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RESEARCH ARTICLE

Effects of monoamine manipulations on the personality and gene expression of three-spined sticklebacks

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ABSTRACT

Among-individual behavioral differences (i.e. animal personality) are commonly observed across taxa, although the underlying, causal mechanisms of such differences are poorly understood. Animal personality has been correlated with physiological functions as well as fitness-related traits. Variation in many aspects of monoamine systems, such as metabolite levels and gene polymorphisms, has been linked to behavioral variation. Therefore, here we experimentally investigated the potential role of monoamines in explaining individual variation in personality, using two common pharmaceuticals that respectively alter the levels of serotonin and dopamine in the brain: fluoxetine and ropinirole. We exposed three-spined sticklebacks, a species that shows animal personality, to either chemical alone or to a combination of the two chemicals, for 18 days. During the experiment, fish were assayed at four time points for the following personality traits: exploration, boldness, aggression and sociability. To quantify brain gene expression on short- and longer-term scales, fish were sampled at two time points. Our results show that monoamine manipulations influence fish behavior. Specifically, fish exposed to either fluoxetine or ropinirole were significantly bolder, and fish exposed to the two chemicals together tended to be bolder than control fish. Our monoamine manipulations did not alter the gene expression of monoamine or stress-associated neurotransmitter genes, but control, untreated fish showed covariation between gene expression and behavior. Specifically, exploration and boldness were predicted by genes in the dopaminergic, serotonergic and stress pathways, and sociability was predicted by genes in the dopaminergic and stress pathways. These results add further support to the links between monoaminergic systems and personality, and show that exposure to monoamines can causally alter animal personality.

KEY WORDS: Animal behavior, Cocktail effects, Dopamine, Ecotoxicology, Fish, Serotonin

INTRODUCTION

Consistent among-individual behavioral differences (i.e. animal personality) have now been widely described across taxa (Wilson, 1998; Gosling, 2001; Dall et al., 2004; Coppens et al., 2010; Wolf and Weissing, 2012; Carere and Maestripieri, 2013). These consistent behavioral differences are a conundrum for behavioral ecologists as they challenge traditional optimality theory that behavior should be flexible and situation specific (Krebs and Davies, 1997; Dall et al., 2004). Despite research demonstrating that animal personality can have important fitness, ecological and evolutionary consequences (Dall et al., 2004; Smith and Blumstein, 2008; Carere and Maestripieri, 2013; Roche et al., 2016; de Boer et al., 2017), the factors shaping and maintaining variation in personality are still poorly understood. To better comprehend how these underlying factors may shape personality, studies using experimental manipulations of different mechanistic pathways are needed (van Oers and Mueller, 2010; Roche et al., 2012). Differences in individual personality traits have been linked to differences in gene expression, which provides a basis for their further investigation (e.g. Bell et al., 2016).

Monoamine neurotransmitters (dopamine, serotonin, adrenaline) are released from neurons in both the brain and the peripheral nervous system, with links to behavioral variation (Winberg and Nilsson, 1993; Coppens et al., 2010; Koolhaas et al., 2010; Caramaschi et al., 2013; Bell et al., 2016; Soares et al., 2018). Variation in metabolite levels, methylation and gene polymorphisms for dopamine and serotonin have been associated with animal personality (Caramaschi et al., 2013). Specifically, low serotonin levels are linked to increased aggressiveness in several species (Bell et al., 2007; Shaw and Øverli, 2012; Caramaschi et al., 2013; Abbey-Lee et al., 2018a), polymorphisms in serotonin transporter genes are associated with aggression, anxiety and impulsivity in primates (Caramaschi et al., 2013), and aggressive behavior in some fish populations is controlled by raphe serotonergic neurons (Elipot et al., 2013). Fluoxetine, a commonly prescribed antidepressant in the family of selective serotonin reuptake inhibitors (SSRIs), has been shown to affect animal behavior. SSRI drugs inhibit the reuptake of the neurotransmitter serotonin from the synaptic cleft during signaling between neurons, by blocking the serotonin transporter; this results in serotonin remaining in the cleft for longer, prolonging the stimulation of serotonin receptors (Vaswani et al., 2003). Exposure to fluoxetine reduces aggression (Perreault et al., 2003; Bell et al., 2007; Clotfelter et al., 2007; Carere and Maestripieri, 2013; Eisenreich et al., 2017; Theodoridi et al., 2017; Barbosa et al., 2018; Kellner et al., 2018). 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; Egan et al., 2009; Filby et al., 2010; van Oers and Mueller, 2010; Shaw and Øverli, 2012; Caramaschi et al., 2013; Carere and Maestripieri, 2013; Holtmann et al., 2016; Abbey-Lee et al., 2018a). Ropinirole, prescribed to treat restless leg syndrome and Parkinson's disease (Connolly and Lang, 2014), is a dopamine agonist at D2 and D3 dopamine receptors (Shill and Stacy, 2009).

Importantly, research suggests that the monoamine systems are not fully independent, with many chemicals that can alter behavior

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having affinities for both serotonin and dopamine receptors (Bischoff et al., 1986; Lejeune and Millan, 1998; Lawler et al., 1999; Borroto-Escuela et al., 2010; Martínez-Clemente et al., 2012; de Bartolomeis et al., 2013). For example, mephedrone, a synthetic stimulant drug of the amphetamine and cathinone classes, interacts with both serotonin and dopamine transporters (Martínez-Clemente et al., 2012). The monoamine systems can work in conjunction, with both serotonin and dopamine involved in physiological stress responses (Winberg and Nilsson, 1993; Øverli et al., 1999; Höglund et al., 2001; Lillesaar, 2011; Melnyk-Lamont et al., 2014). Additionally, fluoxetine can influence more than just the serotonin system; it has been shown to decrease whole-body cortisol concentrations in zebrafish (Egan et al., 2009) and upregulate gene expression of serotonin receptor 5HT-2B, as well as glucocorticoid brain receptors (GRs) and mineralocorticoid brain receptors (MRs) (elements of the stress response system) in proactive zebrafish (Theodoridi et al., 2017). Thus, monoamine profiles can be linked to changes in stress response, indicating an interaction between monoaminergic systems and the hypothalamic–pituitary–adrenal (HPA) axis [hypothalamic–pituitary–interrenal (HPI) axis in fish]. Specifically, levels of serotonin also potentiate production of corticotropin-releasing factor and thus influence the stress response axis (Jørgensen et al., 2002; Aubin-Horth et al., 2012). These links between monoamines, stress physiology and behavior are the basis of coping style theory (Koolhaas et al., 1999). Proactive individuals tend to have low HPA reactivity, while reactive individuals have high HPA reactivity. Previous work shows that reactive animals have higher concentrations of both serotonin and dopamine after stress than proactive individuals (Øverli et al., 2001a,b; Koolhaas et al., 2007). Additionally, the stress response system has been linked to a variety of behavioral responses in fish, from anti-predator response (Fürtbauer et al., 2015) to social dominance behavior (Sloman et al., 2001), and maternal exposure can influence offspring behavior (Sloman, 2010). However, there is limited research on the functional relationship between monoamines and stress mediators. The stress response is facilitated by GRs and MRs. MRs are activated by low concentrations of stress hormone and are linked to amplification of the stress response, while GRs are activated at higher doses and decrease neuronal excitation and mediate adaptation to the stress stimulus (Joëls, 2009; ter Heegde et al., 2015). Therefore, the MR/GR ratio may play a role in phenotypic expression of a coping style, with low GR and MR levels and a low MR/GR ratio associated with a reactive coping style (de Kloet et al., 2005; Oitzl et al., 1995; Vindas et al., 2017). From studies on mental disorders, higher expression of both GRs and MRs is potentiated by antidepressants (Seckl and Fink, 1992). GR and MR are also associated with fear and anxiety, and blocking MR has been show to alleviate fear-related behavior (Korte et al., 1995; Oitzl et al., 1995). Interestingly, the serotoninergic system seems to be able to influence expression of both glucocorticoid and mineralocorticoid receptors (Semont et al., 1999; Zhou et al., 2008), although the exact mechanism is still unknown. Despite research showing promising links between monoamines and behavior, we still lack critical empirical studies that test causational hypotheses and that examine the system more holistically by examining multiple genes.

In this study, we aimed to explore the causal link between monoamines and behavior describing animal personality, using pharmaceuticals affecting the serotonergic (by the use of fluoxetine) and dopaminergic (by the use of ropinirole) systems. Because these pharmaceuticals are designed to affect human behavior (Fuller, 1996; Serretti et al., 2010), and monoamine systems are evolutionarily conserved across taxa (Coppens et al., 2010), they are likely to also influence behavioral variation in other species. We addressed the question of how chemical manipulation of monoamines influences behavior and the expression of genes of monoaminergic and stress systems of the three-spined stickleback (*Gasterosteus aculeatus*). Sticklebacks are abundant in freshwater and marine habitats in the northern hemisphere, where they live in coastal shoals, and are an important model 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), making them a good model species for this study.

We designed four treatment groups: serotonin manipulated (exposed to fluoxetine), dopamine manipulated (exposed to ropinirole), dopamine and serotonin manipulated (exposed to serotonin and dopamine concurrently) and unexposed control. We investigated how behavior and gene expression were influenced by chemical manipulations at two time points, after 6 days of exposure and after 18 days of exposure, to compare shorter- versus longer-term effects. Our study design utilized an integrative approach in which behavior and monoaminergic system genes were assessed in the same fish. We predicted that exposure to monoamine-manipulating compounds would generate behavioral and gene expression changes. Specifically, we focused on behavior describing variation in the personality traits exploration, aggression, sociability and boldness. Based on previous work, we predicted that our serotonin manipulation would increase serotonin levels in the brain, thus decreasing individual aggressiveness (Perreault et al., 2003; Bell et al., 2007; Clotfelter et al., 2007; Carere and Maestripieri, 2013; Eisenreich et al., 2017; Theodoridi et al., 2017; Barbosa et al., 2018; Kellner et al., 2018) and increasing boldness and exploration behavior (Egan et al., 2009). Our dopamine manipulation should increase dopamine receptor activity in the brain, and thus we predicted it would decrease exploration, boldness, aggressiveness and sociability (Schinka et al., 2002; Fidler et al., 2007; Egan et al., 2009; Filby et al., 2010; van Oers and Mueller, 2010; Carere and Maestripieri, 2013; Holtmann et al., 2016). We predicted that our manipulations of the two chemicals simultaneously would have a combined effect, although there is not enough research currently on mixed manipulation effects (so called ‘cocktail effects’; Celander, 2011) to make directional predictions of whether this combined effect would be additive, synergistic or antagonistic. According to previous work in three-spined sticklebacks, we expected that fish with more proactive behavior, either in the control group or as a result of the monoamine-altering treatment, would also show upregulated expression of glucocorticoid receptors (Aubin-Horth et al., 2012). Additionally, we predicted that differential gene expression would be the mechanism by which our monoamine manipulations would influence behavior; thus, we predicted that monoamine- and stress-associated gene expression in the brain would vary depending on treatment (Coppens et al., 2010; Bell et al., 2016). Finally, we predicted that the effects would be more pronounced after longer exposure (Caccia et al., 1992).

MATERIALS AND METHODS

Study population

Three-spined sticklebacks, *Gasterosteus aculeatus* Linnaeus 1758, used in this study were caught from Oxelösund (58.6645147, 17.053448) and Sankt Anna (58.365406, 16.829321), Sweden, in October 2017, within the genetically uniform range of coastal populations in the Baltic Sea (Mäkinen et al., 2006). Fish were

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transported to the lab where they were kept in 27 l tanks (ca. 40×27×27 cm) at approximately 12°C and 6‰ salinity, under 9 h:15 h light:dark conditions. All four sides of the tanks were covered in black plastic, and each tank was half-covered with a lid, to visually isolate the fish from each other and to provide isolation from disturbances in the room (Abbey-Lee et al., 2018b). All experimental tanks initially housed 35 individuals, with three replicates of each of the four treatments (N=420). Fish were fed defrosted red bloodworms every other day and water quality was regularly tested and changed when needed. All tanks had a separate water system that included a filter (Eheim 45) and an airstone. New de-chlorinated water was added during the water changes and re-dosing. There was no water circulation between the tanks and they were maintained as independent systems; therefore, there was no cross-contamination between treatments. All experimental procedures were in compliance with Linköping University ethics permit 17-769.

Monoamine manipulations

Fish were exposed to one of four different treatments: dopamine (2500 ng l^{-1} ropinirole hydrochloride, Sigma-Aldrich), serotonin (200 ng l^{-1} fluoxetine hydrochloride, Sigma-Aldrich), both dopamine and serotonin (2500 ng l^{-1} ropinirole hydrochloride and 200 ng l^{-1} fluoxetine hydrochloride), and control (no dopamine or serotonin). Concentrations of ropinirole were selected based on pilot study results (Table S1). Concentrations of fluoxetine were chosen from previous work in fish and values recorded in ecological assessments of natural water (Fick et al., 2010; Silva et al., 2012; Eisenreich et al., 2017). Dosing was done concurrent with 40% water changes every 5 days to maintain the chosen concentrations (Benotti and Brownawell, 2009; Kellner et al., 2018). To prevent cross-contamination, each treatment had separate buckets and other tools needed for water change and re-dosing, color-coded to ensure that researchers remained blind to the treatment. Our sample size was too large to complete behavioral assays in a single day, so dosing of tanks was staggered so that all individuals were behaviorally tested on the same day relative to dosing (i.e. on the 6th and 18th day of drug exposure).

Behavioral assays

On the day before dosing (pre-exposure, day 0), the 6th day of exposure (day 6) and the 18th day of exposure (day 18), fish were individually caught with nets from their home aquarium and subject to two behavioral tests (total duration 10 min, details below) and a subset of fish were used for brain monoamine data collection (see Fig. 1 for schematic diagram). All data were recorded by an observer blind to treatment who stood approximately 2 m from the tank facing the long side. Prior to the start of the experiment, all observers (N=5) were trained using randomly selected pilot fish until behavioral test scores were comparable. At each time point, all alive fish were caught from their home tank and behaviorally tested. Immediately following behavioral assays, 5 fish per tested tank (therefore three replicates, i.e. 15 per treatment) were randomly chosen and killed with an overdose of anesthetic (benzocaine). Thus, a subset of fish were sampled at each time point (day 0, day 6 and day 18) after the behavioral assay to collect brains to measure gene expression, providing us with both behavioral data and gene expression data from three time points, but for each individual fish we only have the brain data from one time point. At this point, fish length was measured and the presence of visible parasites (*Glugea anomala*) was noted (Petkova et al., 2018). Parasite infection, which was due to the fact that the fish were wild-caught, was monitored to account for potential inter-individual differences for statistical analysis; however, as it was impossible to experimentally control the infection, we did not investigate the interaction between pharmaceutical treatment and parasitic infection. Brains were removed and snap frozen within 13 min and stored at −80°C until quantification of gene expression (see below).

Novel area test

To measure boldness and exploration behavior, fish were introduced into a novel area (35.5×21×21 cm) with different substrate (darker and finer grain gravel instead of multi-colored, coarser gravel) and different decorations from those in their home tank. The decorations changed for each testing period to keep the environment novel; on day 0 there was a novel plastic plant, on day 6 there was a novel large stone, and on day 18 there was a novel large piece of wood and a stone. Fish were placed in the upper left corner of the novel tank and were observed for 5 min. The long side of the tank was divided into four equal lower and four upper square areas with a fine marker on the outside of the tank. Exploration was recorded based on fish moving through these squares, when the eye of the fish was observed to pass the line that divided two different squares (number of square changes). Initial response (latency to move in the novel area), and the proportion of time spent in the upper middle squares (time in upper mid zone) were scored as measures of boldness.

Mirror test

To measure aggression and sociability, fish were exposed to a mirror immediately following the novel area test. Mirror tests are often used to study aggression and sociability 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). This test has thus been recommended for use with sticklebacks and other fish (Peeke et al., 1969; Kleszczyńska et al., 2012). In the same test arena, a mirror was placed at the end of the tank nearest the fish. Fish were observed for an additional 5 min, and the initial response (latency to swim during the aggression test, used as a measure of boldness), the number of times the fish approached and attacked the mirror (number of attacks, used as a measure of aggression), and the time the fish spent close to the mirror, within 1 body length (time close to mirror, with no obvious aggressive action, used as a measure of sociability) were recorded.

Molecular analyses

In order to quantify gene expression of selected genes in the brain monoaminergic and stress response systems, we performed quantitative PCR (qPCR) analyses of different monoamine

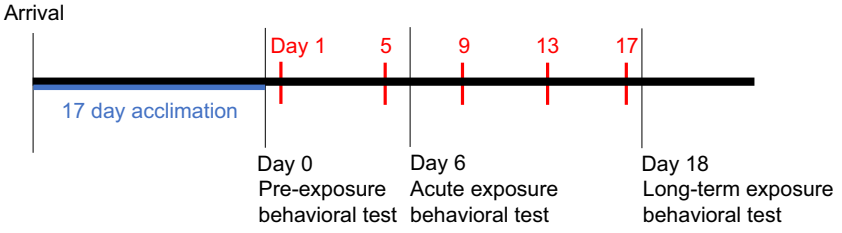

Fig. 1. Schematic diagram of dosing and behavioral test protocol. On arrival in the lab following capture, fish were permitted a 17 day acclimation period. Red indicates the day on which water changes were made and pharmaceutical dosing occurred. Behavioral tests were performed on days 0, 6 and 18.

receptors: two serotonin receptor subtypes (5-hydroxytryptamine receptor 2A, *5-HTR2A*; and 5-hydroxytryptamine receptor 2B, *5-HTR2B*); two dopamine receptor subtypes (dopamine receptor 1, *DRD1Bl*; dopamine receptor 2, *DRD2*); an adrenergic receptor (beta-2 adrenergic receptor, *ADRB2A*); a MR (*NR3C2*); and a GR1 (*NR3C1*) (primer information is given in Table S2). Total RNA was extracted from whole brains using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA quality and quantity were checked with a Nanodrop 1000 (Thermo Fisher). RNA purity and integrity were checked in a Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). RNA integrity number (RIN) was larger than 8.0 in all samples used in further analyses. A 1 µg sample of the DNAse-treated RNA was converted to cDNA using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher). The reactions were performed in a Light Cycler 480 (Roche Diagnostics, Basel, Switzerland). Each reaction included 2 µl nuclease-free water, 1 µl each of forward and reverse primers (0.5 µmol l^{-1}), 1 µg cDNA diluted in 1 µl nuclease-free water and 5 µl SYBR green 1 Master (Roche Diagnostic). Primers were accepted if they resulted in a single product of correct size as determined by a melt curve run on cDNA pooled from all samples. Plates were designed such that one plate contained two replicates of each gene for four individuals, as well as two replicates of a housekeeper control, made of cDNA pooled from all samples. qPCR was performed in a thermocycler with the following protocol: 5 min at 95°C acclimation, followed by 45 cycles of 10 s at 95°C, 10 s at 60°C and 20 s at 72°C. The program was terminated with a melt curve from 65 to 95°C to make sure a single product was amplified, and a final cooling to 40°C. The crossing point values (Cp) were normalized over a housekeeping gene (Pfaffl, 2001), chosen because it did not differ between our treatment groups and had the least overall variation. The relative expression difference between individuals was measured according to the methods in Pfaffl (2001).

Statistical analyses

All statistical analyses were performed with R version 3.3.1 (http://www.R-project.org/). We applied generalized linear mixed-effects models to analyze our data (detailed below), for which we used the 'glmer' function (package lme4; Bates et al., 2014). Additionally, we used the 'sim' function (package arm; https://CRAN.R-project.org/package=arm) 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 used visual assessment of the residuals to evaluate model fit.

Behavioral assays

To test the effect of pharmaceutical manipulations on behavior, we ran univariate generalized linear mixed-effects models. All behavioral measures were Poisson distributed, and thus were modeled using Poisson distributions. Type of manipulation (dopamine, serotonin, both, or control; categorical variable with four levels) and parasite status (binary, yes/no) were added as fixed effects. Parasite status was included to control for the fact that our fish were wild caught and some were parasitized, and previous studies show that parasite infection and personality can co-vary (Petkova et al., 2018), and that monoamine variation and parasite status can be linked (e.g. Øverli et al., 2001a,b). Additionally, size (length; continuous) and housing tank (tank ID, categorical with 12 levels) were included as random effects. As we expected the duration of manipulation to potentially differentially influence behavior, we ran additional models for each time point separately (i.e. three models per behavior, one each for: day 0, day 6 and day 18). Owing to sample design and mortality, final sample sizes for analyses differ: N=187, 150 and 81, respectively.

Molecular analyses

To test the effect of the different pharmaceutical treatments on receptor gene expression, we ran univariate linear mixed models (N=64). These models followed the same structure as above, with a single model for each gene, type of manipulation as a fixed effect and tank ID as a random effect. There were not enough parasitized fish to include parasite status in these models. All measures of gene expression are reported in the ΔCT method, where high values indicate lower expression.

Additionally, to determine whether natural variation in gene expression was related to observed variation in behavior, we ran univariate generalized linear models with data only from our control fish (N=15). Each behavioral measure was used as a response variable, and the expression levels of each of our measured genes were included as fixed effects. We were unable to run a model for our variable 'latency to move in a novel area' as our 15 individuals did not show enough variation (most moved immediately and had a score of 0).

RESULTS

Behavioral assays

We found that, overall, both dopamine- and serotonin-manipulated fish were bolder (i.e. had a shorter latency to move in a novel area) than control fish (Fig. 2, Table 1). When looking at the different time points separately, we confirmed that our treatment groups did not differ during the pre-exposure period, and that significant behavioral differences did not arise until day 18 (Table 1). Additionally, parasitized fish, regardless of treatment, were less bold (i.e. had a longer latency to move in both tests, and spent less time in the upper middle squares), more active (i.e. made more square changes) and more sociable (i.e. spent more time near the mirror), than fish without visible parasites (Table 1).

Molecular analyses

We found that gene expression was not changed by our pharmaceutical manipulations (Fig. 3, Table 2). However, we found that a number of our behavioral measures in control fish were predicted by gene expression (Table 3). Stress-related receptor genes (*NR3C2*, *ADRB2A*), serotonin receptor genes (*HTR2A*, *HTR2B*) and dopamine receptor genes (*DRD1B*, *DRD2*) were all predictors of exploration and boldness, and stress-related receptor genes (*NR3C2*, *NR3C1*) and a dopamine receptor gene (*DRD1B*) were predictors of aggression and sociability (Table 3). Specifically, stress-related receptor genes were positively related to exploration (*NR3C2*, *ADRB2A*), both positively and negatively related to boldness (*NR3C1*, *NR3C2*, *ADRB2A*), and positively related to sociability and aggression (*NR3C2*). Serotonin receptor genes were negatively related to exploration (*HTR2A*, *HTR2B*), both positively and negatively related to boldness (*HTR2A*, *HTR2B*), and positively related to sociability (*HTR2B*). Dopamine receptor genes were both positively and negatively related to exploration, boldness and sociability (*DRD1B*, *DRD2*).

DISCUSSION

We experimentally manipulated monoaminergic systems of fish and explored concurrent changes in behavior and brain gene expression. Our results add support to the hypothesis that manipulation of

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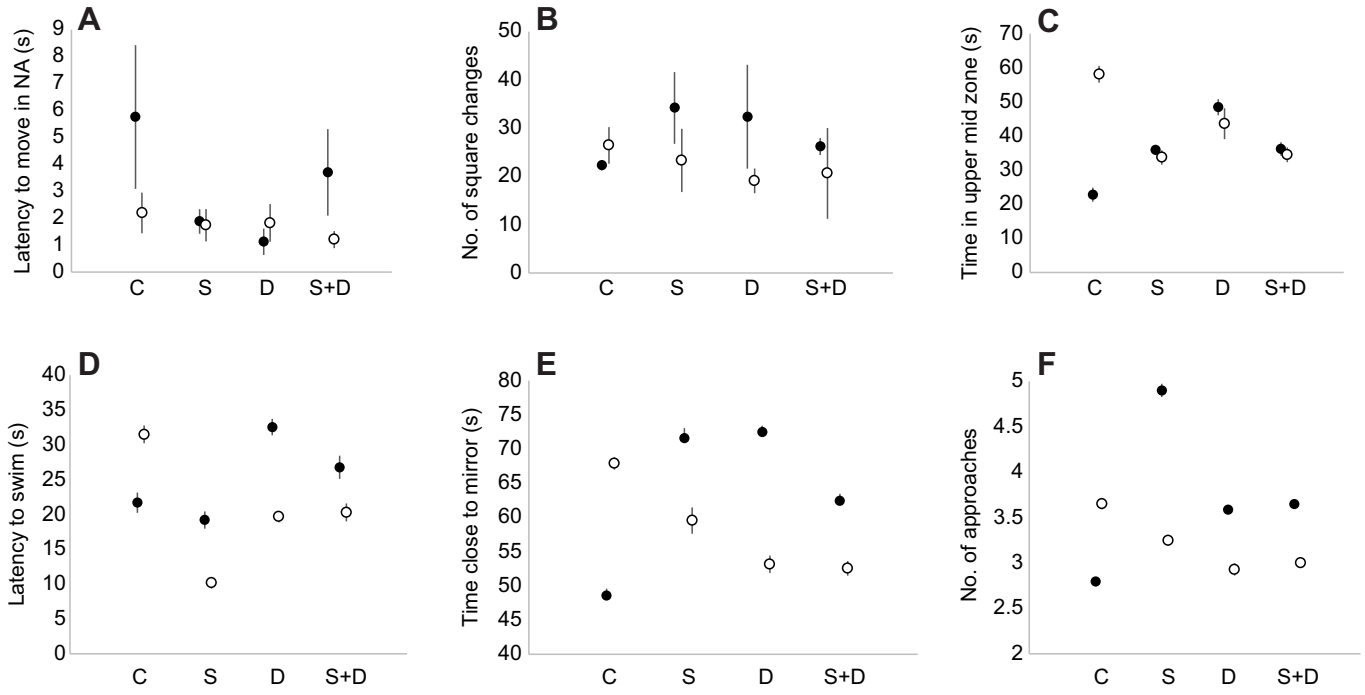

Fig. 2. Influence of monoamine manipulation on stickleback behavior. Fish were divided into control (no monoamines; C), serotonin (fluoxetine hydrochloride; S), dopamine (ropinirole hydrochloride; D) or serotonin and dopamine together (S+D) groups. Means±s.e.m. of raw data for each treatment (control, serotonin manipulated, dopamine manipulated and serotonin+dopamine manipulated; *N*=418) are shown for the following behaviors: (A) latency to move in a novel area (NA), (B) number of square changes in a novel area, (C) time in the upper mid zone of the novel area, (D) latency to swim during the mirror test, (E) time spent close to the mirror and (F) number of times the fish attacked the mirror. Circle color is used to denote parasite status; uninfected fish are represented by open circles, visibly parasitized fish are represented by filled circles.

serotonin and dopamine via pharmaceuticals causally makes fish bolder compared with control fish, with effects appearing after 18 days of exposure. Despite these behavioral changes, we did not detect concurrent alteration of gene expression of monoamine- or stress-associated genes in response to our manipulations. However, we confirmed that natural variation in gene expression co-varied with behavior: exploration and boldness were predicted by genes in dopaminergic, serotonergic, adrenergic and stress pathways, aggression was predicted by a stress-associated gene, and sociability was predicted by genes in the dopaminergic and stress pathways. These observations are consistent with previous work on natural variation in brain gene expression and behavior in wild stickleback populations where a similar subset of genes was studied (Di Poi et al., 2016). Specifically, bolder, more active fish had a higher gene expression of adrenergic (*ADRB2A*), serotonergic (*HTR2A*) and dopaminergic (*DRD2*) receptors, but a lower expression of the gene encoding the *HTR2B* receptor, while variation in aggression was associated with a GR gene (*MC2R*) (Di Poi et al., 2016). In another study, boldness and aggressiveness formed a behavioral syndrome and were positively correlated with expression of glucocorticoid receptors GR1 and GR2 (Aubin-Horth et al., 2012). Together, our results support the link between monoamine systems and behavior, by showing that exposure to monoamines causally alters behavior describing variation in animal personality, and gene expression co-varies with our observed behaviors.

Our finding that exposure to fluoxetine increased boldness matches findings in previous work on sticklebacks (Grécias et al., 2017) and on other fish species (Wong et al., 2013; Ansai et al., 2016; Singer et al., 2016; Sinyakova et al., 2018). Fluoxetine has been shown to also decrease the predator response in sticklebacks (Sebire et al., 2015) and in the fathead minnow (Painter et al., 2009; Weinberger and Klaper, 2014). Additionally, exposure to a similar serotonin-manipulating pharmaceutical, citalopram, increased boldness in fish (Kellner et al., 2016) and other animals (Bergey et al., 2016; Holtmann et al., 2016). Our findings that detectable differences in behavior did not arise until after 18 days of exposure confirms that long-term exposure to human chemicals can have increasingly detrimental effects on animals (Nash et al., 2004; Iñiguez et al., 2010; Kania et al., 2012).

Interestingly, our results show that the combined effect on behavior of two drugs at once seems less strong than the effect of either drug alone. Little is known about how combinations of drugs influence organisms (a so-called ‘cocktail’ effect). Because the monoamine systems are linked, it is possible that the drugs used in our experiment interfered with each other at binding sites, or that the systems naturally mediate each other’s activity (Lejeune and Millan, 1998; Doly et al., 2009, 2017), meaning that in the combined treatment, despite a higher overall exposure to pharmaceuticals, there was an overall lower effect on observed behavior. This situation could potentially occur if the two drugs stimulate the same receptor system, possibly causing individuals exposed to the combined treatment to have a high enough overall receptor stimulation to cross a threshold and trigger a self-regulating mechanism that downregulated the response. Individuals exposed to only one drug would, alternatively, have lower stimulation of the monoaminergic systems and therefore potentially not cross the critical threshold for downregulation, keeping the signaling pathway ‘activated’, and thus resulting in an overall higher and more long-lasting effect (Stephenson, 1956; Kohn and Melnick, 2002; Welshons et al., 2003; Vandenberg et al., 2012). Considering that in

Table 1. Influence of monoamine manipulation on stickleback behavior

	Latency to move in NA		No. of square changes		Time in upper mid zone		Latency to swim		No. of attacks		Time close to mirror	
Fixed effects	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
All exposure time periods pooled												
Intercept	1.07 (0.31, 1.84)	<0.001	3.07 (2.71, 3.45)	<0.001	3.83 (3.44, 4.21)	<0.001	2.75 (1.79, 3.72)	<0.001	1.11 (0.78, 1.44)	<0.001	3.84 (3.42, 4.25)	<0.001
Serotonin	**−1.33 (−2.40, −0.26)**	**0.02**	0.08 (−0.42, 0.57)	0.75	−0.49 (−1.05, 0.05)	0.07	−0.49 (−1.82, 0.84)	0.47	0.15 (−0.32, 0.61)	0.52	0.23 (−0.34, 0.81)	0.43
Dopamine	**−1.22 (−2.29, −0.10)**	**0.03**	−0.02 (−0.52, 0.49)	0.94	−0.12 (−0.66, 0.41)	0.65	−0.12 (−1.46, 1.23)	0.86	−0.28 (−0.74, 0.20)	0.24	−0.05 (−0.63, 0.52)	0.85
Serotonin+dopamine	−0.89 (−2.01, 0.18)	0.11	0.03 (−0.50, 0.56)	0.92	−0.13 (−0.64, 0.41)	0.64	−0.26 (−1.64, 1.01)	0.70	−0.05 (−0.49, 0.41)	0.85	0.08 (−0.49, 0.66)	0.77
Parasite status	**1.25 (1.07, 1.42)**	**<0.001**	**0.13 (0.07, 0.19)**	**<0.001**	**−0.40 (−0.45, −0.35)**	**<0.001**	**0.56 (0.50, 0.63)**	**<0.001**	0.09 (−0.06, 0.25)	0.25	**0.05 (0.01, 0.09)**	**0.02**
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	0.58 (0.39, 0.88)		0.13 (0.09, 0.19)		0.15 (0.12, 0.22)		0.91 (0.73, 1.37)		0.08 (0.04, 0.13)		0.17 (0.14, 0.26)	
Pre-exposure only												
Intercept	0.52 (0.19, 0.85)	<0.001	3.17 (2.92, 3.55)	<0.001	3.82 (3.64, 4.02)	<0.001	3.52 (2.59, 4.42)	<0.001	1.15 (0.84, 1.44)	<0.001	4.10 (3.63, 4.57)	<0.001
Serotonin	0.08 (−0.36, 0.54)	0.74	−0.04 (−0.57, 0.47)	0.88	−0.02 (−0.30, 0.26)	0.88	−1.17 (−2.51, 0.15)	0.08	0.04 (−0.37, 0.45)	0.85	−0.31 (−0.98, 0.36)	0.37
Dopamine	−0.43 (−0.91, 0.04)	0.08	−0.09 (−0.62, 0.41)	0.71	0.22 (−0.06, 0.49)	0.11	−0.31 (−1.60, 0.99)	0.64	0.20 (−0.20, 0.63)	0.33	0.23 (−0.40, 0.89)	0.51
Serotonin+dopamine	−0.09 (−0.55, 0.36)	0.7	−0.06 (−0.57, 0.45)	0.81	−0.16 (−0.44, 0.11)	0.25	−0.80 (−2.08, 0.48)	0.23	0.06 (−0.35, 0.48)	0.78	−0.21 (−0.90, 0.47)	0.53
Parasite status	0.08 (−0.16, 0.32)	0.54	0.00 (−0.07, 0.07)	0.94	**−0.19 (−0.24, −0.14)**	**<0.001**	0.00 (−0.06, 0.06)	0.98	0.03 (−0.13, 0.20)	0.73	**0.04 (0.00, 0.08)**	**0.04**
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	0.05 (0.02, 0.09)		0.13 (0.10, 0.19)		0.04 (0.02, 0.05)		0.86 (0.70, 1.27)		0.06 (0.3, 0.10)		0.23 (0.19, 0.34)	
Day 6 only												
Intercept	0.77 (−0.13, 1.67)	0.09	2.81 (2.43, 3.19)	<0.001	3.8 (3.34, 4.25)	<0.001	3.08 (1.74, 4.43)	<0.001	0.96 (0.58, 1.34)	<0.001	3.72 (3.17, 4.26)	<0.001
Serotonin	−0.27 (−1.57, 1.00)	0.68	0.29 (−0.23, 0.84)	0.29	0.00 (−0.63, 0.64)	0.99	−0.34 (−2.18, 1.61)	0.73	0.05 (−0.50, 0.59)	0.87	0.09 (−0.71, 0.88)	0.81
Dopamine	−0.47 (−1.79, 0.80)	0.47	0.14 (−0.41, 0.67)	0.62	0.21 (−0.42, 0.86)	0.52	−0.32 (−2.21, 1.60)	0.74	−0.05 (−0.56, 0.48)	0.86	0.09 (−0.63, 0.86)	0.82
Serotonin+dopamine	−0.5 (−1.77, 0.68)	0.44	0.17 (−0.39, 0.72)	0.55	−0.07 (−0.71, 0.56)	0.82	−0.56 (−2.44, 1.43)	0.56	−0.07 (−0.61, 0.49)	0.81	−0.02 (−0.78, 0.73)	0.97
Parasite status	**0.68 (0.46, 0.89)**	**<0.001**	**0.21 (0.13, 0.28)**	**<0.001**	**−0.55 (−0.61, −0.49)**	**<0.001**	**0.32 (0.25, 0.40)**	**<0.001**	0.18 (−0.03, 0.39)	0.09	**0.15 (0.10, 0.20)**	**<0.001**
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	0.74 (0.51, 1.17)		0.14 (0.11, 0.22)		0.20 (0.15, 0.30)		1.81 (1.43, 2.67)		0.10 (0.05, 0.17)		0.30 (0.24, 0.46)	
Day 18 only												
Intercept	0.2 (−1.47, 1.94)	<0.001	3.38 (3.01, 3.78)	<0.001	3.30 (2.40, 4.20)	<0.001	1.43 (0.74, 2.18)	<0.001	1.31 (0.98, 1.63)	<0.001	3.76 (3.00, 4.50)	<0.001
Serotonin	**−3.13 (−5.76, −0.59)**	**0.02**	−0.27 (−0.84, 0.28)	0.33	−1.13 (−2.40, 0.16)	0.09	−0.25 (−1.28, 0.79)	0.64	0.14 (−0.31, 0.59)	0.54	0.44 (−0.62, 1.47)	0.41
Dopamine	−1.45 (−3.90, 0.97)	0.24	−0.23 (−0.78, 0.27)	0.4	−0.31 (−1.62, 0.96)	0.63	0.18 (−0.88, 1.21)	0.73	**−0.76 (−1.28, −0.23)**	**<0.01**	−0.82 (−1.87, 0.24)	0.13
Serotonin+dopamine	−1.28 (−3.70, 1.13)	0.29	−0.2 (−0.76, 0.33)	0.47	0.33 (−0.96, 1.59)	0.61	0.08 (−0.94, 1.11)	0.87	−0.07 (−0.53, 0.36)	0.74	0.38 (−0.71, 1.42)	0.48
Parasite status	**2.18 (1.92, 2.43)**	**<0.001**	**0.18 (0.09, 0.28)**	**<0.001**	**−0.2 (−0.29, −0.11)**	**<0.001**	**0.64 (0.47, 0.83)**	**<0.001**	0.14 (−0.10, 0.41)	0.3	0.00 (−0.07, 0.06)	0.88
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	2.41 (1.49, 3.95)		0.14 (0.10, 0.21)		0.80 (0.58, 1.22)		0.47 (0.30, 0.75)		0.04 (0.02, 0.08)		0.55 (0.42, 0.83)	

Fish were divided into control, serotonin (fluoxetine hydrochloride), dopamine (ropinirole hydrochloride) or both serotonin and dopamine treatments. Estimated effect sizes and 95% credible intervals (CI) around the mean of predictors of the measured behaviors: latency to move in a novel area, number of square changes in a novel area, time in the upper mid zone of a novel area, latency to swim during the mirror test, number of times the fish approached and attacked the mirror and time spent close to the mirror. Data are for individuals from all exposure time periods pooled (day 6 and day 18; *N*=442), pre-exposure only (day 0; *N*=187), day 6 only (*N*=150) and day 18 only (*N*=181). Significant differences (CI does not cross zero) are in bold. Parasite status was used as a statistical control, and the interaction between parasite status and treatment was not measured.

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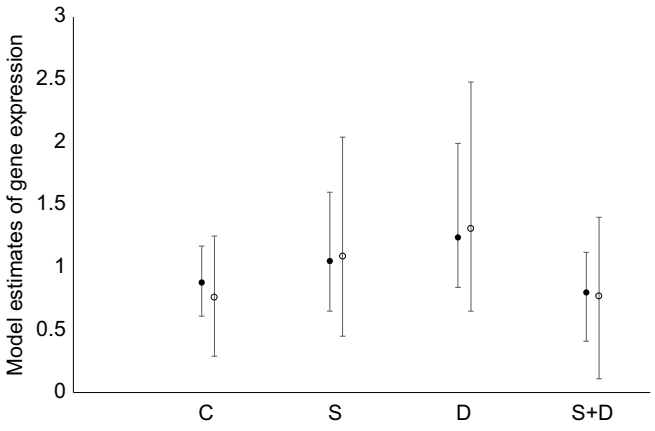

Fig. 3. Influence of monoamine manipulation on expression of dopamine and serotonin receptor genes in sticklebacks. Effect of control (C), serotonin (fluoxetine hydrochloride; S), dopamine (ropinirole hydrochloride; D) and serotonin+dopamine (S+D) treatment on expression of dopamine (DRD1B, open circles) and serotonin (HTR2B, filled circles) receptor genes. Points are univariate linear mixed model estimates with 95% CI, generated from models reported in Table 2. Data are from wild-caught individuals from all time periods pooled (day 0, day 6, day 18; *N*=64), with three replicate tanks per treatment group.

our combined treatment, the overall drug dose was actually higher than in the single treatments (combined individuals got both the full serotonin and full dopamine doses), this could result in a lower pharmacological response in the combined treatment compared with the single drug treatment. Fluoxetine can affect nest quality in sticklebacks with no concentration response (Sebire et al., 2015), suggesting the existence of a plateau in the dose–response curve. Many studies dealing with the effects of pharmaceuticals in the environment are restricted by the use of only one or a couple of different drug concentrations, thus making it difficult to evaluate whether the observed effects are concentration dependent (Sumpter et al., 2014). The ways in which ropinirole could potentially stimulate the serotoninergic system or fluoxetine could potentially stimulate the dopaminergic system remain unclear. Contrary to first-generation ergoline-derived dopamine agonists, ropinirole is weakly active at the 5-HT2 receptors and has almost no affinity for the 5-HT1 receptors (Eden et al., 1991; Borovac, 2016). Fluoxetine is known to have a low affinity for the dopamine transporter (Owens et al., 2001) and thus is thought to have a minimal effect on dopamine reuptake at therapeutic doses. Conversely, acute systemic administration of fluoxetine resulted in increased synaptic concentrations of both serotonin and dopamine in the rat prefrontal cortex (Bymaster et al., 2002), but at fluoxetine concentrations significantly higher than that used in our study. In the light of these observations, how drugs interact is still uncertain and further testing is needed to fully understand the differences between single and combined drug exposure. Specifically, repeating our study using a wide range of concentrations for both drugs would allow us to evaluate the concentration–response relationship and potentially overcome some of the limitations of many studies in this new field (Sumpter et al., 2014).

Our study confirms the importance of parasites in terms of animal behavior, matching previous findings particularly showing that infected sticklebacks are more sociable than uninfected fish (Ward et al., 2005; Barber and Scharsack, 2010; Petkova et al., 2018). Parasitism could thus be another contributing mechanism explaining variation in host personality (Poulin, 2010). In the current study we were unable to compare gene expression in

Table 2. Influence of monoamine manipulation on stickleback brain gene expression

	NR3C2		*NR3C1*		*HTR2A*		*HTR2B*		*DRD2*		*DRD1B*		*ADRB2A*	
Fixed effects	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
Intercept	0.86 (0.52, 1.20)	<0.001	0.99 (0.69, 1.31)	<0.001	0.99 (0.55, 1.44)	<0.001	0.88 (0.61, 1.17)	<0.001	0.93 (0.49, 1.40)	<0.001	0.76 (0.29, 1.25)	0	0.90 (0.43, 1.37)	<0.001
Serotonin	0.16 (−0.28, 0.61)	0.48	0.02 (−0.42, 0.42)	0.93	0.05 (−0.55, 0.64)	0.86	0.17 (−0.23, 0.55)	0.38	−0.1 (−0.73, 0.56)	0.76	0.33 (−0.31, 0.95)	0.32	0.06 (−0.58, 0.69)	0.86
Dopamine	0.30 (−0.14, 0.75)	0.18	−0.06 (−0.52, 0.39)	0.8	0.16 (−0.48, 0.76)	0.6	0.36 (−0.04, 0.75)	0.06	0.30 (−0.30, 0.94)	0.35	0.55 (−0.11, 1.17)	0.09	0.23 (−0.42, 0.85)	0.45
Serotonin+dopamine	0.08 (−0.36, 0.53)	0.71	0.08 (−0.38, 0.50)	0.73	−0.17 (−0.77, 0.45)	0.57	−0.08 (−0.47, 0.32)	0.67	0.00 (−0.65, 0.63)	0.99	0.01 (−0.65, 0.63)	0.98	0.10 (−0.56, 0.74)	0.76
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	0.00 (0.00, 0.00)		0.00 (0.00, 0.00)		0.02 (0.00, 0.04)		0.00 (0.00, 0.00)		0.08 (0.03, 0.16)		0.06 (0.02, 0.12)		0.00 (0.00, 0.00)	

Fish were divided into control, serotonin (fluoxetine hydrochloride), dopamine (ropinirole hydrochloride) or both serotonin and dopamine treatment groups. Estimated effect sizes and 95% credible intervals (CI) around the mean of predictors of the measured genes encoding: a glucocorticoid receptor (GR, *NR3C1*); a mineralocorticoid receptor (MR, *NR3C2*); two serotonin receptor subtypes (*HTR2A* and *HTR2B*); two dopamine receptor subtypes (*DRD1B* and *DRD2*); and an adrenergic receptor (*ADRB2A*). Data are from individuals from all time periods pooled (day 0, day 6, day 18; *N*=64).

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Table 3. Influence of natural expression levels of monoamine-associated genes on stickleback behavior

	No. of square changes		Time in upper mid zone		Latency to swim		No. of approaches		Time close to mirror	
Fixed effects	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
Intercept	**3.27 (2.59, 3.96)**	**<0.001**	**7.15 (6.09, 8.33)**	**<0.001**	−2.82 (−13.3, 7.07)	0.57	−2.75 (−6.44, 0.90)	0.15	**−1.11 (−2.04, 0.13)**	**0.03**
NR3C1	−0.03 (−1.01, 0.93)	0.95	**−2.18 (−4.19, −0.12)**	**0.04**	6.09 (−6.29, 18.3)	0.32	0.62 (−1.99, 3.25)	0.66	**2.34 (1.63, 3.00)**	**<0.001**
NR3C2	**1.21 (0.62, 1.83)**	**<0.001**	**−1.48 (−2.40, −0.60)**	**<0.001**	4.96 (−2.35, 12.6)	0.18	**4.34 (1.29, 7.21)**	**<0.01**	**2.64 (1.89, 3.33)**	**<0.001**
HTR2A	**−1.53 (−2.43, −0.65)**	**0.001**	**3.00 (1.36, 4.67)**	**<0.001**	−6.73 (−14.6, 0.78)	0.09	−2.54 (−6.06, 0.89)	0.16	−0.14 (−0.86, 0.56)	0.69
HTR2B	**−0.57 (−1.09, −0.03)**	**0.04**	**−6.07 (−7.75, −4.41)**	**<0.001**	−7.98 (−19.4, 3.80)	0.17	1.43 (−0.25, 2.99)	0.09	**0.94 (0.56, 1.34)**	**<0.001**
DRD2	**2.00 (1.04, 2.91)**	**<0.001**	**2.31 (0.60, 3.98)**	**<0.01**	6.99 (−1.19, 15.2)	0.09	−0.66 (−3.90, 2.63)	0.69	**−1.31 (−2.22, −0.48)**	**<0.01**
DRD1B	**−2.02 (−2.81, −1.22)**	**<0.001**	**−2.98 (−4.57, 1.41)**	**<0.001**	−4.37 (−12.3, 3.48)	0.28	0.65 (−1.83, 3.24)	0.62	**1.21 (0.50, 1.94)**	**<0.001**
ADRB2A	**0.41 (0.24, 0.58)**	**<0.001**	**0.76 (0.43, 1.10)**	**<0.001**	**3.43 (0.95, 5.98)**	**<0.01**	0.28 (−0.24, 0.78)	0.28	0.05 (−0.09, 0.19)	0.47

Estimated effect sizes and 95% credible intervals (CI) around the mean of the expression level of each gene, as a predictor of the measured behaviors: number of square changes in a novel area, time in the upper mid zone of a novel area, latency to swim during the mirror test, number of times the fish approached and attacked the mirror and time spent close to the mirror. Measured genes encoded: a glucocorticoid receptor (GR, *NR3C1*); a mineralocorticoid receptor (MR, *NR3C2*); two serotonin receptor subtypes (*HTR2A* and *HTR2B*); two dopamine receptor subtypes (*DRD1B* and *DRD2*); and an adrenergic receptor (*ADRB2A*). Data are from control individuals only (N=15). Significant differences (CI does not cross zero) are in bold.

infected and uninfected fish; however, other studies in sticklebacks have found that infected fish had lower levels of serotonin in the brain (Ness and Foster, 1999; Øverli et al., 2001a). Future studies should test whether the link between parasite infection and altered behavior is via changes in expression of genes in the monoamine systems (which is likely; e.g. Øverli et al., 2001a).

We found behavioral responses to our manipulations of monoaminergic systems, and confirmed that natural variation in expression of genes of monoamine and stress systems co-varied with behavior. However, we failed to find treatment-specific differences in the expression of stress- and monoamine-associated genes. Taken together, our results show a clear link between monoamines and behavior, but also highlight that gene expression, at least of our measured genes in whole brain tissue, may not be influenced by exposure to our tested pharmaceuticals. Work in other populations of sticklebacks has had similar results, confirming correlations between behavior and monoamine systems, but not finding monoamines as underlying mechanisms (Abbey-Lee et al., 2018b). Potentially, earlier steps of the HPI axis or other unmeasured aspects of physiology may be responsible for the observed behavioral differences, and therefore our manipulations of serotonin and dopamine levels were ineffectual. Alternatively, gene expression differences may be limited to specific brain regions, such that our analysis of whole brains masked an effect. Follow-up work using a wider range of manipulative compounds, and analyzing more varied potential mechanisms, will help to further elucidate the links between monoamines, stress and behavior.

Conclusions

We have demonstrated a causal link between monoamine manipulation (via exposure to human pharmaceuticals) and stickleback behavior, adding support to monoamines being key mechanisms for behavioral differences. However, we did not detect differences in gene expression of monoamine- and stress-associated genes with our monoamine manipulations, indicating that this may not be the pathway by which monoamine systems are altered. 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.N.A.-L., H.L.; Methodology: R.N.A.-L., A.K., X.F.S., I.P., H.L.; Formal analysis: R.N.A.-L.; Investigation: R.N.A.-L.; Resources: R.N.A.-L.; Data curation: R.N.A.-L.; Writing - original draft: R.N.A.-L., A.K., X.F.S., H.L.; Writing - review & editing: R.N.A.-L., A.K., X.F.S., I.P., H.L.; Visualization: R.N.A.-L.; Supervision: R.N.A.-L., H.L.; Funding acquisition: H.L.; R.N.A.-L.

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Data availability

Data are available from the open access DiVA portal of Linkoping University: http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-160555.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.211888.supplemental

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