hybridization buffer contains 60% formamide and has a sodium ion (Na^+) concentration of 0.825 moles (from 5X SSC). Alternatively, ULTRAhyb Ultrasensitive Hybridization Buffer can be used with a 50% formamide concentration, but the T_m calculation used in step 3a.1 will need to be adjusted. There are many formulations of hybridization buffers; we did not test alternative mixtures. To facilitate binding, probes need to be denatured (linearized) via pre-heating before being placed on the samples.

4a. Place Place 6-20 µl of RNA probe (225-375ng) in 250 µl of hybridization solution and heat 5 minutes in a heat block at 80°C. This can be done while slides are drying (3f.6)

4b. Place heated probe/hyb mix on ice to cool while loading slides.

4c. Add probe/hyb mix to slides, being careful to avoid bubbles. Pipette probe solution lengthwise onto the top edge of a plastic hybri-slip (remove plastic covers from both sides), then lower one long edge of the slide down onto the cover slip, so that the solution wicks up onto the tissue and the hybri-slip sticks onto the slide. Alternatively, pipette the probe mix directly onto the samples and slowly lower the hybri-slip.

4d. Seal the hybri-slip in place using rubber cement along the edges and place in a humidified chamber. Hybridize overnight at T_{hyb}.

5. Post-Hybridization

5a. Stringency Wash

Post-hybridization, stringency washing eliminates non-specific hybridization, thereby boosting the signal to noise ratio. It also removes any excess unbound probe.

5a.1. Set water bath to T_{wash} calculated in step 3a.1 and pre-warm the 4 stringency solutions.

5a.2. Take slides out of hybridization chamber and remove hybri-slips by prying them off with a pair of forceps. If a cover is stuck because the cement fully dried, a 1 minute soak in 5X SSC should soften the cement.

5a.3. Place slides in a glass Coplin jar with 5X SSC for 10 minutes at room temperature.

Important: From this point forward, do not let the tissue dry out completely.

5a.4. Place slides in pre-warmed 5X SSC and place in water bath for 10 minutes.

5a.5. Wash in pre-warmed Wash I (5X SSC/50% Formamide) for 20 minutes.

5a.6. Wash in pre-warmed Wash II (0.5X SSC/50% Formamide) for 20 minutes.

5a.7. Wash in pre-warmed 0.5X SSC for 10 minutes.

5a.8. Wash at room temperature in 0.5X SSC for 10 minutes.

5b. Background Reduction

At this point, the mRNA and probe are bound and inaccessible to RNases. Iodoacetamide treatment reduces non-specific antibody binding by reacting with disulphide bridges and sulphydryl groups (ALLEN Mouse Brain Atlas, n.d.).

Note: Use deionized water from this step forward for solutions; RNase-free water is no longer necessary.

5b.1. Place slides in 20 mM Iodoacetamide (0.148 g in 40 mL water) for 5 minutes.

5b.2. Rinse in 1X Tris Buffered Saline, with Tween (TBST) for 10 minutes.

6. Detection

6a. Blocking

6a.1. Remove slide from Coplin jar, dry back and edges and draw a rectangle with wax pen around tissue to contain the antibody solution. Add 100-200 µl of TNB block buffer.

6a.2. Incubate for 30 minutes at room temperature. Make sure the slides are level so that sections don't dry out. Place in a closed box to reduce evaporation.

6b. Antibody

Using a separate nuclear label is preferable to a mounting medium combined with nuclear label (e.g. Vectashield w/ DAPI) as the intensity of the nuclear dye can be adjusted.

6b.1. With a sharp flick of the wrist, shake off the TNB and add 100-200 µl of Anti-Dig-POD diluted 1:300 in TNB. Optionally, add a nuclear stain to the antibody mix. We recommend using Hoechst at 1 µl (10 µg) for up to 1 mL of solution.

6b.2. Incubate for 1 hour at room temperature in a closed box.

Important: Check halfway to insure that slides do not dry out. Add more antibody mix as required.

6b.2. Wash slides in a Coplin jar 3 times in 1X TBST for 5 min.

6c. TSA Reaction

Probe signal is enhanced using Tyramide Signal Amplification (TSA) for better detection of genes expressed at low levels. The final deionized water washes remove ions that cause a blue glow under fluorescent light sources.

6c.1. Pull out slides from TBST 2-3 at a time and dry the back and edges as before while avoiding the wax outline. Quickly add 100 µl of TSA with Rhodamine, diluted 1:100 in 1X amplification buffer provided in the kit. Incubate for 20-30 minutes. Alternatively, follow the manufacture's recommendation of a 1:50 dilution with a 6-minute incubation.

6c.2. Wash slides in a Coplin jar 3 times in TBST for 5 minutes.

6c.3. Wash slides 2x for 5 minutes in deionized water.

6d. Mounting

Prior to mounting, you can check the signal quality of both the TSA reaction and the nuclear staining by covering samples with water and a coverslip. If additional staining is needed, remove coverslip, wash in TBST and repeat the necessary reaction (Hoechst or TSA) followed by the final washes. While Vectashield is used in this protocol, any anti-fading medium can be used, including Prolong Gold.

6d.1. Lay glass cover slips on the counter and place a thin smear of mounting media (Vector H-1000) lengthwise on the top edge of each slip. Take out the slides from DI water, dry back and edge as before, making sure excess water has dripped off. Lower slides onto the cover slip and

allow media solution to wick along the tissue without any air bubbles. If there are air bubbles on top of the tissue, remove them by gently lifting and replacing the coverslip.

6d.2. Store slides protected from light. Scan slides as soon as possible, since weak signals will fade.

Supply Information

Table 1 Primers

Gene Ensembl ID	Type	Primers
Adenylate cyclase activating polypeptide 1b (pituitary) receptor type I *(PAC1b-R*) ENSGACG00000005402	Outer	5'- CATGTCCCGGAGACACAAGT -3' 5'- TTGTCCTGCATGTAGCGGAT -3'
	Inner (Cloning)	5'- ATTCAGTGACGTGGAACCCG -3' 5'- TTTGAGCCTCGAACCCGATG -3'
	Inner (PCR)	5'- ATTTAGGTGACACTATAGATTCAGTGACGTGGAACCCG -3' 5'- TAATACGACTCACTATAGGGTTTGAGCCTCGAACCCGATG -3'
Glial fibrillary acidic protein *(GFAP)* ENSGACG00000009804	Outer	5'- CGAATTGGCAGCCTTTCTTCC -3' 5'- GGGCTCCTTCCCCTTAAACT -3'
	Inner (Cloning)	5'- GATCACCTTGGGCTCAACCA -3' 5'- GTTCTGCTGCTCCAAAAGGC -3'
	Inner (PCR)	5'- ATTTAGGTGACACTATAGGATCACCTTGGGCTCAACCA -3' 5'- TAATACGACTCACTATAGGGGTTCTGCTGCTCCAAAAGGC- 3'
Oxytocin receptor *(OXTR)* ENSGACG00000000914	Complete	5'- CGAACCCTCTCAAGCGGAAT -3' 5'- ACAGCATGTCTGGTCCGTAG -3'
Tyrosine hydroxylase *(TH)* ENSGACG00000011104	Complete	5'- AGTGAATACCTCGTGGCCCT -3' 5'- GGAGTGACGGATGTACTGGG -3'

Table 2 Solution purchase options and equivalent recipes

Solution	Manufacturer	Catalog #	Recipe
mRNA *in situ* hybridization buffer	Ambion ULTRAhyb	AM8669	12 mL 20X SSC 30 mL DI formamide 12 mL Dextran sulfate, 50% w/v 300 µL 50X Denhardt's solution 900 µL yeast tRNA, 10 mg/mL 1200 µL salmon sperm DNA, 10 mg/mL
20X SSC	Invitrogen	AM9763	0.15 M NaCl 0.015 M Sodium citrate
10X Target retrieval solution	DAKO	S1699	10 mM Sodium citrate 0.05% Tween 20, pH 6.0
10X TBST	DAKO	S3306	0.5 M Tris-HCL, pH 7.5 3.0 M NaCl 1% Tween 20 *Dilute 1:10 for working solution*
TNB blocking buffer	(make from Perkin Elmer FP1020)		0.1 M Tris-HCl, pH 7.5 0.15 M NaCl 0.5% w/v TSA Blocking Reagent

Table 3 Communal equipment

Item Name	Manufacturer	Item #
Embedding station	Leica	EG1150 H
Tissue-Tek VIP	Sakura	3000
Microtome	Leica	RM2255
Cryostat	Leica	CM1850
Slide heater	TBS	SD-11-120

Table 4 Purchasable materials and equipment

Item	Manufacturer	Catalog #
Acetic Anhydride	Sigma	A6404
Anti-Digoxigenin-POD	Roche	11-207-733-910
Clearene	Leica Surgipath	3803600
Formamide	Sigma	F7508
Hoechst	Molecular Probes	H3570
Hybri-slips (plastic) 24X20mm	Sigma	H1034
30% Hydrogen Peroxide	Sigma	H1009-100 mL
Iodoacetamide	Sigma	I1149-25g
Micromesh Biopsy cassette	Simport	M507-2
Proteinase K Solution (PCR grade)	Roche	03115828001
RNaseZap	Ambion	AM9780
Roche DIG RNA Labeling Kit (SP6/T7)	Roche	11175025910
Slides (for cryo sectioning)	Fisher Superfrost Plus	12-550-15
Slides (for paraffin sectioning)	Leica Surgipath X-tra	38002052
TEA (Triethanolamine)	Sigma	T1377
Tissue freezing media	Leica	14020108926
Tissue-Tek base mold 7 x 7 x 5mm	EMS	4161
Tissue-Tek Cryomold	EMS	4566
TOPO TA Cloning Kit, Dual Promoter	Invitrogen	45-0640
TSA blocking reagent	Perkin Elmer	FP1020
TSA with rhodamine (Tetramethyl Rhodamine Tyramide)	Perkin Elmer	NEL742B001KT (250-750 slides total)
Vectashield mounting media	Vector Labs	H1000
Wax Pen (Super Pap Pen)	Invitrogen	00-8899

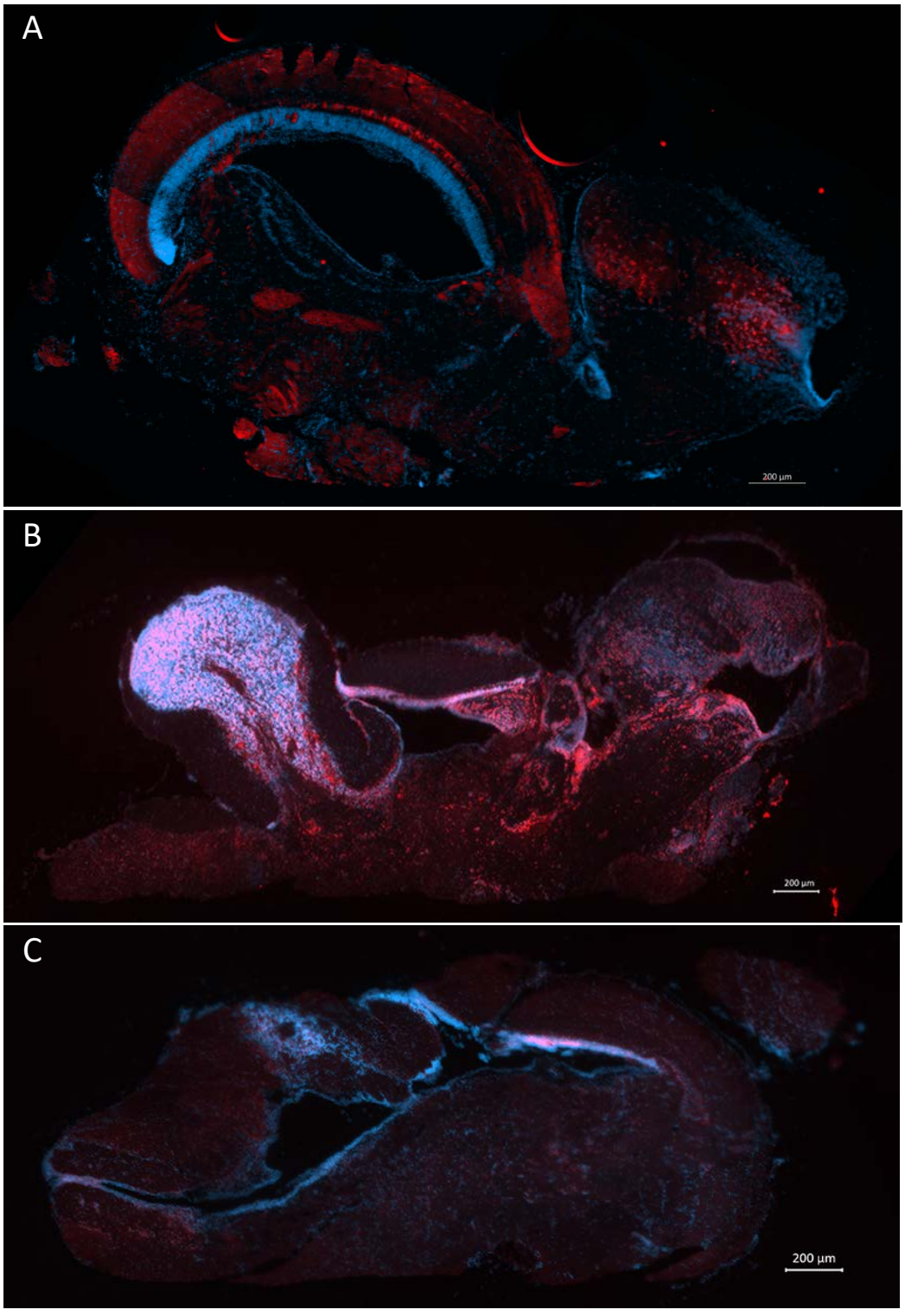

Figure 5 Sagittal sections with the anterior to the right and dorsal oriented upwards. The gene of interest is labeled in red and nuclei are blue. A) Glial fibrillary acidic protein (*GFAP*) is a marker of astrocytes. B) Oxytocin receptor (*OXTR*) is the receptor for the neuropeptide oxytocin. C) Tyrosine hydroxylase (*TH*) is the rate-limiting enzyme in the synthesis of catecholamines.

Representative Results

We further tested the protocol using several different probes on paraffin-embedded adult female stickleback brain tissue, as this was our preferred embedding method. The FISH protocol was sensitive, as we detected signal with concentrations as low as 1 $^{\mu g}/_{mL}$, and used 5-10 $^{\mu g}/_{mL}$ for most probes. Conserved neuronal markers were synthesized into probes and a sample expression pattern for an astrocyte marker (*GFAP*) is shown in Figure 5a. The expression patterns of select genes relevant to social behavior including oxytocin receptor (*OXTR*) and tyrosine hydroxylase (*TH*) are shown in Figure 5b and 5c respectively.

Discussion

We established a protocol for fluorescent *in situ* hybridization (FISH) to localize gene expression on sectioned stickleback brain tissue. Brain dissection technique (fresh or frozen) did not affect morphology or final signal. Both cryo- and paraffin embedding resulted in successful FISH signal throughout the brain. The cryo-embedding process was faster, taking 4 days from dissection to labeled slides, compared to a minimum of 5 days with paraffin embedding. However, paraffin embedding resulted in better tissue preservation and thinner sections.

An increasing number of studies (Colosimo et al., 2005; Greenwood et al., 2013; Kitano et al., 2010; Liu et al., 2014; Miller et al., 2007; Shapiro et al., 2004) have identified QTL or genes relevant to ecologically-important traits in sticklebacks. Neurogenomic studies have identified hundreds of genes whose expression levels are influenced by the biotic environment, including the social environment (Greenwood & Peichel, 2015; Rittschof et al., 2014; Sanogo et al., 2011). By allowing us to visualize gene expression patterns in complex tissue, FISH will help stickleback biologists attempting to mechanistically link genes to phenotypes.

CHAPTER 2: NEST CONSTRUCTION AND PRESENCE DO NOT ALTER TERRITORIAL AGGRESSION IN MALE STICKLEBACK

Territoriality is widespread in the animal kingdom, drastically influencing fitness by allowing animals to gain sole access to resources such as food and mating opportunities. Although holding a territory provides clear benefits, it also carries time and energy costs, as well as a risk of injury in territorial conflicts. The choice to engage in territorial conflict, either as a defending resident or as an intruder, is a complex decision composed of many subprocesses (Arnott & Elwood, 2008; Injaian & Tibbetts, 2015; Reichert & Quinn, 2017), including motivation. The amount of effort invested into defending a territory can be used to measure an animal's subjective appraisal of the territory's value (Hinsch & Komdeur, 2017; Hollander, Titeux, & van Dyck, 2012; O'Connor et al., 2015).

Resource value (RV) can be divided into two factors: *subjective* and *objective* value (Arnott & Elwood, 2008). *Objective* value reflects intrinsic or fundamental qualities of the territory, such as size in m^2, calories of food provided, number of potential mates, and so forth. Because these measures are empirical, the objective value of a territory is the same for all contestants. However, not all contestants will assign identical importance to each quality. For example, new parents evaluating potential homes might favor adjacency to an elementary school, while retirees sensitive to noise might find that same quality unfavorable. These s*ubjective* valuations are specific to the contestant, reflecting differences in perceived value, typically based on prior experience or investment in exploiting a resource (Stockermans & Hardy, 2013). Different internal states further shift individual subjective valuations (Enquist & Leimar, 1987). Knowledge of the territory differs between competitors, typically to the resident's distinct advantage. Subjective valuations can even differ over time for a given individual, reflecting fluctuating motivations or other inconstant facets of internal state.

Reproduction is key to fitness, and becoming a parent clearly shifts physiological state. Parenthood has repeatedly been shown to increase animal perception of risk and value, reviewed in Arnott & Elwood (2008). In rodents, maternal aggression toward intruding, potentially infanticidal males is greater during late pregnancy and early lactation. The concern of mitigating risk to offspring also extends to maintaining a safe living space. In crayfish (*Procambarus clarkia*), maternal females are more likely to initiate shelter-related aggression and to win compared to non-maternal females or males. In American lobsters, maternal females outcompeted nonmaternal females for shelters, whether the maternal females were residents or intruders. Furthermore, gaining access to mating opportunities can influence the perceived or subjective value of a resource. In house crickets, males who are isolated from females are more likely to initiate aggression and win more fights (Brown, Smith, Moskalik, & Gabriel, 2006). Current monopolization of females causes escalation in male cottonwood borers (*Plectrodera scalator*), though in these non-territorial insects, prior pairing does not influence aggression (Goldsmith, Stewart, Adams, & Trimble, 1996). In blackbirds, as competition for nesting sites increases, so does the frequency of conspecific aggression (Diniz, Oliveira, Marini, & Duca, 2018).

Holding a mating or breeding territory has clear objective value, as it implicitly affords the chance to reproduce. A nest, often required for mating, represents a substantial investment into improving a territory. It is a self-initiated change to the territory with inherent, objective value to the resident. The literature is mixed, however, as to whether animals' individual subjective valuations account for the likelihood of reproducing within a nesting territory and correspondingly increase with the presence of a nest. Nest proximity was positively correlated with winning aggressive encounters in house finches (Jonart, Hill, & Badyaev, 2007) and with increased aggression in stickleback fish (Theo C. M. Bakker, 1994). In house finches, nesting stage was correlated with winning (Jonart et al., 2007). However, for the many species across

taxa that do not reuse nests between reproductive attempts, a nest's value is transient – more analogous to a pitched tent than to a furnished house. If the major limiting factor is gaining the territory, then the addition of a reproduction-exclusive nest might be subjectively inconsequential despite the objective value the nest provides. Blackbirds, who typically don't reuse their nests (Ellison, 2008), significantly decreased levels of female-female aggression during advanced stages of nesting (i.e. having eggs or nestlings), without altering male-male aggression (Diniz et al., 2018). Furthermore, fitness is determined by actually reproducing, not just having the opportunity to do so. Nest-holding desert goby fish, who use their nests for both shelter and breeding, did not alter aggression in relation to actual mating opportunities, i.e. recent access to a female (Svensson, Lehtonen, & Wong, 2012). In loons, males with previous mating success and longer tenure in the breeding territory spent less time guarding it, although this may merely have reflected increased efficiency; those with prior success showed increased guarding leading up to egg laying (Spool, Riters, & Piper, 2017).

To examine how subjective value of a mating territory is influenced by nests exclusively used for breeding purposes, we used threespine stickleback fish (*Gasterosteus aculeatus*), a classic system for the study of behavior. Males defend nesting territories during the summer breeding season and do so exclusively for reproductive purposes. However, nests themselves are not reused between reproductive attempts (Rushbrook, Dingemanse, & Barber, 2008). Territorial establishment precedes nesting (W. J. Rowland, 1994; van Iersel, 1953), allowing us to assess the subjective value of the territory in the absence of a nest. Stickleback males initiate mating-specific behaviors upon completion of a nest (Wilz, 1975), revealing a corresponding internal or physiological state change. Male territorial defense presents stereotypically, is easily induced in the lab, and is representative of field studies (A M Bell, 2005). Accordingly, stickleback fish are an ideal system in which to investigate how nests alter subjective value of a breeding territory.

Specifically, our goal was to determine whether individual rates of aggression toward a territorial intruder differ depending on the presence of a nest. Resident male sticklebacks' aggression scales with the proximity of an intruder to the resident's nest (reviewed in Bolyard & Rowland, 2000), suggesting that nests are subjectively valuable. Additionally, previous work by Wootton (1970) indicated that sticklebacks who do nest are marginally more aggressive after nesting than those that do not nest. However, since reasons some fish do not nest remain unknown, the difference in territorial aggression could have been due to some other difference between groups – for example, those that nest may at all times be more aggressive than non-nesters, or the nesters' territories may have been more objectively valuable to begin with, etc. Changes in aggression following nesting within an individual stickleback have not been investigated, leaving open the question of how much subjective value, if any, a completed nest adds to a male's territory.

We hypothesized that residents' aggression during territorial defense would increase with completion of a nest, driven by an increased subjective value of the territory. The objective value increases with the investment on construction and with the immediate mating opportunity afforded by the nest; a subsequent increase in aggression would indicate a corresponding increase in the perceived, subjective, value. While most resource value studies use a between group design, we sought to directly examine subjective value by comparing aggression within the same individuals before and after completing their nests.

Methods

Animals

Freshwater adult fish were collected from Putah Creek, CA in summer 2017 and housed in the lab in 83L (107x33x24cm) group tanks containing ~20 individuals with recirculated freshwater (5ppm salt). The room was maintained at 18° C on a 16:8 (L:D) "summer"

photoperiod. Males showing signs of nuptial coloration were weighed and measured (standard length from nose to caudal peduncle), and then moved to individual, visually-isolated 9.5L (32x21x19cm) tanks lined with gravel and containing a synthetic plant. Each was allowed to acclimate for 24 hours prior to any behavioral measurements. All animal work was done in compliance with IACUC protocol (#15077) at the University of Illinois at Urbana-Champaign.

Response to a territorial intrusion (Flask Assay)

Aggression in male sticklebacks was measured by recording the response of a resident fish to a simulated territorial intrusion on three occasions, with one day between trials (Figure 6). In each trial, an intruder, confined to a glass flask, was placed in the resident's tank. Visual cues are sufficient to elicit an aggressive response in sticklebacks; no water exchange or physical contact is necessary (Felicity Ann Huntingford, 1976; H. V. S. Peeke, 1969; Tinbergen, 1951). The times to orient toward and first bite at the intruder were recorded (respectively TTO and TTB), as well as the number of approaches, bites, and charges (lunges) during the five minutes immediately following orientation. Alternatively, the trial was terminated if the resident fish did not orient to the intruder within five minutes. Fish were not dropped from the study for failure

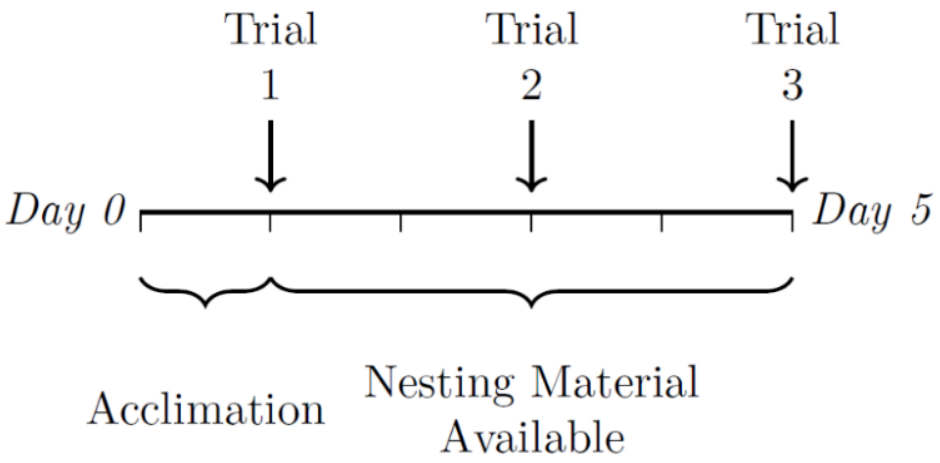

Figure 6 Experimental Timeline. Fish were given nesting materials following Trial 1.

to orient. Accordingly, all fish received all three trials. Individual intruders were not used more than four times per day. All behavioral assays were carried out between 1530-1800 hours in September and October 2017. After the third trial, males were removed from the experiment and their nesting materials and nests were removed from the tank. Water cycled for ~36 hours before tanks were reused.

Nesting Phase

The first trial occurred in the absence of nesting material to serve as a pre-nesting control for all fish. Immediately following the first trial, nesting materials in the form of algae and a sand-filled nesting box were added to the tank. The second and third trials took place three and five days after the first intrusion, in consideration of previous work (unpublished, data available on request) suggesting a median time of 3-4 days for males to complete a nest. Nesting progress was recorded prior to each trial, noting the presence or absence of a completed nest or whether the resident had exhibited nesting behavior, i.e. digging in sand or interacting with algae. A nest is considered complete if it features a visible tunnel (van Iersel, 1953).

Data Analysis

Data analysis was performed in R (v3.5.1). Descriptive statistics (mean ± standard deviation) were generated by the `psych` (v1.8.4) and `FSA` (v0.8.21) packages. Behavioral repeatability is reported as ICC3 from the `psych` package and correlation was analyzed with the `stats` package (v3.5.1). Nonparametric analyses of homogeneity of variances and Wilcoxon rank sum tests were conducted with the `stats` package. As the behavior data were non-normal, analyses of time and nesting effects used the `nparLD` package (v2.1), which supports correction for repeated measures. This is a nonparametric rank-based method requiring no model assumptions that is robust to outliers and small sample sizes. We report the ANOVA-Type

Statistic (ATS) and the adjusted degrees of freedom using the recommended $F_{(\hat{f},\infty)}$ instead of $F_{(\hat{f},\hat{f}_0)}$ (Noguchi, Gel, Brunner, & Konietschke, 2012). To compensate for multiple comparisons, the ANOVA-Type Statistic, Wilcoxon test, and *t* test p-values were minimally adjusted using the Holm method of the `stats` package's `p.adjust` function. Post-hoc Dunn tests through `FSA` similarly used its default Holm correction.

Data Availability

The complete dataset and the final R-scripts used for analysis are publicly available on the Open Science Framework (https://osf.io/y47ur).

Results

In all, 90 males completed the experiment, totaling 270 trials. Ten trials involving nine individual males were halted after the resident failed to orient, requiring the exclusion of these males from analyses not compatible with missing data. Fish that did not bite during at least one trial were removed from analyses involving Time to Bite. Specific sample sizes are given for all analyses. Of the remaining 81 fish, 31 completed nests: 16 "early nesters" between trials 1–2 and 15 "late nesters" between trials 2–3. The "nesters" collectively comprise these two nesting outcomes. A small number of males (N = 6) showed "nesting behaviors", i.e. digging in the nesting box or moving algae around, but did not complete a nest before trial 3 and so were not considered "nesters." The final 44 males fall in the last nesting outcome, "non-nesters."

Intruders provoked aggressive behavior

Resident males were responsive to the intrusion, quickly orienting to the intruder at a mean of 40 seconds (SD = 59) and first biting at 92 seconds (SD = 71) after orienting. Even without a nest, male sticklebacks aggressively defended their territories, biting an average of

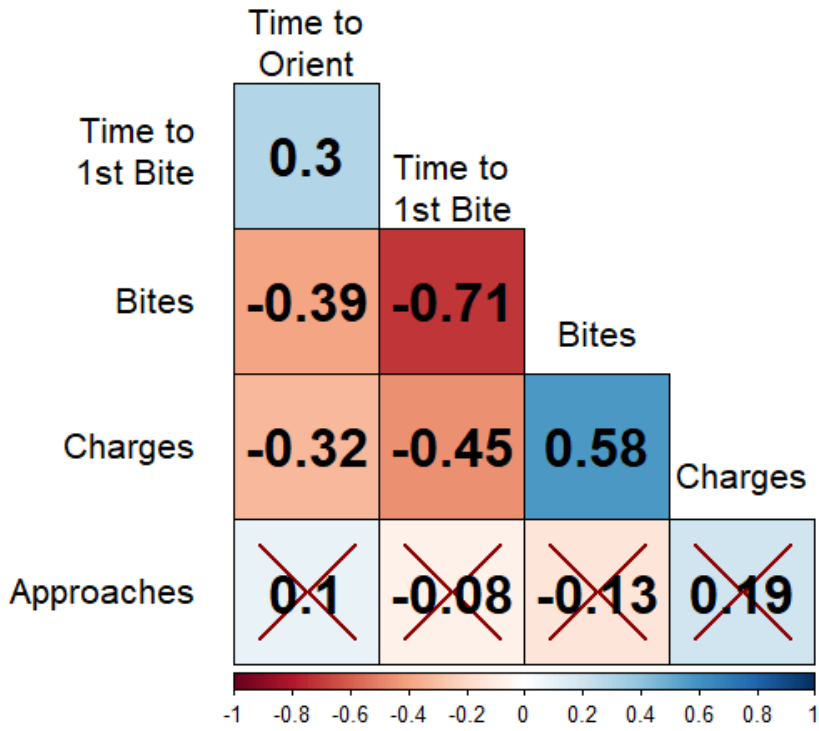

Figure 7 Behavioral measures of territorial aggression were significantly correlated (*N* = 81).

11.0 times per trial (*SD* = 13.6) and charging 6.0 times per trial (*SD* = 7.3). Across all fish and outcomes, bites and charges were significantly positively correlated (Figure 7). Time to bite was significantly negatively correlated with number of bites (Figure 7), as expected: fish that started biting sooner bit more times overall.

Nest presence does not alter aggression

The presence or absence of a nest was not associated with any differences in aggression during any trial (Figure 8). Within trial 2, males who completed their nests (*N* = 16) compared to those who had not nested (*N* = 64) were not more aggressive (Wilcoxon signed rank test: bites Z = -0.45, P_{adj} = 1; charges: Z = -1.95, P_{adj} = 0.20). Within trial 3, males who completed their nests (*N* = 35) compared to those who had not nested (*N* = 47) were not more aggressive (Wilcoxon signed rank test: bites Z = -0.01, P_{adj} = 1, charges Z = -1.03, P_{adj} = 0.91).

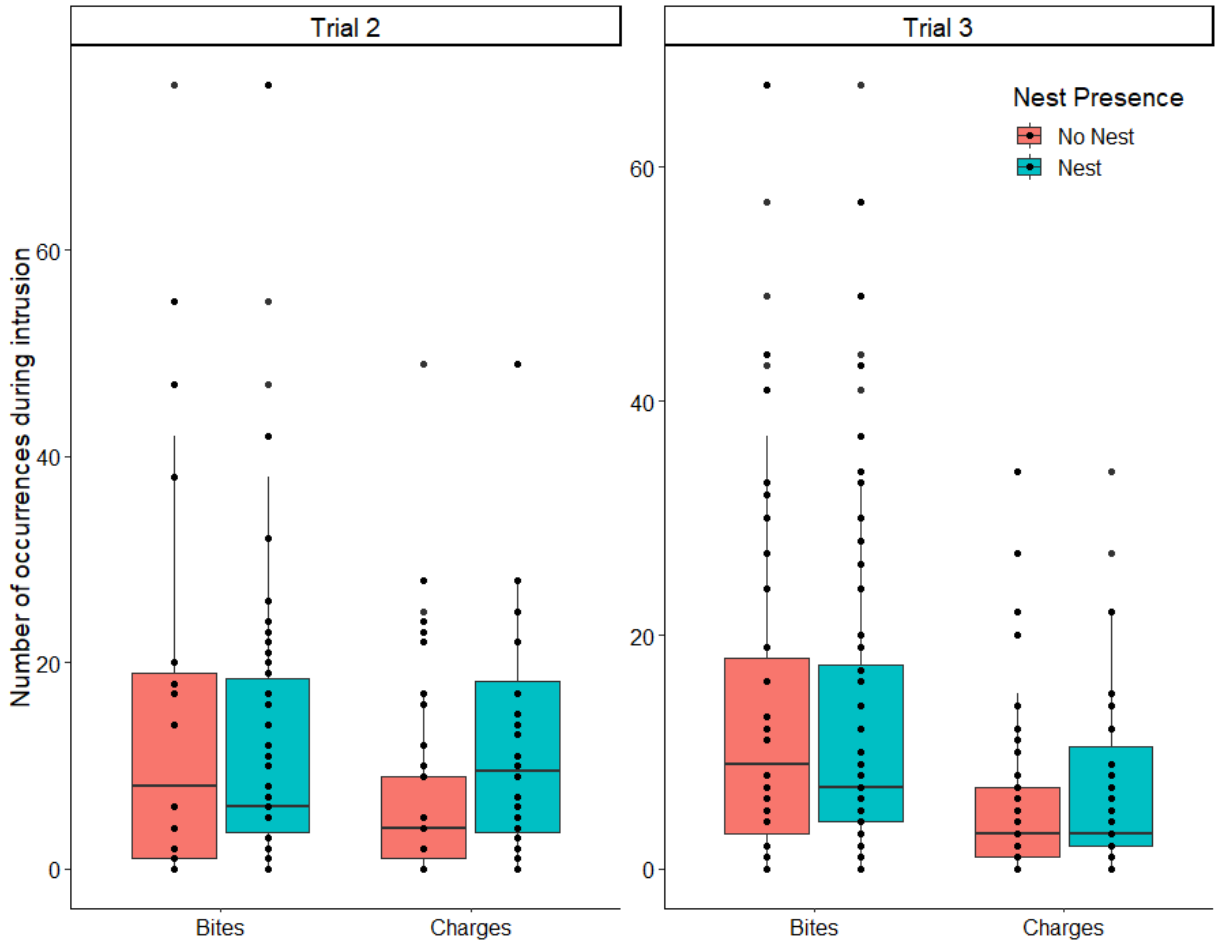

Figure 8 Males with (blue) or without (red) nests did not differ in behavioral measures of territorial aggression during trials in which nesting materials were available.

Aggressive behavior did not change after completing a nest

Completing a nest did not alter a fish's aggressive response to an intruder. Early nesters (N = 16) had no increase in aggression between trials 1 and 2 (Wilcoxon signed rank test: bites Z = -0.63, P_{adj} = 1, Figure 9a, charges: Z = -0.03, P_{adj} = 1, Figure 9b). Late nesters (N = 14) had no increase in aggression between trials 2 and 3 (Wilcoxon signed rank test: bites Z = -1.38, P_{adj} = 0.34, Figure 9a, charges Z = -0.40, P_{adj} = 1, Figure 9b). When considering all nesters (N=33), there was no increase in aggression between the trial prior to starting a nest and the trial after finishing a nest (Wilcoxon signed rank test: bites Z = -0.23, P_{adj} = 1, Figure 9c, charges Z = -0.39, P_{adj} = 1, Figure 9d).

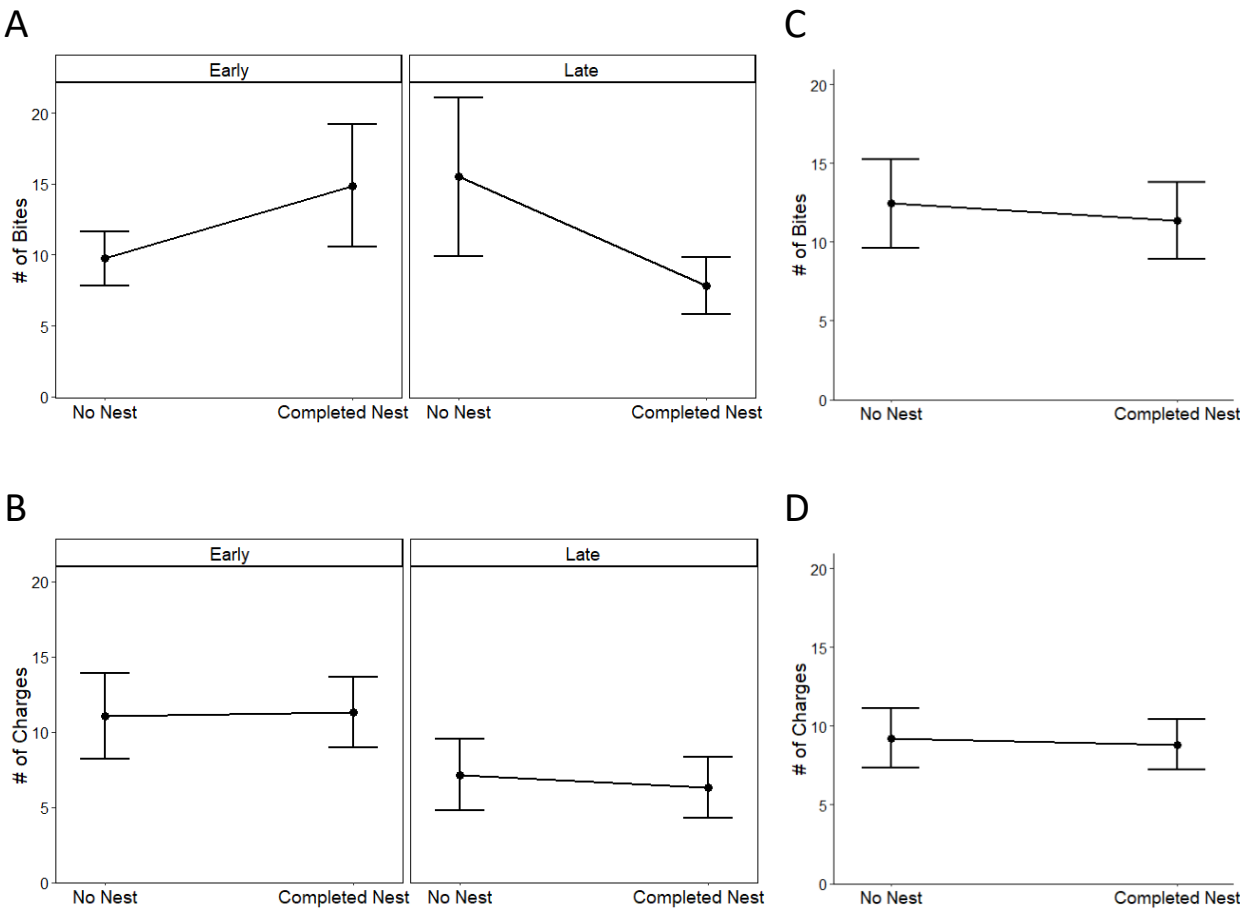

Figure 9 Aggressive behavior counts (mean ± se) versus nest presence among fish who completed a nest. Bites and charges are shown for the trials immediately before and after constructing a nest. There were no significant changes in bites (A) or charges (B) after completing the nest in either early or late nesters. Collectively, nesters (N = 35) did not increase territorial aggression (C & D) following nest completion.

No difference in territorial aggression between early and late nesters

The timing of nest building was not associated with any differences in aggression. Early nesters (N = 16) and late nesters (N = 14) did not differ in aggression (Wilcoxon signed rank test: bites Z = -0.23, P_{adj} = 0.82, charges Z = -1.25, P_{adj} = 0.42) during the trial immediately preceding nest completion, i.e. comparing early nesters in trial 1 versus late nesters in trial 2. Similarly, no difference in aggression was observed in the trial after finishing their nest

(Wilcoxon signed rank test: bites Z = -0.75, P_{adj} = 0.45, charges Z = -1.25, P_{adj} = 0.42). In other words, we found no difference in aggression between the early nester and late nester groups.

No difference in Resource Holding Potential (RHP) between nesters and non-nesters

There was no difference in size (standard length: Z = -0.36, P_{adj} = 0.94, mass: T_{82} = 1.02, P_{adj} = 0.94) between fish who eventually completed a nest (standard length: 43 mm ± 3, mass: 1.13 g ± 0.20) and those who did not (standard length: 43 mm ± 5, mass: 1.11 g ± 0.26). Resident males ranged from 10% smaller to 24% larger than intruders. Size differences between the resident and intruder fish were similar (T_{82} = -0.89, P_{adj} = 0.93) between nesters (7% ± 5%) and non-nesters (6% ± 5%). There was no correlation between size (length, mass, or size difference to intruder) and any measured behavior.

Trial effect for non-nesters only

Due to the correlation between the various measures of aggression (Figure 7) each was analyzed for change across trials separately via univariate non-parametric analysis of variance (Table 5, post hoc analysis: Supplemental Table 1). Only bites and charges changed significantly over time (Figure 10 & Supplemental Figure 1) and only in non-nesters. Bites increased a small but significant amount (all fish: $VDA_{1\text{-}3}$ = 0.34; non-nesters: $VDA_{1\text{-}3}$ = 0.34, Supp. Table 1) after trial 1, peaking at trial 3, but did not change significantly between trial 2 and 3. Charges also increased a small but significant amount (all fish: $VDA_{1\text{-}2}$ = 0.35; non-nesters: $VDA_{1\text{-}2}$ = 0.34, Supp. Table 1) after trial 1, peaking at trial 2, with no significant change between trial 2 and 3. The significant non-nester trial effect (Table 5) drives an overall trial effect, as there is no significant trial effect for nesters. There was no main effect of nesting outcome on bites (ATS: $F_{1.6}$ = 0.96, P = 0.37) or charges (ATS: $F_{1.7}$ = 0.90, P = 0.39), and no interaction of trial by nesting outcome on bites (ATS: $F_{2.0}$ = 0.46, P = 0.63) or charges (ATS: $F_{2.0}$ = 0.43, P = 0.65).

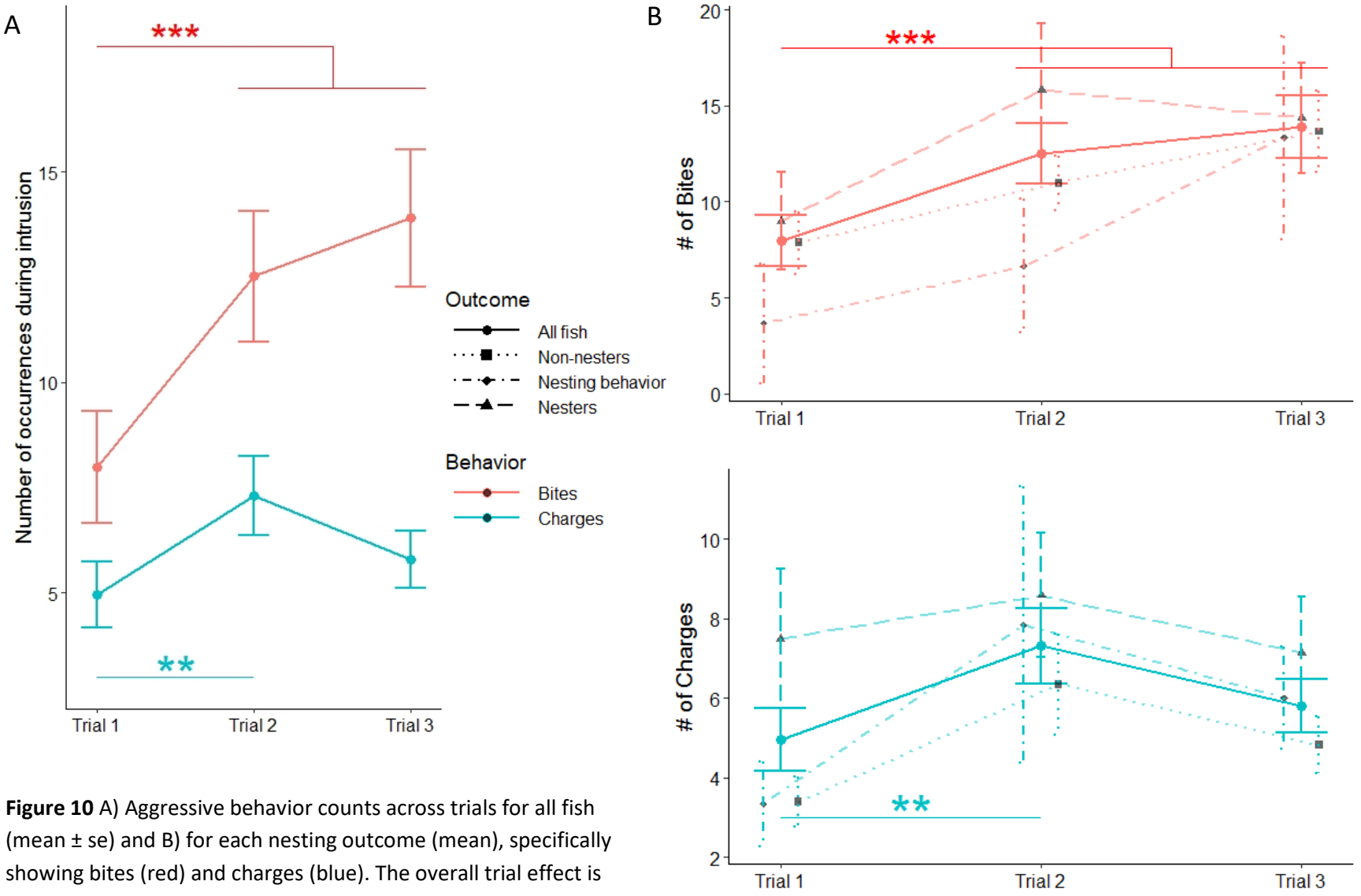

Figure 10 A) Aggressive behavior counts across trials for all fish (mean ± se) and B) for each nesting outcome (mean), specifically showing bites (red) and charges (blue). The overall trial effect is significant for non-nesters only, but the trend is similar for all outcomes. ** $P < 0.01$, *** $P < 0.001$.

Table 5 Trial effect (ANOVA-Type F-statistic main effect) for each behavior. P-value adjusted using Holm to correct for multiple tests.

Behavior	All Fish			Non-nesters			Nest Behavior			Nesters		
	F	P_{adj}	N	F	P_{adj}	N	F	P_{adj}	N	F	P_{adj}	N
Bites	15.1	3.00E-6	81	9.6	4.63E-4	44	2.5	0.45	6	3.7	0.14	31
Charges	7.6	0.003		6.5	0.009		1.3	1		1.0	1	
Approaches	0.9	1		0.6	1		2.2	0.58		0.9	1	
Time to Orient	2.7	0.39		1.9	0.78		2.9	0.34		2.0	0.74	
Time to 1st Bite	4.0	0.126	51	11.4	8.36E-5	30	3.0	0.42	2	0.04	1	19

Significant repeatability of behavior within individuals

Regardless of nesting outcome, all measured behaviors exhibited moderate and significant repeatability (Table 6). Charging had the highest repeatability (*ICC* = 0.56) of all measured behaviors.

Table 6 Behavioral repeatability

Response to a territorial intruder was significantly repeatable across all trials, demonstrating the reliability of the flask-confined intruder paradigm.

Behavior	All Fish			Non-nesters			Nesters		
	ICC	95% CI	P_{adj}	ICC	95% CI	P_{adj}	ICC	95% CI	P_{adj}
Bites	0.39	0.26,0.52	1.83E-09	0.45	0.27, 0.61	8.38E-07	0.36	0.15, 0.57	4.56E-04
Charges	0.56	0.44, 0.66	2.14E-18	0.45	0.28, 0.62	6.98E-07	0.63	0.46, 0.77	3.61E-10
Approaches	0.38	0.25, 0.51	3.46E-09	0.36	0.18, 0.54	5.81E-05	0.43	0.23, 0.63	3.67E-05
Time to Orient	0.34	0.21, 0.48	9.61E-08	0.25	0.07, 0.44	2.17E-03	0.55	0.36, 0.72	8.72E-08
Time to 1st Bite	0.20	0.08, 0.34	6.80E-04	0.37	0.19, 0.55	4.46E-05	0.24	0.04, 0.46	8.62E-03

Discussion

The presence of a nest did not increase any metric of aggression in territorial male sticklebacks. Given the robustness and repeatability of the various behaviors, especially bites and charges, we can be confident in our measurement of aggression. Biting and charging behaviors were consistent under all comparisons, both within-individual (pre-nest vs post-nest), and between groups (early vs late timing, nester vs non-nester outcome). Neither nest presence, timing of construction, nor outcome were associated with differences in territorial aggression. We therefore conclude aggression is neither predictive of nor altered by nesting in threespine stickleback fish.

Our results show that territory-holders did not attack more intensely after nesting, indicating no change in the subjective resource value of their territory. Though the state of the territory is objectively different following nest construction, whether the male takes into consideration the presence of the nest, i.e. whether it influences his behavior, is subjective. In this experiment we directly assayed the subjective value of a nest by measuring territorial aggression before and after nest building. During each measurement, we knew the objective state of the territory: we observed the environment and recorded the presence or absence of a nest. We found, against our hypothesis, that the subjective value to the male appears to remain unchanged, because the male was equally aggressive toward an intruder in both cases.

Previous work found nest removal did not alter biting frequency (Symons, 1966) in threespine sticklebacks, while Wootton (1970) did find a difference in bites and charges between nesters and non-nesters in stickleback fish. Addressing the tension between these results with a larger sample size and more direct analysis was a distinct benefit of our experiment. The Wootton study, in particular, did not enjoy modern ideals regarding publication of its design and analysis. In addition to its limited sample size, that study appears to have used parametric *t* testing, though bites and charges are considered non-parametric throughout the literature. The

observed difference between groups in that prior study may simply reflect unfortunate sampling; analyzing our data in the same fashion yields a 95% CI that contains Wootton's reported T. Finally, that study relied on a between-groups comparison; our experimental protocol, in contrast, additionally directly tests nesters within-group through a repeated measures design.

The increase in bites and charges across trials observed in this study may be explained by several potential factors. The most immediate of these is that the increase may represent a sensitization to intrusion. However, habituation leading to reduced aggression towards a specific territorial intruder is typical in the short term (H. V. Peeke, 1982; H. V Peeke, 1983; W. J. Rowland, 1982, 1988) and persists for 3 to 5 days (H. V. S. Peeke, Blankenship, & Figler, 1979). Alternatively, the increase may suggest that the 24 hour acclimation period following transfer to an empty tank was insufficient for males to become fully territorial. Residency time is well known to influence fight investment across taxa (dos Santos & Peixoto, 2017). The addition of nesting materials may have contributed to the increase in aggression observed during the second trial in particular. However, the significant repeatability in aggressive behaviors suggests that if it did, the effect was equivalent in both nesters and non-nesters. In any case, early nesters were not distinct from late nesters (Figure 9b), indicating that regardless of the increase in territorial aggression over time, there was no change in aggression from the nest's presence.

In consideration of these factors, we recommend a longer acclimation period in future designs. Typical acclimation times for juvenile fish have previously been reported at two days (Alison M. Bell, Backström, Huntingford, Pottinger, & Winberg, 2007; Niels J. Dingemanse et al., 2007) to three (Lacasse & Aubin-Horth, 2013). This timing is corroborated by *in situ* observations in which wild males reach sexual coloration within one to three days (W. J. Rowland, 2000). There is no doubt that nests are subjectively valuable, as previous studies (Theo C. M. Bakker, 1994; W. J. Rowland, 1994) show more vigorous conflict with closer

proximity to the nest. There was no ceiling on aggression in the course of this experiment, as bites and charges increased across trials (Figure 10). Resource holding potential was the same for both nesters and non-nesters and was not correlated with any measure of aggression. In combination, these suggest that the plot of land itself is the largest determining factor in territorial defense and that the addition of a mating-exclusive nest is inconsequential despite its objective value. These results are not only useful for understanding the ecological process of territory conflict, but also have important implications for game theory modeling (Hinsch & Komdeur, 2017).

These results dovetail with recent work showing the relative importance of subjective resource valuation to resource holding potential in other species (ciclids devalue multiple territories: O'Connor et al., 2015; gobies discount mating oppertunities: Svensson et al., 2012) and the current methods in trout (Johnsson & Näslund, 2018; Sundström, Lõhmus, & Johnsson, 2003), suggesting assessment of territorial aggression in the absence of a nest may be practicable for fish in general. To our knowledge, we additionally provide the first quantification of behavioral repeatability for the common flask-style intruder assay in sticklebacks using the established measures of aggression.

Finally, we were able to directly examine how subjective value of a mating territory was influenced by nests used exclusively used for breeding purposes. Since fitness is determined by successful reproduction, not merely the opportunity to mate, the expectation is that the subjective value of a territory would account for the progress towards mating contributed by the nest. However, since many species across taxa do not reuse nests even between reproductive attempts, a nest represents at most a transient increase in value. By using sticklebacks, we were able to examine the subjective value of the nest without the confound of shelter. Though building a nest is an investment into improving a territory as well as a necessary precondition to mating, it does not alter how aggressively the territory is defended.

Practically, this experiment shows that researchers examining territorial aggression in sticklebacks do not need to control for nest building. Accordingly, future studies can now disregard the nesting cycle to employ faster, simpler experimental designs compared to current stickleback methods (T. C. M. Bakker, Bruijn, & Sevenster, 1989; Alison M Bell, Bukhari, & Sanogo, 2016; Felicity Ann Huntingford, 1976; H. V. S. Peeke & Bell, 2012; H. V. S. Peeke et al., 1979; Rittschof et al., 2014; Van Den Assem & Van Der Molen, 1969). With the constraint of nest construction removed, future experiments will enjoy substantially reduced dropout rates, directly providing increased sample sizes and thus statistical power. Happily, animal welfare is also advanced by the use of such a "reduction alternative strategy," (Fenwick, Griffin, & Gauthier, 2009) as sufficient data to answer research questions in territorial aggression may now be obtained using fewer animals.

CHAPTER 3: NEUROSURGICAL INJECTION FOR EXAMINING MOLECULAR MECHANISMS OF BEHAVIOR IN STICKLEBACK

Complex behaviors have been repeatedly shown to be heritable (reviewed in Dochtermann et al., 2019), yet establishing a causal relationship between genes and social behavior remains challenging. Partially, this difficulty arises from limitations of the primarily correlative methods for examining the interplay between genes and behavior (Charney, 2017), such as QTL, GWAS, and RNAseq studies. These types of studies are certainly a necessary step generating many candidate genes. Pharmaceutical manipulation can be used to investigate the molecular effects that drive behavior. However, the isolation imposed by blood-brain barrier prevents many molecules from acting unless directly injected into a targeted region of the brain. To fully characterize how a gene contributes to behavior, it is necessary to consider not just sequence differences, but also regulatory and epigenetic influences. Therefore, to demonstrate and fully characterize a causal relationship between a gene and behavior, it is crucial to have a method for manipulating gene expression at a specific time and location. To enable direct manipulation of these candidate genes and thereby examine how they contribute to behavior, we developed a neurosurgical method to deliver either pharmacological agents or transgenic elements directly into the threespine stickleback (*Gasterosteus aculeatus*) brain.

Stickleback fish are an emerging model system with a fully sequenced genome and growing molecular toolkit. Already one of the best-studied animals for behavior (F. A. Huntingford & Ruiz-Gomez, 2009), sticklebacks are now are gaining popularity in other fields including evolution, physiology, and comparative genomics (Fang et al., 2018). they have been used in comparative cross-taxa studies looking for a conservation in the molecular underpinnings of social behavior (Rittschof et al., 2014; Saul et al., 2019) with both emerging and classic model systems. Sticklebacks enjoy well-established behavioral assays (W. J.

Rowland, 1982; van Iersel, 1953) that are amenable to automation (Ardekani, Greenwood, Peichel, & Tavaré, 2013; Norton & Gutiérrez, 2019).

There is a dearth of information, however, on surgical methodology in small (3-4 cm) fish, necessitating a refining of the anesthesia process (Neiffer & Stamper, 2009; Sladky & Clarke, 2016) and building a custom surgical rig before intercranial injection itself could be explored as a drug delivery technique To maximize animal welfare, we additionally needed to identify clear warning signs of failure to recuperate by establishing a normal recovery pattern in stickleback similar to the work in koi by Harms et al., (2005). To facilitate future behavioral work with this method, we examined methods to minimize post-surgical downtime such as supplemental oxygenation, and we verified behavioral recovery via a simulated territorial intrusion immediately after physiological recovery.

To confirm the efficacy of pharmaceutical manipulations via brain injections, we compared brain and systemic injection of the small hormone molecule, vasotocin. Vasotocin has been used in many teleosts and other vertebrates to alter behavior, both via intercranial and systemic administration (Goodson & Bass, 2001), and has a dosage based response (Gonçalves & Oliveira, 2011; Moore & Miller, 1983; Santangelo & Bass, 2006).Vasotocin can pass through the blood-brain barrier (Banks, Kastin, Horvath, & Michals, 1987; Yaeger et al., 2014). Therefore, unless brain trauma from intercranial injection rendered the technique unsuitable, we expected vasotocin to produce similar effects when administered either by intercranial or intraperitoneal (IP) injection.

Methods

Animals

Freshwater adult fish were collected in spring to summer from Putah Creek, CA in 2016, 2017, and 2018. In addition, F1 fish from crosses generated in 2015 and 2016 and reared in the

lab were also used. All fish were housed in the lab in 83 L (107x33x24 cm) group tanks with recirculated freshwater (5 ppm salt). The room was maintained at 18 °C on a 16:8 (L:D) "breeding" photoperiod from April to October and otherwise an 8:16 (L:D) "non-breeding" photoperiod. Fish were one to two years old at the time of their surgery. Individuals were weighed and measured (standard length from nose to caudal peduncle), and then moved to individual 9.5 L (32x21x19cm) tanks lined with gravel and containing a synthetic plant. All animal work was done in compliance with IACUC protocol (#15077 & 18080) at the University of Illinois at Urbana-Champaign.

Surgical Rig

In a ten-minute neurosurgical procedure, a suspension of foreign material (saline, HSV-1 w/ construct, or pharmacological agents) was administered to the telencephalon or anterior diencephalon of the brain via transcranial injection. For this procedure, we developed a custom-built surgical rig (Figure 11). To provide continuous oxygenation and anesthesia to the fish while out of water, a low pressure and flow rate cannula pump was necessary. No preassembled, standalone products offered satisfactory specifications. The complete parts list along with assembly instructions are publicly available through the Open Science Framework (https://osf.io/sgpvm).

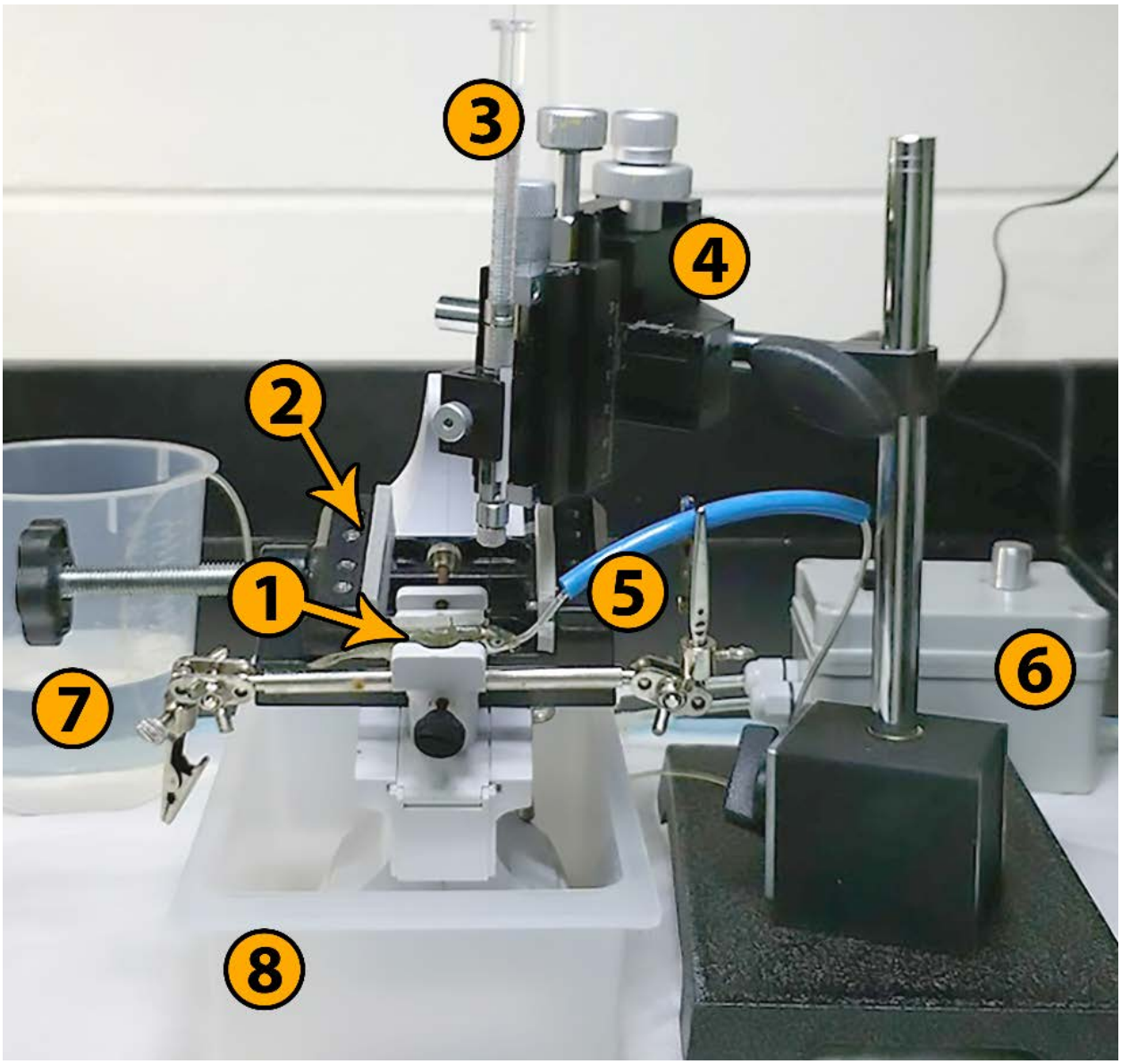

Figure 11 Custom-built surgical rig

1. Threespine stickleback in padded clamp
2. Alternative padded clamp for larger fish
3. Neuros syringe, 5 µL
4. Three-axis manipulator
5. Oral cannula and guide tube
6. Peristaltic cannula pump, 100 mL/min
7. Pump source reservoir
8. Drip tray

Anesthesia

Prior to anesthetization, a pre-surgical baseline breathing rate was taken by counting opercular beats per 20 seconds. Initial anesthetization was done in 0.02% buffered MS-222 (Tricane-S, Western Chemical) for no more than five minutes (188.4 sec ± 74.0), until movement ceased and the fish was unresponsive. A properly sedated fish had:

1. no tail movement
2. decreased but regular breathing (opercula beating)
3. came to rest on the bottom of the soaking container
4. did not respond to touch nor move when removed from the bath

Anesthetization time was not correlated with any physiological measure (Supplemental Figure 2). Fish were rinsed in freshwater (5ppm salt) to remove any residual anesthetic then moved to the surgical rig. In the rig, an oral cannula supplied constant water flow with 0.01% maintenance anesthetic over the gills for the duration of the surgical procedure (233s ± 79, mean ± SD). The speed of water delivery was adjusted to each fish to allow a steady low flow rate over the gills.

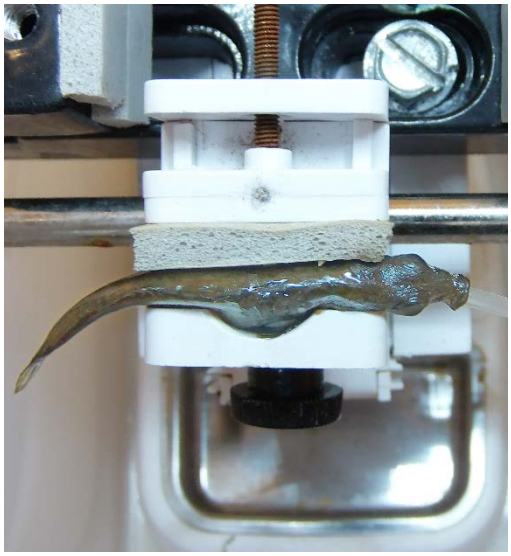

Figure 12 Surgeon's view of a properly clamped fish. Note the eyes are not clamped.

Neurosurgical Procedure

Fish were gently clamped into the surgical rig (Figure 12) behind the eyes, keeping the skull firmly in place. The stickleback brain is visualizable through the skull (Figure 13), allowing injection sites to be selected with moderately high precision. In each injection, the needle, either a 5 µL borosilicate syringe (Hamilton Neuros model 75, #65460-02) with a 33G (0.210 mm OD) needle or an insulin

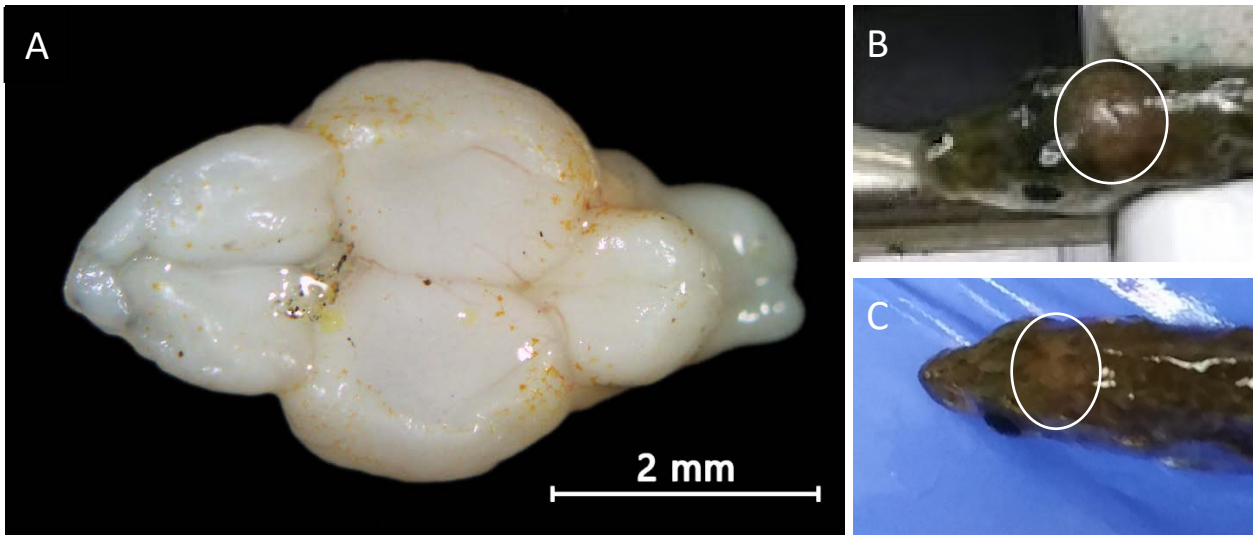

Figure 13 A) The brain of a stickleback with olfactory bulbs (anterior, far left), telencephalon (left), diencephalon and mesencephalon (center), cerebellum (right), and brain stem (posterior, far right). The telencephalon contains much of Social Behavioral Network (amygdala, hippocampus, etc). The preoptic area (POA), hypothalamus, and periaqueductal gray/central gray (PAG) are in the diencephalon. B & C) Example visualizations of the stickleback brain through the skull. Circled is a lighter area corresponding to the diencephalon.

syringe (BD 328431) with a 30G (0.337 mm OD), was inserted transcranially through the thinnest portion of the skull. The finer 33G needle had a hard stop set at 2.5mm. Each transcranial injection delivered ~300 nL of liquid at one or three depths to moderate the area transfected, with one depth being the most restrictive. Bilateral transcranial injections delivered a total of ~600 nL. Any notable accumulation of blood was associated with poor outcome.

Following the procedure, fish were returned to their individual tanks and monitored continuously until clear breathing (opercular movement) was seen, typically within 30 seconds. In the case of shallow breathing, forced movement of fresh water over the gills was used to promote survival by manually "swimming" the fish in a submerged figure eight using only forward motion. Breathing rate and the fish's position in the water column was recorded every 15 minutes for two hours following the injection. Additional checks were performed at three hours and one-day post-injection for all fish. Out of 183 total fish receiving brain injections, 19

did not survive this initial three-hour recovery period; nine did not survive anesthetization and ten were euthanized.

Pharmacological treatments

Exogenous [Arg8]-Vasotocin (Genscript RP10061) was administered either directly into the brain via injection using the neurosurgical protocol described above or systemically via intraperitoneal (IP) injection, already a well-established method in sticklebacks, using a 30G (0.312 mm OD) insulin needle. Because behavioral response has been reported to differ in teleosts based on dosage (Gonçalves & Oliveira, 2011; Santangelo & Bass, 2006), a dose-response curve (0.5, 5, and 10 μg per gram body weight) was tested. Manning compound (Bachem H-5350.0001), a potent V_1 receptor antagonist (anti-vasopressor) was administered systemically via IP injection at a dosage of 3 μg per gram body weight. Both pharmacological agents were freshly diluted on the day of injection from a pre-suspended concentrated stock solution such that all IP injections delivered 10 μL per gram body weight. Behavioral assays were performed 48 hours prior to pharmacological manipulation for baseline measurements, and then at 30 minutes after IP injection or 2 hours after brain injection. Preliminary saline injections showed that 30 minutes was sufficient for both physiological and behavioral recovery from the IP injection procedure.

Viral construct injections

Three promoters were piloted to drive gene expression in stickleback – a long-term promoter (hCMV, $N = 43$, Figure 14) resulting in fluorescent signal 2-5 weeks after injection, a short-term promoter (mCMV, $N = 10$) with expression between 4 and 7 days post-injection, and a retrograde promoter (hEF1a, $N = 7$) which did not result in a detectable fluorescent signal. Promoters were tested for their ability to drive a fluorescent protein (EGFP, EYFP, GCaMP6f, or

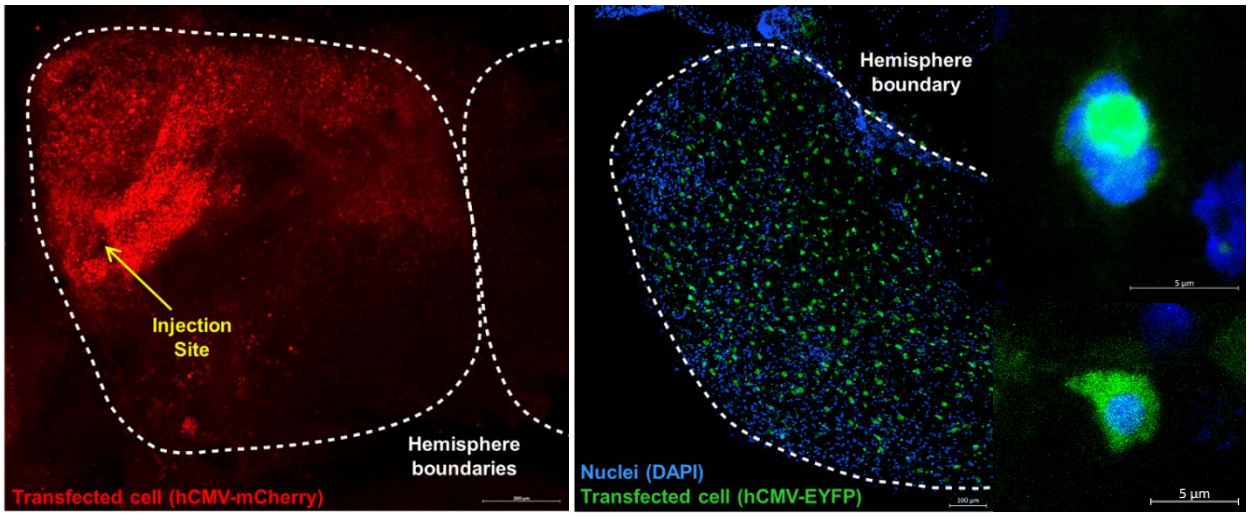

Figure 14 Transfection results visualized
A) Single injection resulting in local expression, limited to a portion of one hemisphere of the telencephalon. B) Broad expression throughout left hemisphere of the diencephalon, typical for injections with delivery at multiple depths. C & D) Successful transfection of entire cells by the long term hCMV-EYFP construct in the lateral left diencephalon three weeks after injection.

mCherry). The long-term promoter (hCMV) was selected as the most useful due to its longer window of effect and was used in all experimental gene transfection trials.

Mammalian cDNA ORF clones were used for *AVP* (human, HG17671-UT, NCBI Ref Seq: NM_000490.4, Sino Biological) and *MAOA* (mouse, MG57436-U, NCBI Ref Seq: NM_173740.3, Sino Biological). These were cloned into the pDONR221 backbone (Epoch Life Science) and then packaged (Gene Delivery Technology Core, Massachusetts General Hospital) with an IRES-GFP backbone in replication deficient Herpes Simplex 1 (HSV-1). Stock hCMV-EYFP (RN12) was used for control injections. All males were randomly assigned to one of the three constructs. The final viral solutions were used undiluted except for the addition of a trace amount of pigment (brilliant blue FCF or tartrazine, i.e. FD&C Blue No. 1 and Yellow No. 5) to allow the solution to be visualized against the gradations of the syringe. These constructs are episomally expressed; the payload genes, packaged as a plasmid, remain in the cytoplasm and neither integrate into nor replicate with the genome.

Behavioral assays during recovery

Breathing rate was determined prior to the territorial challenge by averaging two separate non-continuous counts of opercular beats per 20 seconds taken within a 5 minute period. This ensured that individual variations due to stress from the researcher's arrival were minimized. Territorial aggression was measured by recording the individual's response to an intruder confined to a glass flask. The times to orient toward and to first bite at the intruder (TTO and TTB, respectively) were recorded, as well as the total number of bites, charges (lunges), and trips (approaches) during the five minutes following initial orientation. Intruders (N = 9) were 5-10% smaller conspecific males.

R analysis and data availability

Descriptive statistics are presented as mean ± standard deviation. All data analysis was carried out in RStudio (v1.1.383) with R version 3.5.1. All scripts and data are publicly available on the Open Science Framework (https://osf.io/v56zt) as "Neurosurgical Protocol scripts.R". Survival rate differences for the neurosurgical optimization were calculated using the chi squared function with continuity correction. Survival rate differences for the neurosurgical optimization were calculated using the chi-squared function with continuity correction. A nonparametric-compatible repeated-measures ANOVA was done via the `MANOVA.RM` (v0.3.2) package with the ANOVA type statistic (ATS) reported because the assumption of sphericity could not be met for breathing rate over time (Mauchly tests for sphericity = 0.02, *p-value* =6.02e-81). We report the ANOVA-Type Statistic (ATS) and the adjusted degrees of freedom, the latter of which are based on the number of treatment levels, number of observations, and the variance of ranks in each treatment (Shah & Madden, 2004). For interaction effects, we report the recommended $F_{(\hat{f},\infty)}$ instead of $F_{(\hat{f},\hat{f}_0)}$ (Noguchi et al., 2012). Post-hoc calculation by time point was done via Wilcoxon rank sum test with continuity correction and the `rcompanion`

(v2.2.1) `wilcoxonR` function. P-values were then adjusted for false discovery rate (fdr method). Spearman correlations were calculated using `Hmisc` (v4.1-1). Wilcoxon tests were done with the base `stats` package and effect size was calculated with the `rcompanion` package (v2.2.1).

Results

Direct transcranial injection with an unbeveled ultrafine needle proved simpler and more effective than other piloted techniques, including craniotomy. Fish generally returned to normal swimming and water column use within 15 minutes after removal of anesthesia. Initial piloting (N = 62) revealed breathing rate followed a typical pattern after the operation which we called a recovery curve (Figure 15). Breathing rate peaked about 30 minutes post-surgery and returned to baseline levels by two hours after the neurosurgical procedure. There was no

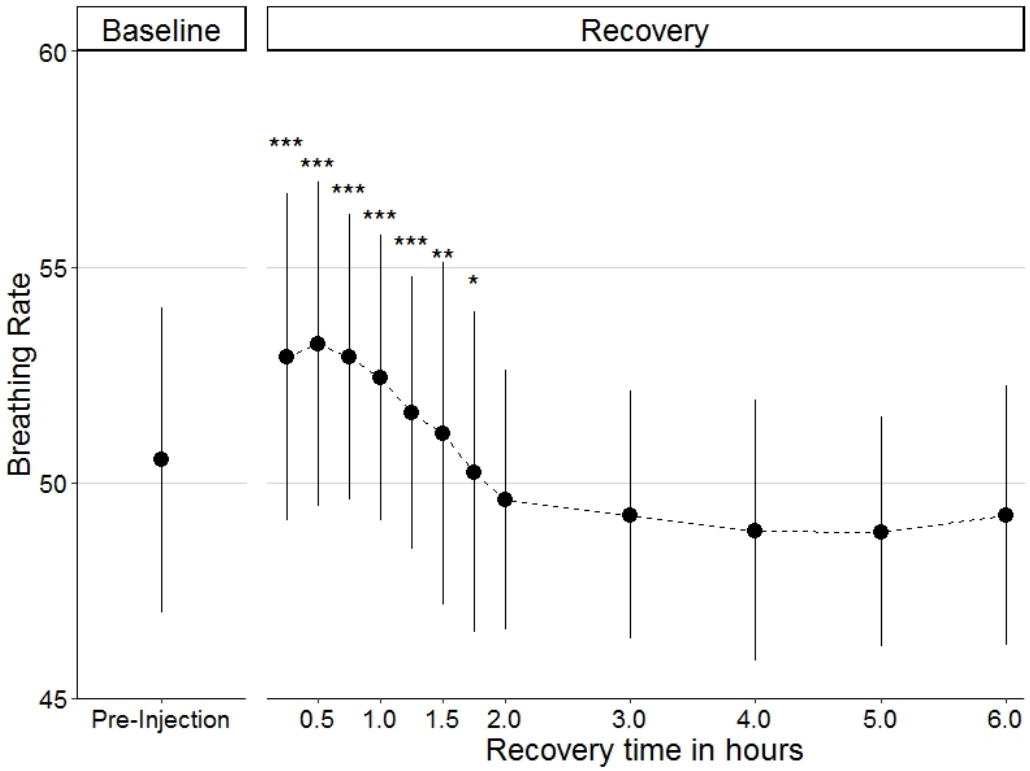

Figure 15 Average recovery curve of breathing rate (opercular beats per 20 seconds) following transcranial brain injections (N = 62). Breathing rate returned to baseline levels by two hours post-injection and remained stable following the surgery. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

difference in territorial aggression two hours after surgery in saline injected controls, compared to the day before surgery in any aggressive behavior (bites: Z = -0.54, *p-value* = 0.29; charges: Z = -0.05, *p-value* = 0.48; time to 1st bite: Z = -0.35, *p-value* = 0.36). Full mating behavior occurred within 3 days for males, determined by nesting behavior and 9 days for females, determined by the presence of eggs.

Needle size

Needle diameter influenced survival rate (χ^2 (1, N_{30G} = 43, N_{33G} = 183) = 23.9, *p-value* = 1.02e-6) with a 54% survival rate with the larger 30G needle compared to the 87% survival rate with the finer needle. Additionally, the finer 33G needle resulted in consistently lower breathing rates ($ATS_{1, 2613}$ = 6.54, *p-value* = 0.01, Figure 16) during the six-hour window following the neurosurgery than the larger diameter 30G needle. However, the effect was small ($r \leq 0.29$,

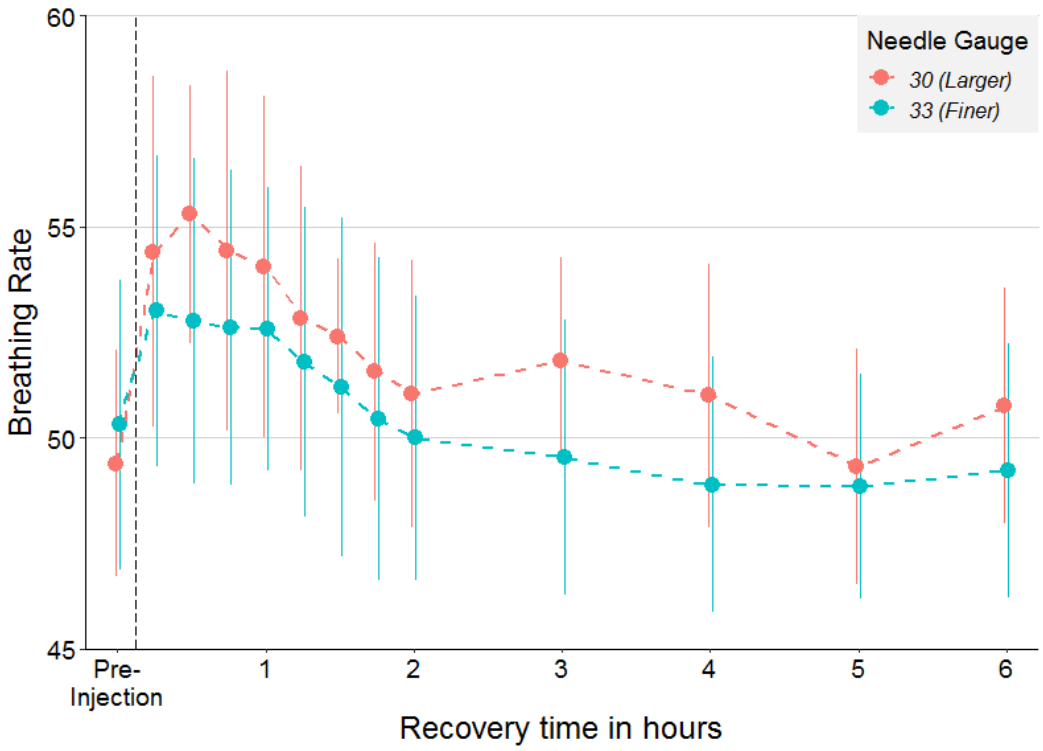

Figure 16 Breathing rate (opercular beats per 20 seconds) following injection with either a 30G or 33G needle for the first six hours following the neurosurgical procedure. The large needle (N = 23) resulted in consistently increased breathing rate compared to the finer needle (N = 62).

Supplemental Table 2a) and non-significant at each time point. There was not a significant time by treatment (needle size) effect ($ATS_{1,\infty} = 1.36$, *p-value* = 0.23). This suggests that larger 30G needle was more traumatic but did not alter the recovery trajectory – i.e. fish injected with the larger 30G needle had an upshifted but similar recovery curve. The finer 33G needle resulted in a return to pre-injection breathing rates by 1.5 hours, while fish injected using the larger 30G needle took at least two hours to recover (Supplemental Table 2b).

Supplemental oxygenation

Supplemental oxygenation for up to two days following surgery did not improve survival (χ^2 (1, $N_{\text{Extra O2}} = 94$, $N_{\text{Normal}} = 89$) = 0.02, *p-value* = 0.89) nor recovery ($ATS_{1,7465} = 0.94$, *p-value* = 0.33, Figure 17).

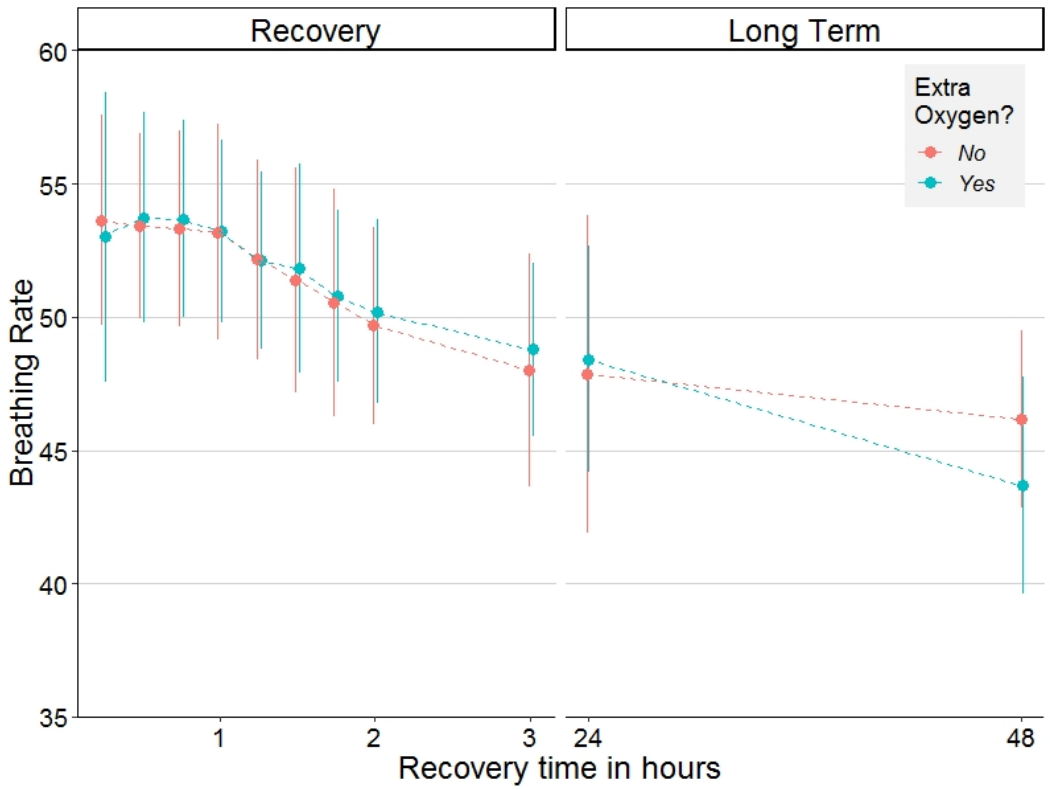

Figure 17 Breathing rate (opercular beats per 20 seconds) following injection either with (N = 83) or without (N = 77) supplemental oxygenation for one day following the neurosurgical procedure. Supplemental oxygenation did not alter recovery rates.

Multiple transcranial injections

Broad expression required multiple transcranial injections – one into each hemisphere of the brain. Bilateral transcranial injections did not alter survival rates (χ^2 (1, $N_{\text{Unilateral}}$ = 64, $N_{\text{Bilateral}}$ = 119) = 0.46, *p-value* = 0.50) compared to a unilateral injection. Overall, breathing rate during recovery was higher in fish with bilateral injections ($ATS_{1,\,4874}$ = 11.61, *p-value* = 0.001) in the three hours following the surgery. However, this comparison was largely confounded by year of capture, with the fish caught in 2018 all receiving two transcranial injections and showing poor health in general. When analyzing only years (2017 & 2019) that received both unilateral and bilateral injections, allowing for direct comparison, there is no difference in breathing rate during recovery between unilateral and bilateral injections ($ATS_{1,\,312}$ = 0.92, *p-*

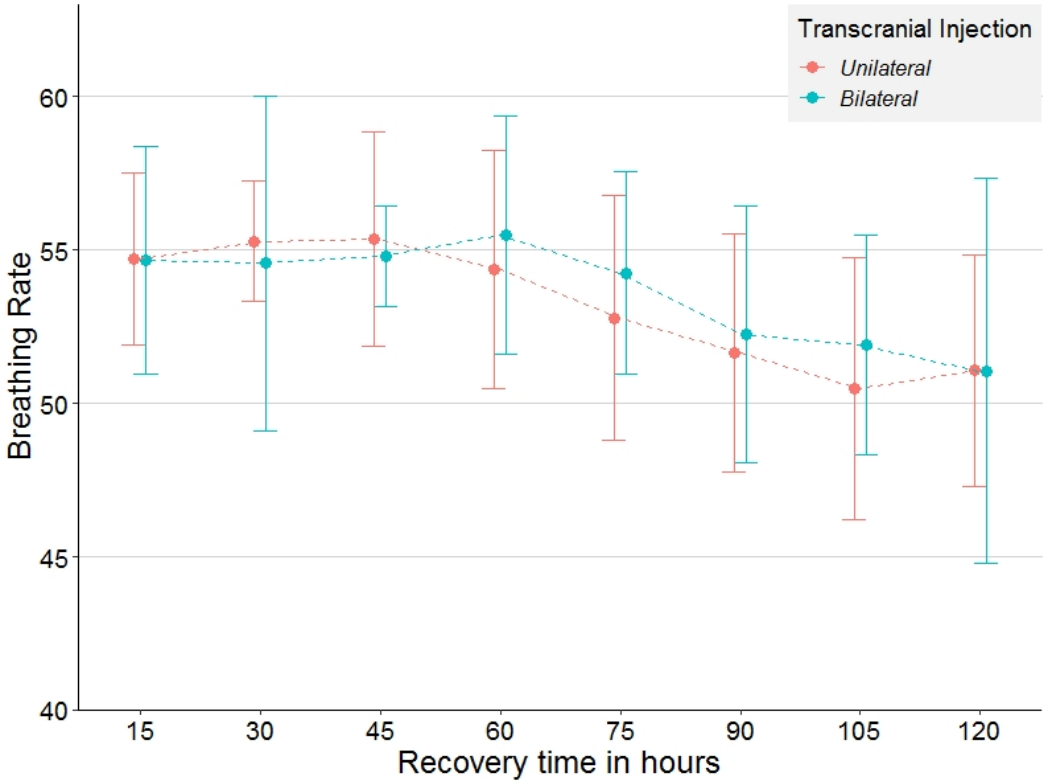

Figure 18 No difference in breathing rate base on number of transcranial injections – one (unilateral) or two (bilateral).

value = 0.34, Figure 18). This suggests that the population differences in recovery overwhelmed any effect that multiple injections might be having.

Injection material

Fish had comparable survival rates regardless of the injected materials (χ^2 (3, $N_{HSV\text{-}1}$ = 113, N_{Pharma} = 22, N_{Saline} = 48) = 2.30, *p-value* = 0.32). After the surgical technique was refined, the procedure's survival rate was approximately 90%. In total, 113 fish were injected with one of three constructs utilizing replication deficient herpes-simplex 1 for transfection; 101 survived. The fish injected with pharmaceutical agents fared similarly, with 20 of 22 fish surviving. Control fish injected with saline naturally fared least well as they were used to initially pilot and

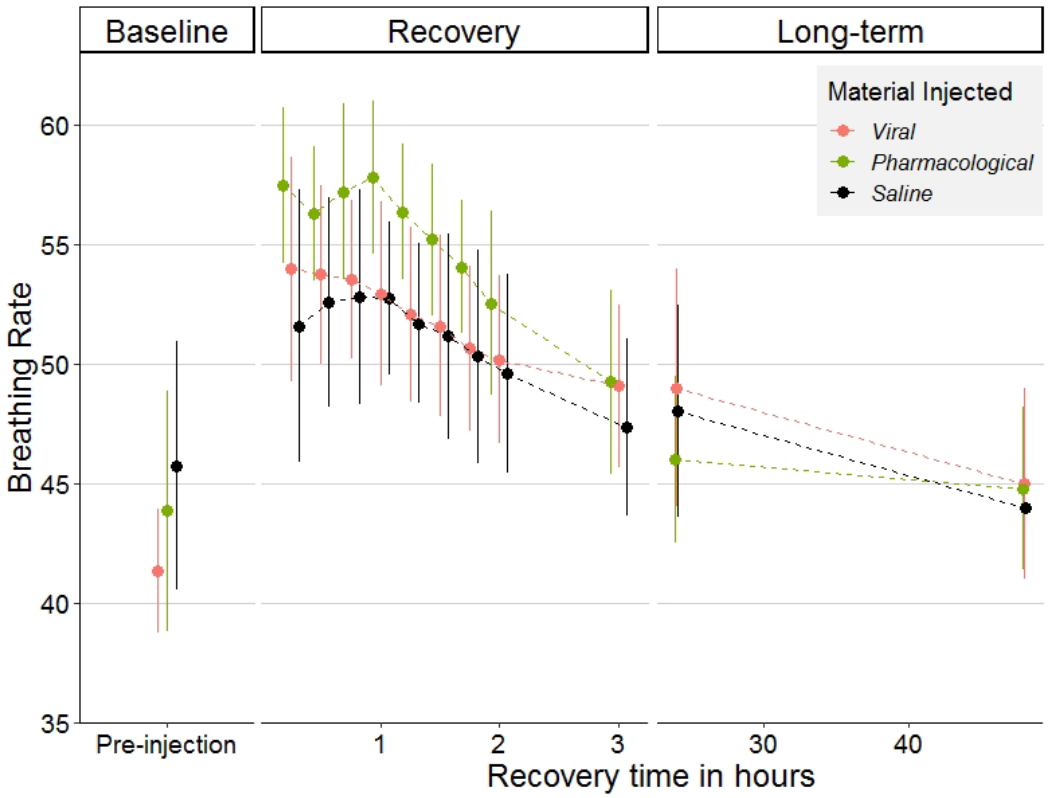

Figure 19 Differences in breathing rate following injection between the various injected materials. There was no difference between saline injected controls and those injected with the herpes virus containing a construct. However, the fast-acting pharmacological agent (vasotocin) did alter the recovery curve, but not survival rate.

refine the surgical technique; they counted 39 survivors among 48 fish, an 81% survival rate. However, there was a time by injected material effect ($ATS_{7.5,\infty} = 9.43$, *p-value* < 0.001, Figure 19) driven entirely by the short-term effects of the pharmaceuticals (Supplemental Table 3).

Viral payload

The time for recovery of fish injected with a replication deficient herpes-simplex 1 (HSV-1) did not differ from that of saline injected controls ($ATS_{3.7,\infty} = 1.83$, *p-value* = 0.12, Supplemental Table 3). Three promoters were piloted to drive gene expression based on work in zebrafish (Zou, De Koninck, Neve, & Friedrich, 2014) – a long-term promoter (hCMV, $N = 43$, Figure 14) resulting in fluorescent signal 2-5 weeks after injection, a short-term promoter (mCMV, $N = 10$) with expression between 4 and 7 days post-injection, and a retrograde promoter (hEF1a, $N = 7$) which did not result in a detectable fluorescent signal. The choice of promoter did not alter survival rate (χ^2 (2) = 1.38, *p-value* = 0.50, Table 7a), nor was there a main effect of promoter ($ATS_{2,143} = 0.33$, *p-value* = 0.70) on the recovery curve. The long-term promoter (hCMV) was selected as the most useful due to its longer window of effect and was thus used in all subsequent viral-mediated transfections. Finally, the specific gene being expressed had no effect (χ^2 (3) = 2.16, p = 0.54, Table 7b) on survival rates relative to saline injected controls. The recovery rate was also unaffected by the gene expressed ($ATS_{2,267} = 1.19$, *p-value* = 0.31, Supplemental Figure 3).

Table 7 A) Survival rate was not significantly affected by the choice of promoter. B) No significant difference in the survival rate for any expressed genes relative to control fish injected with saline.

A

	Survival %	N	Died	Survived
hCMV	86.4	59	8	51
mCMV	100	3	0	3
hEF1a	100	6	0	6

B

	Survival %	N	Died	Survived
AVP	90.9	22	2	20
MAO	91.3	23	2	21
Fluorescent	88.2	68	8	60
Saline control	81.2	48	9	39

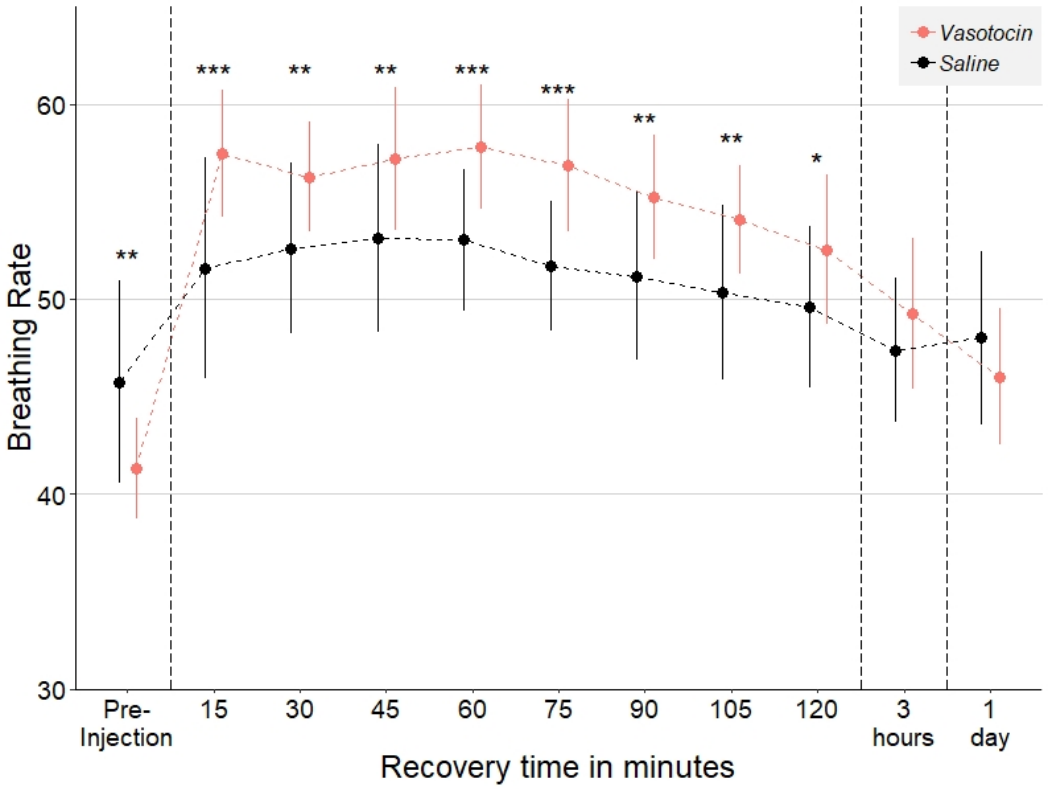

Figure 20 Differences in breathing rate following injection between brain injection of exogenous vasotocin (N = 15) and saline injected controls (N = 39). Fish injected with vasotocin had an elevated breathing rate compared to saline injected controls for more than two hours following injection. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Vasotocin pharmacological treatment

Exogenous vasotocin injection into the brain increased breathing rate rapidly relative to saline injected controls ($ATS_{1, 599} = 8.74$, *p-value* = 0.003, Figure 20). This effect began within 15 minutes and persisted for more than two hours post-injection. The most pronounced difference in breathing rate was between 0.75- to 1.5-hour post-injection (Supplemental Table 3). Intraperitoneal injection of exogenous vasotocin also resulted in a rapid increase in breathing rate (Figure 21). Behaviorally, brain and IP injections of exogenous vasotocin produced parallel results (Supplemental Table 4), in which only the highest dosage (10 µg per gram body weight) altered the number of charges directed at the intruder.

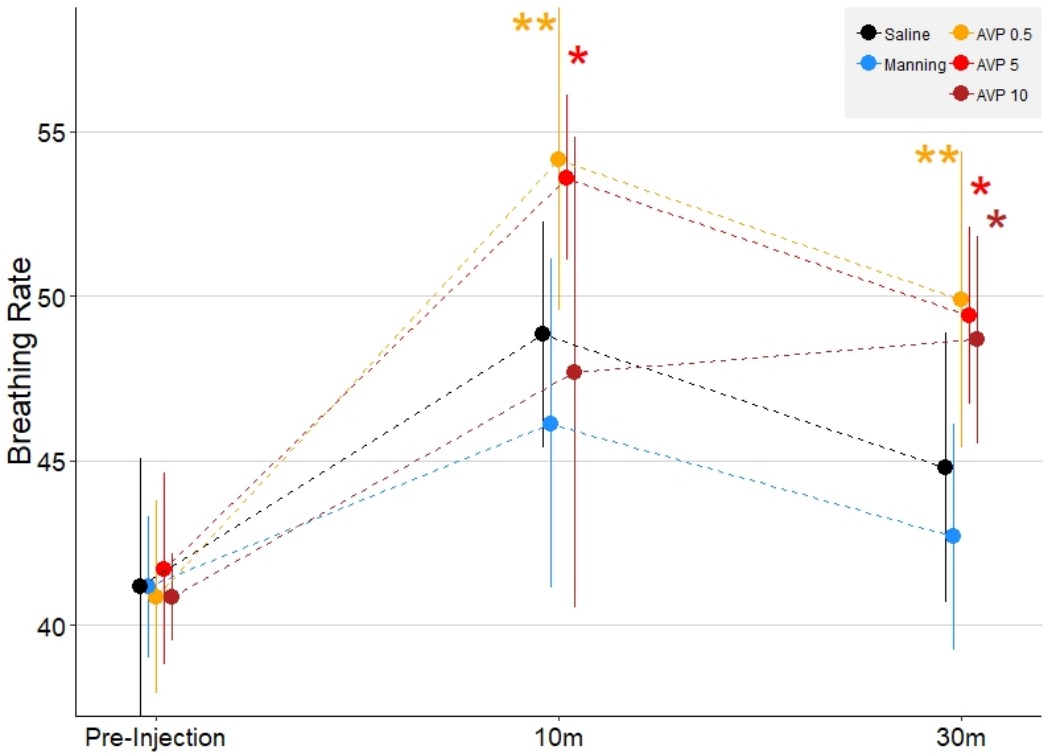

Figure 21 Differences in breathing rate following IP injection of different pharmaceutical agents. Fish injected with vasotocin had breathing rates elevated compared to saline injected controls, paralleling the pattern seen during recovery of brain injection. * $p \leq 0.05$; ** $p \leq 0.01$

Discussion

We present a new method for direct injection of transgenic or pharmaceutical material into the brains of the small teleost fish threespine stickleback. Developing a minimally invasive neurosurgical protocol required 1) refining the anesthesia process, 2) building a custom surgical rig, and 3) determining the normal recovery pattern allowing us to clearly identify warning signs of failure to thrive. Our surgical rig and optimized anesthetization methods (Neiffer & Stamper, 2009; Sladky & Clarke, 2016) resulted in high (90%) survival rates and quick behavioral recovery. Mating behavior also recovered promptly: males completed nests at three days post-surgery, and females were gravid at nine days – suggesting almost no delay in the egg

development time (Baker, Heins, & Susan, 2008) after losing any ripe eggs to clamping during surgery.

Establishing a typical recovery curve (Figure 15) allowed us to identified post-surgical warning signs of failure to thrive. Behavioral manifestations of discomfort or problems included listing (>45º off central axis), assuming a nose-up position, and loss of positional control (twirling). The presence of any of these markers for greater than an hour suggested a poor prognosis and thus we recommend euthanasia. Survival to 24 hours indicated a successful procedure, as 23 out of 24 fish injected with the larger 30G needle and 159 of 160 fish injected with the smaller 33G needle survived to 1 week. Thus, this minimally invasive neurosurgical method is quite reliable.

Exogenous vasotocin administered directly to the brain produced physiological and behavioral responses mirrored in fish receiving vasotocin through IP injections (Supplemental Table 4). These pharmacological results were similar to those seen in other fish (Filby, Paull, Hickmore, & Tyler, 2010; Lema & Nevitt, 2004; Santangelo & Bass, 2006). This indicates that brain injection is now a feasible delivery route for drugs that do not pass through the blood brain barrier (Cook, Mieure, Owen, Pesaturo, & Hatton, 2009).

Viral-mediated transgenesis is a method to alter a gene's expression in a specific location or during a controlled timeframe. This approach has already proved essential in the functional testing of genes related to behavior in rodents (Simonato, Manservigi, Marconi, & Glorioso, 2000) and in the dissection of neural circuits (Luo, Callaway, & Svoboda, 2008). In addition to the experimental uses demonstrated here, viral-mediated transgenesis can also be used to knockdown gene expression using CRISPR or shRNA in the same backbone (Anesti, Peeters, Royaux, & Coffin, 2008). We successfully used multiple promoters to drive expression, allowing tailored expression profiles through time. Additionally, while we used ubiquitous promoters with differing timings, cell-specific targeting can be done by using an alternate promoter (see

Ingusci et al., 2019). Our use of HSV-1 enables larger payloads than adeno-associated viruses (AAVs), making this protocol the first step toward using chemogenetics such as DREDDs (designer receptors exclusively activated by designer drugs, reviewed in Roth, 2016) and optogenetics in sticklebacks.

While sticklebacks are a non-traditional genetic model system, they are one of the best studied behavioral systems, with well described intra-specific variation in aggression, antipredator behavior, and parental care (Fang et al., 2018; Hendry, Peichel, Matthews, Boughman, & Nosil, 2013; F. A. Huntingford & Ruiz-Gomez, 2009). Previous studies have identified hundreds of genes that are differentially expressed in the brain in response to a social interaction (Alison M Bell et al., 2016; Syed Abbas Bukhari et al., 2017; Greenwood & Peichel, 2015; Greenwood et al., 2013; Laine, Primmer, Herczeg, Merilä, & Shikano, 2012; Mommer & Bell, 2014; Sanogo et al., 2011). However, most of these studies are correlative, and thus the direction of the causal relationship – much less the mechanisms by which changes in gene expression underlie behavior – are still not clear. This method will allow us to rigorously test how these genes contribute to future behaviors on every level, from detailed mechanistic protein analyses to broad whole-organism phenotypic studies.

CHAPTER 4: VIRAL-MEDIATED TRANSGENESIS OF AVP & MAOA INCREASES TERRITORIAL AGGRESSION IN STICKLEBACK

Establishing a causal relationship between genes and social behavior is challenging, despite the plethora of candidate genes already identified by various correlative methods (Alison M Bell et al., 2016; Syed Abbas Bukhari et al., 2017; Greenwood & Peichel, 2015; Greenwood et al., 2013; Laine et al., 2012; Mommer & Bell, 2014; Sanogo et al., 2011). To examine the molecular mechanisms underlying complex behavior it is necessary to have a method for manipulating gene expression at a specific time and location as laid out in chapter 3. We developed one such method, viral-mediated transgenesis, for the classic ethological system of threespine stickleback (*Gasterosteus aculeatus*).

As previously discussed, stickleback fish are an emerging model system with a growing molecular toolkit. In addition to having a fully sequenced genome, they have been used in comparative cross-taxa studies looking for a conservation in the molecular underpinnings of social behavior (Rittschof et al., 2014; Saul et al., 2019) as well as in the evolution of behavior (Di Poi, Bélanger, Amyot, Rogers, & Aubin-Horth, 2016; N J Dingemanse, Barber, Wright, & Brommer, 2012; Fang et al., 2018; F. A. Huntingford & Ruiz-Gomez, 2009). Indeed, there are already hundreds of previously identified candidate genes for social behavior waiting to be characterized (Alison M Bell et al., 2016; Syed Abbas Bukhari et al., 2017; Greenwood & Peichel, 2015; Greenwood et al., 2013; Laine et al., 2012; Mommer & Bell, 2014; Sanogo et al., 2011).

As the first test of this method in this species, we chose to focus on territorial aggression for three reasons: 1) it is easy to score, 2) aggression is important for fitness, 3) there are good candidate genes for aggression based on studies in other vertebrates (Sanogo et al., 2012; Saul et al., 2019; Takahashi & Miczek, 2014). Here we employ the neurosurgical method developed in

Chapter 3 to test the function of two conserved candidate genes related to aggression in stickleback. With a repeated measures, within-subjects design, we show that this method can be used to induce and detect changes in behavior with reasonable samples sizes even with outbred animals from a natural population. Thus, it is now possible to examine changes in gene expression as a mechanism underlying behavioral plasticity in this system.

Aggression is a well-studied, complex behavior with important social and fitness repercussions (Freudenberg, Carreño Gutierrez, Post, Reif, & Norton, 2016; Malki et al., 2016; Takahashi & Miczek, 2014; Waltes, Chiocchetti, & Freitag, 2016). Many subprocesses including perception, motivation, and cognition (L. A. O'Connell & Hofmann, 2011; O'Connor et al., 2015; Reichert & Quinn, 2017) must function together to determine when and how aggressively an individual should behave. The integration of these processes occur within the Social Behavioral Network (SBN) of the brain (L. a. O'Connell & Hofmann, 2011), which has good functional homology across vertebrate taxa. Finally, aggression is experimentally tractable in stickleback fish as it is heritable (Theo C.M. Bakker, 1994; A M Bell, 2005), repeatable (Wootton, 1971), and quick to measure. We selected arginine-vasopressin and monoamine oxidase as candidate genes in this study.

Arginine-vasopressin (*AVP*) and its nonmammalian homolog arginine-vasotocin (*AVT*) are highly conserved (Moore, 1992) and pleiotropic (Balment, Lu, Weybourne, & Warne, 2006). Vasopressin and vasotocin are distinguished by only a single amino acid change between mammals (human) and teleosts (sticklebacks), and their respective V_{1a} receptors have similar specificity, signaling mechanisms, and amino acid sequences (Goodson & Bass, 2001). Both vasopressin and vasotocin were found to have similar physiological effects in rats (Feuerstein, Zerbe, & Faden, 1984). Additionally, vasotocin signaling has been shown to influence aggression in various contexts in both fish and mammals (reviewed in Goodson, 2013) and has been characterized throughout the SBN (Albers, 2015). In fact, nonapeptide hormones

(vasopressin/vasotocin, isotocin/mesotocin, and oxytocin) in all taxa interact with sex steroids to influence behavior (Goodson & Bass, 2001; Stoop, 2012), making them quintessential behavioral candidate genes.

In sticklebacks, vasotocin peaks during the start of the breeding season in both males and females (Gozdowska, Kleszczyńska, Sokołowska, & Kulczykowska, 2006). Nesting male sticklebacks have an increase in vasotocin levels in their brains following a mirror (aggression) challenge (Kleszczyńska, Sokołowska, & Kulczykowska, 2012). The arginine-vasopressin-like (*avpl*) gene showed the greatest overexpression in dominant verses subordinate zebrafish (Filby et al., 2010), further supporting its role in aggressive behavior in teleosts. Vasotocin in adult teleosts is mainly located in the preoptic area (POA) of the hypothalamus (Albers, 2015; Huffman et al., 2012; Kagawa et al., 2016), where it is an active regulator in the hypothalamic-pituitary-adrenal (HPA) axis (Arnett, Muglia, Laryea, & Muglia, 2016). Therefore, we hypothesized that supplemental expression of arginine vasopressin (*AVP*) within the socio-behavioral network of the stickleback brain would increase aggression because vasotocin regulates the HPA axis through adrenocorticotropic hormone (ACTH) signaling to gonadal hormones.

Monoamine oxidase, our other candidate gene, has a longstanding association with aggression (Godar, Fite, McFarlin, & Bortolato, 2016), not only in model systems but also in humans (Brunner, Nelen, Breakefiels, Ropers, & van Oost, 1993). In humans, *MAOA* levels are inversely correlated with aggression (Alia-Klein et al., 2008). Further, low *MAOA* activity is associated with increased aggressive response following provocation (Gilad, Rosenberg, Przeworski, Lancet, & Skorecki, 2002), antisocial outcomes (Ouellet-Morin et al., 2016), and sex-related aggressive crimes in the case of complete deficiency (Brunner et al., 1993). Mice with no *MAOA* activity showed increased fearfulness as juveniles and increased aggression in adult males (Cases et al., 1995). Recent work has suggested that *MAOA* allelic variants may be related

to the domestication of dogs, a process which included a marked decrease in aggression (Sacco, Ruplin, Skonieczny, & Ohman, 2017). Teleosts have only one monoamine oxidase gene (*MAO*) as opposed to the two found in mammals (*MAOA* and *MAOB*). Stickleback *MAO* (ENSGACT00000012444.1) and mouse *MAOA* (NP_776101.3) have 68% conservation at the protein level. Despite the low level of conservation, the teleost monoamine oxidase gene has been shown to influence aggression (Freudenberg et al., 2016; Malki et al., 2016; Quadros, Costa, Canzian, Nogueira, & Rosemberg, 2018) and is functionally comparable (Arslan & Edmondson, 2010; Shih, Chen, & Ridd, 1999). Since a low level of monoamine oxidase is associated with increased aggression, increased expression of *MAOA* was expected to decrease aggression through serotonin turnover.

Methods

Animals

Freshwater adult fish were collected from Putah Creek, CA and housed in the lab in 83 L (107x33x24 cm) group tanks with recirculated freshwater (5 ppm salt). The room was maintained at 18 ℃ on a 16:8 (L:D) "breeding" photoperiod. Males were identified by nuptial coloration (secondary sexual characteristics) and by sexing via PCR (Peichel et al., 2004). They were weighed and measured (standard length from nose to caudal peduncle), and then moved to individual, visually-isolated 9.5 L (32x21x19 cm) tanks lined with gravel and containing a synthetic plant. Each individual was allowed to acclimate, undisturbed for three days prior to any behavioral measurements. All animal work was done in compliance with IACUC protocols (#15077 & #18080) at the University of Illinois at Urbana-Champaign.

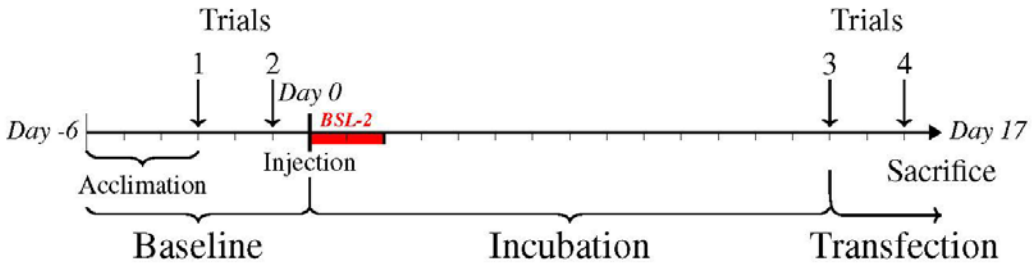

Figure 22 Experimental timeline with the injection of constructs on day 0. Fish were injected with a randomly assigned construct of either an aggression-related gene (*MAOA* or *AVP*) or a control fluorescent protein (*EYFP*). All trials were conducted double-blind to the transfected gene. Each trial had two breathing rate measurements followed by a territorial challenge.

Behavioral assays

All behavioral data were gathered double-blind to the transfected gene. Males' breathing rate and behavioral response to a territorial challenge were recorded four times (Figure 22): twice before and twice after injection, respectively considered baseline and transfected. Breathing rate was determined prior to the territorial challenge by averaging two separate non-continuous counts of opercular beats per 20 seconds taken within a 5 minute period. This ensured that individual variations due to stress from the researcher's arrival were minimized. Territorial aggression was measured by recording the individual's response to an intruder confined to a glass flask. The times to orient toward and to first bite at the intruder (TTO and TTB, respectively) were recorded, as well as the total number of bites, charges (lunges), and trips (approaches) during the five minutes following initial orientation. Intruders (N = 9) were 5-10% smaller conspecific males. Each focal male except one was confronted by the same intruder during all four territorial challenges. In the exception, the initially paired intruder died between trials two and three and was replaced with a new male of the same length.

Constructs

Mammalian cDNA ORF clones were used for *AVP* (human, HG17671-UT, NCBI Ref Seq: NM_000490.4, Sino Biological) and *MAOA* (mouse, MG57436-U, NCBI Ref Seq:

NM_173740.3, Sino Biological). These were cloned into the pDONR221 backbone (Epoch Life Science) and then packaged (Gene Delivery Technology Core, Massachusetts General Hospital) with an hCMV promoter and IRES-GFP backbone in replication deficient Herpes Simplex 1 (HSV-1). Stock hCMV-EYFP (RN12) was used for control injections. All males were randomly assigned to one of the three constructs. The final viral solutions were used undiluted except for the addition of a trace amount of pigment (brilliant blue FCF or tartrazine, i.e. FD&C Blue No. 1 and Yellow No. 5) to allow the solution to be visualized against the gradations of the syringe. These constructs are episomally expressed; the payload genes, packaged as a plasmid, remain in the cytoplasm and neither integrate into nor replicate with the genome.

Neurosurgical injection and surgical rig

In a ten-minute neurosurgical procedure, the construct was injected into the anterior diencephalon of the brain via a transcranial injection. Fish were transferred into a new tank in the surgery room the morning of the injection. Initial anesthetization was done in 0.02% buffered MS-222 (Tricane-S, Western Chemical) for no more than five minutes (188.4 sec ± 74.0), until movement ceased and the fish was unresponsive. The fish was transferred to the surgical rig and held securely in a small clamp, lined on one side with foam tape for padding. A cannula delivered fresh water with maintenance level anesthesia throughout the procedure. The speed of water delivery was adjusted to each fish to allow a steady low flow rate over the gills.

Each fish received two bilateral transcranial injections delivering a total of ~600 nL of construct to the anterior diencephalon. During each injection, ~100 nL was delivered at three different depths (≤ 2.5mm), ensuring broad expression throughout the diencephalon. Viral construct was injected using a 5 µL borosilicate syringe (Hamilton Neuros model 75, #65460-02). In each injection, the 33G (0.210 mm OD) needle was inserted transcranially through the thinnest portion of the skull.

Breathing rate and the fish's position in the water column was recorded every 15 minutes for two hours following the injection. Additional checks were performed at three hours and one-day post-injection for all fish. After two days, fish were removed from the ABSL-2 surgical room to individual tanks.

Sacrifice

Fish receiving each construct were randomly and evenly assigned to be sacrificed at either 1, 8, or 15 days after the 4th and final behavioral trial to validate long term expression. Day of sacrifice did not correlate with any measure of expression. Fish were photographed, patted dry, and rapidly beheaded between 11:30 and 1pm. Blood was immediately collected from near the bisected heart via capillary action into a heparinized glass microhematocrit tube (HT9H, Statspin, Westwood, MA). The presence and quality of testis were confirmed, and the tail was clipped as a DNA sample. The brain was then dissected out and stored in RNAlater. The entire process took between 10 and 30 minutes per day with each fish taking between 4 and 8 minutes to process.

R analysis and data availability

Descriptive statistics are presented as mean ± standard deviation. All data analysis was carried out in RStudio (v1.1.383) with R version 3.5.1. All scripts and data are publicly available on the Open Science Framework (https://osf.io/v56zt) as "Behavioral experiments scripts for release.R". Repeatability is reported as ICC3,1 calculated using `Desctool` (v0.99.25) and confirmed with the nonparametric concordance package `nopaco` (v1.0.6). Significance was similar between the ICC and concordance tests. Spearman correlations were calculated using `Hmisc` (v4.1-1). Wilcoxon and Mann-Whitney tests were done with the base `stats` package and effect size was calculated with the `rcompanion` package (v2.2.1). Finally, sample size calculations utilized the `WMWssp` package (0.3.7) with the defaults of 0.05% for two-sided type I error rate and 0.8 power.

Results

Behavioral repeatability and intercorrelations

Repeatability was analyzed across the two trials at each timepoint (baseline and transfected) to ensure that behavior had stabilized following transfection. Only behaviors that were consistently repeatable, i.e. at both baseline and following transfection but not necessarily between the timepoints (Table 8), were considered in subsequent analysis.

Table 8 Repeatability of territorial aggression behaviors and breathing rate across the two trials at each timepoint using two-way mixed, single score ICC (type 3,1) for all fish (N = 54). EYFP is the control, *AVP* and *MAOA* are genes of interest.

Measure	Condition	Baseline			Transfected			Repeatable at both timepoints
		ICC	95% CI	p-value	ICC	95% CI	p-value	
Breathing Rate	*EYFP*	0.49	0.01, 0.78	0.02	0.91	0.76, 0.97	2.51E-07	Yes
	AVP	0.38	-0.09, 0.71	0.05	0.73	0.42, 0.89	1.76E-04	Yes
	MAOA	0.53	0.12, 0.78	0.007	0.35	-0.09, 0.68	0.06	
Time to Orient	*EYFP*	0	-0.48, 0.48	0.50	0.13	-0.37, 0.58	0.30	
	AVP	0	-0.46, 0.46	0.50	0	-0.46, 0.46	0.50	
	MAOA	0.04	-0.40, 0.47	0.43	0.21	-0.24, 0.59	0.18	
Time to 1st Bite	*EYFP*	0.77	0.47, 0.91	1.32E-04	0.47	-0.01, 0.78	0.03	Yes
	AVP	0.51	0.07, 0.78	0.01	0.28	-0.20, 0.65	0.12	
	MAOA	0.48	0.06, 0.75	0.01	0.74	0.45, 0.89	6.96E-05	Yes
Bites	*EYFP*	0.88	0.68, 0.95	2.0E-06	0.78	0.47, 0.92	1.17E-04	Yes
	AVP	0.45	0, 0.75	0.03	0.73	0.42, 0.89	1.69E-04	Yes
	MAOA	0.62	0.25, 0.83	0.001	0.59	0.21, 0.82	0.002	Yes
Charges	*EYFP*	0.64	0.23, 0.86	0.003	0.69	0.31, 0.88	0.001	Yes
	AVP	0.55	0.12, 0.80	0.008	0.54	0.11, 0.80	0.01	Yes
	MAOA	0.39	-0.06, 0.70	0.04	0.83	0.62, 0.93	1.61E-06	Yes
Trips	*EYFP*	0.75	0.41, 0.90	2.82E-04	0.32	-0.19, 0.70	0.10	
	AVP	0.39	-0.08, 0.72	0.05	0.19	-0.29, 0.59	0.22	
	MAOA	0.61	0.24, 0.82	0.002	0.66	0.31, 0.85	6.18E-04	Yes

Aggression measures were generally equally repeatable compared to the physiological measure of breathing rate. However, time to orient was not repeatable at baseline nor following transfection for any construct (Table 8). The number of trips was not repeatable for control fish (EYFP), nor for any construct across all four trials (ICC_{EYFP} = 0.01, ICC_{AVP} = 0, ICC_{MAOA} = 0.01). Time to first bite was not significantly different for any construct (Supplemental Figure 4). Therefore, subsequent analyses focus on bites and charges.

Total number of bites and charges were strongly correlated ($r \geq 0.7$) in the control group (Figure 23a) and following transfection of either *AVP* (Figure 23b) or *MAOA* (Figure 23c). Time to first bite was negatively correlated with total number of bites and charges as well – i.e. fish that bit sooner also attacked more overall.

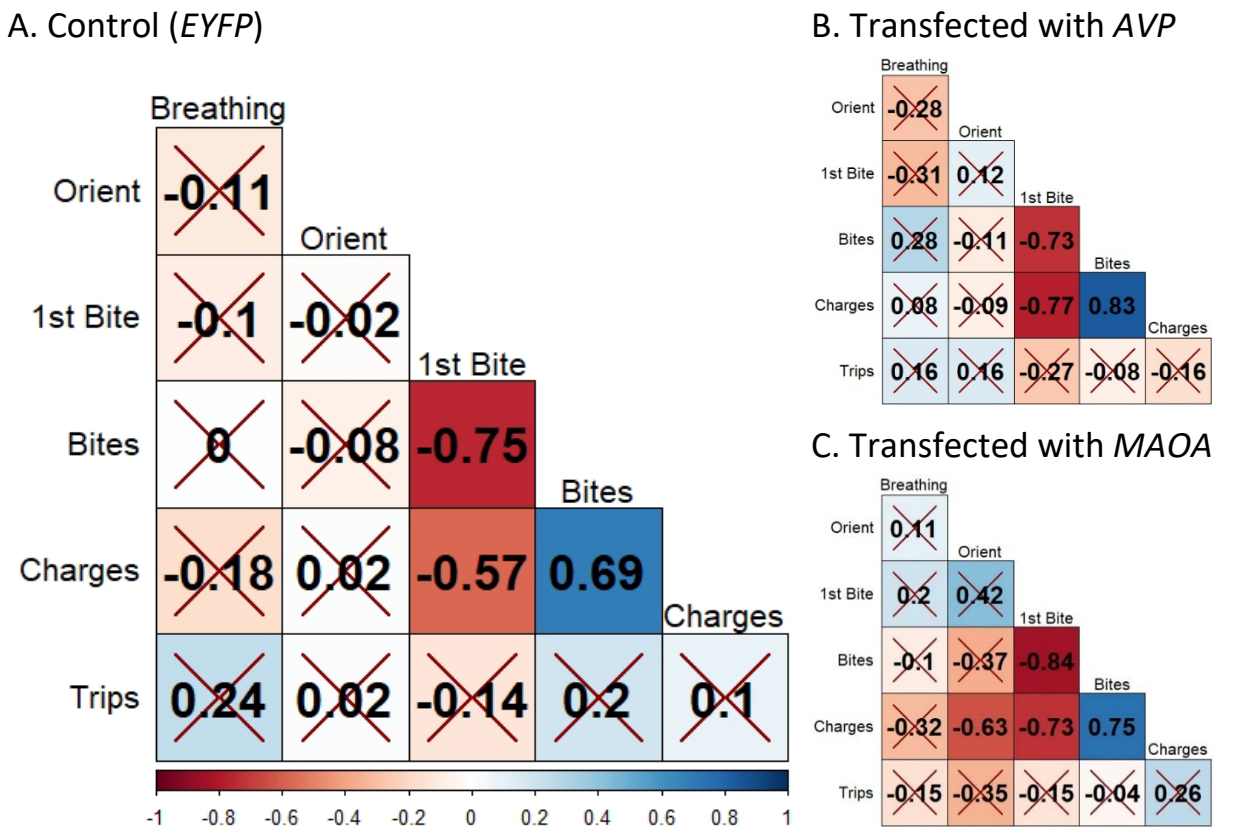

Figure 23 A) Correlation between all measured behaviors in control (*EYFP*) fish averaged across all four trials (*N* = 16). Bites, charges, and time to first bite remain correlated following transfection of B) *AVP* (*N* = 18) and C) *MAOA* (*N* = 20). Numerical values are the strength of the correlation with crossed out boxes indicating non-significance.

Increased aggression from transfection of MAOA or AVP

Aggressive behavior (charges) increased in fish transfected with either *AVP* or *MAOA*. *AVP* had a large effect on the number of charges (paired Wilcoxon signed rank test: *rs* = 0.79, *Z* = -3.07, *p-value* = 0.001) with 16 of the 18 individuals increasing the average number of charges compared to their baseline. In magnitude, this represented an almost 100% increase in average number of charges, from 9.7 (*SD* = 5.1) at baseline to 18.8 (*SD* = 10.6) following transfection.

Transfection with *MAOA* also caused a large increase in the average number of charges (paired Wilcoxon signed rank test: *rs* = 0.53, *Z* = -2.10, *p-value* = 0.018) relative to baseline. However, the effect of *MAOA* was less drastic than that of *AVP* and had more variation in individual response (Figure 24), with 13 of 20 individuals increasing their average number of

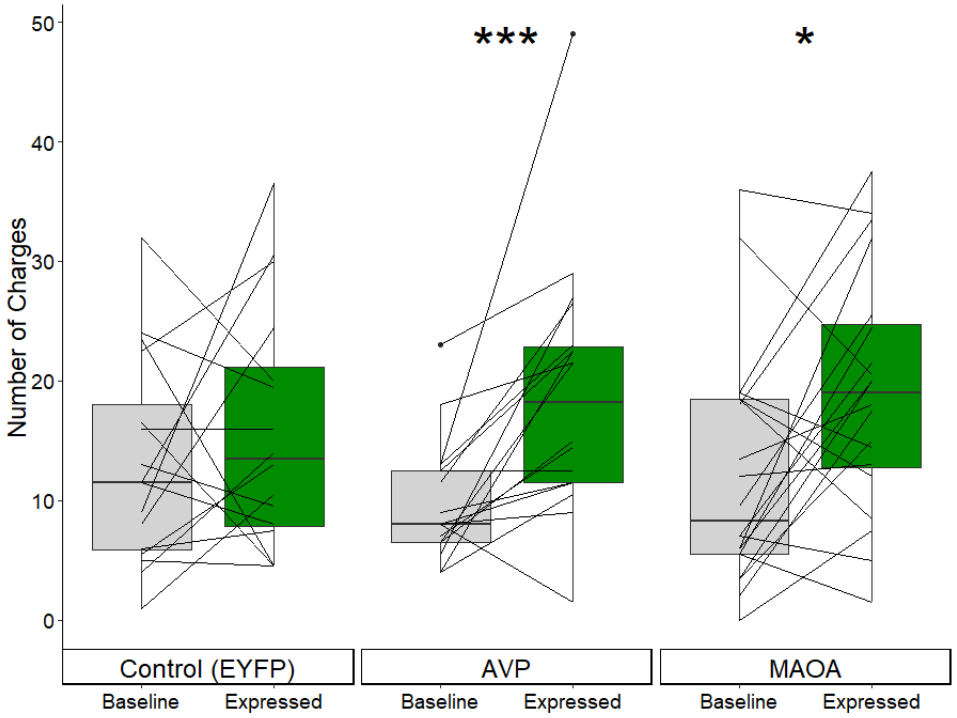

Figure 24 Number of charges (averaged across the two trials as each timepoint) before and after transfection for each construct. Each line represents an individual showing their change in behavior following transfection of the gene of interest. Transfection with *AVP* resulted in a substantial and consistent increase in the number of charges; note that only one individual exhibited decreased charging behavior. Transfection with *MAOA* resulted in an increase of large effect size in charges, although there was more variation in individual response. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

charges. Despite this, *MAOA* still resulted in an approximately 50% increase from 12.1 (*SD* = 9.7) charges at baseline to 19.1 (*SD* = 10.0) following transfection.

MAOA decreased breathing rate

Only the *MAOA* construct altered breathing rate (Figure 25). Compared to baseline, *MAOA* strongly and significantly (*N* = 20, *rs* = 0.85, *Z* = -3.62, *p-value* = 0.0001) lowered breathing rate, with 19 of the 20 individuals experiencing a decrease in resting breathing rate. They dropped from an average of 40.8 (*SD* = 1.9) to 38.1 (*SD* = 2.0) breaths per 20 seconds. Additionally, when comparing the breathing rates between the fish transfected with *MAOA* and the *EYFP* controls (*N* = 16, *mean* = 40.8, *SD* = 3.9), the decrease was still significant, though

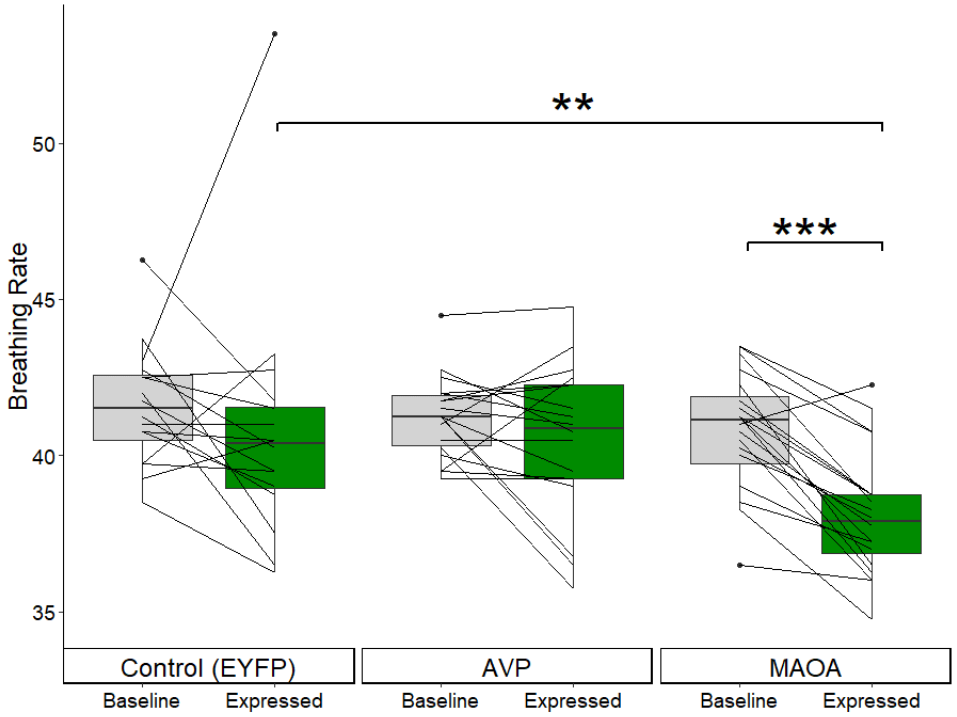

Figure 25 Breathing rate measured by opercular beats for the three constructs. Each line represents an individual showing their change in breathing rate, averaged across the two trials at each timepoint, before and after transfection. Only *MAOA* altered breathing rate; a drastic decrease compared to both baseline (within-subject comparison, *N* = 20) and to the control group (*N* = 16). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

reduced to a moderate effect size (Mann-Whitney test: *rs* = 0.44, *Z* = -2.65, *p-value* = 0.009). There was not significant change in breathing rate compared to baseline due to either the control *EYFP* (*Z* = -1.11, *p-value* = 0.13) or *AVP* (*Z* = -0.92, *p-value* = 0.18) constructs.

Discussion

Viral-mediated transgenesis resulted in altered territorial aggression specifically for fish receiving candidate genes related to aggression, but not for a fluorescent protein. Additionally, we found that construct selection is not limited to native genes; widely available mammalian plasmids successfully altered behavior. This method is therefore accessible to a broad array of users, a feature especially important in a system with roots in ethology. Furthermore, transfection enables within-subject experimental designs, reducing sample sizes required for the same statistical power and making behavioral experiments viable, as detailed below.

Viral-mediated transgenesis allows statistically powerful repeated measures design

Complex phenotypes that emerge at the whole organism level, such as behaviors like aggression, are difficult to assay due to their subtleties and time-intensive screening. Social behaviors are influenced by many genes of small effect (Spencer, Su, Donnelly, & Marchini, 2009; Wahlsten, 2012) and social psychology generally has smaller effect sizes (*r*) relative to other psychological sub-disciplines (Schäfer & Schwarz, 2019). Indeed, the neuroscience, psychiatry, psychology, and behavioral ecology fields are plagued with reports of overestimates of effect sizes (Button et al., 2013; Fanelli & Ioannidis, 2013; Forstmeier & Schielzeth, 2011; Schäfer & Schwarz, 2019). Additionally, behavior in natural populations tends to have high inter-individual variation, further reducing statistical power (Taborsky, 2010). For sticklebacks, who have a generation time of approximately one year, the traditional approach of breeding a stable transgenic line is not always practical. Here, by using within-subject design, we

successfully examined two behaviorally relevant genes for effects on aggression in wild-caught fish.

By using repeated measures on the same fish before and after transfection, we were able to drastically reduce the necessary sample size needed to detect significant changes in behavior. In this study we found large effect sizes for both behavior and breathing rate, a typical physiological measure. However, variation following transfection with *MAOA* was about 25 times larger for charging behavior ($\sigma^2 = 99.9$) compared to breathing rate ($\sigma^2 = 3.9$). A between group comparison would have required an impractical sample size of as many as 300 fish (Table 9) to detect the difference in charges, even though these genes have a large magnitude ($rs > 0.5$) of effect on behavior. However, by using these methods we were able to reduce the sample size down to merely 20 fish, a far more manageable sum. Thus, viral-mediated transgenesis enables the study of genetic effects on natural behavior in wild-caught animals, because it makes possible a repeated measures design comparing within the same individuals, increasing sensitivity.

Table 9 Necessary sample sizes assume a statistical power of 0.8 and are based on the effect sizes observed in this study – i.e. 8 out of 10 experiments using samples this large will detect the difference. Viral-mediated transgenesis makes possible a repeated measures design comparing within the same individuals, increasing sensitivity. Note that *AVP* and *MAOA* are known to have a large effect on behavior, so this should be viewed a minimum for future candidate genes being screened for behavioral phenotypes.

	Behavior *(Charges)*		**Physiology *(Breathing)***
	AVP	***MAOA***	***MAOA***
Necessary sample size (between groups, vs. control)	277	187	35
Actual sample size	18	20	20
Effect size (*rs*)	0.79	0.53	0.85
Variance (σ^2, post-expression)	113	99.9	3.9

Genetic underpinnings of territorial aggression

To demonstrate the practicality of viral-mediated transgenesis to examine candidate gene function on behavior, we looked for behavioral changes from two aggression related candidate genes. Both vasopressin (*AVP*) and monoamine oxidase (*MAOA*) are well established as influencing aggression (Goodson, 2013).

The effects of pharmacological manipulation of vasotocin or vasopressin on aggression in teleosts has been mixed (Gonçalves & Oliveira, 2011; Santangelo & Bass, 2006), purportedly depending on dosage, species, or receptor localization. Here, we found that viral-mediated transfection was more robust than our previous work using either IP or direct brain injection of exogenous vasotocin (Chapter 3). The effect following transfection was consistent, with 16 of the 18 fish experiencing an increase in aggression, one remaining constant and only one decreasing aggression, and was of stronger magnitude than pharmacological manipulation (Figure 24). This is not entirely surprising given vasopressin's extremely short half-life (<1 minute in the rat brain (Stark, Burbach, Van Der Kleij, & De Wied, 1989)). Transfection allows for a continual, natural production of vasotocin that may bypass immediate biofeedback mechanisms and allows for stabilization of the HPA axis following treatment.

A more detailed, cross-population study of vasotocin in stickleback would present an ideal opportunity to investigate the evolutionary constraints or trade-offs between behavior and physiology for pleiotropic genes. In addition to being associated with behavior, vasotocin plays a key role in osmoregulation via the AVP V2 receptors. Vasotocin anatomy and aggression differed in concordantly with salinity and osmoregulation challenges in pupfish (Lema, 2006). A more nuanced examination is possible in stickleback as there are numerous freshwater and several anadromous populations that are independently evolved from ancestral-like marine population. In addition, population differences in aggression have already been documented in sticklebacks

(A M Bell, 2005; Niels J. Dingemanse et al., 2007; Keagy, Lettieri, & Boughman, 2016). However, an examination of the integration of these two evolutionary concerns has not yet been undertaken, despite a relationship between aggression and kidney size having already been discovered in stickleback bred for extremes of territorial aggression (Theo C.M. Bakker, 1986). This makes stickleback uniquely suited system to address the relationship of physiological ecology, anatomy, and social behavior (Goodson, 2013).

We also looked at aggression following overexpression of *MAOA*, a gene which is well established to increase aggression when downregulated (Godar et al., 2016). Therefore we expected overexpression of *MAOA* in this study to produce the opposite behavioral effect of decreased expression. However, counter to our hypothesis, and similar to *AVP*, aggression also increased following transfection of *MAOA* (Figure 24). In mice, increased *MAOA* levels resulting from a knockout of Rines E3 ubiquitin ligase produced emotional behavior abnormalities, namely heightened anxiety and increased social interactions with an unfamiliar conspecific in both an unfamiliar space and during a resident-intruder test (Kabayama et al., 2013). In social rodents like mice, non-social stress has been found to promote affiliative behavior (Beery & Kaufer, 2015). Thus, their increased affiliation is potentially explained as a response to stress rather than a direct effect of changes in monoamine oxidase levels. Stickleback do not naturally affiliate when stressed, but emotional behavior abnormalities and heightened anxiety could manifest as increased aggression.

Our finding that transfection of *MAOA* increased aggression is consistent with a decrease in serotonin, which is enzymatically cleaved by monoamine oxidase. Indeed, the clear and unambiguous decrease in breathing rate we observed (Figure 25) is strong evidence of *MAOA* functioning as expected physiologically. Breathing rate correlates positively with serotonin and norepinephrine concentration (Hodges & Richerson, 2008; Whelan & Young, 1953); monoamine oxidase enzymatically lowers levels of both neurotransmitters. Additionally, in

accordance with our findings, previous RNAseq data from nesting male sticklebacks (Syed Abbas Bukhari et al., 2017) shows a similar if non-significant positive correlation between aggression and *MAO* expression (Figure 26). It is worth noting that in this previous study, aggression was measured with only bites, time to orient and time to first bite at the intruder, which we found to have greater variance both within and between individuals than charging, which marks voluntary initiation of aggression (van Iersel, 1953); the decreased statistical power of these measures compared to number of charges may explain the lack of significance. Additionally, trout monoamine oxidase has been found to be equivalently effective to human monoamine oxidase in metabolizing 5-HT and PEA (Shih et al., 1999), making it unlikely that the increase in aggression is an off-target effect of using mammalian *MAOA*. Further characterization of anxiety levels following transfection, pharmacological rescue (Godar et al., 2014), and quantification of the downstream neurotransmitters remain as potential avenues to a better mechanistic understanding of this result.

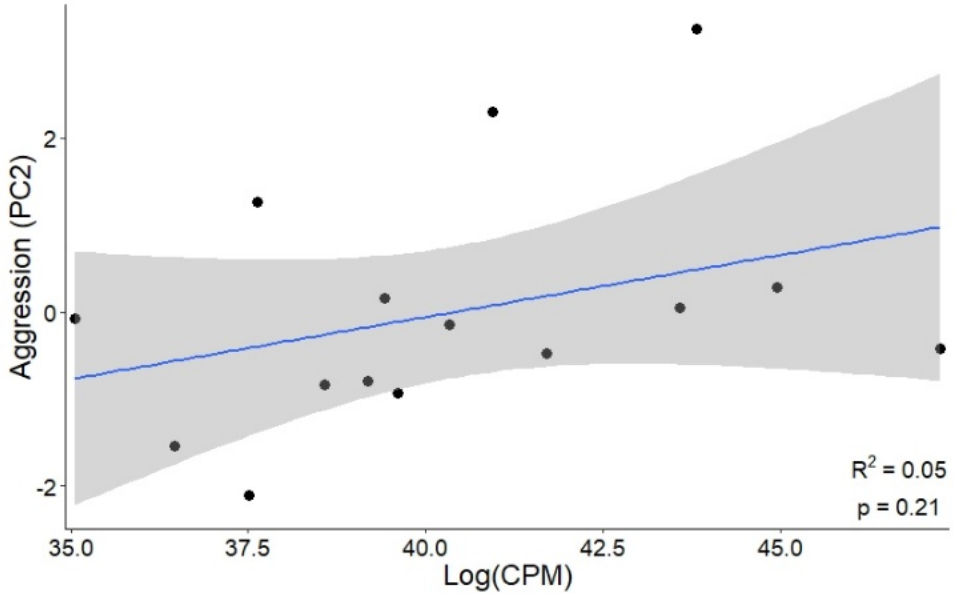

Figure 26 Correlation between increased aggression and increased expression of *MAO*.

Conclusion

We present a method for viral-mediated transgenesis that enables a more direct examination of the genetic mechanisms underlying behavior in wild-caught animals from natural populations. This method is appealing because it is flexible, fast, and allows us to compare individual behavior before and after transgenesis, maximizing statistical power. It further enhances the growing molecular toolkit in threespine stickleback, a classic ethological system. Overall, our experimental results show that viral-mediated transgenesis is a promising method for testing the function of candidate genes in this system and confirm the importance of *MAOA* and *AVP* for aggression in teleost fish. Transfection with a human-based *AVP* construct demonstrates that widely available, ready-to-use mammalian plasmids are viable with this method, lowering the barrier of entry. Finally, the unexpected result that increasing *MAOA* resulted in increased aggression indicates the need for a more complete characterization of monoamine oxidase's role in aggression at high levels.

CONCLUSION

Viral-mediated transgenesis is a method to alter a gene's expression in a specific location or during a controlled timeframe. This approach has already proved essential in the functional testing of genes related to behavior in rodents (Simonato et al., 2000) and in the dissection of neural circuits (Luo et al., 2008). While I used ubiquitous promoters with differing timings, cell-specific targeting is possible using alternate promoters (reviewed in Ingusci et al. 2019), such as using GFAP to target astrocytes. Specific cellular targeting can limit increased expression to cells that naturally produce the neurotransmitters. For instance, while GAD targets glutamatergic neurons, TH targets dopaminergic neurons. Ongoing efforts to identify targeted promotors at the individual cell level (Tasic et al., 2018) will allow for more carefully manipulation in ecologically relevant regions or cells, for instance targeting GABAergic neurons in the hypothalamus. Further, the use of HSV as the viral vector enables larger payloads than adeno-associated viruses (AAVs), making this protocol the first step toward using chemogenetics and optogenetics in sticklebacks.

This initial foray into developing viral-mediated transgenesis focused on ubiquitous promoters that are expressed in every cell both within and outside the brain. While this maximizes the likelihood of seeing a behavioral effect, the outcome may not reflect naturally evolved mechanisms. In the contentious case of vasopressin receptor's (V1aR) role in the ventral pallidum in prairie vole monogamy, for example, forced expression increases affiliation and monogamy, a result uncorroborated by field studies (Albers, 2015; Insel, 2010; Ophir, Wolff, & Phelps, 2008). It is important to pair manipulative studies that elucidate proximate mechanisms with naturalistic field studies focused on ultimate evolutionary mechanisms. Sticklebacks have numerous freshwater populations that are independently evolved from an ancestral marine population, making it possible to use extant field studies to pin down molecular mechanisms.

For instance, paternal care, a hallmark behavior of sticklebacks, appears to be lost in a few populations of 'white' sticklebacks (Haley, 2018). Current efforts by Colby Behrens in the Bell lab to determine candidate genes using linkage association will be followed up with manipulative experiments.

My results show increased aggression with increased expression of monoamine oxidase (Figure 24), preliminarily suggesting an ideal functional range similar to dopamine and serotonin's inverted-U functionality (Cano-Colino, Almeida, Gomez-Cabrero, Artigas, & Compte, 2013; Floresco, 2013). However, limitations in our approach leave further work to truly support this hypothesis. It remains possible that these results are due to the unnatural ubiquitous expression and broad targeting of the injection to an entire hemispheres of the brain, which may explain the variation in individual response seen behaviorally, though would not be expected to produce the consistent changes in breathing rate.

Additionally, it is important to bear in mind that within the social behavioral decision making network, neurotransmitters likely function in a balanced system (Albers, 2015; Newman, 1999) in which different social behaviors are associated with differing relative amounts of those neurotransmitters.. Thus, my gross manipulation of an upstream regulator of neurotransmitter levels is akin to taking a sledgehammer to a finely-tuned machine, resulting in potentially unpredictable downstream effects. Future efforts should focus on identifying the responsible downstream neurotransmitters (serotonin, dopamine or norepinephrine) rather than region-specificity for better efficiency. After determining the causal neurotransmitters, then the focus should shift to specific regions in the social-decision making network.

In this case, the clear and unambiguous decrease in breathing rate (Figure 25) is strong evidence of *MAOA* functioning as expected – enzymatically lowering levels of dopamine and norepinephrine. Additionally, in accordance with this ideal functional range, previous stickleback RNAseq study (Syed Abbas Bukhari et al., 2017) also found increased aggression

with increased *MAO* expression (Figure 26) suggesting my manipulation reflects natural processes. These results highlight the need to examine both extremes of expression for all candidate genes to fully characterize the molecular mechanisms at play.

In chapters 3 and 4 I used both pharmacological and transgenic manipulations on vasotocin/vasopressin levels. In every case of vasotocin signaling manipulation that we tested – pharmacological IP inhibition (Manning compound) or supplementation, exogenous brain injection, and transfection – number of charges at the intruder was the most responsive aggressive behavior. However, depending on the type of manipulation we saw opposing effects on the amount of charges – pharmacological supplementation of exogenous vasotocin resulted in a decrease while transfection with *AVP* resulted an increase. In examining this contrariety it is natural to question if both manipulations actually succeeded. However, there is reason to hold high confidence in each. It is extremely unlikely that the difference is due to a failure of transfection; there are multiple pieces of evidence indicating that we achieved successful transgenesis. The most visible, literally, is the presence of novel fluorescent proteins, detailed in chapter 3. Additionally, in chapter 4, I showed consistent changes in both physiological and behavioral phenotypes following transfection. Finally, transcripts specifically of the injected mammalian *MAOA* homolog remain detectable for up to 4 weeks after transfection (Figure 27).

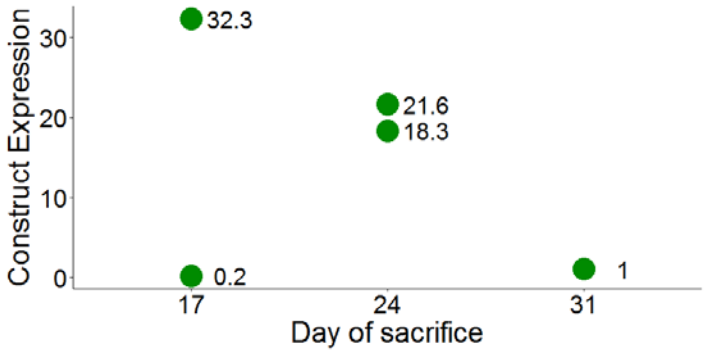

Figure 27 Expression of mouse *MAOA* detected by qPCR at 17, 24, and 31 days after injection. Note that the primers were specific to the construct copy and did not detect the native *MAO* gene.

The effects of pharmacological manipulation of vasotocin or vasopressin on aggression in teleosts has been mixed, purportedly depending on dosage, species, or receptor localization. For example,

damselfish show no change or an increase in aggression, depending on dosage, while knife- and pup-fish treated with AVT showed decreased aggression (Gonçalves & Oliveira, 2011; Santangelo & Bass, 2006). In this experiment, similar physiological effects were produced throughout the dose-response curve (Figure 21). In conjunction, although behavioral effects were produced only at the highest dosage, they were similar for both routes of administration (Supplemental Table 4). These consistencies suggest that the pharmacological manipulation did in fact work. It is possible that systemic treatment of a peptide via intraperitoneal injection caused an off-target immune/inflammatory response, one possible explanation for the decrease in aggression. However, this is a widely-used route of administration in animal studies. Furthermore, the brain injections which largely bypass the circulatory system produced a similar decrease in aggression. Summarily, no explanation focusing mechanically on only one of these experiments is likely to fully explain both results.

The difference in the exposure timing has more power to explain the difference in results. Vasopressin has an extremely short half-life of less than a minute (Stark et al., 1989), making pharmacological manipulation very rapid. In contrast, transfection is much longer lasting. Transgenesis allows for a continual, natural production and secretion of vasotocin that may bypass immediate biofeedback mechanisms such as the immune system and allow for long-term stabilization of the HPA axis. Additionally, vasotocin signaling is mainly constrained by receptor type and location (Albers, 2015; Goodson, 2013; Huffman et al., 2012) further emphasizing the potential of long-term homeostasis to influence the behavioral outcome. To mimic this with pharmacological manipulation, it would be best to use an implanted cannula in the brain to deliver repeated low doses of exogenous vasotocin. This would allow a more direct comparison between treatment methods clarifying if the opposing effects of pharmacological manipulation and transgenesis are due to homeostatic balancing from long-term exposure or a potential side effect from an immune response to the injection of small molecules.

Overall, I achieved my major goal of demonstrating changes in behavior caused by increased expression of candidate genes. Furthermore, this was done in wild caught adult fish, maximizing the ecological relevance of my findings. These fish had a completely natural rearing and natural levels of social & genetical diversity. This type of experiment is only possible due to the statistical power of the within-subject repeated measures design. However, future work should focus on a more targeted manipulation via promoter specificity as well as tracking where the secreted neurotransmitters are functioning. Additionally, ongoing work aims to combine the major benefit of sticklebacks – their adaptive radiation – with viral-mediated transgenesis to look at ultimate evolutionary mechanisms that give rise to population level variation in behavior.

In my effort to address how genes contribute to behavior, I developed new tools for a classic behavioral system while bearing in mind that this is a cross-field undertaking. In chapters 1 and 3, I presented my new methods in stickleback – *in situs* for localizing gene expression, pharmacological manipulation bypassing the blood-brain barrier, and transgenesis to manipulate gene expression. These methods are accessible and useful to many the many fields currently using the stickleback system including neuroscience, behavioral genetics, ethology, and ecology & evolution. Finally, I discussed my interesting finding on high levels of monoamine oxidase which increased aggression suggesting an ideal functional range and highlighting the need to examine both extremes of expression even for classic behavioral genes.

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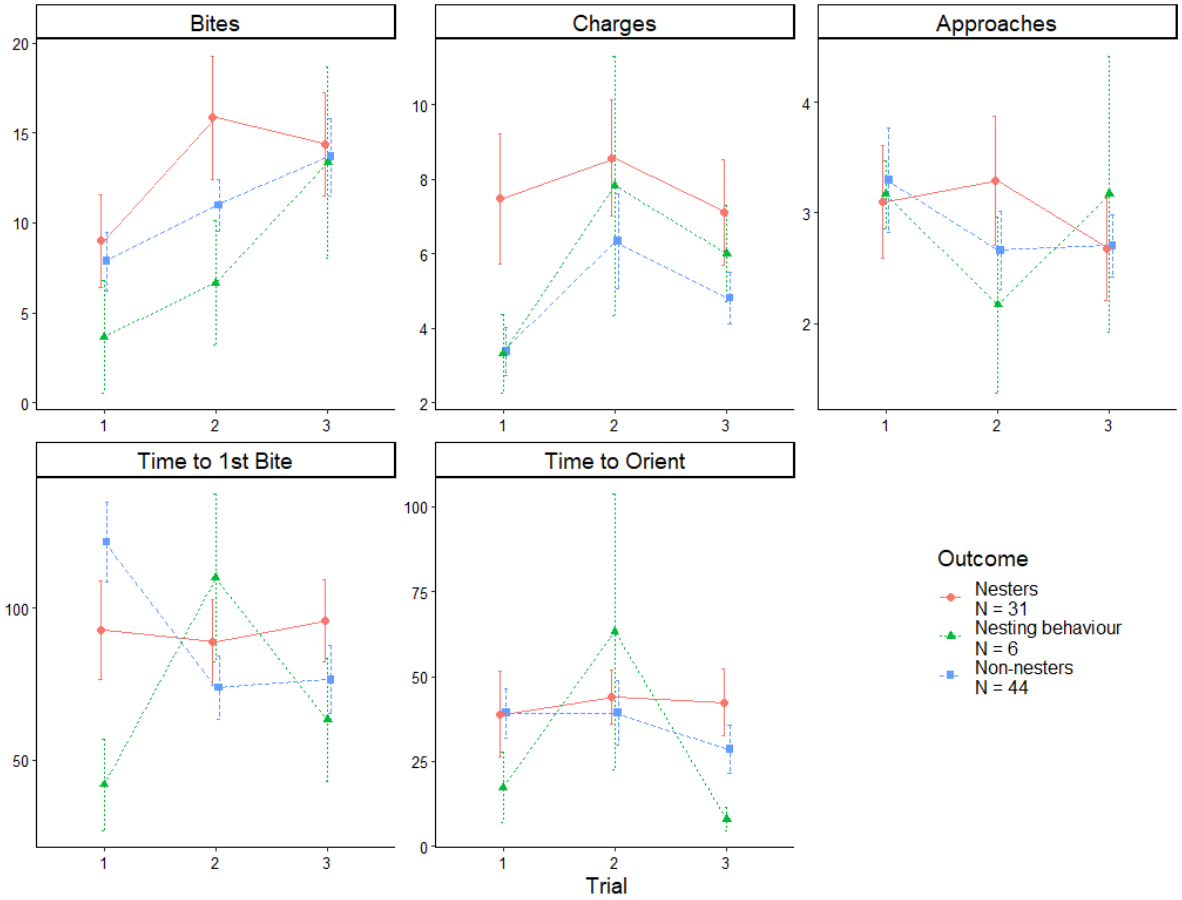
APPENDIX A: Supplemental Data

Supplemental Table 1 Post-hoc comparison across trials for significant territorial aggression measures

	All Fish		Non-nesters		
	Bites P-value (adj)	**Charges P-value (adj)**	**Bites P-value (adj)**	**Charges P-value (adj)**	**Time to 1st Bite P-value (adj)**
Trials 1 & 2	0.01	0.02	0.14	0.06	0.02
Trials 1 & 3	0.003	0.13	0.07	0.16	0.01
Trials 2 & 3	0.65	0.41	0.62	0.55	0.88



Supplemental Figure 1 Counts of each behavioral measure of territorial aggression (mean ± se) across all trials for each nesting outcome.

Supplemental Table 2 A) Comparison of breathing rate for the larger 30G (N = 23) versus the smaller 33G needle (N = 61). There is a main effect of needle size (ATS1, 2613 = 6.54, p-value = 0.01) by a repeated measures ANOVA; the larger needle slowed recovery. Following FDR correction, effect size ® is is small and insignificant but consistent at each time point. B) Comparison against pre-injection breathing rate for each needle. Given are the adjusted p-values for the Wilcoxon signed rank test comparing the pre-injection breathing rate at each time point. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

A

Time (h)	Z	p-value		p (adj)	r
0	1.6	0.95		0.95	-0.01
0.25	-1.5	0.07	.	0.22	0.20
0.5	-1.4	0.08	.	0.22	0.19
0.75	-1.2	0.12		0.22	0.17
1	-1.3	0.10	.	0.22	0.18
1.25	-1.0	0.16		0.26	0.16
1.5	-0.9	0.19		0.28	0.14
1.75	-0.8	0.22		0.28	0.13
2	-0.7	0.24		0.28	0.13
3	-2.2	0.02	*	0.12	0.27
4	-2.1	0.02	*	0.12	0.29
5	-0.1	0.47		0.51	0.09
6	-1.2	0.12		0.22	0.20

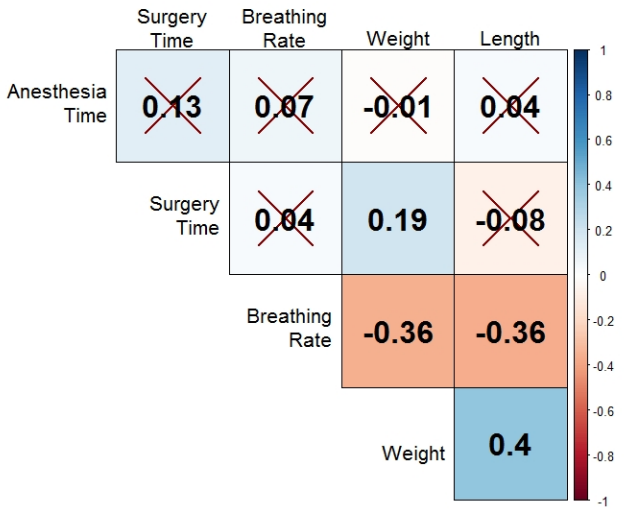
B

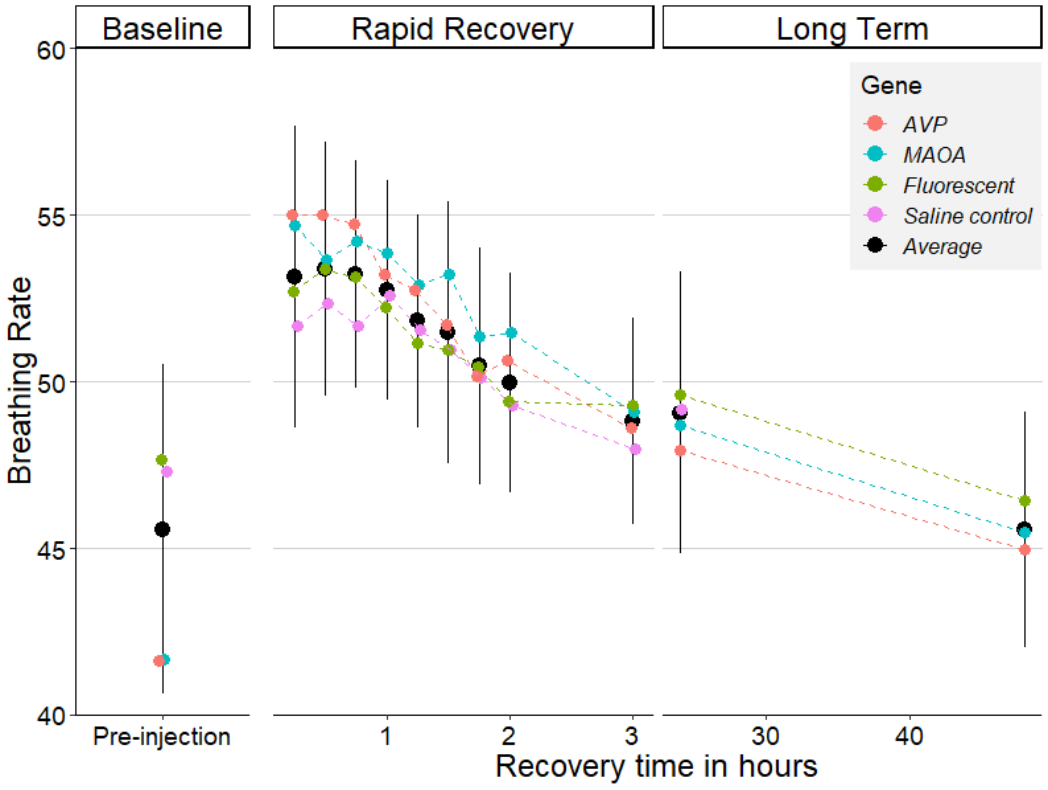
Time (h)	30G (Large)		33G (Fine)	
0.25	0.003	**	6.28E-04	***
0.5	0.003	**	5.28E-05	***
0.75	0.003	**	9.65E-06	***
1	0.003	**	1.21E-05	***
1.25	0.003	**	5.43E-05	***
1.5	0.005	**	0.01	*
1.75	0.03	*	0.09	.
2	0.05	*	0.34	
3	0.24		0.83	
24	0.07	.	0.23	

Supplemental Table 3 Comparison of breathing rate (opercular beats per 20 seconds) to all saline injected controls (*N* = 39) throughout the recovery curve. The injection of porcine vasopressin resulted in increased breathing rate for about two hours following injection. * *p* ≤ 0.05; ** *p* ≤ 0.01; *** *p* ≤ 0.001

	HSV-1 w/ Gene			Vasotocin		
Time (h)	Z	p (adj)	r	Z	p (adj)	R
0	-2.1	0.08	-0.22	-2.8	0.005 **	0.43
0.25	-2.3	0.08	0.23	-3.5	0.001 ***	-0.50
0.5	-1.1	0.43	0.13	-2.7	0.006 **	-0.40
0.75	-0.4	0.61	0.08	-2.7	0.006 **	-0.40
1	2.2	0.99	0.00	-3.8	0.0004 ***	-0.54
1.25	-0.2	0.61	0.07	-4.0	0.0003 ***	-0.57
1.5	0.0	0.62	0.06	-2.8	0.005 **	-0.42
1.75	-0.8	0.55	0.11	-2.6	0.006 **	-0.39
2	-1.3	0.34	0.15	-2.4	0.01 *	-0.36
3	-2.5	0.08	0.25	-0.9	0.18	-0.20
24	-0.3	0.61	0.08	-1.0	0.18	0.20

Supplemental Figure 2 Spearman correlations between time to anesthetization (*N* = 148), time for neurosurgical procedure (*N* = 124), breathing rate prior to surgery (*N* = 153), weight (*N* = 157) and length (*N* = 87). Anesthetization time was not correlated

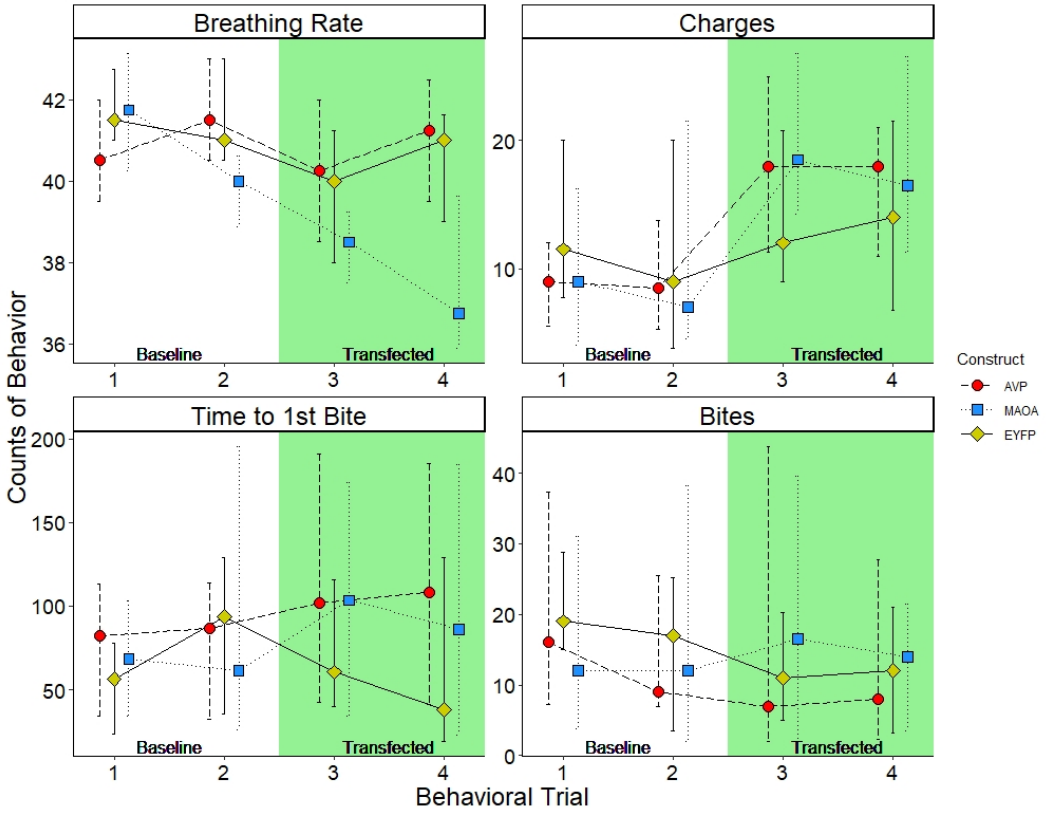




Supplemental Figure 3 The choice of HSV-1 construct did not result in any significant differences in breathing rate during recovery.

Supplemental Table 4 Within-subject comparison of territorial aggression following pharmaceutical manipulation of vasotocin signaling compared to baseline. Brain and IP injection results were similar with only the highest dosage altering behavior. No group (including the Manning treatment) significantly differed from saline-injected controls. * $p \leq 0.05$; ** $p \leq 0.01$

		Bites				Charges			
	N	Change	Z	r	p	Change	Z	r	p
AVP (Brain, 0.5 $^{\mu g}/_{gbw}$)	3	-29.3	-1.3	0.93	0.18	-2.0	-0.9	0.82	0.35
AVP (IP, 0.5 $^{\mu g}/_{gbw}$)	16	-10.0	-1.6	0.41	0.11	1.8	-1.1	-0.29	0.25
AVP (Brain, 1 $^{\mu g}/_{gbw}$)	2	-16.5	-0.9	0.95	0.37	-24.5	-0.9	0.95	0.37
AVP (IP, 5 $^{\mu g}/_{gbw}$)	5	-2.4	-1.4	0.66	0.18	-6.3	-0.8	0.43	0.41
AVP (Brain, 10 $^{\mu g}/_{gbw}$)	10	-17.8	-1.5	0.52	0.12	-8.2	-2.0	0.66	0.04 *
AVP (IP, 10 $^{\mu g}/_{gbw}$)	9	-2.4	-0.3	0.12	0.80	-6.3	-2.0	0.67	0.05 *
Manning (IP)	16	-32.4	-2.6	0.67	0.008 **	-5.8	-2.5	0.64	0.01 *
Saline (Brain)	8	-8.1	-0.6	0.25	0.53	1.5	-0.4	-0.15	0.73
Saline (IP)	19	6.9	-0.7	0.17	0.46	-1.9	-1.2	0.27	0.23



Supplemental Figure 4 Breathing rate and repeatable behaviors across all trials & constructs. Breathing rate decreased significantly following transfection of only *MAOA*. Charges increased following *AVP* or *MAOA* transfection but not in control *EYFP* fish. Graph presents median with interquartile range bars.

APPENDIX B: Experimental Oversight Approval

Institutional Animal Care and Use Committee
University of Illinois

TO: Alison Bell
FROM: Josh Gulley, PhD
DATE: Friday, May 11, 2018
SUBJECT: Approval of Animal Use Protocol

Your animal use protocol submission entitled, "Maternal and paternal effects on behavior in threespined sticklebacks," was approved by the Institutional Animal Care and Use Committee (IACUC) on Friday, May 11, 2018. The IACUC approval number for this protocol is 18080.

Please note that changes in the protocol, animal numbers, or personnel must receive approval by the IACUC.

This approval is valid for a three-year period, which expires on 5/10/2021. If work will continue beyond the expiration date, a new protocol will need to be submitted and approved by the IACUC prior to 5/10/2021. Additionally, federal regulations and campus policy require annual administrative review of protocols. You will receive notification from the IACUC prior to the deadlines for these reviews as well as for the protocol expiration.

If you have any questions, please do not hesitate to contact the IACUC staff.

Sincerely,

Josh Gulley, PhD
Chair, IACUC
University of Illinois at
Urbana-Champaign

Institutional Biosafety Committee
Division of Research Safety
102 Environmental Health and Safety Building
101 South Gregory Street
Urbana, Illinois 61801-3070

TO: Bell, Alison Marie, Evolution Ecology Behavior
FROM: Institutional Biosafety Committee
DATE: 4/12/2017
SUBJECT: Approval – Developing methods for viral-mediated transfer into the brain of sticklebacks, IBC-4140

Reason for Review:

Viral Recombinant or Synthetic Nucleic Acids: replication deficient HSV-1

Transgenic Vertebrate Animals: none

Human/Animal Pathogens: replication defective HSV-1

NIH Category(ies):

Section III-D:

- D-3 Use of Infectious recombinant or synthetic nucleic acids or RNA Viruses or Defective DNA or RNA Viruses in the Presence of Helper Virus in Tissue Culture Systems. (recombinant or nucleic acids with http://osp.od.nih.gov/sites/default/files/NIH_Guidelines.html#_Toc351276297 and NIH Guidance document on Lentiviral Vectors: http://osp.od.nih.gov/sites/default/files/resources/Lenti_Containment_Guidance_0_0.pdf.)
- D-4 Experiments that involve the transfer of recombinant or synthetic nucleic acids into any non-human vertebrate or invertebrate animal. Experiments generating transgenic animals (except rodents at BL-1: Section III-E-3) or testing viable recombinant or synthetic nucleic acids microorganisms on whole animals.

Required Containment Level(s) for This Project:

Laboratory Work:

- Biosafety Level 1
- Biosafety Level 2

Vertebrate Animal Work:

- Animal Biosafety Level 1
- Animal Biosafety Level 2