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Abbey-Lee, R. N., Uhrig, E., Zidar, J., Favati, A., Almberg, J., Dahlbom, J., Winberg, S., Løvlie, H., (2018), The Influence of Rearing on Behavior, Brain Monoamines, and Gene Expression in Three-Spined Sticklebacks, *Brain, behavior, and evolution*, 91(4), 201-213.
https://doi.org/10.1159/000489942

The influence of rearing on behavior, brain monoamines and gene expression in three-spined sticklebacks

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Running title: Rearing alters behavior and monoamine gene expression

Highlights:

- early environment conditions influence behavior later in life
- early environment conditions alter monoamine associated gene expression
- mechanistic pathways linking these responses are still unclear

Abstract

1. The causes of individual variation in behavior are often not well understood, and potential underlying mechanisms include both intrinsic and extrinsic factors, such as early environmental, physiological, and genetic differences.
2. In an exploratory laboratory study, we raised three-spined sticklebacks (*Gasterosteus aculeatus*) under 4 different environmental conditions (simulated predator environment, complex environment, variable social environment, and control). We investigated how these manipulations related to behavior, brain physiology and gene expression later in life, with focus on brain dopamine and serotonin levels, turnover rates, and gene expression.
3. The different rearing environments influenced behavior and gene expression, but did not alter monoamine levels or metabolites. Specifically, compared to control fish, fish exposed to a simulated predator environment tended to be less aggressive, more exploratory, and more neophobic; and fish raised in both complex and variable social environments tended to be less neophobic. Exposure to a simulated predator environment tended to lower expression of dopamine receptor DRD4A, a complex environment increased expression of dopamine receptor DRD1B, while a variable social environment tended to increase serotonin receptor 5-HTR2B and increased serotonin transporter SLC6A4A expression. Despite both behavior and gene expression varying with early environment, there was no evidence that gene expression mediated the relationship between early environment and behavior.
4. Our results confirm that environmental conditiaons early in life can affect phenotypic variation. However, the mechanistic pathway of the monoaminergic systems translating early environmental variation into observed behavioral responses was not detected.

Keywords: dopamine, fish, novel arena, novel object, personality, serotonin

1. Introduction

Aspects of the environment faced by young animals (from embryos to juveniles) can have important subsequent effects on the phenotype [Jonsson, and Jonsson, 2014], with influences on reproductive success and fitness [Harrison et al., 2011]. A classic example is the 'silver spoon' effect where positive conditions during development have long-term positive effects on adult life [Monaghan, 2008]. Recently, research has focused on how such early environmental aspects, particularly aspects of environmental complexity and stress, can lead to consistent differences among individuals [Stamps, and Groothuis, 2010; Gudsnuk, and Champagne, 2011]. Exposure to predators can cause a variety of behavioral responses in their prey [Lima, 1998], such as aggressiveness [Bell, and Sih, 2007] and decreasing activity levels [Lima, and Dill, 1990; Lima, 1998]. Additionally, more complex physical or social environments can alter individuals' behavior [van Praag et al., 2000; Christensen, and Nielsen, 2004; Lazic et al., 2007; Patzke et al., 2009], where individuals exposed to more complex environments tend to increase in exploratory behavior and decrease in neophobia [Christensen, and Nielsen, 2004]. Further, aspects of early social environment can influence exploration [Naguib et al., 2011]. Despite these findings that a variety of environmental aspects influence behavioral responses, little is known about the mechanisms translating early environmental variation into behavioral differences later in life.

Of increased focus in animal behavior is individual variation that shows among-individual consistency (a.k.a. animal personality, coping styles, behavioral types [Dall et al. 2004; Carere, and Maestripieri, 2013]). Understanding the mechanisms by which such behavioral types arise and are maintained is of fundamental interest to behavioral ecologists because such differences challenge the traditional view that individuals should adopt situation-specific, adaptive responses [Krebs, and Davies, 1997; Dall et al., 2004; Stamps, 2007; Carere, and Maestripieri, 2013]. However, such mechanisms remain largely unclear although both environmental and genetic factors have been postulated to influence behavior [Dall et al., 2004; Stamps, 2007; van Oers, and Mueller, 2010]. Thus, in order to understand the underlying mechanisms, integrative experimental studies relating environmental variation to phenotypes to (epi)genetics and physiology are needed [van Oers, and Mueller, 2010; Roche et al., 2012].

Monoamine neurotransmitters (dopamine, serotonin, adrenaline) are released from neurons in both the brain and peripheral nervous system. All functions of these monoamines are still not clear, but they are thought to be important to many aspects of behavior, and limited previous work suggests that at least some consistent behavioral variation is linked to monoaminergic systems [Winberg, and Nilsson, 1993; Coppens et al., 2010; Carere, and Maestripieri, 2013]. Variation in metabolite levels, methylation, and gene polymorphisms for dopamine and serotonin have been linked to consistent behavioral variation [Carere, and Maestripieri, 2013]. Specifically, low serotonin levels are negatively associated with aggressiveness in several species [Bell et al., 2007; Carere, and Maestripieri, 2013] and polymorphisms in serotonin transporter genes are related to aggression, anxiety, and impulsivity in primates [Carere, and Maestripieri, 2013]. Dopamine levels, polymorphisms in dopamine receptor and transporter genes, and differential methylation of dopamine associated genes are related to novelty-seeking and exploratory behavior in mammals and birds [Schinka et al., 2002; Fidler et al., 2007; van Oers, and Mueller, 2010; Carere, and Maestripieri, 2013; Holtmann et al., 2016]. Additionally, these neurotransmitters have long been linked to reward and motivation systems across taxa, and are the primary proposed mechanism [Zuckerman, 1996]. Despite these promising links, empirical studies, particularly in non-mammalian species, are still rare, as are studies that examine more than one gene or test causational hypotheses.

We designed an experiment to explore how aspects of early environment influence behavior, brain monoamine levels, metabolites, and expression of genes of the dopaminergic and serotonergic systems of the three-spined stickleback (*Gasterosteus aculeatus*) later in life. We designed three treatment groups capturing three of the main environmental effects postulated to influence behavior and personality: simulated predation-induced stress, increased habitat complexity, and a more variable social environment. We investigated how behavior and brain monoamines of individuals, as well as their relationship, are shaped by environmental manipulations. Our study design utilized an integrative approach in which behavior, brain monoamines, and monoaminergic system genes were assessed in the same phenotyped fish.

Sticklebacks are important models for behavioral research [Hendry et al., 2013], including research on animal personality [Bell, and Stamps, 2004; Dingemanse et al., 2007], physiology [Winberg, and Nilsson, 1992; Kitano et al., 2012], and genetics [Hohenlohe et al., 2010]. They are abundant in freshwater and marine habitats in the northern hemisphere where they live in coastal shoals outside of the breeding season. Stickleback are well suited to lab-based

research, making them the ideal model species for this study. We predicted that environmental enrichment will generate behavioral differences later in life. Specifically, we focused on biologically relevant behaviors. Sticklebacks are a mobile species and exploration is required to find both food and mates; aggression is likely important for both social interactions and for males to establish breeding burrows and attract mates; and neophobia is important for avoiding predators [e.g. Ostlund-Nilsson et al., 2007]. Based on previous work, we predict that exposure to predation stress increases aggressiveness [Lima, and Dill, 1990; e.g. Bell, and Sih, 2007] and neophobia [e.g. Elvidge et al., 2016], and decreases exploration [e.g. Abbey-Lee et al., 2016; Moses, and Sih, 1998; Hedrick, and Kortet, 2006]; exposure to habitat complexity increases exploration behavior and decreases neophobia [e.g. Christensen, and Nielsen, 2004]; and exposure to variable social environment increases aggression and decreases neophobia [e.g. Bannier et al., 2017, Naguib et al., 2011]. Additionally, we predict that monoamine levels, their metabolites, and the expression of genes related to monoaminergic transmission will vary in the brain depending on early environmental treatment, but, as the relationship between environment and monoaminergic systems are not well understood, we have no directional predictions [Winberg, and Nilsson, 1992; Carere, and Maestripieri, 2013]. Finally, we expect that monoaminergic systems will be the mechanistic link between early life environmental variation and behavioral variation [Coppens et al., 2010].

2. Material and Methods

2.1 Study population

For this study, two families of experimental three-spined sticklebacks were generated in the lab from wild caught parents. To avoid inbreeding and ensure genetic diversity of offspring, parents came from different regions in Sweden, within the genetically uniform range of coastal populations in the Baltic Sea (F_{ST} = 0.003 (0-0.01)) [Mäkinen et al., 2006]. Fry hatched in July 2013 and were kept within families without parental care, and in small aquariums (ca 10 x 20 cm) at approximately 19°C under 16:8 light:dark conditions with 0.05 % salinity. At two weeks of age, within families, the fish were moved to groups of 15 individuals in 27 L tanks (ca 40 x 27 x 27 cm). Tanks had sand substrate, two plastic plants, filters, and were half-covered with black lids and siding providing isolation from the environment (basic conditions). Tanks initially housed 15 individuals from the same family,

but due to early fry mortality individuals were re-allocated to smaller groups, maintaining 4 replicates (two per family) of each of the 4 treatments (fish were 23-26 days old; $N_{family1} = 31$, $N_{family2} = 49$). Fish were fed newly-hatched live artemia and, at 12 weeks of age, were fed defrosted frozen red bloodworms. Water quality was regularly tested and changed bi-weekly, but more often if necessary. From week 20, the light:dark cycle was changed to 12:12. After the first set of behavioral tests around 6 months of age (see below), all fish were tagged with elastomer implants (Northwest Marine Technologies, Shaw Island, Washington) of unique color combinations to facilitate individual identification. Fish were also measured in length to the nearest mm. Salinity, temperature, and day length were all kept within the natural range sticklebacks are exposed to in Sweden.

2.2 Treatments

The fish were kept under 4 different conditions for their entire rearing from two weeks of age until sacrifice: simulated predator stress, complex environment, social stress, and control. For the predator stress treatment ('predator stress'), sticklebacks were chased with a dip net three times a week for approximately 2 minutes at random points during the day in order to simulate predator-related stress. For the complex environment treatment ('complex'), fish were kept in enriched tanks with additional aquarium decorations (stones, tree branches, plastic plants, and standard aquarium decorations) changed weekly to maintain environmental novelty and complexity. The number of pieces in the tank remained constant (4-5), but the type of decoration varied weekly. For the social stress treatment ('social stress'), three fish were switched between tanks, within the same family, weekly creating changes to their social environment. The aquaria with the control treatment ('control') were kept in the basic conditions described above. All fish were identifiable via unique elastomer tags (Northwest Marine Technologies, Shaw Island, Washington) so that the identity of fish was known.

2.3 Behavioral assays

At approximate 6 months of age (January 2014), a subsample of fish (N = 47, 10 complex, 7 predator stress, 15 social stress, 15 control; $N_{family1} = 16$, $N_{family2} = 31$) were individually caught with nets from the home aquarium and immediately placed in a novel arena and monitored for 10 minutes. Immediately after this, aggressive behavior was assessed (see below) for an additional 10 minutes. At 7 months of age the process was repeated on the same individuals and a measure of neophobia was also measured immediately after aggression (see below). All data were recorded by an observer blind to rearing treatment. The observer stood

approximately 2 m from the tank facing the long side, not disturbing the behavior of the fish. After the second round of behavioral tests, all fish were anesthetized (Benzocaine), weighed, and measured. In order to ensure all fish were in a similar physiological state at the time of sacrifice, and thus brains were directly comparable, all fish were stressed for >2 minutes via chasing with a dip net immediately prior to benzocaine application. We had mortality throughout our experiment, particularly in the predator and complex environment, thus sample sizes for these groups is lower for behavioral analyses. Additionally, since exposure to behavioral tests is itself as a form of enrichment, we kept a subset of untested fish in order to determine the general effect of exposing the fish to the behavioral testing on brain monoamines.

2.3.1 Novel arena test

Fish were introduced into a novel arena (NA, a test with similarities to open field tests), a tank similar to their home tank, but with differently colored and structured substrate (white finer sand instead of multi-colored brown, coarser sand, different types of plant decorations. Fish were dropped at one end of the test tank and observed for 10 minutes. The arena was visually divided into 6 lower and 6 upper regions and an 'area change' was recorded when the eye of the fish passed the imaginary line that divided two different areas. Initial response (Latency to swim NA) and proportion of time active (Rate active) were scored.

2.3.2 Mirror test

An aggression test took place in the aquarium used for the novel arena test. After the novel arena test, a mirror was placed in the middle of the tank, facing the fish. Mirror tests are often used to study aggression as individuals treat their mirror image as a conspecific that is size and behaviorally matched to the focal individual [e.g. Gallup, 1968; Andrews, 1996] and has been recommended for use in sticklebacks [Peeke et al., 1969; Kleszczynska et al., 2012]. Fish were observed for an additional 10 minutes, and the initial response (Latency to swim Aggression test) and the number of times the fish poked/attacked its reflection (Number of pokes) were recorded.

2.3.3 Novel object test

During the second round of behavioral tests at age 7 months, we added an additional test to determine neophobia of the fish. Immediately after the mirror test, fish were exposed to a novel object (NO, a multicolored plastic cup, approximately 10 cm h x 7 cm d), placed at the

opposite end of the aquaria from the fish. Fish were observed for 5 minutes. The initial response (Latency to swim NO) and time spent in the interaction zone (Time close NO) were recorded. The interaction zone was defined as 2 body lengths distance from the novel object. For this test, the sample size for control fish was only 10, due to time constraints.

2.4 Molecular analyses

After the second set of behavioral assays, all fish were sacrificed with an overdose of benzocaine (N = 76; 38 female, 38 male; 14 predator stress, 12 complex, 24 social stress, 26 control). To enable dissections within minutes after the fish was dead to reduce breakdown of monoamines, while still enable multiple molecular analyses of the same brain, brains were separated into forebrain and hindbrain, keeping the diencephalon with the forebrain [Dahlbom et al., 2012]. Brains were quickly snap-frozen (<3 min) on dry-ice and stored at -80^{o}C until quantification of monoamines. Genetic sex was determined via individual genotyping for a male-specific genetic marker using DNA extracted from tissue samples taken postmortem [Peichel et al., 2004]. This was done to avoid the additional stress of finclipping during the experiment.

2.4.1 Monoamine and metabolite variation

Hindbrains were used to quantify monoamine levels (serotonin: 5-hydroxytryptamine 5HT, dopamine: DA, norepinephrine: NE) and their metabolites (serotonin: 5-hydroxyindoleacetic acid 5HIAA, dopamine: 3,4-dihydroxyphenylacetic acid DOPAC). This tissue sectioning (hind- vs fore-brain) was done because all monoaminergic systems are displayed in all major brain areas of fish [Kaslin, and Panula, 2001]. We used high performance liquid chromatography with electrochemical detection (HPLC-EC) analyzes. Additionally, we calculated the ratio of metabolites to monoamines as a measure of turnover rates and activity of monoaminergic systems, where higher rates indicate a quicker metabolic pathway due to higher biosynthetic enzyme activity (for serotonin: 5HIAA/5HT, for dopamine: DOPAC/DA). Frozen hindbrains were homogenized with a Sonifier cell disruptor B-30 (Branson Ultrasonics, Danbury, CT, USA) in ice-cold perchloric acid 4% (weight/volume) containing 100 ng/ml 3,4-dihydroxybenzylamine (DHBA, the internal standard). To separate proteins from monoamines, the samples were centrifuged at 20 000 × g for 10 minutes at 4^{o}C. We carried out the HPLC-EC as described by [Overli et al., 1999]. In short, the HPLC-EC system consisted of a solvent delivery system model 582 (ESA, Bedford, MA, USA), an autoinjector Midas type 830 (Spark Holland, Emmen, the Netherlands), a reverse phase column (Reprosil-

Pur C18-AQ 3 µm, 100 mm × 4 mm column, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) kept at 40°C and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at reducing and oxidizing potentials of −40 mV and +320 mV. A guarding electrode with a potential of +450 mV was employed before the analytical electrodes to oxidize any contaminants. The mobile phase consisted of 75 mM sodium phosphate, 1.4 mM sodium octyl sulphate and 10 µM EDTA in deionized water containing 7% acetonitrile brought to pH 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations. DHBA was used as internal standard to correct for recovery using HPLC software Clarity™ (DataApex Ltd, Prague, Czech Republic). Brain monoamine and metabolite concentrations were normalized against protein weight, which was determined using Bicinchoninic acid protein determination (Sigma Aldrich, Sweden) following the manufacturer's protocol. The assay was read on a Labsystems multiskan 352 plate reader (Labsystems, Thermo Fisher Scientific) at a wavelength of 570 nm.

2.4.2 Monoaminergic system gene expression variation

Total RNA was extracted from forebrains for quantification of gene expression of various genes in the brain monoaminergic systems.We performed quantitative polymerase chain reaction (qPCR) analyses of serotonin and dopamine transporter genes (solute carrier family 6 member 4, SLC6A4A/B; dopamine active transporter 3, DAT3), receptors (5-hydroxytryptamine receptor 2B, 5-HTR2B; dopamine receptor 1, DRD1B, and dopamine receptor 4, DRD4A), and synthesizing enzymes (tryptophanhydroxylase 2, TPH2, the gene coding for the rate limiting enzyme in serotonin synthesis; tyrosine hydroxylase, TH, the gene coding for the rate limiting enzyme in dopamine synthesis). Total RNA was extracted using a Masterpure RNA purification kit (Epicentre, Nordic Biolabs, Sweden) following the manufacturer's protocol. RNA quality and quantity were checked with a Nanodrop 1000 (Thermofisher, Sweden). Four to five µg total RNA were DNAse treated (Turbo DNA free, Ambion, Invitrogen, Sweden). The RNA was again quantified with Nanodrop 1000 and an estimate of quality through absorbance ratios 260/280 and 230/280 nm was made. One µg of the DNAse-treated RNA was converted to cDNA using Maxima First Strand cDNA Synthesis Kits for RT-qPCR (Thermofisher, Sweden).

For the qPCR reaction, Dynamo Flash SYBR green qPCR kit (Thermofisher, Sweden) was used. Primer concentrations were 0.4 µM and primers were designed using primer3plus

(http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with 'qPCR' as special settings. Primers were accepted if they resulted in a single product of correct size as determined by a melt curve, as well as an agarose electrophoresis gel (1.5% in 0.5×TAE buffer, 45 min). All products were of expected/correct size except DRD4A, which was sent to Macrogen for sequencing and confirmation. Transcript sequences were taken from ensembl.org or NCBI. See supplementary information (S1) for primer sequences, amplicon lengths and accession numbers. The qPCR was run on an ABI Applied Biosystems 7900HT Fast Real-Time PCR system with the following protocol: 7 min at 95^{o}C, 40 cycles of 10 s at 95^{o}C, 20 s at 60^{o}C. A melt curve was done to make sure a single product was amplified. For relative quantification, the ΔCT method was used, comparing the target gene with the reference genes B-actin and ubiquitin.

2.5 Statistical analyses

All statistical analyses were performed with R version 3.3.1 (R Core Team, 2016). We applied linear mixed-effects models to analyze our data (detailed below), for which we used the 'lmer' function (package lme4) [Bates et al., 2014]. Additionally, we used the 'sim' function (package arm) [Gelman, and Su, 2016] to simulate the posterior distribution of the model parameters, and values were extracted based on 2000 simulations [Gelman, and Hill, 2007]. The statistical significance of fixed effects and interactions were assessed based on the 95 % credible intervals (CI) around the mean (β). We did not correct for multiple testing because we had clear predictions based on previous experiments [Nakagawa, 2004]. We consider an effect to be "significant" when the 95 % CI did not overlap zero [Nakagawa, and Cuthill, 2007]. Repeatability of behavioral responses were calculated using the 'rpt' function (package rptR) [Stoffel et al., 2017]. We used visual assessment of residuals to evaluate model fit.

2.5.1 Early environment and behavior

To test the effect of early environmental manipulation on behavior, we ran univariate linear and generalized linear mixed-effects models. Count data (number of pokes) was modeled using a Poisson distribution. All other variables were modeled using Gaussian distributions, latencies were log transformed to meet normality assumptions. Type of treatment (a single categorical variable with one of four levels: predator stress, complex, social stress, control), sex (m/f) and family (1/2) were added as fixed effects. Individual fish identity, observer, and tank of rearing were included as random effects. Individual identity was not added in models

analyzing Latency NO and Time close NO where we only have one measure per fish. Correlations between behavioral response variables and other potentially confounding factors such as test sequence (categorical variable, assay time 1 or 2) and fish size (length, continuous) were checked, and not included as they were very weak.

2.5.2 Early environment and brain monoaminergic systems

To test the effect of the different environmental treatments on monoamine levels, metabolites, and gene expression, we ran univariate linear mixed models. Again, type of treatment (predator stress, social stress, complex, control; categorical variable with four levels), sex (m/f) and family (1/2) were included as fixed effects. Additionally, size (length; continuous) and if the fish were behaviorally tested (binary) were included as fixed effects as these have been shown to influence behavior [Abrahams, and Cartar, 2000; Zidar et al., 2017]. Tank of rearing (Tank ID) was included as a random effect. All measures of gene expression are reported in the ΔCT method, where high values indicate lower expression.

2.5.3 Monoaminergic systems as mechanisms

To explore if monoaminergic systems were the mechanism translating early environmental differences into behavioral differences, we used mediation analyses and the 'mediation' package for R [Tingley et al., 2014]. We compared the relative weights of two pathways: a direct relationship between early environment and behavior, and an indirect relationship between early environment and behavior via monoamines (or their metabolites, or genes). This type of comparison allowed us to determine which pathway was more important. If the indirect pathway was stronger, we would state that the monoamines were important in translating environmental differences into behavioral differences. To decrease the number of tests run, we tested only behaviors and monoamines that were already found to be influenced by our environmental treatments (see results, Table 1). Specifically, we tested the effect of the predator treatment on aggression (Number of pokes) and tested if DRD4A mediated this relationship. We also explored the effect of the complex treatment on neophobia (Time close NO), and tested if DRD1B mediated this relationship. Finally, we explored the effect of the social treatment on neophobia (Time close NO), and if 5-HTR2B or SLC6A4A mediated this relationship.

3. Results

3.1 Early environment and behavior

We demonstrated that rearing environment tended to influence behavioral responses later in life (Table 1, Figure 1). Compared to control fish, fish exposed to simulated predator stress tended to have a shorter latency to swim in a novel arena and a longer latency to swim when presented with a novel object. Fish that were raised in a complex environment were more aggressive (performed more pokes at their mirror image) and less neophobic (spent more time near the novel object) than control fish. Fish raised in socially stressful environment tended to be less neophobic (spent more time near the novel object) than fish raised in a control environment. Males were more active than females and were quicker to swim in the novel arena. Families differed in latency to swim in a novel arena. We had no *a priori* predictions amongst manipulated groups, only between each manipulation and the control. However, our model structure allows for comparison among all treatment groups simultaneously in a single model, and thus we were able to determine that fish raised in a complex environment had a significantly longer latency to move in the novel arena than fish exposed to predator stress (relative β(95%CI) = -1.26 (-2.28, -0.22)).

As part of our models we could calculate the repeatability (ratio of within to among individual variance) of the traits measured twice (Table 1). We found that the proportion of time a fish was active in the novel area and the latency to swim during the mirror test were both repeatable, while the latency to first swim in the novel area was slightly repeatable. The number of pokes was not repeatable. The latency to swim with a novel object, and the time spent near the novel object were only measured once, thus repeatably could not be estimated.

3.2 Early environment and brain monoaminergic systems

Rearing environment did not significantly influence brain monoamine levels, or metabolites (Table 2) based on HPLC analysis of hindbrains. However, qPCR analysis of forebrains found that expression of genes in the monoaminergic systems tended to vary depending on early environmental experience. Specifically, DRD4A (dopamine receptor) tended to have lower expression in fish exposed to simulated predator stress. DRD1B (dopamine receptor) tended to have higher expression in fish raised in a complex environment. Finally, 5-HTR2B (serotonin receptor) and SLC6A4A (serotonin transporter) tended to have higher expression in fish raised with increased social stress. Additionally, comparisons amongst manipulated groups revealed that DRD4A had higher expression in fish raised in a complex environment relative to both fish raised with predator stress (relative β(95%CI) = +0.91 (0.25, 1.56)) and fish raised with social stress (relative β(95%CI) = +0.76 (0.23, 1.32)); and SLC6A4A had

higher expression in fish raised in a stressful social environment relative to fish raised with predator stress (relative β(95%CI) = +1.10 (0.43, 1.78)). (Note, values presented in Table 2 come from ΔCT method, where high values indicate lower expression.)

Sex, size, family, and if an individual was behaviorally assayed were related to differences in brain monoamine levels, metabolites, and gene expression (Table 2). Adrenaline levels tended to be lower in males, and in larger fish. Gene expression of the genes DRD4A, TH (dopamine synthesizing enzyme), and TPH2 (serotonin synthesizing enzyme) were lower in males. DRD4A and 5-HTR2B expression tended to decrease with increasing fish size. 5-HTR2B had higher expression, and TH tended to have higher expression in fish not behaviorally tested. Families tended to differ in SLC6A4A, 5-HTR2B, and DRD1 expression.

3.3 Monoaminergic systems as mechanisms

There was no evidence that the expression of monoamine related genes in the brain influenced the observed relationship between early environmental manipulations and behavioral responses later in life. All models only found support for the direct path between environmental treatment and behavior, with no support for the indirect path via gene expression (Table 3).

4. Discussion

Fish exposed to different early environment conditions differed in behavior later in life. Further, differential rearing tended to alter the expression of genes related to monoaminergic systems. However, our results did not support that the aspects of the brain monoaminergic systems measured were the mechanistic link between environmental manipulations and behavioral response in our study.

4.1 Early environment and behavior

Our results add to the growing body of research supporting that conditions in early life can alter behavior [reviewed by e.g. Stamps, and Groothuis, 2010]. More specifically, based on previous work on sticklebacks and other animals, we predicted neophobia would decrease for fish raised with increased simulated predator stress, habitat complexity, or social stress [e.g. Elvidge et al 2016, Christensen and Nielsen 2004, Naguib et al 2011, Bannier et al 2017]. We found that fish raised in in complex physical or socially stressful environments

were less neophobic, confirming our predictions, and matches findings also in birds [Christensen, and Nielsen, 2004]. Numerous other studies find that early social environment matters: birds raised in 'dynamic' flocks with varying individuals had larger singing networks and had greater mating success than individuals raised in stable flocks [White et al., 2010], rodents raised in social groups were less anxious [Curley et al., 2009; Cirulli et al., 2010], and social aspects of group composition during rearing influenced fish neophobia and cognition [Bannier et al., 2017]. Additionally, many psychological studies using the reverse of our treatment (exposing individuals to negative or no early environmental stimulus) found comparable results: primates with negative early social experience were less explorative and more neophobic [Fairbanks, and McGuire, 1988], and rodents with no early social experience showed reduced overall attention [Lovic et al., 2011]. Additionally, aggression was predicted to increase for fish raised with increased simulated predator stress or increased social stress [e.g. Lima and Dill 1990, Bell and Sih 2007, Naguib et al 2011, Taborsky and Oliveira 2012]. We found that fish raised in complex physical environments were more aggressive, and fish in our other early environmental treatments did not differ in aggression relative to control fish. This is in opposition with our predictions but matches findings in rodents [e.g. Marashi et al., 2003]. Exploration was predicted to be increased in fish raised in complex environments and decreased in fish raised with increased simulated predator stress [Abbey-Lee et al 2016, Moses and Sih, 1998, Hedrick and Kortet 2006, Christensen and Nielsen 2004]. We found that our measures of exploration behavior did not differ across our early environmental treatment groups, indicating that, at least in our study, this prediction was not supported. There are many potential explanations to why there are differences observed across studies, which can for example be due to differences among the biology of species and methods used. To understand the generality of differences in underlying contributions to behavioral variation via ontogenetic environment thus warrant further research.

The behaviors we measured have been used to describe variation in behavioral types or personality, in the same or other species [Reale et al 2007, Carere, and Maestripieri, 2013]. We confirm that several of these behaviors showed repeatability also in our population, and that the measured values were within the range for other behavioral traits [Bell 2009]. That repeatability differs in behaviors across populations or studies is commonly observed, and adds to the discussion of what explains variation in behavioral types.

Overall, we confirm that early environmental differences can lead to behavioral differences later in life. Nevertheless, our study is only based on reductionist tests measured in the lab on two families of fish, therefore further work is needed to confirm similar behavioral changes in

the wild, and the fitness consequences of such changes. Additionally, further work is needed to determine the specific aspect of our treatments that elicited the observed responses, for example, our increased social stress treatment included social re-grouping, but also the additional aspects of capturing and moving fish.

4.2 Early environment and monoamines

We aimed to link physiological and genetic variation in monoaminergic systems predicted to be of importance for behavior to early rearing environment manipulation. We found that dopamine receptor DRD4A tended to have lower expression in fish exposed to simulated predator stress and DRD1B (also a dopamine receptor) tended to have higher expression in fish raised in a more complex environment, at least in our limited study using two families of fish. Previous work has linked dopamine to motor control, motivation, and reward [Graybiel et al., 1994; Carere, and Maestripieri, 2013]. Motor control is important for effectively avoiding predators and navigating a complex physical environment, therefore our results linking these environmental factors to dopaminergic gene expression add support. Additionally, previous studies found that polymorphisms in dopamine receptor genes were related to novelty-seeking and exploratory behavior in open field tests and its variants in mammals and birds [Schinka et al., 2002; Fidler et al., 2007; van Oers, and Mueller, 2010; Carere, and Maestripieri, 2013; Holtmann et al., 2016]. Our finding that fish raised in complex environments tend to have higher expression of dopamine associated genes indicates that the process of exploring itself may lead to higher dopamine expression in our fish. Previous work on the serotonin system has found that individuals with atypical social behavior have serotonin deficiencies [Carere, and Maestripieri, 2013]. Our finding that fish raised in a more socially stressful environment tended to have higher expression of serotonin related genes (5-HTR2B and SLC6A4A), indicates that a complex social environment early in life may help increase serotonin levels, a necessity for developing important social skills. Interestingly, all fish exposed to behavioral tests tended to have decreased expression of the serotonin receptor gene 5-HTR2B relative to fish not behaviorally tested. This supports previous studies in birds showing that early cognitive stimulation influenced neuroendocrinology [Clayton, and Krebs, 1994; Freire, and Cheng, 2004], and indicates that forms of early cognitive stimulation (in our case exposure to behavioral tests) can potentially affect neuronal function. However, for other aspects of the monoaminergic systems investigated (monoamine levels and metabolites), we did not find any relationship with early environment. Our findings show that despite the observed alteration of gene expression in our

study, actual levels of brain monoamines did not vary. This is likely because we found expression to be altered only for receptor and transporter genes, not for synthesizing enzyme genes. Thus, this suggests that this may be a mechanism by which the efficacy of monoamines may be selectively altered in specific brain regions, as the same level of monoamines can have stronger or less strong effects in specific regions if the number of receptors or transporters there are altered. This finding is supported by other research on sticklebacks finding similar changes to gene expression but not monoamine levels [Di Poi et al., 2016].

4.3 Monoaminergic systems as mechanisms

We did not find support for our prediction that monoaminergic systems were the mechanism translating early environmental variation into behavioral differences in our population. We expected monoaminergic systems to be important because prior work has shown that variation in metabolite levels, methylation, and gene polymorphisms for dopamine and serotonin is linked to behavioral variation [Carere, and Maestripieri, 2013]. However, our analysis did not find any direct relationship between our measured aspects of the monoaminergic systems and behavior. The lack of observed relationship between environment and the multiple aspects of the monoaminergic systems measured suggests that the monoaminergic systems may not be the main mechanism. Instead, earlier steps of the HPA axis or other aspects of underlying physiology may be responsible for observed differences. We did nevertheless observe variation in aspects of the monoaminergic system in relation to other traits: fish differed in monoamine levels and gene expression depending on sex, size, and exposure to behavioral tests. Thus, our lack of relationship between monoamines and environment is not merely an artifact of low genetic variation because of only two sample populations, but rather indicates that monoamines are not the main mechanism for these relationships. Specifically, we expected to find that serotonin levels were negatively related to aggressiveness [Carere, and Maestripieri, 2013]. However, we observed that only the simulated predator stress treatment altered aggression, and we saw no link between our predator stress environment and any aspects of the serotonergic system. Serotonin has also been shown to be related to anxiety in humans, and therefore may be linked to aspects of neophobia [Carere, and Maestripieri, 2013]. We found that both the complex and social stress treatments altered neophobic behavior, but the link between these environmental manipulations and expression of genes for serotonin transmitters, receptors, or synthesizing enzymes was too weak to mediate the relationship between our environmental treatments and the resulting behavioral differences.

Further, we expected dopamine levels to be positively related to exploratory behavior and negatively related to neophobia [Carere, and Maestripieri, 2013]. However, exploration behavior did not vary with any of our environmental manipulations. We suggest further studies with the ability to measure monoamines in further detail, in a wider range of populations, before ruling out the role of monoamines in translating environmental variability into behavioral variation.

Our experiment ran for over 6 months mortality was related to environmental conditions (higher in the predator stress and complex treatments). Thus, it is possible that there was selective mortality within environmental conditions, reducing behavioral variation and masking our ability to detect differences. Additionally, our treatments involved exposure on average once per week. It is thus possible that the low level of exposure and long treatment duration lead to habituation and the lack of observed behavioral differences. Since monoamines change relatively quickly and all fish were sampled at 7 months, perhaps monoamine levels are more different earlier in life when behavioral types are being established and our sampling point missed this key period. Future studies would benefit from a design that allows for sampling and sacrificing individuals throughout ontogeny, before ruling out the role of monoamines in translating environmental variability into behavioral variation.

5. Conclusions

By the use of an integrative approach, exploring early rearing, brain physiology and gene expression of the same phenotyped fish, we demonstrate that early environment conditions influenced behavior and monoamine associated gene expression later in life. However, neither early environmental manipulations nor observed behavioral differences were clearly related to brain monoamine levels or turn-over rates in our study. Follow up studies should be carried out to confirm these findings, including investigating a broader range of mechanistic pathways to better understand the inter-relatedness of behavior, physiology and gene expression and the causal nature of these relationships.

6. Acknowledgements

We are grateful to Bertil Borg for providing stickleback eggs, to P-O Thörnqvist for advice during analyses of brain monoamines, and to Louise Hedlund for animal care. Funding was provided to HL from ‘Längmanska Kulturfonden’, ‘The Royal Physiographic Society of

Lund', and the LiU program 'Future research leaders'; to HL and RNAL by Center for Systems Neurobiology; and to JZ from 'The Lars Hierta's Memorial Foundation'.

The authors have no conflicts of interest.

7. Data Accessibility

Data will become available in the Dryad Repository upon acceptance of the paper (www.datadryad.org).

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Figure Legends

Figure 1. The influence of early environmental manipulation of stickleback, on their behavior. Means and standard errors for each early environmental manipulation group (Control, Predator, Complex, Social) of behaviors assayed in a novel arena: (A) initial response (Latency to swim NA, plotted on log scale) and (B) proportion of time active (Rate active); when exposed to a mirror: (C) initial response (Latency to swim, plotted on log scale) and (D) the number of times the fish poked its reflection (Number of pokes); and when exposed to a novel object: (E) initial response (Latency to swim NO, plotted on log scale) and (F) the time spent in within two body lengths of the novel object (Time close NO, plotted on log scale).

Figure 1

Tables

	Latency to swim NA	Proportion of time active	Latency to swim Aggression	Number of pokes	Latency to swim NO	Time close NO
Fixed Effects	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Intercept[a]	3.60 (2.55, 4.63)	0.10 (0.04, 0.16)	2.57 (1.86, 3.28)	4.21 (3.22, 5.23)	0.79 (-0.47, 2.15)	1.08 (0.29, 1.93)
Predator[b]	-0.86 (-1.91, 0.09)	0.06 (-0.01, 0.12)	0.15 (-0.72, 1.10)	0.13 (-1.02, 1.41)	1.24 (-0.16, 2.52)	0.44 (-0.60, 1.47)
Complex[c]	0.39 (-0.49, 1.24)	0.04 (-0.01, 0.09)	0.17 (-0.65, 0.96)	**1.33 (0.32, 2.37)**	0.52 (-0.64, 1.69)	**1.26 (0.35, 2.17)**
Social[d]	-0.06 (-0.85, 0.70)	0.04 (-0.01, 0.10)	0.19 (-0.54, 0.90)	0.22 (-0.71, 1.16)	0.27 (-0.69, 1.19)	0.86 (0.01, 1.68)
Sex[e]	**-0.62 (-1.28, 0.00)**	**0.05 (0.02, 0.09)**	-0.09 (-0.66, 0.47)	0.02 (-0.73, 0.79)	-0.13 (-0.89, 0.60)	0.24 (-0.44, 0.94)
Family	**-1.07 (-1.75, -0.37)**	-0.01 (-0.05, 0.04)	-0.01 (-0.67, 0.65)	0.62 (-0.25, 1.46)	0.05 (-0.79, 0.87)	0.22 (-0.48, 0.96)
Random Effects	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)
Tank ID	0.00 (0.00, 0.00)	5.0E-4 (1.8E-4, 1.1E-3)	0.03 (0.01, 0.07)	0.00 (0.00, 0.00)	0.01 (0.00, 0.02)	0.00 (0.00, 0.00)
Observer ID	0.45 (0.15, 0.93)	5.4E-4 (1.7E-4, 1.2E-3)	0.00 (0.00, 0.00)	0.11 (0.01, 0.33)	0.79 (0.11, 2.92)	0.00 (0.00, 0.00)
Fish ID	0.26 (0.15, 0.42)	2.2E-3 (1.5E-3, 3.3E-3)	0.40 (0.26, 0.63)	0.00 (0.00, 0.00)	-	-
Residual	1.18 (0.93, 1.82)	1.6E-3 (1.1E-3, 2.1E-3)	0.58 (0.43, 0.82)	3.74 (2.98, 4.65)	1.00 (0.69, 1.82)	0.80 (0.56, 1.53)
Repeatabilty	0.18 (0.00, 0.47)	**0.43 (0.19, 0.69)**	**0.37 (0.08, 0.63)**	0.00 (0.00, 0.27)	-	-

[a]Reference category; female control individuals

[b]Difference between the treatments (predation - control)

[c]Difference between the treatments (complex - control)

[d]Difference between treatments (social - control)

[e]Males

Table 1. The influence of early environmental manipulation on stickleback behavior. Estimated effect sizes and 95 % credible intervals (CI) around the mean of predictors of the measured behaviors. In a novel arena: initial response (Latency to swim NA) and proportion of time active. Exposed to a mirror: initial response (Latency to swim Aggression) and the number of times the fish poked its reflection (Number of pokes).

Exposed to a novel object: initial response (Latency to swim NO) and the time spent in the interaction zone (Time close NO). Estimates that do not cross zero (significant in the frequentist sense) are in bold.

a)	NE	DOPAC	5HIAA	DA	5HT	5HIAA/5HT	DOPAC/DA
Fixed Effects	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Intercept[a]	-4.72 (-10.74, 1.15)	0.00 (-0.03, 0.03)	-0.42 (-3.09, 2.16)	-0.14 (-1.13, 0.84)	3.38 (-4.85, 11.42)	0.20 (-0.80, 1.17)	-0.02 (-0.09, 0.06)
Predator[b]	0.63 (-1.09, 2.42)	0.00 (-0.005, 0.005)	0.18 (-0.51, 0.90)	0.06 (-0.23, 0.34)	-0.14 (-1.50, 1.34)	0.08 (-0.12, 0.23)	0.00 (-0.01, 0.01)
Complex[c]	-0.75 (-2.32, 0.80)	0.00 (-0.004, 0.006)	-0.15 (-0.73, 0.45)	-0.05 (-0.30, 0.19)	-0.70 (-1.91, 0.54)	0.07 (-0.09, 0.25)	0.00 (-0.01, 0.01)
Social[d]	0.41 (-0.94, 1.77)	0.00 (-0.004, 0.003)	0.04 (-0.46, 0.55)	0.03 (-0.19, 0.24)	0.28 (-0.68, 1.33)	-0.01 (-0.16, 0.15)	0.00 (-0.01, 0.01)
Sex[e]	0.82 (0.04, 1.58)	0.00 (-0.006, 0.002)	0.09 (-0.24, 0.41)	0.07 (-0.05, 0.19)	0.00 (-1.01, 1.03)	-0.05 (-0.18, 0.06)	0.00 (-0.01, 0.01)
Length	1.81 (0.49, 3.13)	0.00 (-0.006, 0.008)	0.35 (-0.24, 0.96)	0.14 (-0.07, 0.37)	-0.11 (-1.95, 1.71)	0.07 (-0.15, 0.30)	0.01 (-0.01, 0.02)
Tested[f]	-0.18 (-0.76, 0.39)	0.00 (-0.003, 0.003)	-0.12 (-0.36, 0.11)	-0.07 (-0.16, 0.01)	-0.24 (-1.00, 0.57)	-0.06 (-0.16, 0.03)	0.00 (-0.01, 0.01)
Family	0.33 (-0.90, 1.48)	0.00 (-0.006, 0.006)	0.22 (-0.23, 0.72)	0.03 (-0.17, 0.22)	0.05 (-0.94, 1.08)	0.04 (-0.09, 0.17)	0.01 (0.00, 0.01)
Random Effects	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)
Tank ID	0.74 (0.35, 1.34)	0.00 (0.00, 0.00)	0.08 (0.04, 0.15)	0.02 (0.01, 0.03)	0.10 (0.04, 0.24)	0.004(0.001, 0.01)	0.00 (0.00, 0.00)
Residual	1.34 (0.96, 1.92)	0.00 (0.00, 0.00)	0.25 (0.18, 0.37)	0.03 (0.02, 0.05)	2.53 (1.88, 3.80)	0.04 (0.03, 0.05)	0.00 (0.00, 0.00)

b)	DAT3	DRD1B	TH2	5-HTR2B	DRD4A	SLC6A4A/B	SLC6A4B	TPH2
Fixed Effects	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Intercept[a]	-9.36 (-12.12, -6.35)	6.54 (3.92, 9.25)	8.06 (5.24, 10.83)	6.83 (5.27, 8.51)	5.97 (3.11, 8.78)	5.18 (1.44, 8.90)	12.08 (7.34, 17.16)	4.82 (-1.22, 10.72)
Predator[b]	0.25 (-0.87, 1.29)	-0.19 (-0.66, 0.25)	0.01 (-0.44, 0.44)	-0.12 (-0.43, 0.15)	0.62 (-0.02, 1.27)	0.46 (-0.15, 1.04)	0.07 (-0.71, 0.85)	0.53 (-0.63, 1.72)
Complex[c]	-0.66 (-1.47, 0.24)	-0.42 (-0.83, -0.01)	0.01 (-0.41, 0.42)	-0.01 (-0.28, 0.26)	-0.35 (-0.91, 0.20)	-0.02 (-0.56, 0.52)	0.25 (-0.42, 0.98)	-0.24 (-1.23, 0.79)

Social[d]	0.28 (-0.47, 1.02)	-0.19 (-0.54, 0.14)	-0.25 (-0.57, 0.08)	-0.22 (-0.44, 0.01)	0.41 (-0.09, 0.90)	-0.47 (-0.91, -0.04)	-0.12 (-0.70, 0.48)	-0.41 (-1.28, 0.44)
Sex[e]	0.09 (-0.27, 0.44)	0.10 (-0.22, 0.43)	**1.13 (0.80, 1.49)**	0.13 (-0.08, 0.33)	**0.88 (0.53, 1.24)**	0.41 (-0.06, 0.87)	-0.18 (-0.80, 0.48)	**1.45 (0.72, 2.22)**
Length	0.17 (-0.51, 0.82)	0.29 (-0.30, 0.92)	0.11 (-0.50, 0.73)	0.37 (0.00, 0.71)	**1.25 (0.62, 1.91)**	0.72 (-0.10, 1.57)	-0.08 (-1.23, 1.01)	0.98 (-0.34, 2.32)
Tested[f]	-0.05 (-0.34, 0.22)	0.13 (-0.11, 0.37)	*0.24 (-0.02, 0.49)*	0.15 (0.00, 0.31)	0.05 (-0.24, 0.32)	0.11 (-0.24, 0.45)	0.03 (-0.44, 0.50)	0.01 (-0.53, 0.56)
Family	-0.02 (-0.72, 0.67)	0.31 (-0.02, 0.62)	0.21 (-0.10, 0.52)	0.21 (0.00, 0.42)	-0.16 (-0.58, 0.24)	0.46 (0.06, 0.88)	0.04 (-0.49, 0.59)	0.28 (-0.50, 1.06)
Random Effects	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)
Tank ID	0.21 (0.10, 0.39)	0.04 (0.01, 0.08)	0.00 (0.00, 0.00)	0.02 (0.01, 0.04)	0.05 (0.02, 0.10)	0.04 (0.02, 0.10)	0.00 (0.00, 0.00)	0.13 (0.05, 0.27)
Residual	0.35 (0.22, 0.45)	0.26 (0.19, 0.37)	0.30 (0.22, 0.44)	0.11 (0.07, 0.14)	0.31 (0.22, 0.44)	0.54 (0.41, 0.79)	1.08 (0.77, 1.48)	1.31 (0.96, 1.88)

[a]Reference category; female control individuals, behaviorally tested

[b]Difference between the treatments (predation - control)

[c]Difference between the treatments (complex - control)

[d]Difference between treatments (social - control)

[e]Males

[f]Fish not exposed to behavioral tests

Table 2. The influence of early environmental manipulation on stickleback monoaminergic systems. Estimated effect sizes and 95 % credible intervals (CI) around the mean of predictors of each of the measured a) brain monoamines (norepinephrine [NE], dopamine [DOPAC], and serotonin [5HIAA], metabolites [dopamine [DA], serotonin [5HT]), and their turn-over rates (serotonin [5HIAA/5HT], dopamine [DOPAC/DA]), and b) gene expression of transporters (dopamine [DAT3], serotonin [SLC6A4A/B]), receptors (dopamine [DRD1B and DRD4A], serotonin [5-HRT2B], and synthesizing enzymes (dopamine [TH], serotonin [TPH2]) in the brain. Estimates that do not cross zero (significant in the frequentist sense) are in bold.

a)

	Number of Pokes, DRD4A
Mediated Effects[1]	-0.07 (-0.52, 0.30)
Direct Effects[2]	0.31 (-0.26, 0.85)
Total Effects	0.23 (-0.33, 0.81)
Proportion Mediated	-0.05 (-8.21, 6.64)

b)

	Time close NO, DRD1B
Mediated Effects[1]	-0.02 (-0.31, 0.22)
Direct Effects[2]	0.84 (-0.18, 1.89)
Total Effects	0.82 (-0.22, 1.87)
Proportion Mediated	-0.01 (-1.07, 0.61)

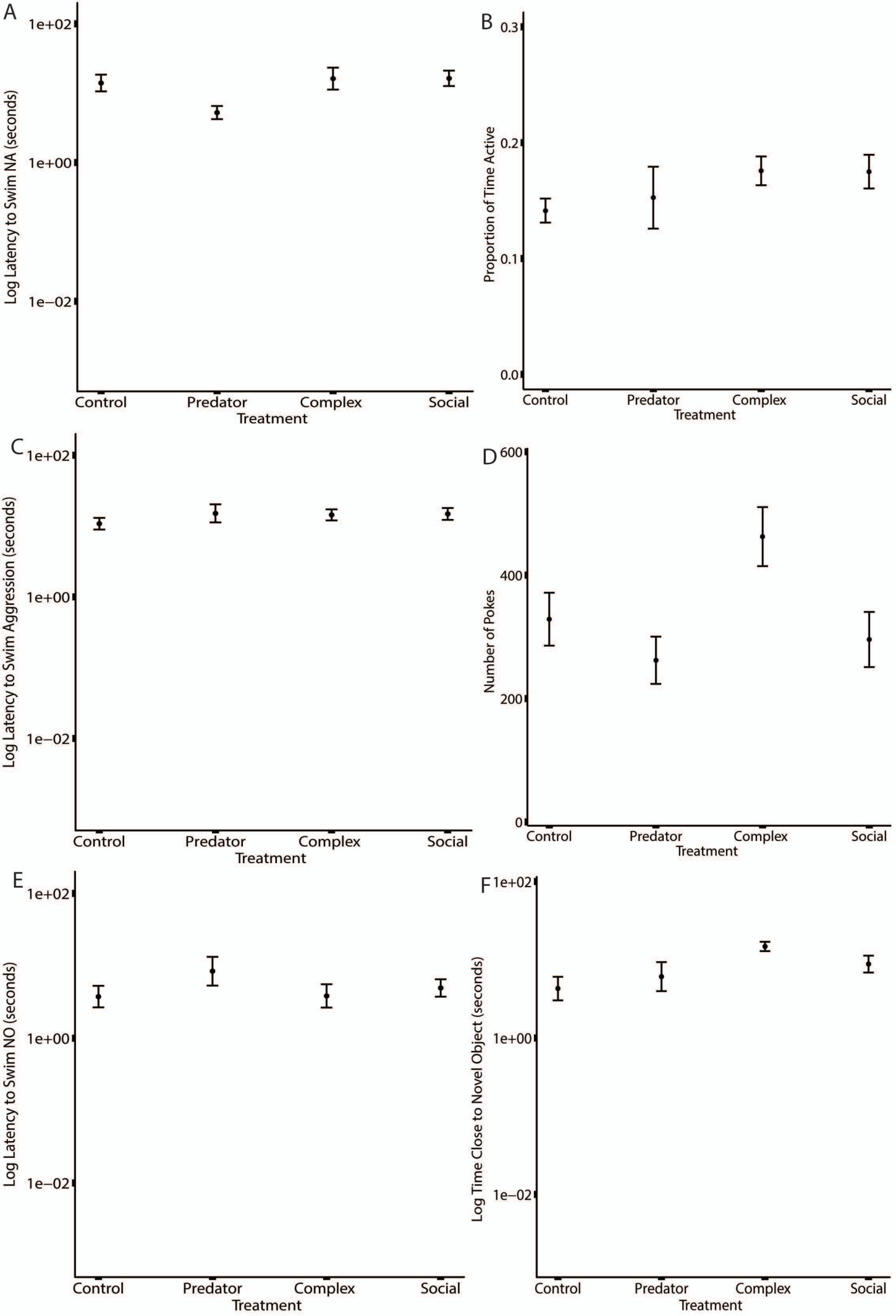
c)

	Time close NO, 5-HTR2B	Time close NO, SLC6A4A
Mediated Effects[1]	-0.01 (-0.26, 0.20)	-0.07 (-0.58, 0.40)
Direct Effects[2]	0.80 (-0.12, 1.92)	0.88 (-0.19, 1.89)
Total Effects	0.79 (-0.13, 1.94)	0.81 (-0.24, 1.72)
Proportion Mediated	0.00 (-0.62, 0.48)	-0.05 (-1.86, 1.32)

[1]The effect of aspects of monoaminergic systems on behavior

[2] The effect of rearing environment on behavior

Table 3. Relationship between early environment, brain monoamine systems, and behavior in sticklebacks. Estimation of causal mediation effects and 95% credible intervals (CI) around the mean for models of how candidate aspects of monoaminergic systems translate exposure to a) a simulated predator environment, b) a complex environment, and c) an alternated social environment to behavioral responses.


A
Log Latency to Swim NA (seconds)
1e+02
1e+00
1e−02
Control
Predator
Complex
Social
Treatment
B
Proportion of Time Active
0.3
0.2
0.1
0.0
Control
Predator
Complex
Social
Treatment
C
Log Latency to Swim Aggression (seconds)
1e+02
1e+00
1e−02
Control
Predator
Complex
Social
Treatment
D
Number of Pokes
600
400
200
0
Control
Predator
Complex
Social
Treatment
E
Log Latency to Swim NO (seconds)
1e+02
1e+00
1e−02
Control
Predator
Complex
Social
Treatment
F
Log Time Close to Novel Object (seconds)
1e+02
1e+00
1e−02
Control
Predator
Complex
Social
Treatment