Viral-mediated transgenesis of MAOA and AVP increases territorial aggression in stickleback

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Running Title

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Key Words

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Major Findings

Forced expression of MAOA or AVP resulted in more attacks, showing a causal link between genes and behavior.

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Introduction: 1158
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Abstract

Establishing a causal relationship between genes and social behavior is challenging. To enable direct manipulation of candidate genes and thereby examine how their expression contributes to behavior, we developed a neurosurgical method to deliver pharmacological agents or transgenic elements directly into the threespine stickleback (*Gasterosteus aculeatus*) brain. Threespine sticklebacks are a classic system for the study of behavior, ecology, and evolution. Male sticklebacks defending nesting territories are highly aggressive toward intruders. Previous studies in stickleback have shown that aggression is heritable, and that hundreds of genes are differentially expressed in the brain following territorial intrusion.

We use viral-mediated transgenesis to test the effects on territorial aggression of overexpression of candidate genes, monoamine oxidase (MAOA) and arginine vasopressin (AVP), in the stickleback brain. Male sticklebacks received transcranial injections of mammalian homolog cDNA packaged in a replication-deficient Herpes Simplex Virus 1 carrier. Animals transfected with either AVP or MAOA constructs were more aggressive in response to a territorial intruder, unlike control animals transfected with a fluorescent protein.

Viral-mediated transgenesis is a promising method to examine genetic underpinnings of behaviors. Our success demonstrates that widely available mammalian plasmids work with this method, lowering the barrier of entry to the technique. This method is flexible, fast, and amenable to statistically powerful within-subject experimental designs, making it practical for use in natural populations. It further enhances the growing molecular toolkit in threespine stickleback, a classic ethological system, and is the first step toward using chemogenetics and optogenetics.

Introduction

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. However, 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. We developed one such method, viral-mediated transgenesis, for the classic ethological system of threespined stickleback (*Gasterosteus aculeatus*).

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 (Huntingford & Ruiz-Gomez 2009), sticklebacks are now are gaining popularity in other fields including evolution, physiology, and comparative genomics (Fang *et al.* 2018). 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) with both emerging and classic model systems. Indeed, there are already hundreds of previously identified candidate genes for social behavior waiting to be characterized (Sanogo *et al.* 2011; Laine *et al.* 2012; Greenwood *et al.* 2013; Mommer & Bell 2014; Greenwood & Peichel 2015; Bell *et al.* 2016; Bukhari *et al.* 2017) and well-established behavioral assays (van Iersel 1953; Rowland 1982) that are amenable to automation (Ardekani *et al.* 2013; Norton & Gutiérrez 2019).

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 stickleback brain. There is a dearth of information 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 (Zou *et al.* 2014). 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).

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. Here we employ this neurosurgical method 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 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 (Takahashi & Miczek 2014; Freudenberg *et al.* 2016; Malki *et al.* 2016; Waltes *et al.* 2016). Many subprocesses including perception, motivation, and cognition (O'Connell & Hofmann 2011b; 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 (O'Connell & Hofmann 2011a), which has good functional homology across vertebrate taxa. Finally, aggression is experimentally tractable in stickleback fish as it is heritable

(Bakker 1994; 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 *et al.* 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 *et al.* 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 *et al.* 2006). Nesting male sticklebacks have an increase in vasotocin levels in their brains following a mirror (aggression) challenge (Kleszczyńska *et al.* 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 (Huffman *et al.* 2012; Albers 2015; Kagawa *et al.* 2016), where it is an active regulator in the Hypothalamic-Pituitary-Adrenal (HPA) axis (Arnett *et al.* 2016). Therefore, we hypothesized that supplemental expression of arginine vasopressin within the socio-behavioral network of the stickleback brain would increase aggression because

123 vasotocin regulates the HPA axis through adrenocorticotropic hormone (ACTH) signaling to gonadal
124 hormones.

125 Monoamine oxidase, our other candidate gene, has a longstanding association with aggression
126 (Godar *et al.* 2016), not only in model systems but also in humans (Brunner *et al.* 1993). In humans,
127 MAOA levels are inversely correlated with aggression (Alia-Klein *et al.* 2008). Further, low MAOA
128 activity is associated with increased aggressive response following provocation (Gilad *et al.* 2002),
129 antisocial outcomes (Ouellet-Morin *et al.* 2016), and sex-related aggressive crimes in the case of
130 complete deficiency (Brunner *et al.* 1993). Mice with no MAOA activity showed increased fearfulness
131 as juveniles and increased aggression in adult males (Cases *et al.* 1995). Recent work has suggested that
132 MAOA allelic variants may be related to the domestication of dogs, a process which included a marked
133 decrease in aggression (Sacco *et al.* 2017). Teleosts have only one monoamine oxidase gene (MAO) as
134 opposed to the two found in mammals (MAOA and MAOB). Stickleback MAO
135 (ENSGACT00000012444.1) and mouse MAOA (NP_776101.3) have 68% conservation at the protein
136 level. Despite the low level of conservation, the teleost monoamine oxidase gene has been shown to
137 influence aggression (Freudenberg *et al.* 2016; Malki *et al.* 2016; Quadros *et al.* 2018) and is
138 functionally comparable (Shih *et al.* 1999; Arslan & Edmondson 2010). Since a low level of monoamine
139 oxidase is associated with increased aggression, increased expression of MAOA was expected to
140 decrease aggression through serotonin turnover.

141

142 **Materials and Methods**

143 *Animals*

144 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 from April to October and otherwise an 8:16 (L:D) "non-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 3 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.

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 1): 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, Beijing, China). These were cloned into the pDONR221 backbone (Epoch Life Science, Missouri City, TX) and then packaged (Gene Delivery Technology Core, Massachusetts General Hospital, Boston, MA) 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.

Three promoters were piloted to drive gene expression based on work in zebrafish (Zou *et al.* 2014) – a long-term promoter (hCMV, $N = 43$, Figure 2) 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 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.

Neurosurgical injection and surgical rig

In a ten-minute neurosurgical procedure, a suspension of foreign material (saline, HSV with construct, or pharmacological agents) was injected into the anterior diencephalon of the brain via a

transcranial injection. For this procedure, we developed a custom-built surgical rig (Figure 3). The complete parts list along with assembly instructions are publicly available through the Open Science Framework (https://osf.io/sgpvm).

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, Fisher) 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 ~600nl of construct to the anterior diencephalon. During each injection, ~100nl was delivered at three different depths (≤ 2.5mm), ensuring broad expression throughout the diencephalon. The stickleback brain is visualizable through the skull (Supplemental Figure 2), allowing injection sites to be selected with moderately high precision. Viral construct was injected using a 5 μL borosilicate syringe (Hamilton Neuros model 75, #65460-02, Reno, NV). 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. 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. After two days, fish were removed from the ABSL-2 surgical room to individual tanks.

Pharmacological treatments

Exogenous [Arg8]-Vasotocin (Genscript RP10061, Piscataway, NJ) was administered either directly into the brain via injection using the neurosurgical protocol described above or systemically via intraperitoneal (IP) injection using a 30G (0.312 mm OD) insulin needle. Because behavioral response has been reported to differ in teleosts based on dosage (Santangelo & Bass 2006; Gonçalves & Oliveira 2011), a dose-response curve (0.5, 5, and 10 µg per gram body weight) was tested. Manning compound (Bachem H-5350.0001, VWR), 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.

R statistical 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” for the injection optimization and as “Behavioral experiments scripts for release.R” for the viral-mediated transgenesis. 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 =1.65e-74). 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). 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

Neurosurgical injection

Fish generally returned to normal swimming and water column use within 15 minutes after removal of anesthesia. Initial piloting and optimization of the transcranial injection procedure ($N = 62$) revealed breathing rate followed a typical pattern after the operation which we called a recovery curve (Figure 4). Breathing rate peaked about 30 minutes post-surgery and returned to baseline levels by 90 minutes after the neurosurgical procedure. 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) or recovery ($ATS_{1,\ 7465} = 0.94$, *p-value* = 0.33, Supplemental Figure 3). 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. Full mating behavior occurred within three days post-surgery for males, determined by nesting behavior, and within nine days for females, determined by the presence of eggs.

Fish had comparable outcomes regardless of the type of injected materials (χ^2 (3, N_{HSV1} = 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 refine the surgical technique; they counted 39 survivors among 48 fish, an 81% survival rate.

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). The choice of promoter did not alter survival rate (χ^2 (2) = 1.38, *p-value* = 0.50, Table 1A), nor was there a main effect of promoter ($ATS_{2,\ 143}$ = 0.33, *p-value* = 0.70) on the recovery curve. Using the hCMV promoter, fluorescent protein expression was detected up to five weeks after injection. Additionally, the specific gene being expressed had no effect (χ^2 (3) = 2.16, p = 0.54, Table 1B) 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 4).

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 5). 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 1). Intraperitoneal injection of exogenous

vasotocin also resulted in a rapid increase in breathing rate (Supplemental Figure 5). Behaviorally, brain and IP injections of exogenous vasotocin produced parallel results (Supplemental Table 2), in which only the highest dosage (10 µg per gram body weight) altered the number of charges directed at the intruder.

Behavioral repeatability and intercorrelations

Repeatability was analyzed across the two trials at each timepoint (baseline and transfected). Only behaviors that were consistently repeatable, i.e. at both baseline and following transfection but not necessarily between the timepoints (Supplemental Table 3), were considered in subsequent analysis. 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 (Supplemental Table 3). 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 orient was not significantly different for any construct (Supplemental Figure 5). 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 6A) and following transfection of either AVP (Figure 6B) or MAOA (Figure 6C). 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.

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: $rs = 0.79$, $Z = -3.07$, $p\text{-value} = 0.001$) 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 7), with 13 of 20 individuals increasing their average number of 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 8). 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 20s. 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 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

Neurosurgical procedure & viral-mediated transgenesis

We present a new method for direct injection of transgenic or pharmaceutical material into the brains of the small teleost fish threespined stickleback. Developing a minimally invasive neurosurgical protocol required 1) refining the anesthesia process, 2) building a custom surgical rig, and 3)

322 determining the normal recovery pattern allowing us to clearly identify warning signs of failure to
323 thrive. Our surgical rig and optimized anesthetization methods (Neiffer & Stamper 2009; Sladky &
324 Clarke 2016) resulted in high (90%) survival rates and quick behavioral recovery. Mating behavior also
325 recovered promptly: males completed nests at three days post-surgery, and females were gravid at nine
326 days – suggesting almost no delay in the egg development time (Baker *et al.* 2008) after losing any ripe
327 eggs to clamping during surgery.

328 Exogenous vasotocin administered directly to the brain produced physiological and behavioral
329 responses mirrored in fish receiving vasotocin through IP injections (Supplemental Table 2). These
330 pharmacological results were similar to those seen in other fish (Lema & Nevitt 2004; Santangelo &
331 Bass 2006; Filby *et al.* 2010). This indicates that brain injection is now a feasible delivery route for
332 drugs that do not pass through the blood brain barrier (Cook *et al.* 2009).

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

341 Viral-mediated transgenesis is a method to alter a gene's expression in a specific location or
342 during a controlled timeframe. This approach has already proved essential in the functional testing of
343 genes related to behavior in rodents (Simonato *et al.* 2000) and in the dissection of neural circuits (Luo
344 *et al.* 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 *et al.* 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 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 (Huntingford & Ruiz-Gomez 2009; Hendry *et al.* 2013; Fang *et al.* 2018). Previous studies have identified hundreds of genes that are differentially expressed in the brain in response to a social interaction (Sanogo *et al.* 2011; Laine *et al.* 2012; Greenwood *et al.* 2013; Mommer & Bell 2014; Greenwood & Peichel 2015; Bell *et al.* 2016; Bukhari *et al.* 2017). 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.

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 *et al.* 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 (Forstmeier & Schielzeth 2011; Button *et al.* 2013; Fanelli & Ioannidis 2013; 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 (σ^2 = 99.9) compared to breathing rate (σ^2 = 3.9). A between group comparison would have required an impractical sample size of as many as 300 fish (Table 2) 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.

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 (Santangelo & Bass 2006; Gonçalves & Oliveira 2011), purportedly depending on dosage, species, or receptor localization. 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 main responding aggressive behavior. This matches the effects of vasotocin seen in pupfishes (Lema & Nevitt 2004). Here, we found that viral-mediated transfection was more robust than either IP or direct brain injection of exogenous vasotocin. 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 7). This is not entirely surprising given vasopressin's extremely short half-life (<1 minute in the rat brain (Stark *et al.* 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 (Bell 2005; Dingemanse *et al.* 2007; Keagy *et al.* 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 (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 7). 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 8) is strong evidence of MAOA functioning as expected physiologically. Breathing rate correlates positively with serotonin and norepinephrine concentration (Whelan & Young 1953; Hodges & Richerson 2008); monoamine oxidase enzymatically lowers levels of both neurotransmitters. Additionally, in accordance with our findings, previous RNAseq data from nesting male sticklebacks (Bukhari *et al.* 2017) shows a similar if non-significant positive correlation between aggression and MAO expression (Figure 9). 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; Keagy *et al.* 2016); 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.

Conclusion

We present a method for brain injection and 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 threespined 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.

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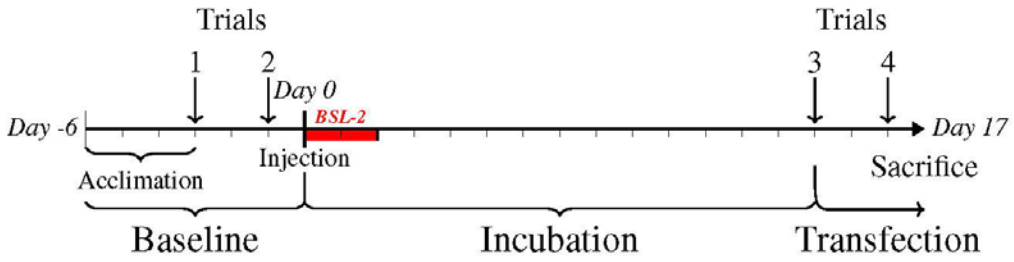
677 **Figures**



678

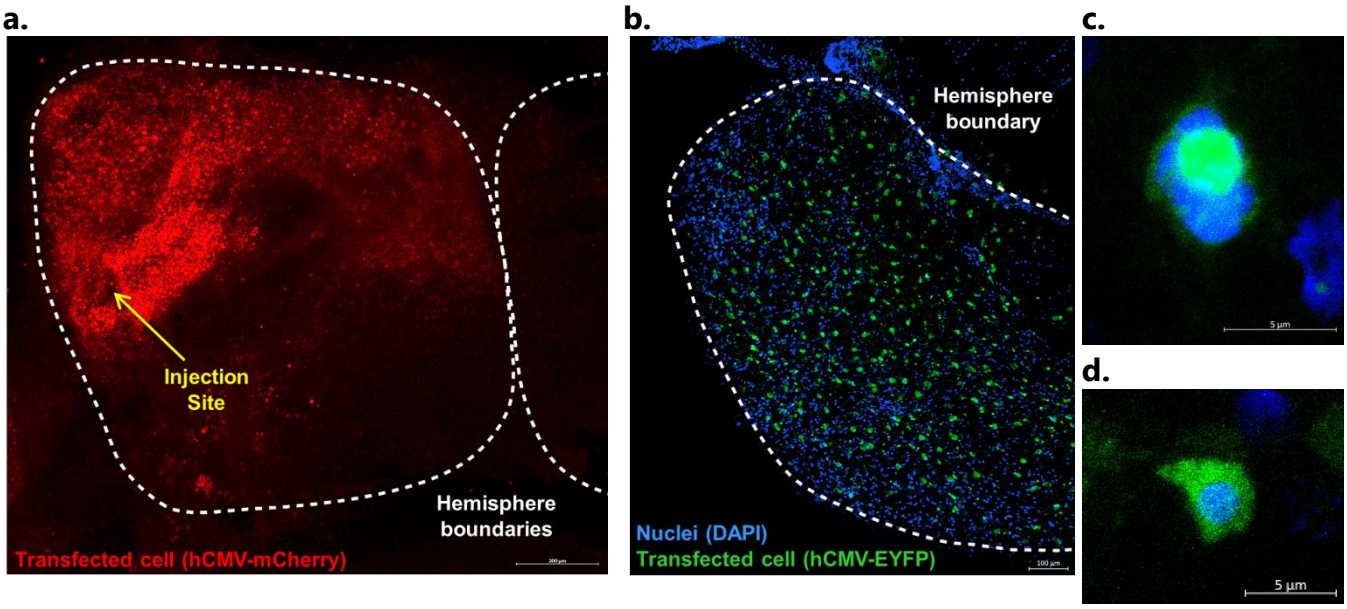
679 **Figure 1**. *Experimental timeline with the injection of constructs on day 0. Fish were injected with a*

680 *randomly assigned construct of either an aggression-related gene (MAOA or AVP) or a control*

681 *fluorescent protein (EYFP). All trials were conducted double-blind to the transfected gene. Each trial*

682 *had two breathing rate measurements followed by a territorial challenge.*

683



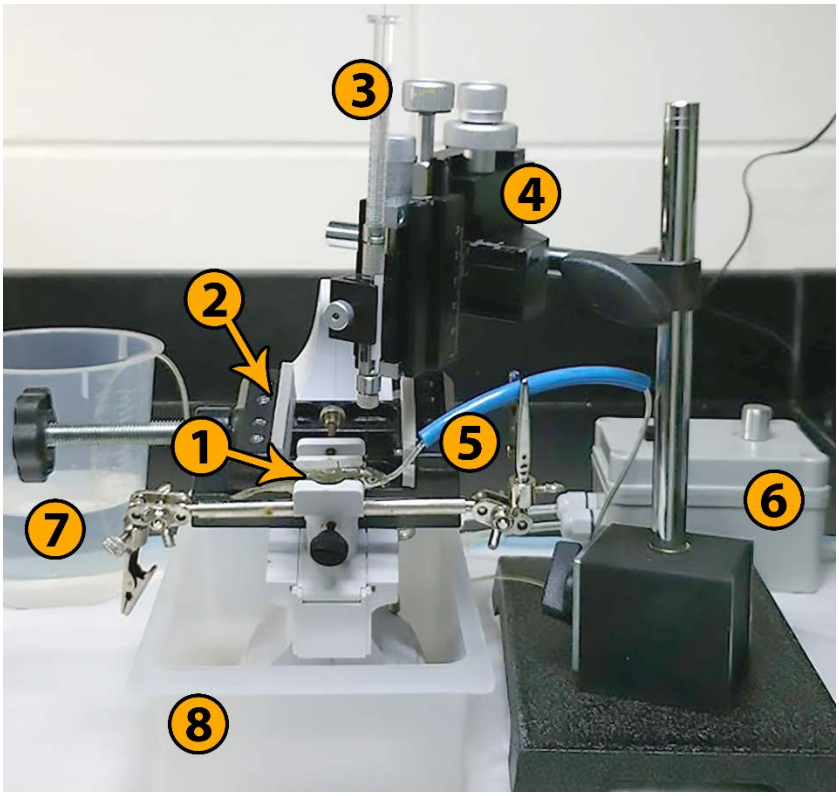
684 **Figure 2.** a) *Single injection resulting in local expression, limited to a portion of one hemisphere of the*

685 *telencephalon.* b) *Broad expression throughout left hemisphere of the diencephalon, typical for*

686 *injections with delivery at multiple depths.* c & d) *Successful transfection of entire cells by the long term*

687 *hCMV-EYFP construct in the lateral left diencephalon three weeks after injection.*

688



689

690 **Figure 3**. *Custom-built surgical rig.* 1) *Threespine stickleback in padded clamp,* 2) *Alternative padded*

691 *clamp for larger fish,* 3) *Neuros syringe, 5 µL,* 4) *Three-axis manipulator,* 5) *Oral cannula and guide*

692 *tube,* 6) *Peristaltic cannula pump, 100 mL/min,* 7) *Pump source reservoir,* 8) *Drip tray*

693

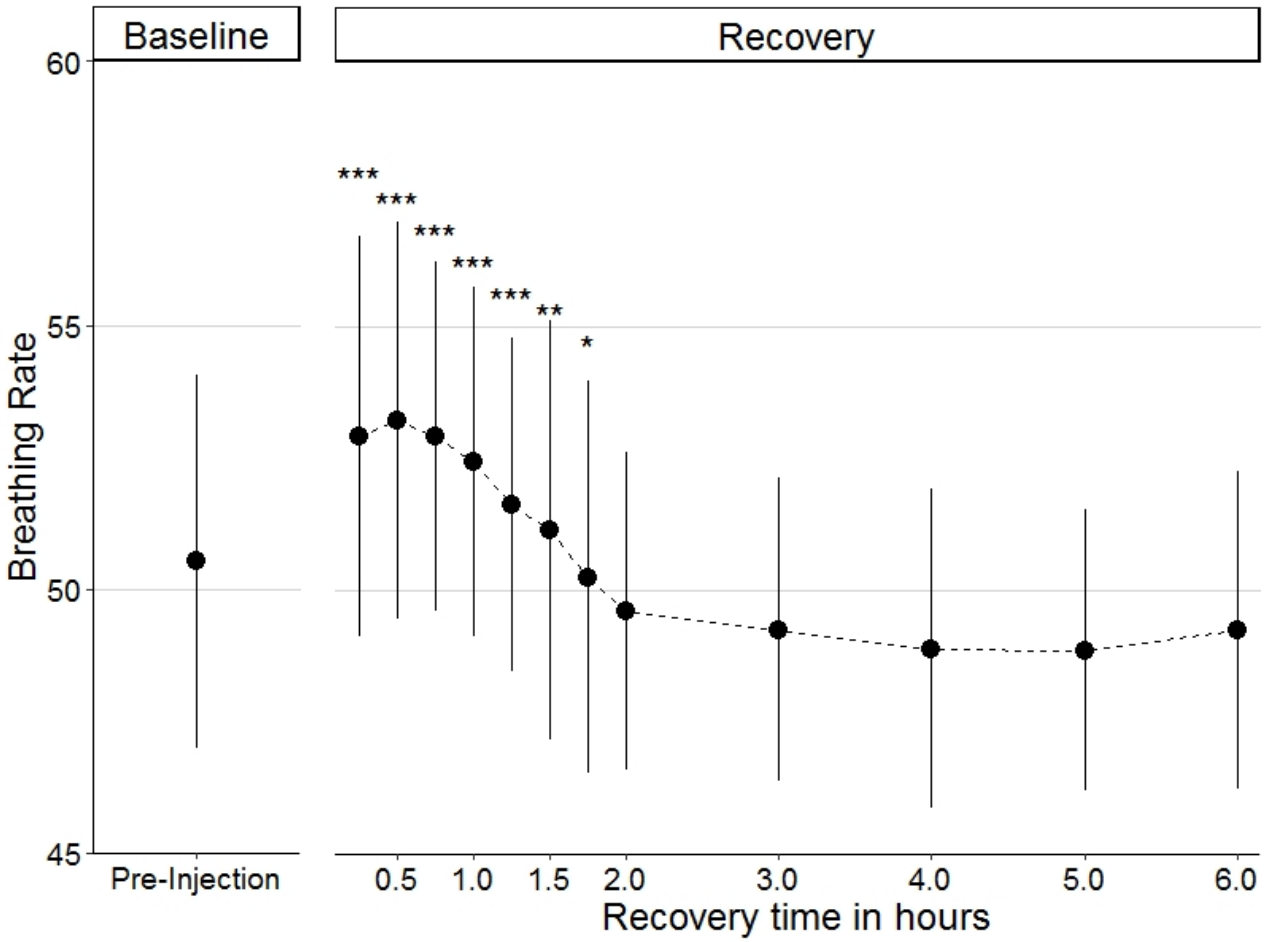


Figure 4. *Average recovery curve of breathing rate (opercular beats per 20s) following transcranial brain injections (N = 62). Breathing rate returned to baseline levels by 1.5 hours post-injection and remained stable following the surgery.*

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

699 **Table 1**. a) *Survival rate was not significantly affected by the choice of promoter*. b) *No significant*

700 *difference in the survival rate for any expressed genes relative to control fish injected with saline.*

701

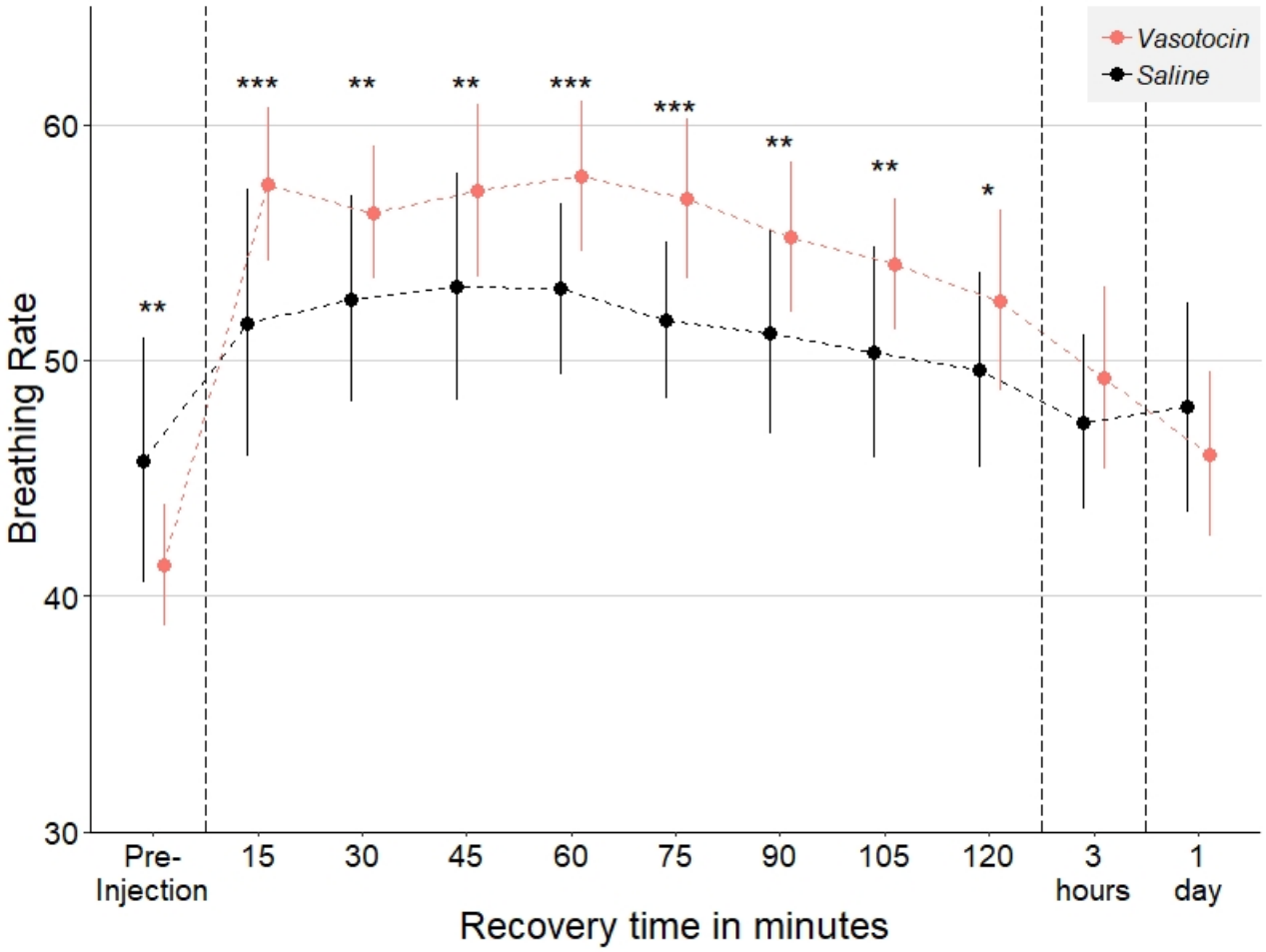


Figure 5. *Differences in breathing rate following injection between brain injection of exogenous vasotocin (N = 15) and saline injected controls (N = 39). Fish injected with vasotocin had an elevated breathing rate compared to saline injected controls for more than two hours following injection.*

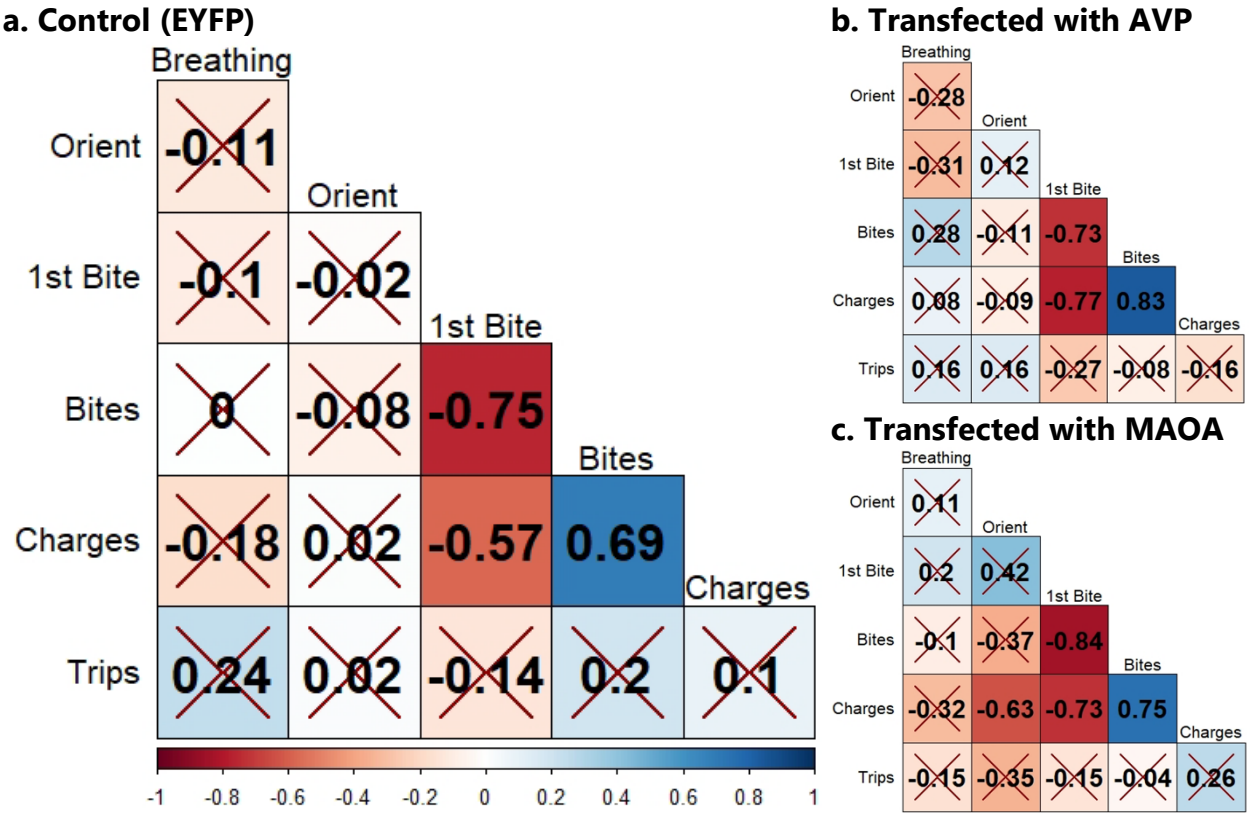


Figure 6. a) *Correlation between all measured behaviors in control (EYFP) fish averaged across all four trials (N = 16). Bites, charges, and time to first bite remain correlated following transfection of* b) *AVP (N = 18) and* c) *MAOA (N = 20). Numerical values are the strength of the correlation with crossed out boxes indicating non-significance.*

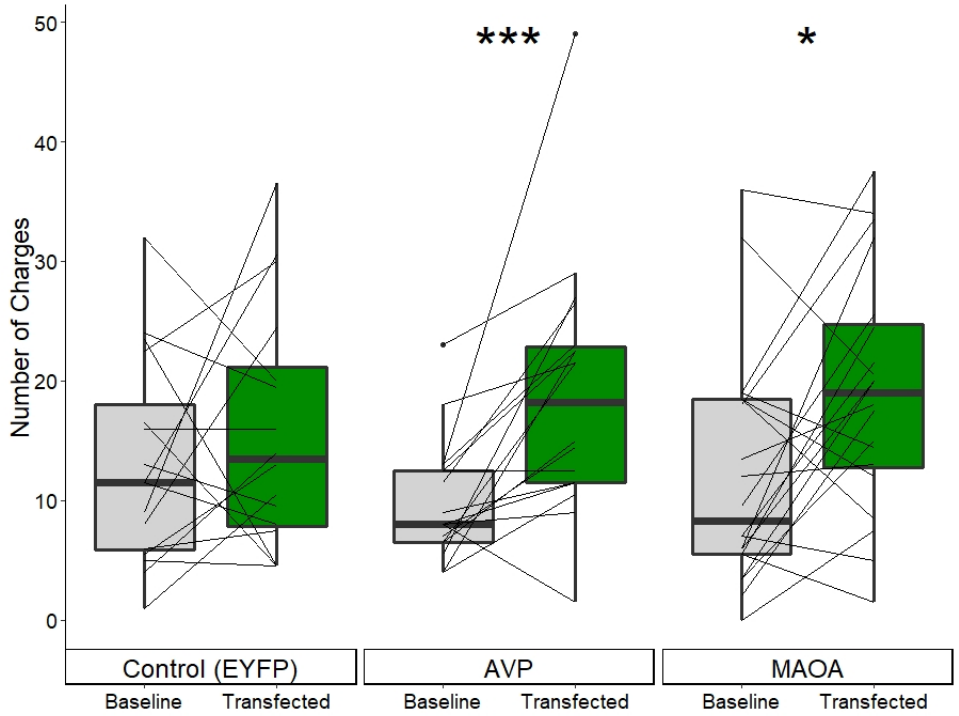


Figure 7. *Number of charges (averaged across the two trials as each timepoint) before and after transfection for each construct. Each line represents an individual showing their change in behavior following transfection of the gene of interest. Expression of AVP resulted in a substantial and consistent increase in the number of charges; note that only one individual exhibited decreased charging behavior. Expression of MAOA resulted in an increase of large effect size in charges, although there was more variation in individual response.*

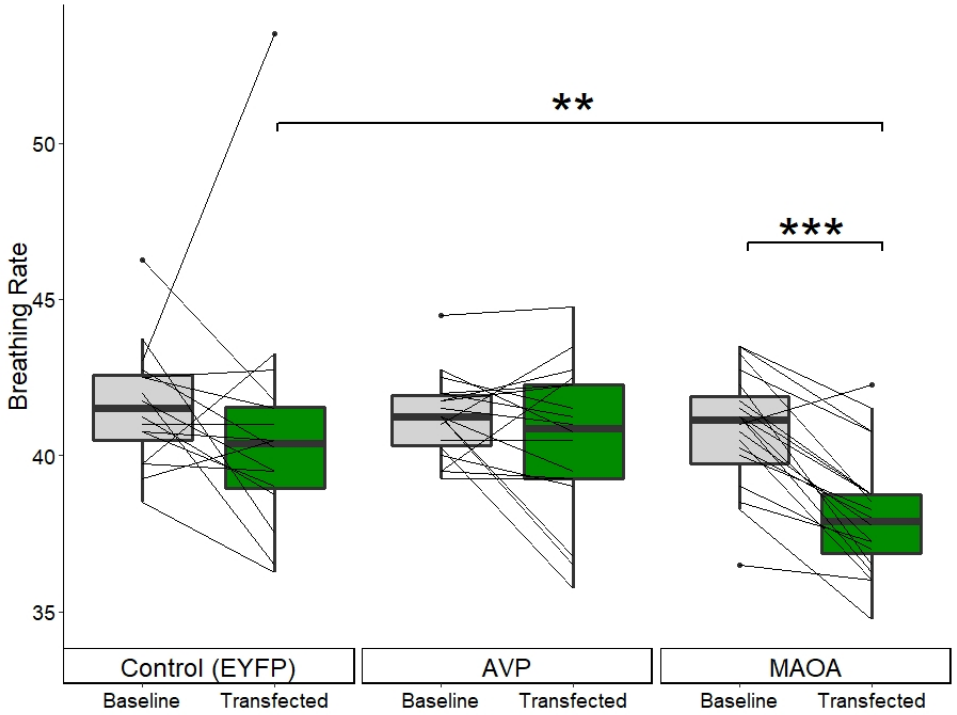
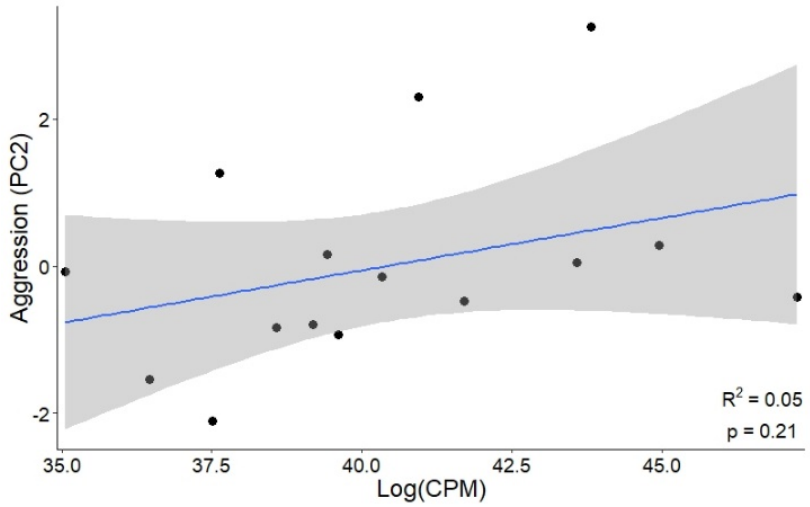


Figure 8. *Breathing rate measured by opercular beats for the three constructs. Each line represents an individual showing their change in breathing rate, averaged across the two trials at each timepoint, before and after transfection. Only MAOA altered breathing rate; a drastic decrease compared to both baseline (within-subject comparison, N = 20) and to the control group (N = 16).*

Comparison	Behavior ***Charges*** **AVP**	**MAOA**	Physiology ***Breathing*** **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

728 **Table 2**. *Necessary sample sizes assume a statistical power of 0.8 and are based on the effect sizes*
729 *observed in this study – i.e. 8 out of 10 experiments using samples this large will detect the difference.*
730 *Viral-mediated transgenesis makes possible a repeated measures design comparing within the same*
731 *individuals, increasing sensitivity. Note that AVP and MAOA are known to have a large effect on*
732 *behavior, so this should be viewed a minimum for future candidate genes being screened for behavioral*
733 *phenotypes.*

734



735

736 **Figure 9**. *Correlation between increased aggression and increased expression of MAO.*

737