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Social context affects behavior, preoptic area gene expression, and response to D2 receptor manipulation during territorial defense in a cichlid fish[1]

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Social context often has profound effects on behavior, yet the neural and molecular mechanisms which mediate flexible behavioral responses to different social environments are not well understood. We used the African cichlid fish, *Astatotilapia burtoni*, to examine aggressive defense behavior across three social contexts representing different motivational states: a reproductive opportunity, a familiar male and a neutral context. To elucidate how differences in behavior across contexts may be mediated by neural gene expression, we examined gene expression in the preoptic area, a brain region known to control male aggressive and sexual behavior. We show that social context has broad effects on preoptic gene expression. Specifically, we found that the expression of genes encoding nonapeptides and sex steroid receptors are upregulated in the familiar male context. Furthermore, circulating levels of testosterone and cortisol varied markedly depending on social context. We also manipulated the D2 receptor (D2R) in each social context, given that it has been implicated in mediating context-dependent behavior. We found that a D2R agonist reduced intruder-directed aggression in the reproductive opportunity and familiar male contexts, while a D2R antagonist inhibited intruder-directed aggression in the reproductive opportunity context and increased aggression in the neutral context. Our results demonstrate a critical role for preoptic gene expression, as well as circulating steroid hormone levels, in encoding information from the social environment and in shaping adaptive behavior. In addition, they provide further evidence for a role of D2R in context-dependent behavior.

Keywords: Aggression, cortisol, dopamine, nonapeptides, preoptic area, sex steroid receptors, social context, territorial defense, testosterone

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In social animals, individual behavior is powerfully affected by interactions with conspecifics. Even the presence or absence of an audience can cause dramatic changes in behavior during social interactions (Zajonc 1965). For example, subordinate male cichlid fishes will increase aggressive displays when dominant males are not paying attention (Desjardins *et al.* 2012). Male zebra finches respond differently to calls of their female partner depending on whether the audience is a mated or unmated pair (Vignal *et al.* 2004). Furthermore, male budgerigars are more likely to seek extra-pair copulations when their mate cannot see them (Baltz & Clark 1997). Despite our appreciation of behavioral flexibility in animals, our understanding of the neural and molecular mechanisms by which animals assess and respond to such social information is still incomplete (Hofmann *et al.* 2014).

There are several neurochemical pathways that may be involved in mediating context-dependent behavior. The nonapeptides oxytocin (OT) and arginine vasopressin (AVP) and their receptors have been well studied for their roles in modulating social behavior, including aggression, social recognition, pair-bonding and parental care (Goodson & Thompson 2010). However, they are often found to have inconsistent effects on behavioral phenotypes across species, suggesting the way in which they modulate behavior depends to some extent on the social context. For example, in the violet-eared waxbill, treatment with arginine vasotocin 1a receptor antagonist reduced mate-competition aggression but not resident-intruder aggression (Goodson *et al.* 2009). Similarly, the effects of OT and AVP antagonists in the lateral septum on play behavior of juvenile rats differ between novel and familiar environments (Bredewold *et al.* 2014).

Steroid hormones have also been implicated in mediating behavioral flexibility in response to the social environment (Oliveira 2009). They have pleiotropic effects that serve to integrate the whole organism physiological response to the environment. Circulating levels of testosterone (T) respond to social challenges but can be decoupled from aggressive behavior depending on the social context (Pinxten *et al.* 2002; reviewed in: Trainor *et al.* 2006). Circulating levels of glucocorticoids, such as cortisol (Cort), are involved in the stress response and promote the memory and perception of risk (Hayden-Hixson & Ferris 1991; McEwen & Wingfield 2003). The behavioral responsiveness to circulating steroid hormone levels can be modulated by changes in the density

of androgen (AR) and estrogen (ER) receptors in brain regions that mediate social behavior. For example, the expression levels of AR and ER in the forebrain of cichlid fish differ between dominant and subordinate males in a manner that suggests dominant males may be more sensitive to circulating steroids (Burmeister *et al.* 2007).

The neurotransmitter dopamine (DA) can also mediate behavioral flexibility. Dopamine plays a fundamental role in encoding the salience and rewarding properties of social stimuli, and may mediate social context-dependent behavior by changing the motivational state between different contexts (Lucas *et al.* 2004; Riters 2012; Trainor 2011; Young *et al.* 2011). The D2 receptor (D2R) pathway in particular plays an intriguing role in modulating motivation related to social context (Choleris *et al.* 2011; Young & Wang 2004). In male Syrian hamsters, intraperitoneal treatment with a D2R antagonist reduces the attractive properties of a rewarding sexual cue (Bell & Sisk 2013). In male zebra finches, subcutaneous treatment with D2R agonist inhibits aggressive behavior displayed when competing for mates, but does not affect courtship song (Kabelik *et al.* 2010). In male mice, intraperitoneal treatment with a D2R antagonist decreases defensive behavior and increases social investigation after experiencing defeat, while a D2R agonist produces the opposite effects (Puglisi-Allegra & Cabib 1988). Interestingly, manipulations of D1 and D2 receptors appear to often have opposing effects in a variety of behaviors, where D1 stimulation facilitates and D2 stimulation inhibits the production of sexually-motivated male behavior (Aragona *et al.* 2006; Balthazart 1997; Kleitz-Nelson *et al.* 2010a, 2010b). It has thus been suggested that the ratio of D2/D1 receptors may influence sexual activity (Hull *et al.* 1989).

Social behavior is controlled, in part, by the social decision-making network, a set of brain regions involved in encoding stimulus salience and valence that ultimately results in adaptive behavior (O'Connell & Hofmann 2011). A central node in this network is the preoptic area (POA), which regulates male sexual and aggressive behavior across vertebrates (Hull & Dominguez 2007; Kleitz-Nelson *et al.* 2010a, 2010b). The actions of the nonapeptide, sex steroid, and DA pathways acting within the POA may play a primary role in context-dependent social behavior. For example, the preoptic DA system mediates context-dependent song production in birds (Heimovics & Riters 2008; Riters 2012). Little is known, however, as to how nonapeptide, sex steroid and DA genes all vary in response to social context, particularly in a spatially explicit manner in the brain.

To begin to delineate the role of these candidate pathways in regulating social context-dependent decision-making, we used the African cichlid fish, *Astatotilapia burtoni*, a model system in social neuroscience (Robinson *et al.* 2008). *Astatotilapia burtoni* display extraordinary cognitive abilities in a social context-dependent manner, which can be robustly quantified and manipulated in naturalistic settings in the laboratory (Hofmann 2003). Males reduce their aggressive interactions as they become familiar neighbors (FNs; Weitekamp & Hofmann 2017). Individuals are keenly aware of their social environment and modulate their aggressive and courtship behavior for reproductive and social advantage. Importantly, male-male aggression is higher when in the audience of a reproductive female (Desjardins *et al.* 2012). Finally, the nonapeptide, sex steroid and DA systems have been well characterized in this species (Huffman *et al.* 2012a, 2012b; Munchrath & Hofmann 2010; O'Connell *et al.* 2011, 2013a,2013b).

In the present study, to examine the effect of social context on behavior and gene expression, we conducted a series of four experiments in which we repeatedly presented dominant, territorial *A. burtoni* males with one of three social contexts; either a FN male, a gravid female (Reproductive Opportunity, RO), or a non-reproductive female [Neutral Stimulus (NS)]. After exposure to the social context, we added a novel conspecific male intruder to the territory of the focal male. In Experiment 1, we assessed the effect of social context on defense behavior. In Experiment 2, we examined differences in circulating hormone levels across contexts. In Experiment 3, to assess how behavior may be mediated via changes in POA gene expression, we examined differences in expression of nonapeptide, sex steroid and DA genes across contexts. In addition, we measured expression of two immediate-early genes, which serve as markers of neuronal activation, expecting that overall neural activity in the POA may differ between contexts. Specifically, given the role of sexual reward and the observation of increased territorial aggression in the presence of reproductive females, we predicted all measured genes would be upregulated in the RO context. Finally, in Experiment 4, we manipulated the D2R pathway, expecting to find opposing behavioral effects across contexts, as the motivation underlying territorial defense differs in each context. We predicted the D2R agonist to inhibit aggression in the sexually-motivated context, RO and the D2R antagonist to inhibit territorial aggression in the FN context. Our results suggest that social context has large effects on circulating hormone levels and preoptic gene expression, and provide further evidence for a role of the D2R pathway in mediating social-context-dependent behavior.

Materials and methods

Animals

Astatotilapia burtoni descended from a wild caught stock population were maintained in stable naturalistic communities, as described previously (O'Connell & Hofmann 2012), until transferred to the experimental paradigm. All work was done in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Behavior

For all treatments, the focal tank from a set of adjacent 38 l aquarium tanks was established with one territorial male and two non-reproductive females (Fig. 1). In the RO context, the adjacent tank contained one gravid female and two non-reproductive females. In the FN context, the adjacent tank contained one size-matched territorial male and two non-reproductive females. In the NS context, the adjacent tank contained three non-reproductive females. Females were stripped of their brood immediately before placement in each tank, ensuring that they would remain non-reproductive for the duration of the experiment (Kidd *et al.* 2013). Each tank contained a halved flower pot to serve as a bower, and flowerpot shards and plastic aquarium plants as refuge for the females. To allow fish to acclimate, adjacent tanks were separated by an opaque divider and kept visually

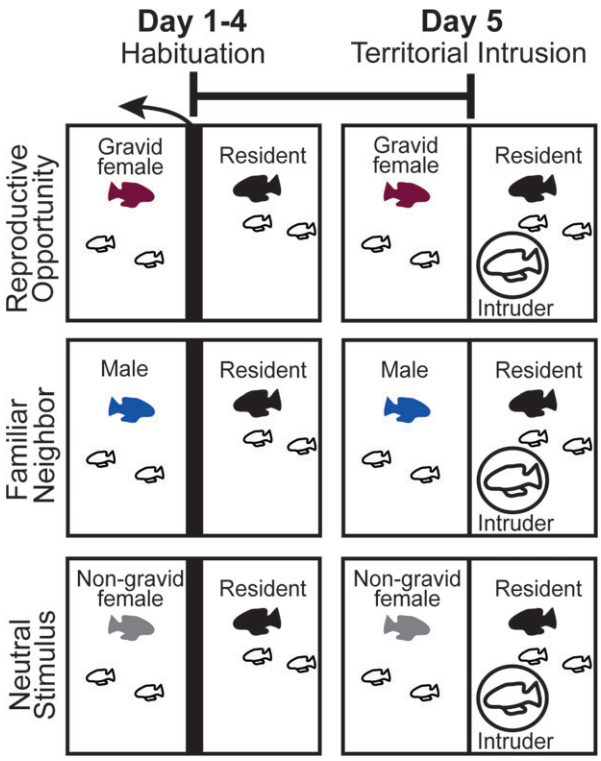

Figure 1: Experimental paradigm. After acclimation, the visual barrier separating tanks was removed for 1 h twice daily for 4 days and served as the habituation phase. On Day 5, the visual barrier was removed immediately prior to the introduction of the intruder. The social context was either a gravid female (Reproductive Opportunity), a male (Familiar Neighbor) or a non-gravid female (Control). All tanks contained additional non-gravid females.

isolated for 1 week. A few males did not show a dominant, territorial phenotype at the end of the acclimation period and therefore were replaced with new fish for the next round of experiments.

At the start of each experiment, we removed the opaque divider between tanks for 1 h each at 1000 h and 1500 h, respectively, for 4 continuous days which served as the habituation phase (Fig. 1). On Day 5, at 1000 h, we placed a territorial intruder contained within a transparent plastic cylinder within the tank of the focal male (Resident), and immediately removed the opaque divider between tanks. The intruder was always larger than the Resident so as to elicit consistent levels of aggression from the Resident. One hour after the introduction of the intruder, the Resident was removed and its body mass and standard length was measured. One hour is sufficient time to detect acute changes in hormone levels (Huffman *et al.* 2012a, 2012b; O'Connell *et al.* 2013b) and immediate-early gene messenger RNA (IEG mRNA) levels (Clayton 2000; Maruska *et al.* 2013). Expression levels of receptor genes and nonapeptides are generally assumed to change more slowly, e.g. in response to social context during the habituation phase. It is, however, possible that these genes are also dynamically regulated.

Behavior was video recorded on Day 5. Video scoring was done blind to treatment. Behavior of the Resident was quantified using JWatcher V1.0 and was scored for a 10 min window 20 min after removal of the opaque divider. Previous studies have shown this window to effectively capture meaningful variation in intruder-directed aggressive behavior (O'Connell *et al.* 2013b). Behaviors recorded included forward displays to the intruder (or partner), lateral displays to the intruder (or partner), chases to the school, and courtship displays. Forward and lateral displays showed similar patterns, and thus were summed to total aggressive displays. The data from chases and courtship displays are not shown because they occurred at low and similar rates across groups. In the FN context, behavior of the partner male was also scored. For a detailed analysis, see (Weitekamp & Hofmann 2017). The behavior of the females was not scored as there was very little variation in female behavior. Sample sizes and male sizes are summarized in Table S1, Supporting Information.

Tissue preparation

Behavior experiments were conducted as described above with no drug treatments (RO: $n=15$; FN: $n=13$; NS: $n=12$). For a subset of individuals ($n=9$ per treatment), after measuring for length and mass, blood was drawn from the dorsal aorta using heparinized 26G butterfly infusion sets (Becton Dickson, Mountain View, CA, USA). Plasma was stored at −80°C for hormone analysis. Males were killed by rapid cervical transection and brains flash frozen in O.C.T. (Tissue-Tek; Fisher Scientific Co., Pittsburgh, PA, USA) and stored at −80°C.

Hormone measurements

Free circulating T and Cort were measured for each Resident male from which tissue was collected ($n=9$ per treatment) using ELISA (Enzo Life Sciences, Farmingdale, NY, USA). Plasma samples were diluted 1:30 and processed as previously described (Kidd *et al.* 2010). We did not measure 11-ketotestosterone, the active AR in many teleost species, as several studies in *A. burtoni* and other haplochromine cichlids have demonstrated that 11-ketotestosterone levels are tightly correlated with T levels, are an order of magnitude lower, and contain more random variation (Dijkstra *et al.* 2012; Kidd *et al.* 2010; Parikh *et al.* 2006).

Quantitative RT-PCR

Brains were sliced on a cryostat in the coronal plane at 300 μm. A 300 μm diameter sample corer tool (Fine Science Tools, Foster City, CA, USA) was used to micro-dissect the POA. The POA was anatomically defined following Maruska *et al.* (2013). Two microdissected punches (left and right hemisphere) were taken from a single brain slice and stored in DNA/RNA Shield (Zymo Research, Irvine, CA, USA) at −80°C until processing. This technique likely does not include the caudal gigantocellular preoptic nucleus (Maruska *et al.* 2013).

To homogenize tissue prior to RNA extraction, ZR BashingBeads (Zymo Research) were added to samples suspended in DNA/RNA Shield and tubes were vortexed. To further lyse tissue, a Proteinase K digestion was done for 2 h at 55 °C. Total RNA was then extracted in accordance with the protocol for the Quick-RNA MicroPrep kit (Zymo Research). To prevent genomic DNA contamination, RNA samples were treated with DNase (Zymo Research) during the isolation procedure. RNA was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega Corporation, Madison, WI, USA).

Quantitative RT-PCR was used to measure the mRNA levels of target genes ($n=9$ per treatment). To investigate how nonapeptides, sex steroids and DA may contribute to regulating context-dependent behavior within the POA, we examined expression of *it*, *avt*, *itr*, *v1ar2*, *erα*, *arα*, *d1r*, *d2r* and tyrosine hydroxylase (*th*; which catalyzes the rate limiting step in catecholamine synthesis and is a neurochemical marker for dopaminergic cell populations in the teleost fore- and midbrain (Levitt *et al.* 1965; O'Connell *et al.* 2011)). In addition, we measured expression of two IEGs, *c-fos* and *egr-1*, expecting that overall neural activity may differ between contexts. Quantitative RT-PCR primers (Table S2) were designed to flank exon-exon boundaries. For each sample, target gene expression was measured in triplicate in the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using GoTaq qPCR Master Mix (Promega Corporation). Amplification efficiency for each primer pair was determined using standard curves made from serial dilutions of cDNA.

Pharmacology

Tanks were established as described above. At 0900 h, i.e. 1 h before the introduction of the intruder on Day 5, the Resident received an intraperitoneal injection of either 2 μg/g body weight D2R agonist quinpirole hydrochloride (Sigma Chemical Co., St. Louis, MO; $n=8$ per treatment), 1 μg/g body weight D2R antagonist metoclopramide hydrochloride (Sigma; $n=8$ for RO and NS, $n=9$ for FN) or 20 μl/g body weight 1×PBS saline ($n=8$ per treatment). Systemic administration of these drugs has demonstrated behavioral effects when given at similar intervals prior to testing (Baker *et al.* 2015; Boulougouris *et al.* 2009). Furthermore, these drugs have been used at similar doses in teleosts (Brzuska *et al.* 2004; Messias *et al.* 2016; Otto *et al.* 1999). At the end of each trial, males were uniquely tagged and returned to community tanks. Injections were performed blind to treatment.

Statistical analyses

All statistical tests were performed using R v. 3.1.0. To determine relative gene expression of each sample, we used the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Specifically, cycle threshold values of the target genes were first normalized by the amount of 18S present in each sample and then calibrated within each target gene.

To examine the effects of social context on baseline behavior, hormone levels and gene expression we used one-way analysis of variance (ANOVA), followed by *post hoc* analysis using Tukey's honest significant difference (HSD) test. To examine whether social context affects gene expression co-variance patterns, we used R package lattice to create clustered correlation matrices for each context. We generated *P*-values for each cluster using multiscale bootstrap resampling from the R package pvclust with the Ward clustering method (Suzuki & Shimodaira 2006) and highlighted clusters for which $P<0.05$ with boxes. For the pharmacology data, planned comparisons were conducted using Welch's two sample t-tests to examine differences between saline treatment and drug treatment. Tests were also separately conducted within each drug treatment group to examine context-specific differences using one-way ANOVA. Data were checked for normality with the Shapiro-Wilk test. Non-normal data were log transformed, resulting in normally distributed residuals. We used generalized linear regression analysis to examine the relationships between aggression and male size, hormone levels and gene expression. We used the R package glmulti, which ranks models by AICc scores, for model selection (Calcagno & de Mazancourt 2010).

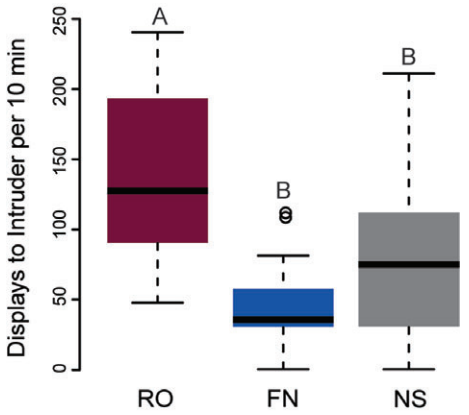

Figure 2: Aggressive response to an intruder challenge differed by social context. Males displayed more frequent aggression in RO, compared to FN or NS. Letters denote homogeneous subgroups.

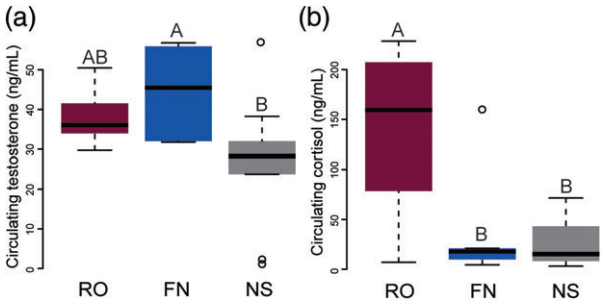

Figure 3: Hormonal response to an intruder challenge differed by social context. Letters denote homogeneous subgroups.

Results

Experiment 1: social context affects territorial aggression

We found that intruder-directed aggression differed significantly by social context ($F_{2,37}=9.72$, $P=4.05\times10^{-4}$; Fig. 2). Males attacked the intruder more frequently in RO compared to FN ($P=3.15\times10^{-4}$) and to NS ($P=0.027$). Aggressive displays to the intruder in the FN and NS contexts did not differ ($P=0.31$). Aggressive displays to the intruder negatively correlate with Resident male size in the RO ($R^2=0.3$, $P=0.035$) and NS ($R^2=0.42$, $P=0.024$) contexts, but not in FN ($R^2=0.16$, $P=0.18$).

Experiment 2: hormonal responses across social contexts

Given the well-known role of ARs and glucocorticoids in regulating social behavior, we examined next whether the hormonal response to an intruder challenge depended on social context by measuring levels of circulating T and Cort. Circulating levels of both T ($F_{2,21}=4.14$, $P=0.03$; Fig. 3a) and Cort differed significantly across contexts ($F_{2,17}=6.56$, $P=7.7\times10^{-3}$; Fig. 3b). Circulating T was higher in FN compared to NS ($P=0.03$). There were no differences in levels of T between the RO and NS ($P=0.15$) and RO and FN ($P=0.59$). Circulating Cort levels were higher in RO relative to both FN ($P=0.046$) and NS ($P=8.2\times10^{-3}$), resembling the pattern seen above with aggressive displays. There was no difference in Cort levels between FN and NS ($P=0.89$). Levels of T ($R^2=0.0024$, $P=0.82$) and Cort ($R^2=0.062$, $P=0.50$) did not correlate with aggressive displays to the intruder across contexts. T and Cort levels themselves were negatively correlated in RO ($R^2=0.84$, $P=0.0039$), positively correlated in NS ($R^2=0.57$, $P=0.03$), but not correlated in FN ($R^2=0.01$, $P=0.90$).

Experiment 3: POA gene expression varies with context and behavior

We find variation in *arα* ($F_{2,24}=5.12$, $P=0.014$), *erα* ($F_{2,24}=4.0$, $P=0.032$), *it* ($F_{2,24}=6.83$, $P=0.0045$) and *avt* ($F_{2,24}=3.5$, $P=0.046$) across contexts (Fig. 4a, see also Figure S1, Table S3). *arα* is higher in FN compared to both RO

($P = 0.035$) and NS ($P = 0.022$). *erα* is higher in FN than in NS ($P = 0.037$) but not significantly higher than RO ($P = 0.089$). *it* is higher in FN compared to NS ($P = 0.0038$) and marginally higher than RO ($P = 0.052$). Finally, *avt* is marginally higher in FN compared to NS ($P = 0.06$). Expression levels of *itr*, *v1ar*, *th*, *d1r and d2r*, as well as the *d2r:d1r* ratio did not differ across contexts (Table S3).

Next, we investigated how candidate gene expression in the POA varies with intruder-directed aggressive behavior both within and across social contexts. Across contexts, the linear regression model that best predicts intruder-directed aggression includes expression levels of *th* ($P = 0.027$) and *erα* ($P = 0.012$; Overall model: $R^2 = 0.25$, $P = 0.033$). Within each context, we examined which candidate gene best predicted aggressive displays to the intruder (Table S4, Figure S2). In RO, aggressive displays to the intruder positively correlated with expression of *th* (Fig. 4b; $R^2 = 0.51$, $P = 0.030$). In FN, intruder-directed aggression negatively correlated with *d2r:d1r* ratio (Fig. 4b; $R^2 = 0.49$, $P = 0.036$).

Finally, to examine whether social context affects gene expression co-variance patterns and to generate novel hypotheses, we examined clustered correlation matrices for each context. Clusters for which $P < 0.05$ differed between each context (Fig. 5). Most notably, in the FN context, expression levels of all measured genes in the POA were positively correlated and fell into one of two clusters, one comprising *egr-1*, *arα*, *it*, and *d1r*, and the other one including *c-fos*, *erα*, *th*, *d2r*, *itr*, *v1ar* and *avt*. Intriguingly, subsets of the latter cluster are maintained in the RO (*th*, *d2r*, *itr*, *v1ar*) and NS (*th*, *itr*, *v1ar*) contexts. Also note that the two IEGs *egr-1* and *c-fos* are significantly associated with different clusters only in the FN context, while in the RO context the activity of these genes appears unrelated to the pathways under study. Interestingly, in the presence of a NS *egr-1* and *c-fos* mRNA levels co-vary strongly.

Experiment 4: context-specific effects of D2R manipulation

Finally, given the association between D2R and context-dependent behavior found in other vertebrates, we pharmacologically perturbed D2R function with either an agonist or antagonist, in addition to a vehicle control. Within each social context, we found that manipulating D2R had effects on intruder-directed aggressive behavior (Fig. 6a). In RO, treatment with both agonist ($t(12.8) = 2.63$, $P = 0.021$) and antagonist ($t(8.1) = 4.36$, $P = 0.0023$) reduced aggressive displays to the intruder compared to saline. In FN, treatment with agonist reduced aggressive displays to the intruder compared to saline ($t(9.4) = 3.04$, $P = 0.013$), while antagonist treatment did not differ from saline ($t(14.2) = -1.04$, $P = 0.32$). In NS, treatment with agonist did not have a significant effect, while antagonist treatment significantly increased aggressive displays to the intruder compared to saline ($t(13.4) = -2.3$, $P = 0.037$). For effects within each treatment, see Table S5.

We also examined whether D2R manipulation affected the target of aggression in the FN context. We found that D2R agonist treatment decreased the proportion of displays directed to the intruder compared to saline ($t(11) = 3.16$, $P = 0.0090$). Saline and D2R antagonist treatment did not differ in this regard ($t(14.9) = 0.49$, $P = 0.64$).

Discussion

In the present study we have shown that social context has powerful behavioral and physiological effects on the aggressive response to an intruder. There were differences in circulating T and Cort across social contexts, with higher T in the FN context and strikingly higher Cort in the RO context. Furthermore, we discovered context-specific differences in the POA in the expression of the nonapeptides and sex steroid receptors. Lastly, we found that treatment with D2R antagonist has social context-specific effects on behavior, suggesting a further role for this pathway in affecting behavioral plasticity. Our data make an important contribution to our understanding of how brain gene expression and hormone levels mediate context-dependent behavior.

Social context and male size affect intruder-directed aggression

Resident territorial males exhibit more frequent aggressive displays to an intruder when in the presence of a gravid, reproductive female compared to a non-reproductive female or a male neighbor. Males may be using territorial aggression to signal their quality to the female (Kidd *et al.* 2013) and/or males may perceive their territory as more valuable in this context and consequently increase the effort to defend it from intruders (Riechert 1998). Future studies should attempt to dissociate these alternative, though not mutually exclusive, hypotheses by removing the stimulus context while the territorial intrusion is in progress. While our experimental design involved long-term exposure to a social context, another study in *A. burtoni* showed that male-male aggression increases when a gravid female is in the audience (Desjardins *et al.* 2012).

We found that small resident males are more aggressive to the intruder than large males in both RO and NS contexts, though not in the FN context. Heightened aggression in small individuals has been observed in other species (Reddon *et al.* 2013; Svensson *et al.* 2012), including invertebrates (Hofmann & Schildberger 2001; Smith *et al.* 1994). Interestingly, we did not observe a relationship with body size in the FN context, likely because in resident male *A. burtoni*, the size and behavior of the neighboring male has a strong influence in determining the frequency of aggression displayed to an intruder (Weitekamp & Hofmann 2017). Furthermore, the presence of a FN may change the odds of winning the encounter, such that a 'Napoleon' strategy (Morrell *et al.* 2005) is no longer advantageous.

Hormone levels respond to social context

We find that T levels in response to a territorial intrusion are higher in the context of a male neighbor when compared to the control social context. Elevated T in response to male-male competition is a well-documented phenomenon (Gleason *et al.* 2009; Wingfield *et al.* 1990). Interestingly,

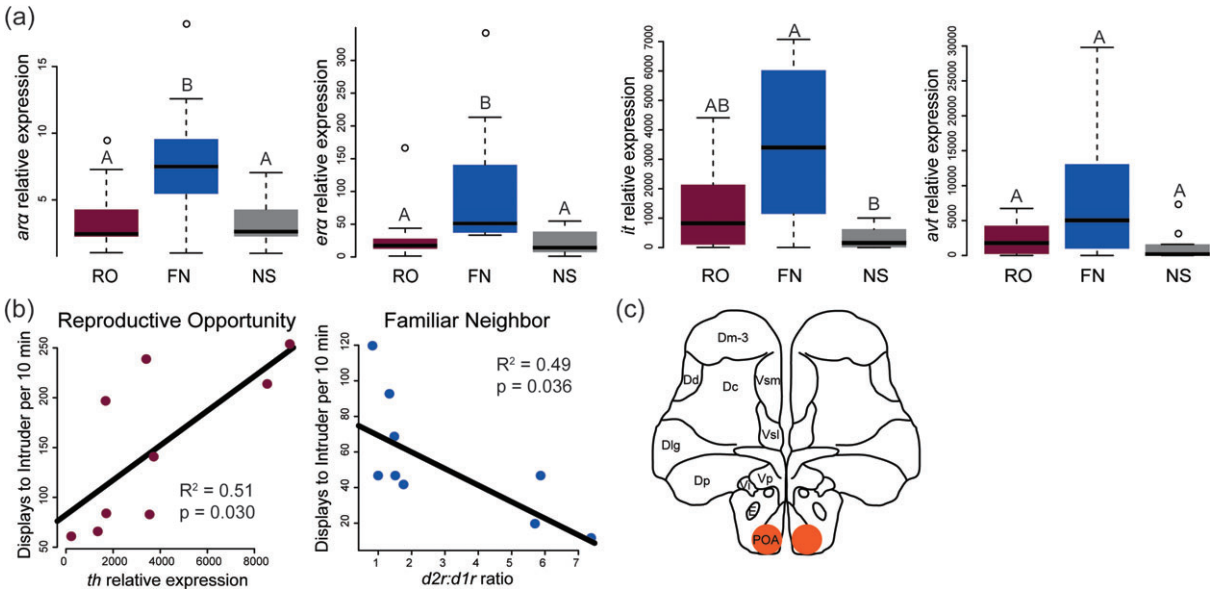

Figure 4: Social context affected POA gene expression. (a) Relative gene expression of *arα*, *erα*, *avt* and *it* differed across social contexts. Letters denote homogeneous subgroups. (b) Aggressive displays to the intruder were best predicted by expression levels of different genes in the POA. In RO, aggression positively correlated with expression of *th*. In FN, aggression negatively correlated with *d2r:d1r* ratio. (c) Representative coronal slice from which the POA was microdissected with the approximate sampled area shown by the circle.

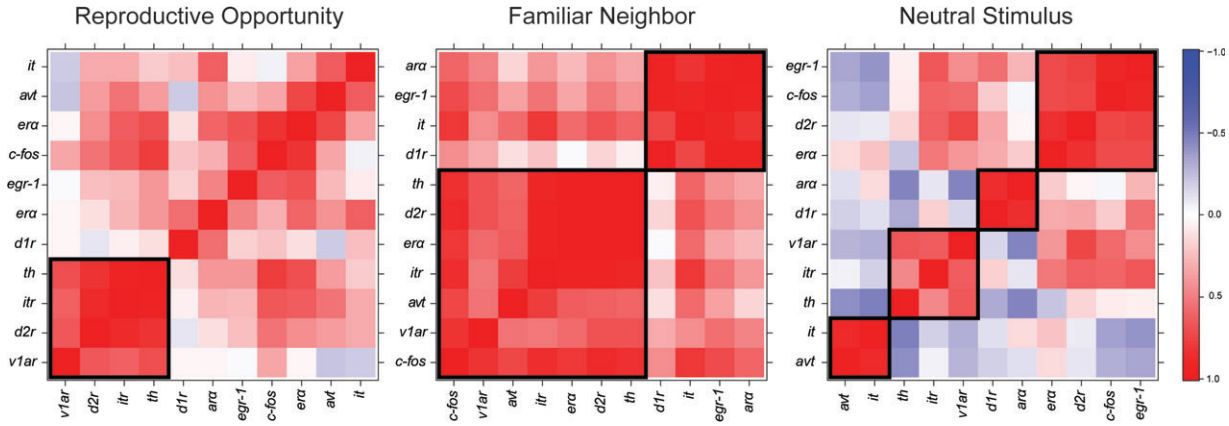

Figure 5: Hierarchically clustered correlation matrices of gene expression within each social context. Boxes indicate significant clusters as determined by bootstrap resampling.

here, all three contexts involve male-male competition in the form of territory defense. A rise in T levels is typically transient (Hirschenhauser & Oliveira 2006; Wingfield & Wada 1989), suggesting that our findings are unlikely a result of elevated T during the initial period of repeated exposures. Furthermore, we previously observed reduced T levels after repeated exposures in the FN context (Weitekamp & Hofmann 2017), though we cannot rule out the possibility that basal levels were higher in the FN context. Indeed, a study in the cichlid fish *Oreochromis mossambicus* showed that males exhibit an AR response in anticipation of territorial challenges (Antunes & Oliveira 2009).

We also find that Cort levels in response to a territorial intrusion are strikingly higher in the RO context, when a gravid female is present, compared to both other social contexts. Interestingly, while aggression to the intruder was also highest in the RO context, Cort levels do not correlate with aggressive behavior. The elevated Cort levels appear to be in response to the social context, rather than directly mediating aggression across contexts. Basal Cort levels in both territorial and subordinate *A. burtoni* males have been reported to range from 0 to 50 ng/ml (Huffman *et al.* 2015). We find a similar range in FN and NS, but find a large increase in Cort in the RO context. Similar to T, aggression elicits a

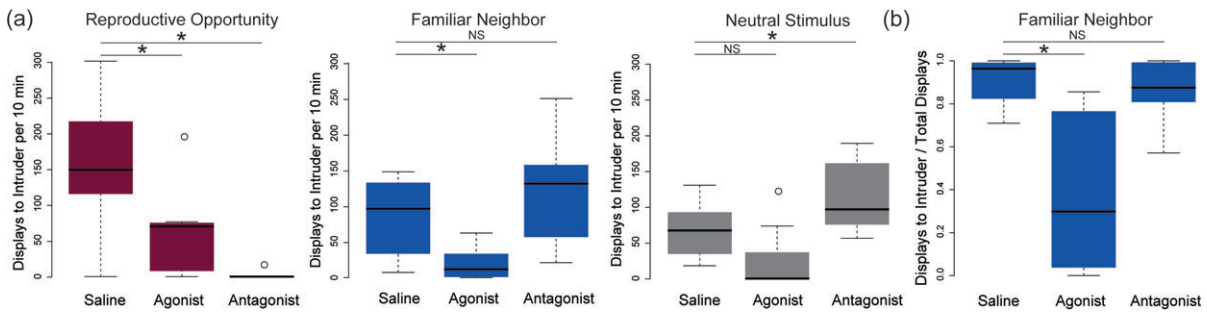

Figure 6: Behavioral response to D2R manipulation was affected by social context. Manipulation of D2R across social contexts affected intruder-directed aggression (a), and changed the proportion of displays directed toward the intruder (b). Letters denote homogeneous subgroups.

rapid and acute glucocorticoid response which can be social context-dependent (Summers *et al.* 2005). Here, the presence of a RO may raise the stakes of territory defense, and circulating Cort may be acting to mediate the relative increase in aggressive displays against the intruder. Interestingly, we also found that T and Cort levels were negatively correlated in the RO context but positively correlated in the NS context, while they were not at all correlated in the FN context. The specific function of the interaction between these systems in modulating the response to social context merits future study.

Social context affects preoptic gene expression

We identified expression differences in the POA, which is critical for the regulation of vertebrate male aggressive and sexual behavior, across social contexts in several candidate genes, including IT, AVT, ERα and ARα. Intriguingly, these genes all show increased relative expression in the FN context. The presence of a FN has important implications for territory defense as it can lead to cooperative defense against an intruder (Weitekamp & Hofmann 2017). Thus, becoming familiar with a territorial neighbor may alter POA gene expression profiles in a way that prepares an animal for more interactions in the future. The repeated exposure to the FN over the course of 4 days might have caused a conditioned response in the nonapeptide and sex steroid pathways we examined in the POA. In anticipation of agonistic interactions, there are often physiological changes that help prepare an animal to fight (Adams *et al.* 1968). For example, in male rats, after confrontation with an opponent for a series of days, DA levels in the nucleus accumbens increased in anticipation of the encounter (Ferrari *et al.* 2003). Following classical conditioning between a light and a male intruder, males of the cichlid fish *O. mossambicus* showed an AR response to the light alone, suggesting hormonal anticipation of aggressive defense which may function to increase vigilance (Antunes & Oliveira 2009). Thus, the gene expression differences we identified may function to prepare males for aggressive encounters. Notably, levels of aggression were not highest in this group, therefore the changes in gene expression may lead to an increase in vigilance, as was observed in *O. mossambicus*, rather than function to improve aggressive abilities.

Dopamine system modulates behavior in a context-dependent manner

Different DA cell populations respond to specific social contexts (Bharati & Goodson 2006; O'Connell *et al.* 2013b). The DA cell group in the POA responds specifically to sexual interaction (Bharati & Goodson 2006). Sexual motivation is also mediated by DA receptors in the POA (Dominguez & Hull 2005). Thus, we expected to find expression differences in the RO context specifically. Surprisingly, we found no differences in expression of D1R, D2R, their ratio, or TH between social contexts. However, our results do suggest a role for behavioral modulation by the DA system in the POA, as we found that aggression to the intruder may be mediated by different dopaminergic genes in a context-dependent manner. Specifically, expression of TH was positively correlated with intruder-directed aggression in RO. As TH serves as a marker for DA synthesis, it is possible that higher DA levels correspond to an increase in perceived sexual reward, resulting in an increase in motivation and higher aggression against an intruder (Hull & Dominguez 2007; Paredes 2009; Wise & Rompre 1989). In FN, the D2R:D1R ratio was negatively correlated with aggression to the intruder. Stimulation of D1 and D2Rs often has opposing effects on behavior (Trantham-Davidson *et al.* 2004). D1R stimulation activates adenylyl cyclase activity, while D2R inhibits it. Furthermore, D1 receptors are typically expressed post-synaptically, while D2Rs can be expressed both pre- and post-synaptically, and can act as autoreceptors (Callier *et al.* 2003). Pharmacological manipulation in the POA demonstrated that the D2R:D1R ratio affects sexual behavior in rats (Hull *et al.* 1989). In the FN context, the interaction between these two receptor types in the POA may serve an important role in regulating aggressive behavior as well.

Functional interrelationships of preoptic DA, sex steroid, and/or nonapeptide signaling

In addition to the role of the DA system discussed above, an extensive literature demonstrates the importance of sex steroid and nonapeptide signaling in regulating male aggressive behavior (Nelson 2005). Much less is known about how these pathways functionally interact (but see Caldwell & Albers 2015; Dominguez & Hull 2005; Love 2014;

Rosell *et al.* 2015). Our finding that co-variance patterns of gene expression in the POA varied significantly according to social context extends these insights and suggests new avenues for research. Specifically, DA production and V1aR/ITR signaling appear tightly co-regulated independent of social context. An interaction between these pathways in the POA has been reported for regulating pair bonding and maternal care in rodents (Liu & Wang 2003; Numan & Stolzenberg 2009). Other genes/pathways might get recruited into this invariant module in a context-specific manner in FN and RO conditions, respectively. Also of note, in the RO context EGR-1 and c-Fos activity seemed unrelated to any of the pathways under study, whereas in the NS context expression of both IEGs was strongly correlated with D2R and ERα. In contrast, in the FN context (where all genes were positively correlated, possibly indicating a global activation or release of inhibition) the two immediate-early genes were associated with two discrete clusters, possibly indicating a distinct role for these IEGs in two different neural processes: c-Fos might orchestrate D2R and nonapeptide signaling with DA and AVT production, whereas EGR-1 coordinates D1R and ARα signaling with IT production. Future experiments that use co-labeling of IEGs with pathway-specific markers in a spatially explicit manner (e.g. O'Connell *et al.* 2013b; Weitekamp & Hofmann 2017) will reveal which cell types are being activated in a coordinated manner and depending on (or independent of) social context. Based on this analysis one could reasonably predict that, in the FN context, EGR-1 mediates activation of neurons expressing ARα, IT, and/or D1R, whereas c-Fos might play this role in neurons expressing ERα and/or AVT, and possibly dopaminergic and nonapeptide-responsive cells as well (although the coordinated activity of these latter cell populations appears to be social context-invariant).

D2R antagonist reduces aggression in RO context

We found that activation of D2Rs with agonist treatment reduced territorial aggression in RO and FN (NS followed a similar trend), while inhibition of D2Rs by treatment with antagonist reduced territorial aggression in RO and increased aggression in NS. D2R antagonist treatment is often reported to reduce sexual motivation (Blackburn *et al.* 1992; Moses *et al.* 1995). This is consistent with our data given that the presence of a gravid female is a source of sexual motivation, and explains the context-specific effects of antagonist treatment that we observed. The RO context may be associated with baseline levels of D2R occupancy that are different compared to the FN and NS contexts. For example, treatment with D1R agonist in the prefrontal cortex of rats had opposite effects on task performance in individuals with differing memory traces, presumably due to differences in pre-existing DA levels (Floresco & Phillips 2001). Dopamine receptor occupancy was likely higher in RO given the repeated visual exposure to a reproductive female, as DA is known to increase in several brain regions before copulation (Dominguez & Hull 2005). Furthermore, intense stimulation of D2Rs may shift the autonomic balance between receptor subtypes, which we may be observing if there are differences in DA release between contexts (Dominguez & Hull 2005).

Studies have reported mixed effects on aggressive defense from D2R agonists. In male zebra finches, aggressive territory defense in the presence of a female audience, similar to our RO context, was reduced by a D2R agonist (Kabelik *et al.* 2010). In the weakly electric fish, *Apteronotus leptorhynchus*, DA treatment modulates agonistic signaling in response to simulated male electric organ discharges (Maler & Ellis 1987). In contrast, in cats, D2R agonist in the POA facilitated aggressive defense behavior (Sweidan *et al.* 1991). Similarly, in male mice, D2R agonist treatment increased defensive behaviors (Puglisi-Allegra & Cabib 1988). Birds and fishes may fundamentally differ in their responses to D2R agonist compared to mammals. Interestingly, previous data have shown that the D2R/D1R ratio in several brain regions differs between birds and mammals, which may, in part, account for the different responses to drug treatment between taxa (Kleitz *et al.* 2009). Notably, several other variables could contribute to differences in effects across studies, including the binding efficiencies of the drugs used and their mode of administration. Furthermore, DA often has sensitive dosage-dependent effects and typically follows an inverted U-shaped response pattern (Monte-Silva *et al.* 2009). To better understand the results observed here, future studies of social context in *A. burtoni* should include dose–response curves. Lastly, given the effects of D2R treatment, it was surprising that there were no differences between contexts in the expression of dopaminergic genes in the POA. It may be that the context-specific effects on behavior that we observed from manipulating the D2R resulted from primary actions in other brain regions where there are differences in D2R density between contexts.

D2R agonist disrupts social recognition in FN context

We find that treating Residents with D2R agonist changes the target of aggression. In untreated males, the majority of aggressive displays are typically directed toward the intruder, while aggression between FNs is very low (Weitekamp & Hofmann 2017). D2 receptor agonist treatment caused relatively more aggression to be directed towards the FN. We suggest that activating D2Rs in the Resident leads to impaired social recognition, possibly by disrupting the retrieval of the memory of the partner or by disrupting the perception of the salience of the intruder. The learning that takes place during the habituation period between two territorial males could be considered to result in a form of emotional memory (Pezze & Feldon 2004). D2 receptor has been well studied for its actions in learning the salience of stimuli and in emotional memory processing (Laviolette 2007). For example, a study in rats found that D2R agonist treatment blocked the retrieval of an emotional memory, measured by the learned association between a conditioned and unconditioned stimulus (Nader & LeDoux 1999).

Conclusions

The social environment has long been known to have important influences on individual social behavior and decision-making. However, the mechanisms by which the brain mediates context-dependent behavior are less well

understood. Here, we used territory defense to demonstrate how social context influences both brain and behavior. We found strong effects on circulating T and Cort, which may be modulating context-specific effects in the brain. In the POA, we found that nonapeptide and sex steroid receptor expression was highest in the FN context. We also found interesting differences in the gene expression co-variance patterns across contexts. Finally, we identified context-dependent responses to D2R manipulation. The mechanisms we identified present a wealth of opportunities for future studies that examine how the social environment shapes behavior.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1: Sample sizes and male sizes for all four experiments. RO, Reproductive Opportunity; FN, Familiar Neighbor; NS, Neutral Stimulus.

Table S2: Primers used for qPCR amplification.

Table S3: Comparison of gene expression across contexts.

Table S4: Aggressive behavior within each context is best predicted by different candidate genes. For completeness, we also show how these genes vary in the other two contexts.

Table S5: Between contexts effects of D2R manipulation.

Figure S1: Comparison of gene expression across contexts.

Figure S2: Aggressive behavior within each context is best predicted by different candidate genes. For completeness, we also show how these genes vary in the other two contexts.

Table S1: Sample sizes and male sizes for all four experiments. RO = Reproductive Opportunity; FN = Familiar Neighbor; NS = Neutral Stimulus.

Treatment	Experiment	N	Focal avg. length ± SD (mm)	Focal length range (mm)	Intruder avg. length ± SD (mm)	Intruder length range (mm)
RO	1; 2&3	15; 9	53.5 ± 4.1	47 - 59	60.2 ± 3.8	55-68
FN	1; 2&3	13; 9	51.8 ± 5.1	44 - 61	57.9 ± 5.9	45-66
NS	1; 2&3	12; 9	56 ± 5.4	45 - 62	61.8 ± 4.0	55-68
RO ag	4	8	55.1 ± 5.6	44 - 60	60.7 ± 4	54-65
RO ant	4	8	52.3 ± 4.5	46 - 58	59.1 ± 3.1	55-64
RO saline	4	8	54.8 ± 4.8	44 - 59	58.9 ± 3.4	53-64
FN ag	4	8	58.1 ± 1.2	56 - 60	64 ± 1.2	62-66
FN ant	4	9	55.8 ± 4	54 - 62	61.3 ± 2.7	57-65
FN saline	4	8	55.6 ± 4	50 - 61	60.9 ± 3.4	55-65
NS ag	4	8	56.6 ± 6.3	50 - 66	62.8 ± 6.4	55-71
NS ant	4	8	55.4 ± 3.8	50 - 60	61 ± 4.3	56-67
NS saline	4	8	59.2 ± 1.8	57 - 62	64.8 ± 2.1	63-68

Table S2: Primers used for qPCR amplification.

Primer Name	Primer Sequence
18S rRNA forward primer	5’- CCCTTCAAACCCTCTTACCC
18S rRNA reverse primer	5’- CCACCGCTAAGAGTCGTATT
ARα forward primer	5’- CAGGAATGCCGCTGTATCTG
ARα reverse primer	5’- TGAGGAATCGCACTTGGGTA
AVT forward primer	5’- AGGCAGGAGGGAGATCCTGT
AVT reverse primer	5’- CAGGCAGTCAGAGTCCACCAT
c-fos forward primer	5’- GAGGAATAAGCAGGCAGCAGCAAA
c-fos reverse primer	5’- TCTCCTTCAGCAGGTTGGCGATA
D1R forward primer	5’- CAGTCAGTGAGAGAGCTGGTG
D1R reverse primer	5’- CAGCAGCTGTGTTCCTCCAA
D2R forward primer	5’- CTGGCTGTCGCTGACCTTCT
D2R reverse primer	5’- GATCTTGCTAAAGCGCCACTC
egr-1 forward primer	5’- CTCTAGCTCTTCCTCCGCAG
egr-1 reverse primer	5’- TGAGATGAGGACGAGGAGGT
ERα forward primer	5’- CTACGAAGTGGGCATGATGAAA
ERα reverse primer	5’- GGTCTTTGGCTGGTTTGTCTCT
IT forward primer	5’- GGAAACAGCTCACTGTGTGGA
IT reverse primer	5’- AGCACAGCGTCCTCCTTCAG
ITR forward primer	5’- GGCTTACATGCTCTGCTGGA
ITR reverse primer	5’- AGCAGCATGGAGATAATGAAGG
TH forward primer	5’- ATGGGCACTCGATCCCCAGAGT
TH reverse primer	5’- TTCACTGCAGGCATGGGTGGTG
V1aR2 forward primer	5’- GAAAGAAGACTCAGACAGTAGCC
V1aR2 reverse primer	5’- ACCATCACTACACACATCTCG

Table S3: Comparison of gene expression across contexts.

	$F_{2,24}$	p	RO:FN	RO:NS	FN:NS
d1r	1.04	0.37	-	-	-
d2r	0.51	0.61	-	-	-
d1r:d2r	1.27	0.30	-	-	-
th	0.40	0.68	-	-	-
c-fos	0.81	0.46	-	-	-
egr-1	1.21	0.32	-	-	-
ara	5.12	**0.014**	**0.035**	0.96	**0.022**
era	4.00	**0.032**	0.089	0.91	**0.037**
avt	3.50	**0.046**	0.10	0.96	0.06
it	6.83	**0.0045**	0.052	0.50	**0.0038**
v1ar	0.59	0.56	-	-	-
itr	1.70	0.21	-	-	-

Figure S1: Comparison of gene expression across contexts.

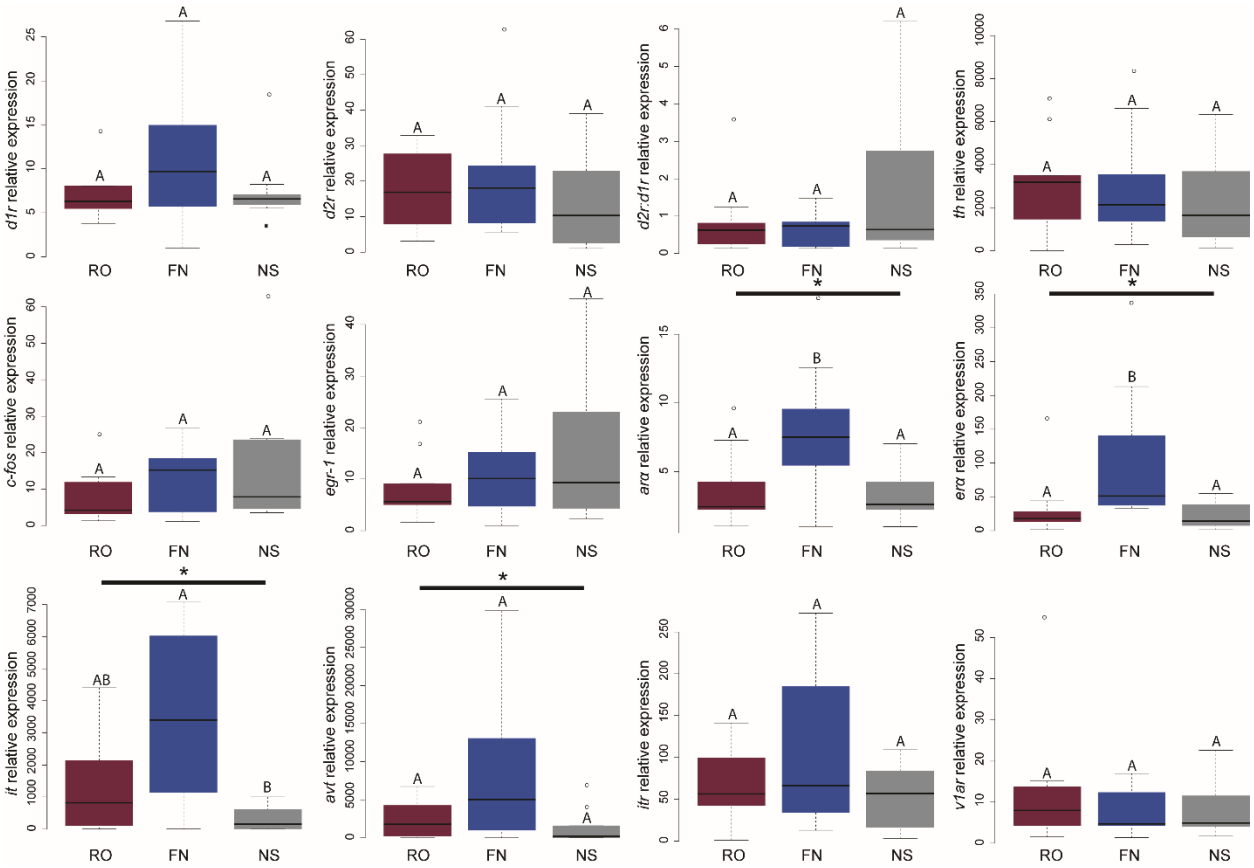

Table S4: Aggressive behavior within each context is best predicted by different candidate genes. For completeness, we also show how these genes vary in the other two contexts.

	TH	*D2R:D1R*	*egr-1*
RO	R^2 = 0.51, p = **0.030**	R^2 = 0.042, p = 0.59	R^2 = 0.032, p = 0.62
FN	R^2 = 0.40, p = 0.068	R^2 = 0.49, p = **0.036**	R^2 = 0.094, p = 0.42
NS	R^2 = 0.035, p = 0.63	R^2 = 0.057, p = 0.54	R^2 = 0.49, p = **0.037**

Figure S2: Aggressive behavior within each context is best predicted by different candidate genes. For completeness, we also show how these genes vary in the other two contexts.

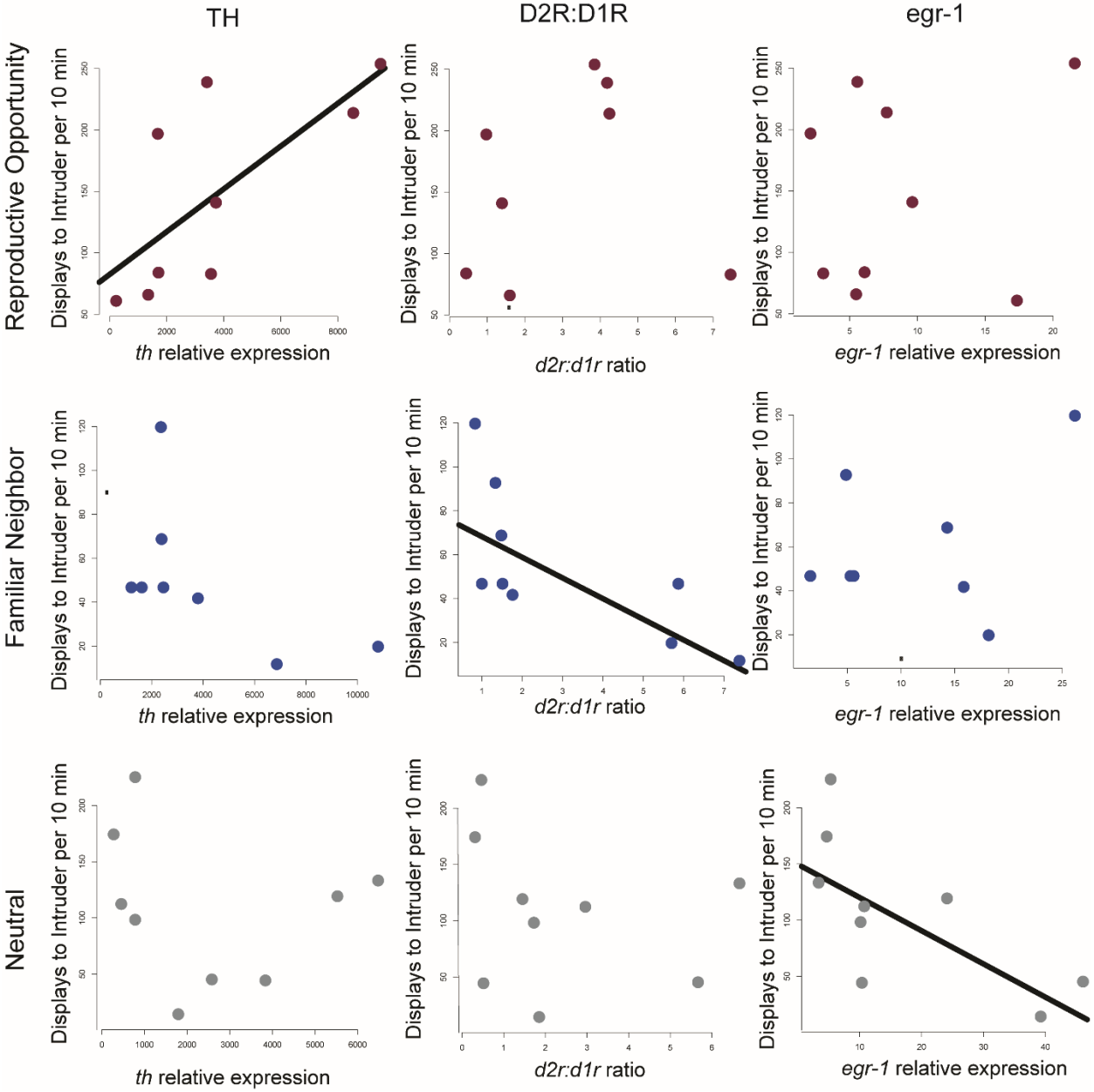

Table S5: Between contexts effects of D2R manipulation.

	$F_{2,24}$	p	RO:FN	RO:NS	FN:NS
Saline	4.27	**0.0278**	0.091	**0.031**	0.85
Agonist	1.18	0.33			
Antagonist	9.09	**1.3×10^{-3}**	**2.8×10^{-3}**	**4.0×10^{-3}**	0.99