Early-life social environment alters juvenile behavior and neuroendocrine function in a highly social cichlid fish

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Abstract

Early-life experiences can shape adult behavior, with consequences for fitness and health, yet fundamental questions remain unanswered about how early social environments and experiences are translated into variation in brain and behavior. The African cichlid fish *Astatotilapia burtoni*, a model system in social neuroscience, is well known for its highly plastic social phenotypes in adulthood. Here, we rear juveniles in either social groups or pairs to investigate the effects of early-life social environments on behavior and neuroendocrine gene expression. We find that both juvenile behavior and neuroendocrine function are sensitive to early-life social effects. Behavior robustly co-varies across multiple contexts (open field, social cue investigation, and dominance behavior assays) to form a behavioral syndrome. Rearing environment shifts pair-reared juveniles towards the end of syndrome that is less active and socially interactive. Pair-reared juveniles also submit more readily as subordinates. In a separate cohort, we then measured neural expression for stress and sex hormone genes, signaling systems known to be developmentally plastic and involved in translating environmental conditions into biological responses and regulating adult social behavior. Rearing environment causes striking differences in neuroendocrine gene co-expression networks. Specifically, expression was tightly integrated in pair-reared juveniles, but not group-reared or isolated juveniles. Glucocorticoid receptor subtypes 1a, 1b, and 2, as well as androgen receptor α, drive the significant differences between treatment groups, which supports a highly conserved role for the stress axis mediating early-life effects. Together, this research demonstrates the important developmental origins of behavioral phenotypes and identifies potential behavioral and neuroendocrine mechanisms.

Key words: early environment, ontogeny, social behavior, behavioral syndrome, hypothalamic-pituitary-adrenal axis, stress hormones, sex hormones

Introduction

Ontogeny has long been recognized as essential to understanding phenotype (Tinbergen, 1963), yet the early-life origins of individual behavioral variation remain understudied. Development reveals the proximate mechanisms by which genes interact with the environment during early life to sculpt the 'machinery of behavior' (Stamps, 2003; Tinbergen, 1963). Current or predicted environmental conditions can trigger developmental plasticity, and the resulting changes are often long-lasting, or even permanent, and can facilitate locally-adapted (e.g., predator resistant, Gilbert, 2001) phenotypes (Kasumovic and Brooks, 2011; Lummaa and Clutton-Brock, 2002; Piersma and Drent, 2003; Snell-Rood, 2013; Stamps, 2003; Stearns, 1989; West-Eberhard, 1989). The developmental mechanisms that shape social behavior via underlying neural regulatory mechanisms should be a particularly important target for natural selection (Taborsky, 2016) because of the direct consequences of social behavior for fitness and health (e.g., Bennett et al., 2006; Meyer-Lindenberg and Tost, 2012; Silk, 2007; Solomon-Lane et al., 2015; Wilson, 1980).

Social stimuli are among the most important attributes of the early-life environment (Taborsky, 2016). Although maternal (and, to a lesser extent, paternal) interactions have largely been the focus (e.g., Champagne & Curley, 2005; McClelland, Korosi, Cope, Ivy, & Baram, 2011), the broader early-life social environment is increasingly recognized for its role in behavioral and neural plasticity (Buist et al., 2013; Creel et al., 2013; Jonsson and Jonsson, 2014; Kasumovic and Brooks, 2011; Taborsky, 2016; White, 2010). For example, the early presence of

brood care helpers, unrelated adult males, and multiple mothers and litters have long-term effects on social behavior in the Daffodil cichlid fish *Neolamprologus pulcher* (Arnold and Taborsky, 2010; Taborsky et al., 2012), brown-headed cowbirds (White et al., 2002), and laboratory mice (Branchi et al., 2013, 2006; D'Andrea et al., 2007), respectively. These features of the social environment alter the quality and quantity of social experiences and sensory cues perceived, which together influence neural function and behavior (Taborsky, 2016). Developmental plasticity may be limited to a single behavior or extend to an entire suite of behaviors (i.e., a behavioral syndrome), and the effects may be context-specific (Bell, 2007; Snell-Rood, 2013; Stamps, 2003; Stamps and Groothuis, 2010).

Neuroendocrine signaling is a primary mechanism by which environmental conditions and experience are translated into physiological responses (Crespi and Denver, 2005; Remage-Healey and Romero, 2000; Wingfield et al., 1990). Hormones are also important sources of individual variation in social behavior (e.g., across seasons, sexes, reproductive tactics) and underlie developmental plasticity relevant to adult behavior. The stress axis, or hypothalamic-pituitary-adrenal (interrenal in fish; HPA/I) axis, is widely implicated as a highly-conserved mechanism of early-life effects (Champagne and Curley, 2005; Francis et al., 1999; McClelland et al., 2011; Taborsky, 2016). In response to an environmental stressor, which includes any external condition that disrupts or threatens to disrupt homeostasis, the HPA/I axis integrates relevant internal and external cues and coordinates a response, such as changes in behavior and physiology. The stress response is initiated by the release of corticotropin-releasing factor (CRF) from the hypothalamus, which signals to the pituitary to release adrenocorticotropic hormone, which then signals the adrenal glands to release glucocorticoids (e.g., cortisol in fish) (Denver, 2009; Lowry and Moore, 2006).

Effects of early-life experiences on HPA/I axis function have been demonstrated in every major vertebrate lineage (e.g., birds: Banerjee, Arterbery, Fergus, & Adkins-Regan, 2012; mammals: Champagne & Curley, 2005; amphibians: Crespi & Denver, 2005; fish: Jonsson & Jonsson, 2014). For example, the presence of brood helpers during early-life affects *N. pulcher* cichlid social behavior via changes in neural expression levels of CRF and glucocorticoid receptor (GR), as well as receptor ratios (Taborsky et al., 2013). Stress axis mechanisms can also mediate the effects of the early-life social environment on human health (e.g., Turecki & Meaney, 2016). Sex steroid hormones (e.g., androgens, estrogens) also play a role mediating the long-term effects of early-life experiences (Adkins-Regan, 2009; Brown and Spencer, 2013; Shepard et al., 2009) and regulating social behavior (Goodson, 2005; Newman, 1999). For example, neural estrogen receptor expression is associated with maternal behavior in mother rats and offspring (Champagne et al., 2003; Champagne and Meaney, 2007), and socially stressed pre- and postnatal female guinea pigs have upregulated neural estrogen and androgen receptor levels, elevated testosterone, and masculinized behavior (Kaiser et al., 2003). Together, these and other neuroendocrine systems interact to affect behavior.

To investigate the effects of the early-life social environment on behavior and its neuroendocrine mechanisms, we used the highly social African cichlid *Astatotilapia burtoni*, a model system in social neuroscience (Fernald and Maruska, 2012; Hofmann, 2003). Adults of this species form mixed-sex, hierarchical communities with males of dominant or subordinate status and females. Dominant males are territorial, reproductively active, and colorful. In comparison, subordinate males shoal with females, are reproductively suppressed, and drab in coloration. Male status is socially regulated, and individuals regularly transition between status phenotypes (Fernald and Maruska, 2012; Hofmann, 2003). Adults, and juveniles (Fernald and

Hirata, 1979), express a suite of highly conserved social behaviors, including aggression, affiliation, courtship, and cooperation (Fernald, 2012; Hofmann, 2003; Weitekamp et al., 2017). Substantial progress has also been made towards understanding variation in stress and sex steroid hormone signaling, including in the regulation of social behavior (Chen and Fernald, 2008; Fox et al., 1997; Greenwood et al., 2003; Munchrath and Hofmann, 2010; O'Connell and Hofmann, 2012a). All GRs (Greenwood et al., 2003), estrogen receptors (ER), and androgen receptors (AR) (Munchrath and Hofmann, 2010) have been studied in the adult *A. burtoni* brain, and neuroendocrine function can vary substantially. Subordinate males, for example, have lower levels of whole brain CRF and GR2 (Chen and Fernald, 2008), higher cortisol, and lower testosterone than dominants (Fox et al., 1997; O'Connell and Hofmann, 2012a), although these patterns can vary dynamically (Maguire and Hofmann, in prep.). The transcriptomic response in the preoptic area (POA) to pharmacological manipulation, such as an ER antagonist, is also status-specific (O'Connell and Hofmann, 2012a).

Given this rich literature on adult *A. burtoni*, it may seem surprising that the developmental origins of adult phenotypic variation remain largely unknown. The few studies that have investigated juveniles demonstrate the importance of early-life. For example, the development of male behavior and nuptial coloration, as well as reproductive maturation, are affected by the early-life social environment (Fernald and Hirata, 1979; Fraley and Fernald, 1982). Gestational cues (e.g., maternal social crowding) also have lasting effects on methylation and transcription of the *gnrh*1 gene in offspring (Alvarado et al., 2015). This result is particularly interesting given that preoptic GnRH1 neurons, which regulate gonadotropin release from the pituitary, are socially modulated in adults (Davis and Fernald, 1990; Hofmann and Fernald, 2001). However, studies of the effects of different early-life experiences on other neuroendocrine

139 pathways or behavior are lacking.

140 In the present study, we conducted two experiments to test the hypothesis that the early-
141 life social environment generates variation in juvenile behavior through neuroendocrine gene
142 expression. We manipulated the early-life social environment, and consequently social
143 experience, by rearing juveniles in either social groups or pairs. In the group condition, social
144 experience implies interactions with more social partners, who also vary in size, sex, experience,
145 and patterns of behavior. Interactions in groups can also involve more than two individuals, and
146 it is possible to observe and learn from interactions of group members as a bystander. Although
147 it has not been tested in juveniles, adults are capable of gaining important social information as a
148 bystander (Desjardins et al., 2012, 2010; Grosenick et al., 2007). In the pair condition, juveniles
149 occupy only one social role in a relationship with just one other individual. Similar
150 manipulations of early-life social complexity have been important for behavioral and neural
151 development in other species (reviewed in Taborsky, 2016). We predicted that rearing
152 environment would affect a suite of social behaviors across contexts, including social
153 investigation, dominant, and subordinate behavior. In the brain, we predicted effects on whole
154 brain gene expression of neuroendocrine systems that mediate early-life experiences. Related to
155 the HPA/I axis, we measured glucocorticoid receptor 1a (GR1a), glucocorticoid receptor 1b
156 (GR1b), glucocorticoid receptor 2 (GR2) (nomenclature from Maruska & Fernald, 2010),
157 mineralocorticoid receptor (MR), and CRF. For sex steroid hormone signaling, we quantified
158 androgen receptor α (ARα) and estrogen receptor α (ERα). By investigating these early-life
159 effects in juveniles, we can identify important intermediary steps that inform how developmental
160 plasticity may shape the adult phenotype.
161

Methods

Animals

Juvenile *A. burtoni* came from a laboratory population descended from a wild-caught stock. The adults that bred the juveniles were housed in naturalistic social groups of males and females. Dominant males court gravid females that then lay eggs in his territory. The female then scoops up the eggs into her mouth, where the male fertilizes them. The mother orally incubates the larvae as they develop for 10-13 days. Under natural (and some laboratory) conditions, juveniles remain close to their mother for the 2-4 weeks following their initial release from her mouth. As they age, juveniles seek shelter in her mouth less and less often. In the first two weeks, juveniles primarily school together, with overt social interactions beginning at 2-3 weeks old (Fernald and Hirata, 1979; Renn et al., 2009). Social behaviors, such as chasing, nipping, territorial displays, emerge in a predictable sequence as juveniles approach reproductive maturity, which can occur as early as 15 weeks, depending on the early-life social conditions (Fernald and Hirata, 1979; Fraley and Fernald, 1982).

We removed juveniles from the mother's mouth 6-12 days post-fertilization. Once sufficiently developed (~day 12, freely swimming with no remaining yolk), juveniles were transferred into experimental rearing environments. Juveniles are all silver (drab) in coloration, and none developed coloration during the study, which would indicate reproductive maturity for males. Sex cannot be determined anatomically until maturation; therefore, the sex ratios of our rearing environments, and the sex of the focal individuals, is unknown. The sex ratio of *A. burtoni* broods is approximately 1:1. All work was done in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

185 *Experimental rearing conditions (Experiments 1 & 2)*

186 As the first study of this kind in this species, we opted to quantify behavior and gene
187 expression in separate experiments in order to capture different developmental time points. In
188 Experiment 1, juveniles for the behavioral assays were reared in social groups of 16 fish (n=12
189 groups) or in pairs (n=9 pairs) for 58-73 days (average 65.76 ± 0.81; ~8-10 weeks), as long of a
190 duration that could be used without juveniles reaching reproductive maturity. In Experiment 2,
191 neural gene expression was measured in a separate cohort of juveniles reared in social groups of
192 16 fish, pairs, or in isolation for 1 week (groups: n=8; pairs: n=8; isolates: n=8) or 5 weeks
193 (groups: n=14; pairs: n=10). Here, we aimed to capture early changes in gene expression that
194 might set individuals along different developmental trajectories. Isolation was included because
195 we expected it to impact gene expression in this highly social species, not as a social control. We
196 cannot distinguish between the effects of chronological age from the treatment duration (i.e., 1
197 vs. 5 weeks) in this study.

198 For both Experiments, juveniles from multiple clutches of the same age and
199 developmental stage (day 12-14 fry) were divided among treatment groups. Group-reared fish
200 were housed in 35 L aquaria with three shards of terracotta pots for a shelter and/or territory.
201 Pairs and isolated fish were housed in small aquaria (22.9 x 15.2 x 15.2 cm) with one terracotta
202 pot shard. The volume of water per fish was similar for the group (2.6 L) and paired (2.7 L)
203 treatments. Juveniles were fed daily with Hikari plankton (Pentair Aquatic Eco-Systems, Cary,
204 NC). The food was mixed in water, and a transfer pipette was used to deliver a set volume to
205 each tank. Groups received eight times more food than pairs. Pairs and isolated fish received the
206 same amount. All juveniles were maintained on a 12:12 light/dark cycle.
207

Experiment 1: Behavioral assays

Behavior for both members of the pairs (n=18 individuals) and two fish from each group (n=24 individuals) was analyzed. To choose focal individuals from the groups, we removed all fish from the aquarium and selected the largest fish. Because size is a strong predicator of social dominance (Alcazar et al., 2014), this individual was very likely to have dominance experience, similar to the larger fish in the pair. A smaller fish was then chosen such that the ratio of large-to-small fish standard length (SL, mm) was approximately equal in the group and a pair from the same cohort of juveniles (same age). Standard length was recorded for all focal fish. Behavior was observed in novel, small aquaria (22.9 x 15.2 x 15.2 cm) without covers. For analysis, the aquaria were divided into 4 zones (Fig 1), delineated with permanent marker. In the middle of each short side, a circle was drawn (28 mm diameter) to indicate the placement of the scintillation vial (see below: social cue investigation). An arc 2.54 cm from the edge of that circle was drawn to form a semicircle. One semicircle was designated the "territory" zone and had a terracotta pot shard for a shelter and/or territory. The other semicircle was designated the "investigate" zone. The "close" zone was between the territory zone and halfway along the long side of the tank. The "far" zone was between the halfway mark and the investigate zone (Fig 1). Video cameras recorded behavior from above so that all areas of the tank, except under the terracotta pot shard, were visible. Solomon Coder was used for analysis (www.solomoncoder.com).

We quantified behavior in four assays, which were always presented in the same sequence (Fig. 1): an open field test that is commonly used in other species to assess activity and anxiety (e.g., Cachat et al., 2010; Prut & Belzung, 2003); a social cue investigation as a measure of social motivation or preference (e.g., Bonuti & Morato, 2018; Moy et al., 2004); and social

231 interactions within either dominant or subordinate status contexts, which regularly occur in
232 social communities of *A. burtoni* (Hofmann, 2003). All observations were made by the same
233 observer who was blind to treatment.
234 Open field test: The focal fish was transferred to the test aquarium with a hand net and
235 remained in the tank alone for 30 min. Movement around the tank was observed from minutes 20
236 to 30. We recorded the number of times a fish crossed into each zone (frequency) and the time
237 (s) spent in each zone. Social cue investigation: Novel juveniles were collected from a
238 community tank and placed into scintillation vials (20 mL). The top of the vial was covered with
239 parafilm with holes to allow water through. A vial containing one cue fish was placed into each
240 test aquarium (n=16 group-reared, n=13 pair-reared). Cue fish were 0-6.4 mm SL (average 3.37
241 ± 0.27) smaller than their focal fish. An empty vial was used as a control (n=8 group-reared, n=5
242 pair-reared). The social cues were in the aquarium for 30 min. Movement around the tank
243 (frequency and time in each zone) was scored from minutes 2 to 12.
244 Dominance behavior: The scintillation vials were removed from the aquaria and a novel
245 smaller fish (by 1-6.4 mm SL, average 3.37 ± 0.25) was immediately added to each aquarium,
246 freely swimming with the focal fish. The pair remained together for 30 minutes, and behavior
247 was scored from minutes 2 to 12. Subordinate behavior: The small cue fish was removed from
248 the aquaria and a novel, larger fish (by 2.4-12 mm SL, average 5.74 ± 0.34) was immediately
249 added to each aquarium, freely swimming with the focal fish. The pair remained together for 30
250 minutes, and behavior was scored from minutes 2 to 12. In the dominance and subordinate
251 behavior assays, we analyzed agonistic interactions between the pair. An approach was defined
252 as one fish swimming directly towards any part of the other fish's body, within 3 body lengths. If
253 the approached fish responded by moving away, in any direction, the behavior was recorded as a

254 displacement for the initiator and a submission for the responder. From these measures, we
255 calculated agonistic efficiency, or the proportion of approaches that led to a displacement
256 (Solomon-Lane et al., 2014), for focal and cue fish. The difference in agonistic efficiency
257 between the focal and cue fish was used as a measure of agonistic asymmetry, which
258 characterizes status relationships (Drews, 1993). We also recorded the frequency of entering and
259 the time spent in the territory, for the focal fish, cue fish, and both together.
260 Importantly, after 8-10 weeks in their respective treatment condition, group-reared
261 juveniles were significantly larger than pair-reared juveniles (see Results). This size difference
262 influenced the size of the fish selected to be the social stimuli. Specifically, the difference in SL
263 (in mm) between the focal fish and the social cue (t-test: $t=3.38$, $p=0.0016$), as well as the focal
264 fish and the small cue fish ($t=3.476$, $p=0.0013$), was significantly greater for group-reared
265 juveniles. The size difference (SL) between the focal fish and the large cue fish was significantly
266 greater for pair-reared juveniles ($t=-3.22$, $p=0.0025$). Relative size differences followed the same
267 pattern as absolute size differences (data not shown).
268
269 *Experiment 2: Whole brain gene expression*
270 Gene expression for two fish from each group (1 week: n=8; 5 weeks: n=14), both
271 members of the pairs (1 week: n=8; 5 weeks: n=10), and every isolate (1 week: n=8) was
272 analyzed. Focal individuals from the group condition were selected haphazardly. Juveniles were
273 removed from their rearing environments with a hand net and rapidly decapitated. The brains
274 were dissected immediately, flash frozen on dry ice, and stored at -80° C until processing. Gene
275 expression was quantified using qPCR and previously validated primers (Supplemental Table 1,
276 Chen and Fernald, 2008; Greenwood et al., 2003; O'Connell and Hofmann, 2012a) for GR1,

GR2a, GR2b, MR, CRF, ARα, and ERα, as well as control genes 18S and G3PDH. RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI), and the Promega GoScript Reverse Transcription System (Promega, Madison, WI) was used for reverse transcription. PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) was used for quantitative PCR. All standard kit protocols were followed. Relative gene expression levels were quantified using ΔΔCT analysis, using 18S and G3PDH as reference genes. The results are largely concordant independent of the reference gene used. Here, we present the analyses for 18S, as this gene has shown very little expression variation across social phenotypes in transcriptome studies of *A. burtoni* (O'Connell and Hofmann, 2012a; Renn et al., 2008).

Statistical analyses

All statistical analyses were conducted using R Studio (version 1.0.143). Results were considered significant at the $p<0.05$ level, and averages ± standard error of the mean are included in the text. The box of the box and whisker plots show the median and the first and third quartiles. The whiskers extend to the largest and smallest observations within or equal to 1.5 times the interquartile range. Comparisons between group- and pair-reared juveniles were conducted using t-tests for fish SL, time and frequency in each tank zone, and rates of agonistic behavior. Mann-Whitney-Wilcoxon tests were used for data that did not meet the assumptions of parametric statistics. Regression analysis was used to identify significant associations between SL and frequency and time in a zone and between SL and agonistic behavior. We used a false discovery rate correction for regressions with focal fish SL (Benjamini and Hochberg, 1995). Two-way ANOVAs were used to identify significant effects of rearing environment, presence of the social cue, or an interaction, on the frequency and time spent in each zone of the tank. We

used Principal Components Analysis (PCA) to identify how behaviors clustered across the four assays and for each assay individually. T-tests were used to compare principal component scores between group- and pair reared juveniles. Correlation analysis was used to identify significant associations among principal components (PCs).

Gene expression data did not meet the assumptions of parametric statistics; therefore, the effects of rearing environment (group, pair, isolated) and treatment duration (1 week, 5 weeks) were analyzed separately using Kruskal-Wallis tests or one-way ANOVA and Mann-Whitney-Wilcoxon tests, respectively. To get a tentative estimate for possible interaction effects, we conducted two-way ANOVAs. These analyses, along with visual inspection of the data, do not suggest any interactions between rearing environment and treatment duration. We also used PCA to identify how the expression of candidate genes clustered. T-tests, or Mann-Whitney-Wilcoxon tests if appropriate, were used to compare group- and pair-reared juveniles and expression following 1 vs. 5 weeks in rearing environments. Dunn's test was used for *post hoc* analysis of significant results. Partial correlation networks were calculated using the "ppcor" package in R and visualized using "qgraph." The nodes of the networks represent the gene. The edges are the partial correlation coefficient, with thicker edges indicating stronger correlations. Only significant correlations are shown. Mantel tests were used to test for pairwise differences between the gene expression networks. A non-significant p-value (> 0.05) indicates that the partial correlation matrices are not related.

Results

Experiment 1

Open field test and social cue investigation

In the open field test (and subsequent assays), juveniles of both treatment groups moved readily around the novel environment with minimal acclimation. We present the data for the frequency of entering each zone. There were no significant effects for the time spent in each zone ($p>0.05$). Group-reared juveniles entered the territory (Mann-Whitney-Wilcoxon test: $W=299$, $p=0.034$), close ($W=293.5$, $p=0.049$), and investigate zones ($W=293.5$, $p=0.049$) significantly more frequently than pair-reared juveniles. There was no significant difference for the far zone ($W=289$, $p=0.064$).

Next, we used a social cue investigation task to examine whether and how rearing environment and/or the presence of the social cue affect locomotor activity. Two-way ANOVA revealed that, following the addition of the social cue, juveniles entered the investigate zone significantly more frequently than controls ($F_{1,36}= 4.91$, $p=0.033$). There was no effect of rearing environment ($F_{1,36}=1.69$, $p=0.20$) and no interaction ($F_{1,36}=0.046$, $p=0.83$). There was no effect of rearing environment ($F_{1,36}=2.68$, $p=0.11$), social cue ($F_{1,36}=0.87$, $p=0.36$), or an interaction ($F_{1,36}=0.84$, $p=0.37$) on frequency of entering the far zone. Group-reared juveniles entered the close zone significantly more than pair-reared juveniles ($F_{1,35}=4.47$, $p=0.042$), but there was no effect of the social cue ($F_{1,35}=0.11$, $p=0.74$) and no interaction ($F_{1,35}=0.44$, $p=0.52$). There was no effect of rearing environment ($F_{1,35}=3.28$, $p=0.079$), social cue ($F_{1,35}=0.17$, $p=0.68$) and no interaction ($F_{1,35}=0.83$, $p=0.37$) on the frequency of entering the territory zone. Linear regression analyses of the relationship between SL and the frequency of entering zones of the tank are shown in Table 1.

Dominant and subordinate behavior

Interestingly, rearing environment did not affect rates of focal fish behavior. As the

dominant fish, there were no differences in approaching (W=242.5, p=0.20) or displacing (W=253, p=0.12) the small cue fish. As the subordinate, there were no differences in approaching (W=205.5, p=0.85), displacing (W=214.5, p=0.62), or submitting to (W=217.5, p=0.56) the large cue fish. In the dominance assay, rearing environment did not affect agonistic efficiency for the focal fish (t=0.83, p=0.41), small cue fish (W=115.5, p= 0.97), or the difference between the pair (t=1.03, p=0.32). In the subordinate assay, although there was no effect of rearing environment on agonistic efficiency for the focal fish (W=169.5, p=0.28) or the large cue fish (W=112.5, p=0.061), the difference in agonistic efficiency was significantly higher for pair-reared juveniles (p=0.022). See Table 1 for linear regression analyses of SL with behavior.

Multivariate analysis across assays

In order to gain more insight into this multivariate dataset, we employed PCA to determine which measures of morphology (i.e., size) and behavior act in concert to explain different aspects of the variability across individuals. We first conducted a PCA that included variables from each of the four assays (focal fish SL; frequency of entering each zone in the open field test and social cue investigation; focal fish social approaches and displacements as a dominant towards the small cue fish; and focal fish approaches, displacements, and submissions as a subordinate with the larger cue fish). We found that principal component (PC) 1 accounts for 43.3% of the variation and differs significantly between group- and pair-reared juveniles (p=0.029, Fig 2A). As the vector plot in Fig 2B shows, variables from the open field test, social cue investigation, and dominance behavior assay all load on PC1, along with focal fish SL. Measures of subordinate behavior do not contribute. There were no significant treatment

369 differences in higher order PCs except for PC6, which accounted for 5.0% of the variation in the
370 data and contained significantly higher values for group-reared compared to pair-reared juveniles
371 (p= 4.082e-05, Fig 2C). Focal fish SL loads most strongly on PC6 (data not shown). This is
372 entirely in accordance with the finding that after 8-10 weeks in their respective early-life
373 environments, group-reared juveniles (16.85 ± 0.32 mm SL) were significantly larger than pair-
374 reared juveniles (13.76 ± 0.40 mm SL) (t=6.00, p=7.25e-07).

375 To better understand how rearing environment affected behavior within the assays that
376 contributed to the treatment difference, we conducted PCAs for the open field, social cue
377 investigation, and dominance behavior assays separately. We expanded these analyses to include
378 all of the measured variables, for the focal and cue fish. The open field test analysis included
379 focal fish SL and the frequency of entering and time in each zone of the tank. The social cue
380 investigation included the same measures, as well as the SL of the cue fish. Finally, the
381 dominance behavior analysis included SL of the focal fish and small cue fish, approaches and
382 displacements of both fish, and the frequency of entering and time spent in the territory by either
383 or both fish. For each analysis, PC1 differed significantly between group- and pair-reared
384 juveniles: open field (43.4% variation, p=0.04, Fig 3A), social cue investigation (37.2%
385 variation, W=102, p = 0.0032, Fig 3B), and dominance behavior (29.8% variation, W=128,
386 p=0.025, Fig 3C). The PC1s were also significantly and linearly correlated with each other (Fig
387 3D, open field x social cue: r^2=0.46, p=5.33e-07; open field x dominance: r^2=0.33, p= 4.69e-05;
388 social cue x dominance: r^2=0.46, p= 4.97e-07, Supplemental Figure 1). We found no significant
389 differences for any higher order PCs in the three analyses. See Supplemental Figure 2 for the
390 proportion of variation explained by each PC.

391 For the open field test, all variables loaded on PC1 except time in the territory and

392 investigate zones. For the social cue investigation, all variables loaded on PC1 except time in the
393 territory, investigate, and close zones. Finally, for dominance behavior, the strongest loadings for
394 PC1 include approaches and displacements by the focal and small cue fish, the frequency of the
395 focal fish entering the territory, the time spent in the territory by the small cue fish, and the SL of
396 both the focal and small cue fish (Supplemental Figure 3).
397
398 *Experiment 2*
399 Neural gene expression patterns
400 Neuroendocrine signaling is a primary mechanism by which early-life experiences are
401 translated into biological changes. To identify potential mediators of the behavioral effects we
402 identified, we measured mRNA levels of genes involved in the stress axis and in sex steroid
403 signaling in the brains of a separate cohort of juveniles. We compared relative expression across
404 rearing environments (isolation, pairs, groups) and time in rearing environment (1 week, 5
405 weeks). Overall, there was little significant variation in neural gene expression with regards to
406 either rearing environment or treatment duration ($p>0.05$, Fig 4, Supplemental Fig 4,
407 Supplemental Table 2). There was a significant effect of rearing environment on GR1a
408 expression (Kruskal-Wallis: $\chi^2_2=16.58$, $p=0.00025$). *Post hoc* analysis showed that expression
409 was significantly higher in groups-reared juveniles than pair-reared ($p=0.0015$) or isolated
410 ($p=0.0015$) juveniles, which did not differ from each other ($p=0.39$). Expression of GR2
411 ($F_{2,43}=4.22$, $p=0.021$) and CRF ($\chi^2_2=6.17$, $p=0.046$) also differed significantly among rearing
412 environments; however, *post hoc* analyses showed there were no significant pair-wise
413 differences ($p>0.05$).
414 Genes function within regulatory networks, rather than in isolation, and they can affect

each other's expression. Similarly, a common upstream regulator may control multiple functional networks of genes. Because of their known effects on physiology and behavior, these candidate genes are likely to function in pathways that interact with each other. To quantify how rearing environment affects gene co-expression, we calculated partial correlation networks (Fig 5). Partial correlations show the associations between gene pairs, independent of other correlations in the network. Comparing the group and pair networks (Mantel test: $p=0.31$), the group and isolate networks ($p=0.61$), and the pair and isolate networks ($p=0.12$) revealed that there was no evidence that any of these networks were similar to any other.

To gain a more holistic understanding of how rearing environment and/or treatment duration affect variation in neuroendocrine gene expression, we used PCA. PC1 accounts for 69.1% of the variation in the data. While there were no differences in PC1 based on rearing environment ($W=113$, $p=0.13$), there was a trend for differences based on treatment duration ($W=99$, $p=0.053$; Fig 6A). There were no differences due to rearing environment for any higher order PCs except for PC4, which accounted for 5.7% of the variation in the data and differed significantly according to rearing environment ($p=0.011$; Fig 6B). Fig 6C shows how the different candidate genes load onto PC1 and PC4.

Discussion

In the present study, we demonstrate that juvenile *A. burtoni* behavior and neuroendocrine gene expression are both sensitive to early-life social effects. By rearing juveniles in different social environments—either in a social group or as a pair, both of which allow individuals to interact freely at all times—we altered the quality and quantity of social experiences and sensory cues perceived and set individuals along different developmental

438 trajectories. Behaviorally, the early-life environment shifted juveniles in a predictable manner
439 along a continuum of a novel behavioral syndrome (i.e., correlated behaviors across contexts, see
440 below) comprised of open field, social cue investigation, and dominance behaviors (Fig 2, Fig 3)
441 and affected patterns of subordinate behavior, a critically important social role for young
442 individuals. In the brain, rearing environment caused striking changes in neuroendocrine gene
443 co-expression patterns, differences driven by the expression of GRs and AR. Together, these
444 experiments provide an essential step towards understanding how developmental plasticity
445 generates the individual variation in behavior and neuroendocrine function that has fitness and
446 health consequences in adulthood (e.g., Champagne, 2010; Turecki and Meaney, 2016). Our
447 results also contribute to an important and growing literature on the impact of early-life social
448 environments beyond parental interactions (Champagne and Curley, 2005; Taborsky, 2016),
449 using a species that, despite its prominence in social neuroscience (Fernald and Maruska, 2012;
450 Hofmann, 2003), has rarely been studied during development (Alvarado, Lenkov, Williams, &
451 Fernald, 2015; Fernald & Hirata, 1979; Fraley & Fernald, 1982).
452
453 Juvenile behavior forms a syndrome affected by early-life social environment
454 Using a battery of four behavioral assays to gain a comprehensive understanding of
455 behavioral phenotype, within and across contexts (Fig 1), we discovered that open field, social
456 cue investigation, and dominance behavior together formed a behavioral syndrome (Fig 3).
457 Syndromes are a population-level metric defined as the correlation between rank-order
458 differences between individuals, across contexts and/or over time (Bell, 2007). The presence of a
459 syndrome indicates consistency in patterns of individual behavior across contexts and/or over
460 time (Bell, 2007; Sih et al., 2004a, 2004b). Our data suggest that how individuals move around

in space is relevant to the social role they play. Specifically, juveniles that were more active in the open field test were more likely to be active in the social cue investigation and more interactive in the dominance assay. Interestingly, behavior from the subordinate assay does not contribute to the treatment effect or syndrome, likely because subordinate focal individuals respond primarily to the dominant fish's behavior. To our knowledge, this is the first behavioral syndrome to be identified in *A. burtoni* at any developmental stage.

Behavior patterns may coalesce into a syndrome due to shared mechanisms (e.g., neuroendocrine regulation), early-life experiences that set individuals along developmentally plastic trajectories, or correlational selection (Bell, 2007; Ketterson and Nolan, Jr., 1999; Stamps, 2003). We found that the behavior of all juveniles was described by the same syndrome, indicating that how the behaviors are related across experimental contexts (i.e., assays) was maintained independently of the early-life social environment. Whether an individual was reared in a group or pair then dictates where along the continuum of the syndrome they fall (Fig 3D). Pair-reared juveniles appear restricted to one end, whereas group-reared juveniles are represented along the full range of behavioral variation. That there are group-reared juveniles that behaviorally resemble the pair-reared individuals suggests there may be social environments within a group (Saltz et al., 2016) that share key elements with the paired experience. Conversely, the range of possible social roles seems much more restricted in the paired treatment. To identify the causal behavioral and/or sensory cues, it will be necessary to conduct detailed observations of individuals within the rearing environments (Taborsky, 2016). Based on our pilot observations, we hypothesize that the complexity of interactions and/or abundance of social sensory cues in groups cause these treatment differences (Taborsky, 2016, e.g., Arnold & Taborsky, 2010).

Activity and social interaction are common components of syndromes in other species, along with bold-shy and proactive-reactive behaviors (Bell, 2007; Conrad et al., 2011; Groothuis and Carere, 2005; Koolhaas et al., 1999; Sih et al., 2004b; Verbeek et al., 1994). For example, large juvenile brown trout are more active and aggressive (Näslund and Johnsson, 2016), similar to our results. Activity-aggression syndromes are also found in a number of other fish species (reviewed in Conrad et al., 2011). For *A. burtoni* juveniles, locomotor activity and social interaction may be causally related. First, active individuals may encounter conspecifics more frequently and, as a result, initiate more interactions. Second, juvenile social interactions appear to be prosocial in that they increase the likelihood of future proximity and interaction. In the dominance behavior assay, approaches and displacements for both the focal and subordinate cue fish load in the same direction on PC1. Correlation analysis (data not shown) confirms that, as one member of the pair initiates social interactions, the other member also initiates, potentially leading to more activity. This may be beneficial by increasing shoaling and reducing the risk of predation. Interestingly, adult dominance behavior does not lead to a prosocial response in subordinates, suggesting that although social behavior appears similar across life history stages (Fernald and Hirata, 1979; Fraley and Fernald, 1982), there are important differences.

Size plays a secondary role in determining juvenile behavioral phenotype

Size is central to understanding juvenile activity, social interactions, and the effects of the early-life social environment. Group-reared juveniles were larger than those reared in pairs, and SL was positively associated with activity in the open field and social cue investigation assays, approaching and displacing as a dominant fish, as well as entering the territory zone, alone or with the cue fish, in the dominance behavior assay. The importance of size in juveniles is

consistent with the research showing that adult *A. burtoni* are highly sensitive to size during social interactions (Alcazar et al., 2014; Weitekamp & Hofmann, 2017). Growth is also socially regulated in both juveniles and adults (Fraley and Fernald, 1982; Hofmann et al., 1999; this study). However, the effect of the early social environment is much larger and more complex than size alone. First, the PCA of behavior from all four assays shows that focal fish SL contributes only moderately to the significant treatment difference for PC1 (Fig 2B), as many other variables load much more strongly on PC1 (i.e., open field, social cue investigation, and dominance behaviors) (see also: Supplemental Fig 3). Second, SL is the strongest contributing variable for PC6, which represents the significant size difference between group-reared and pair-reared individuals discussed above, yet contributes only 5% to the overall variation in the data. Finally, the group-reared juveniles that fall within the range of pair-reared juveniles along the continuum of the behavioral syndrome (i.e., high PCA scores, Fig 3) are not the smallest individuals. While size may be secondary for understanding early-life effects in juveniles, it remains to be tested how individual behavior changes over time in relation to both size and developmental stage, which can be decoupled from chronological age in fish (Jonsson and Jonsson, 2014).

Early-life social experience affects social dynamics when focal juveniles are subordinate

Developmental plasticity can shift behavior in ways that ultimately benefit fitness (Smith and Blumstein, 2008), in part because social behavior has direct consequences for reproductive success (Wilson, 1980, e.g., Henry et al., 2013; Robbins et al., 2007; Young et al., 2006). A majority (64%) of studies show that experimentally increasing the frequency, diversity, or complexity of early-life social experiences enhances social skills or competence (Taborsky,

2016). For example, juvenile *N. pulcher* cichlids reared with brood helpers demonstrated more context-appropriate behavior when establishing status, integrating into novel groups, and competing for a resource (Arnold and Taborsky, 2010; Fischer et al., 2015; Taborsky et al., 2013, 2012). We have no evidence of an advantage for group-reared juveniles; however, juveniles may fill the subordinate role differently. While nearly all focal fish successfully established themselves as subordinate (88%) in the assay, and there were no treatment differences in approaches or displacements, there was a significantly larger asymmetry in agonistic efficiency for pair-reared juveniles. There was also a trend for pair-reared juveniles to submit more readily (measured as large fish agonistic efficiency). Status relationships are defined by asymmetrical agonistic displays (Drews, 1993); therefore, pair-reared juveniles may behave more submissively. The subordinate role is a critically important one for juveniles because all will enter adult communities as subordinates. It will be necessary to measure behavior and reproductive success of these juveniles once they are adults in order to determine whether these phenotypes persist or if one is more successful than another (Pradhan, Solomon-Lane, & Grober, 2015).

Early-life social environment affects neuroendocrine gene co-expression networks

We have shown that early-life environments can determine where individuals will end up along the axis of the newly discovered behavioral syndrome, which raises questions about the underlying mechanisms (e.g., pleiotropic genes and/or neuroendocrine regulation). The behavioral effects we detect as a result of the early-life social environment suggest important variation in the underlying neural regulatory mechanisms. Neuroendocrine stress and sex hormone signaling are likely sites of developmental plasticity in *A. burtoni* because they are

553 sensitive to early-life effects (Champagne & Curley, 2005; Shepard et al., 2009), translate
554 environmental conditions and experiences into biological responses (Crespi & Denver, 2005;
555 Wingfield et al., 1990), and regulate behavior (Adkins-Regan, 2009; Solomon-Lane, Crespi, &
556 Grober, 2013). We focused on steroid hormone nuclear receptors specifically because they
557 regulate the transcription of target genes with a diversity of physiological and behavioral roles
558 (Rochette-Egly, 2005). Overall, gene expression was highly variable, especially among group-
559 reared juveniles, possibly reflecting the considerable variability in experiences within groups.
560 With the exception of GR1a, we found no significant differences when comparing the expression
561 of single genes across rearing environments or treatment durations (Fig 4, Supplemental Fig 4),
562 suggesting individual variation is strongly influenced by other factors, which we did not measure
563 here (e.g., social status, body size, and sex). For example, social experience and status can both
564 affect gene expression (Li et al., 2014). In adult male *A. burtoni*, the expression of ARα, MR,
565 GR1a, and GR2 in the POA of the hypothalamus is higher in dominants, whereas GR1b is higher
566 in subordinates (Korzan et al., 2014). Our behavior data suggest that an individual's position
567 along the behavioral syndrome, as well as its social status, may be critical for understanding gene
568 expression variation.

569 The expression of other genes can also contribute to expression variation.
570 Neuroendocrine systems are dynamic and interact on multiple biological levels, including within
571 gene regulatory networks (e.g., Huffman et al., 2012; Korzan, Fernald, & Grone, 2014;
572 O'Connell & Hofmann, 2012). Based on their co-localization in the POA of *A. burtoni* (Korzan
573 et al., 2014), co-localization and correlation in other species (e.g., Meyer & Korz, 2013), and
574 overlapping physiological effects (Crespi & Denver, 2005; Wingfield et al., 1990), the
575 neuroendocrine pathways represented by our candidate genes are likely to functionally interact.

We identified striking differences in co-expression networks among juveniles reared in different environments. Expression was highly correlated in pair-reared juveniles (Fig 5A), such that every candidate gene was significantly correlated with at least two others. At the center of the network, ARα shares five significant connections. The two sex steroid hormone genes (ARα, ERα) are also integrated with the stress axis genes, which form distinct smaller networks: CRF-GR1a-GR1b and GR2-MR. In contrast, group-reared juveniles have only one significant partial correlation between ERα and GR1b, a connection that is not present in the pair-reared network (Fig 5B). There are no significant partial correlations for isolated juveniles, suggesting that the neuroendocrine regulatory network is dysregulated, possibly due to isolation acting as a stressor (Galhardo and Oliveira, 2014). These network differences might underlie the behavioral differences we identified in the behavioral syndrome, subordinate behavior, or more broadly related to stress response (see below). The differential co-regulation could also serve to make behavior more similar in the face of other neural differences caused by rearing environment, as is the case for some neural sex differences and behavior (De Vries, 2004). These hypotheses can be tested directly using central pharmacological manipulation.

Complex regulation of neural stress and sex steroid signaling by the early-life social environment

From the gene co-expression networks alone, it is challenging to determine whether specific genes drive the significant differences across treatment groups. This is where decomposing the total variance into principal components offers a powerful approach that allowed us to show that the HPI axis plays a central, and likely highly-conserved (Crespi and Denver, 2005), role in responding to the early-life social environment in juvenile *A. burtoni*. First, PCA revealed that the neural expression of ERα, MR, and CRF strongly loaded on PC1,

599 which explains 69.1% of the variance (Fig 6C). The trend for differences in PC1 based on
600 treatment duration (Fig 6A) suggests that we cannot rule out that neuroendocrine gene
601 expression patterns change in important ways over the course of development. Because all
602 juveniles entered treatment at the same age and developmental stage, future work is needed to
603 distinguish between the effects of treatment duration and age (if any), and to identify possible
604 critical periods for early-life effects.

605 Second, scores for PC4 (5.7%) were significantly different between group- and pair-
606 reared juveniles (Fig 6B). All of the GRs, as well as AR, load on PC4 and contribute to the
607 treatment effect (Fig 6C). Many teleosts, including *A. burtoni*, have three glucocorticoid
608 receptors: MR, GR1, and GR2. Receptor 1 has splice variants 1a and 1b, which differ by a nine
609 amino acid insertion in the DNA-binding domain of 1b that reduces transcriptional response
610 (Greenwood et al., 2003; Korzan et al., 2014). Consistent with the distinct roles for the different
611 receptors and splice variants (Greenwood et al., 2003), GR1a and GR2 load in the opposite
612 direction from GR1b and ARα (Fig 6C), suggesting their expression may be antagonistically
613 regulated (e.g., Fig 5A). Changes in HPA/I axis function typically manifest as altered baseline
614 levels of circulating glucocorticoids, a higher or lower glucocorticoid 'peak' in response to an
615 acute stressor, and/or altered efficiency of the negative feedback loop that returns the system to
616 baseline. Negative feedback, in particular, is regulated by neural GR expression and can be
617 affected by early-life experience (Champagne and Curley, 2005; Francis et al., 1999). This
618 suggests either group- or pair-reared juveniles, or both, may have altered negative feedback
619 mechanisms via differential GR expression. In particular, the hippocampus and amygdala (and
620 their non-mammalian homologs), brain regions important in spatial cognition and emotional
621 processing, respectively, are central to negative feedback (Denver, 2009).

622 Finally, none of the endpoints we measured loaded on PC2 (11.3%) or PC3 (7.17%),
623 suggesting that there are likely other molecular pathways involved that we did not assay, which
624 is to be expected for a complex phenotype such as social behavior. A genome-scale analysis of
625 gene expression using RNA-seq can provide novel candidates here.
626
627 Integrating the effects of early-life social environments on behavior and brain
628 Our work demonstrates that early-life social environments shape behavioral phenotype
629 and neuroendocrine gene expression in powerful ways for *A. burtoni* juveniles. In this study, we
630 quantified behavior and gene expression in separate experiments in order to focus on different
631 developmental time points. However, understanding the full scope and consequences of early-
632 life effects requires measuring brain and behavior in the same individuals, throughout
633 development and into adulthood. This work can begin to address the fact that across species,
634 remarkably little is known about the mechanisms that shape the ontogeny of behavior (Taborsky,
635 2016). Our results suggest that brain regions that express GRs and ARα (Greenwood et al., 2003;
636 Korzan et al., 2014; Munchrath and Hofmann, 2010), along with brain regions of the social
637 decision-making network (SDMN) that together regulate social behavior (O'Connell and
638 Hofmann, 2012b), are likely to be sensitive to early-life effects and could cause the observed
639 changes in behavior. Interestingly, the POA—a critical node in the SDMN (O'Connell and
640 Hofmann, 2012b)—contains GR1a, GR1b, GR2, and ARα in adult *A. burtoni* (Korzan et al.,
641 2014; Munchrath and Hofmann, 2010). Additional SDMN nodes, such as the hippocampus and
642 amygdala are also likely sites of overlap. Interactions between the HPI axis and androgen
643 signaling, including in the POA, could be a mechanism for the social regulation of development
644 (Fraley and Fernald, 1982; Korzan et al., 2014; Solomon-Lane et al., 2013; Wada, 2008).

Another hypothesis is that the behavior patterns sensitive to early-life effects and HPI axis function will together form a specific kind of syndrome called a coping style, which ranges from proactive to reactive copers. Proactive copers tend to be more active, aggressive, and less responsive to stress (i.e., lower baseline glucocorticoid levels, faster negative feedback) than reactive copers (Koolhaas et al., 1999). Overall, this research can uncover the neuroendocrine mechanisms by which early-life social experience gives rise to individual variation in adults, which is critical to understanding subsequent disparities in fitness and health.

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947

948 **Table 1:** Linear regressions of focal fish standard length with behavior in the open field, social
949 cue investigation, dominance, and subordinate behavior assays. The zones of the tank refer to the
950 frequency of entering that zone. Adjusted R^2 values are reported. Significant results following a
951 false discovery rate correction are bolded.

Assay	Behavior	r-squared	p-value
Open field	Investigate zone	0.17	**0.0036**
	Far zone	0.15	**0.0059**
	Close zone	0.11	**0.017**
	Territory zone	0.18	**0.0028**
Social cue investigation	Investigate zone	0.035	0.13
	Far zone	0.085	**0.038**
	Close zone	0.096	**0.031**
	Territory zone	0.11	**0.021**
Dominance behavior	Approach	0.089	**0.035**
	Displace	0.10	**0.024**
	Territory zone (focal)	0.11	**0.022**
	Territory zone (subordinate fish)	0.14	**0.011**
	Territory zone (both)	0.11	**0.02**
Subordinate behavior	Approach	-0.02	0.57
	Displace	-0.0044	0.37
	Submit	-0.023	0.72
	Territory zone (focal)	-0.0015	0.34
	Territory zone (subordinate fish)	-0.02	0.67
	Territory zone (both)	-0.024	0.87

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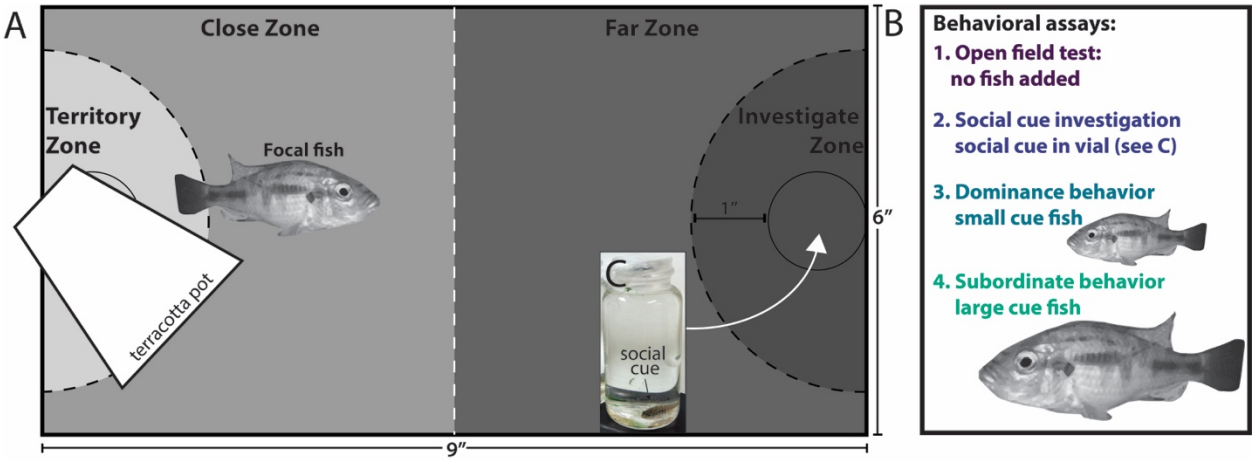
956 **Figures & legends**



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958 **Figure 1: Experimental setup for behavior assays.** Juvenile behavior was observed in a novel
959 experimental tank in four sequential assays administered in the same order, each lasting 30 min.
960 A terracotta shard served as a shelter and/or territory. The black lines (dotted, solid) were drawn
961 on the tank bottom in permanent maker, dividing the tank into four zones: territory, close, far,
962 and investigate. The center dividing line (white) was not drawn (A). The focal fish was alone in
963 the tank for the open field assay, and the time in each zone and frequency of entered each zone
964 was recorded (B, assay 1). For the social cue investigation, a juvenile inside of a scintillation vial
965 was placed in the circle within the investigate zone (see C). The time in and frequency of
966 entering each zone was recorded (B, assay 2). The social cue was removed and a freely
967 swimming, novel cue fish (smaller than the focal) was added to the tank for the dominance
968 behavior assay (B, assay 3). The small cue fish was then removed and a freely swimming, novel
969 cue fish (larger than the focal) was added to the tank for the subordinate behavior assay (B, assay
970 4). Social interactions were recorded for the dominant and subordinate behavior assays. The time
971 in and frequency of entering the territory zone was also recorded for both fish.

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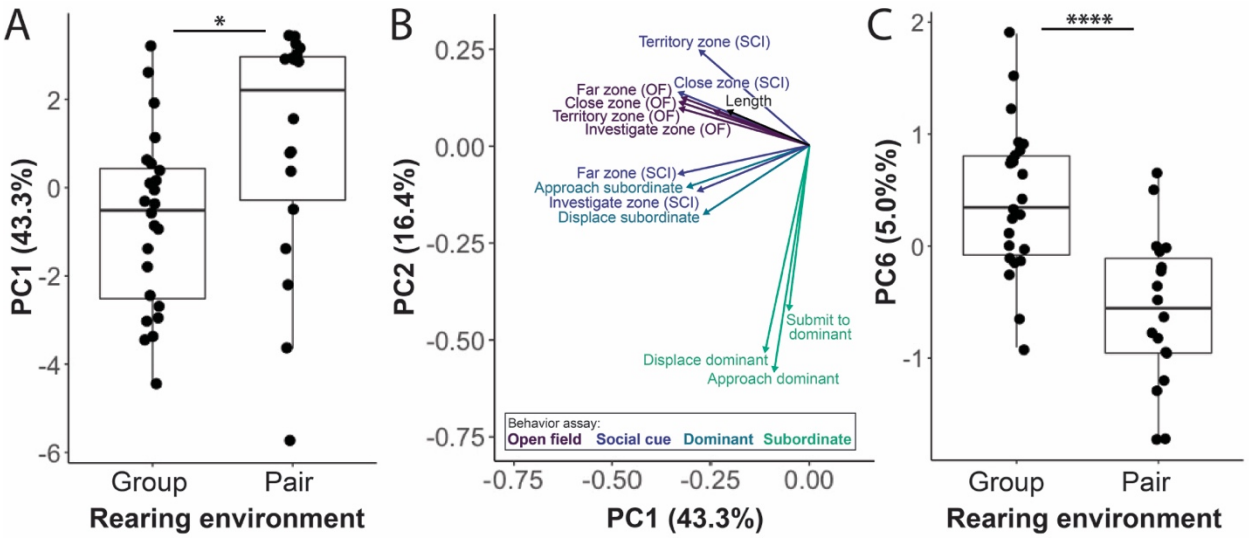
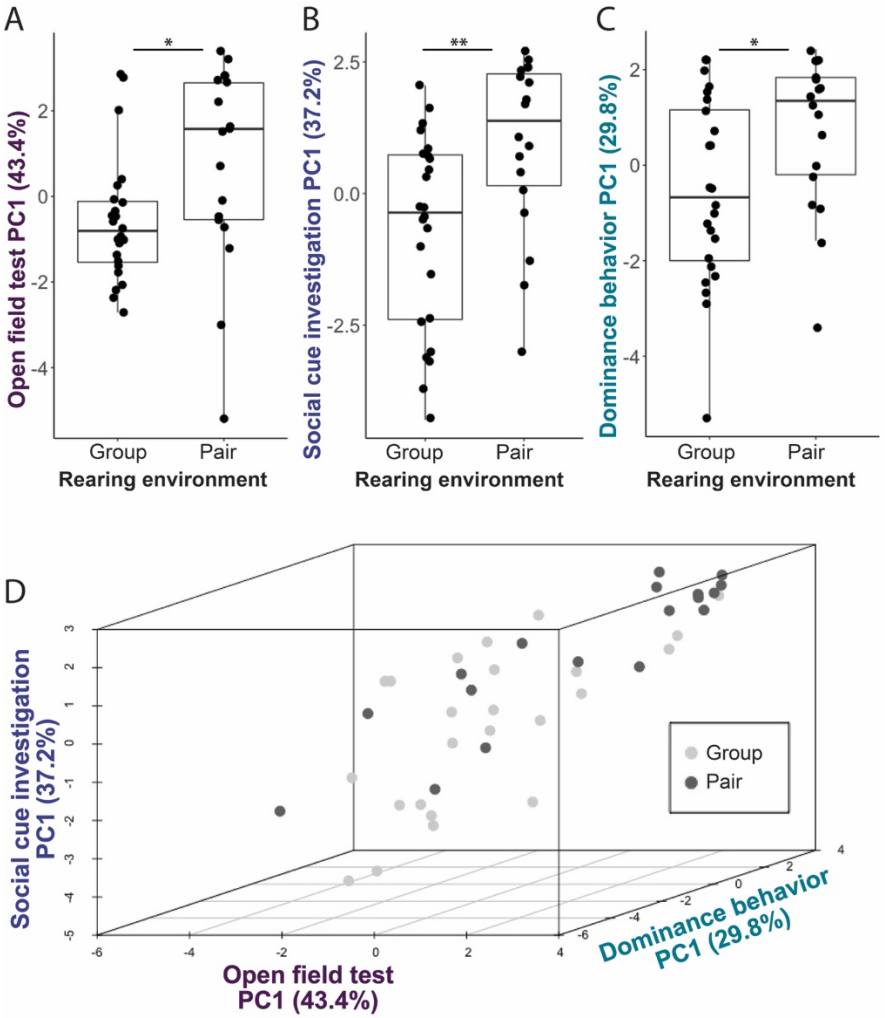


Figure 2: Principal component analysis (PCA) of focal fish behavior from all four assays (open field, social cue investigation, dominance, subordinate behavior). Differences in PC1 between group- and pair-reared juveniles (p=0.029) (A). Vector plot showing the PCA variables that load on PC1 (B). Differences in PC6 between group- and pair-reared juveniles (p= 4.082e-05) (C). Percentages refer to the amount of variation explained by that component. Pair (n=18 individuals). Group (n=24 individuals). Social cue investigation (SCI). Open field exploration (OF). *p<0.05, ****p<0.001.



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984 **Figure 3:** Separate principal component analyses performed for the open field (A), social cue

985 investigation (B), and dominance behavior (C) assays. Both focal and non-focal fish variables

986 (behavior, size). The significant, positive correlations about the PC1s are shown in a three-

987 dimensional plot (D). Percentages refer to the amount of variation explained by that component.

988 Pair (n=18 individuals). Group (n=24 individuals).

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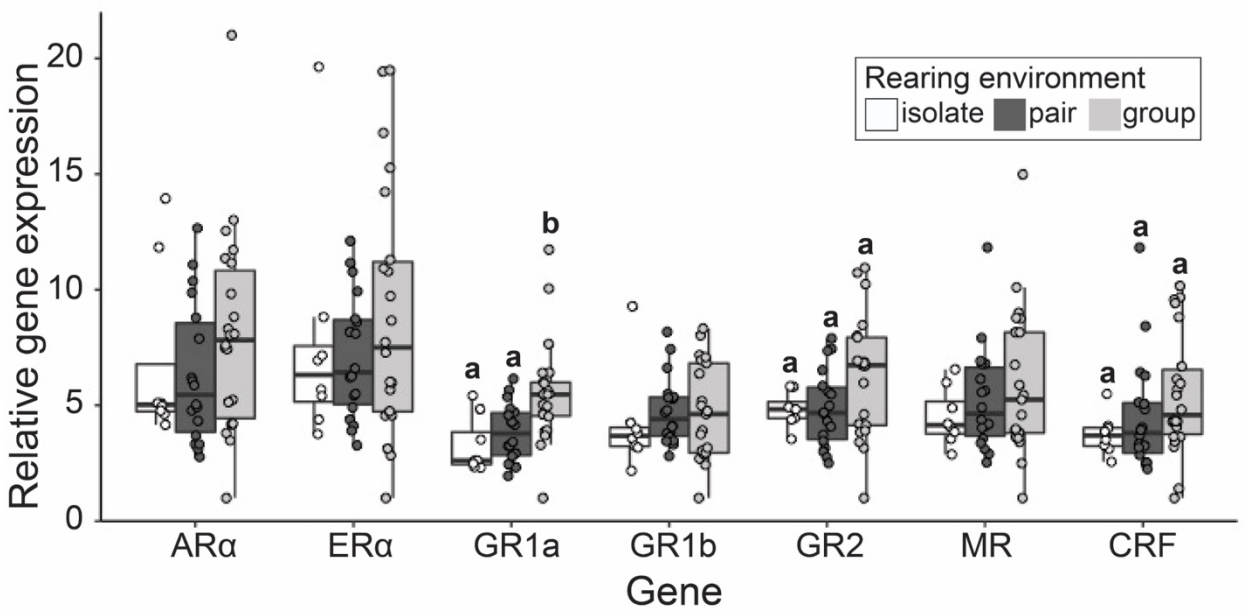

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991 **Figure 4:** Relative gene expression calculated using ΔΔCT analysis (reference gene 18S) for
992 juveniles reared in isolation (1 week, n=8), pairs (1 week or 5 weeks, n=18), and groups (1 week
993 or 5 weeks, n=22). Androgen receptor α (ARα). Estrogen receptor α (ERα). Glucocorticoid
994 receptors (GR). Mineralocorticoid receptor (MR). Corticotropin-releasing factor (CRF). Letters
995 indicate significant *post hoc* differences within a gene ($p<0.05$).

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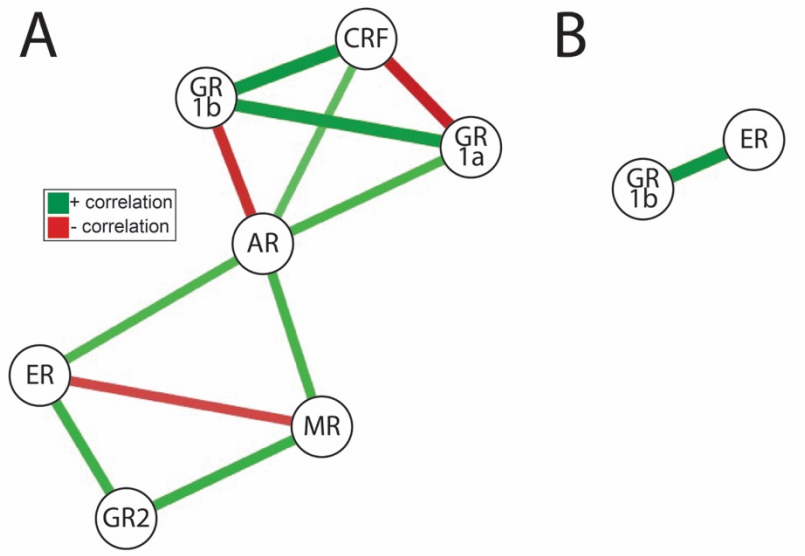

998 **Figure 5:** Partial correlation network of gene expression in pair-reared juveniles (n=18) (A) and
999 group-reared juveniles (n=22) (B). Nodes are the candidate genes. Edges represent partial
1000 correlations between nodes. Only significant partial correlations are shown ($p<0.05$), and edge
1001 thickness indicates correlation strength. There were no significant partial correlations for
1002 juveniles reared in isolation (n=8) ($p>0.05$). Androgen receptor α (AR). Estrogen receptor α
1003 (ER). Glucocorticoid receptors (GR). Mineralocorticoid receptor (MR). Corticotropin-releasing
1004 factor (CRF).

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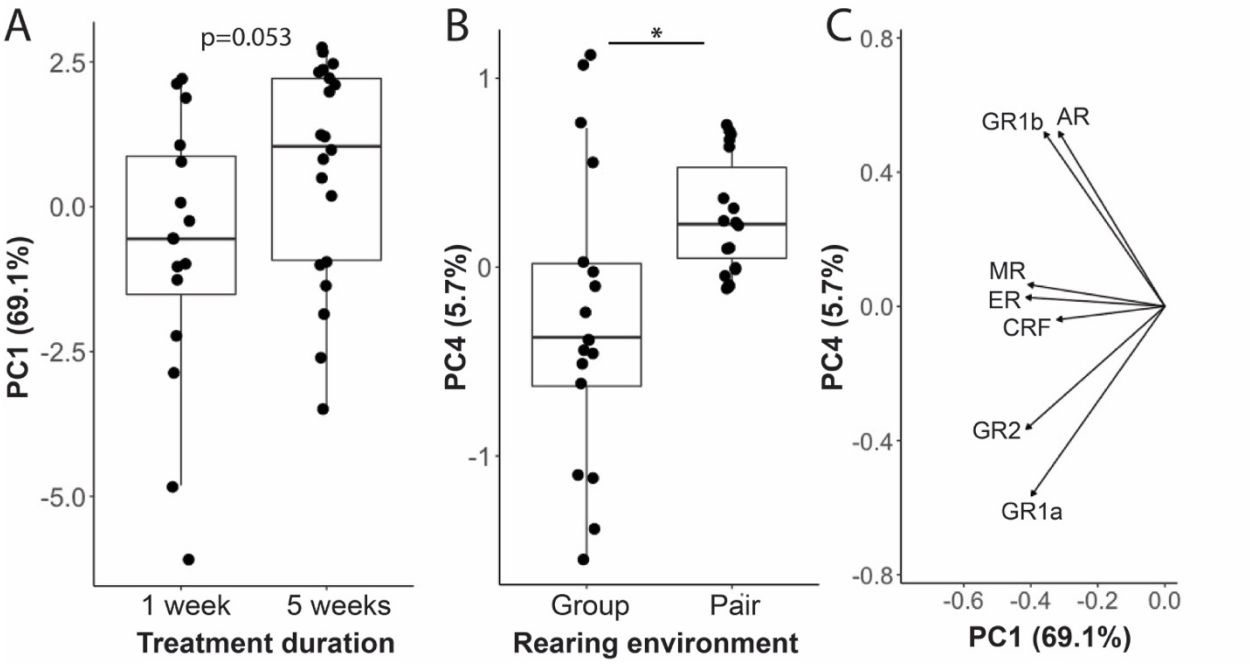

Figure 6: Principal component analysis of relative expression of candidate genes in whole brain from group- (n=22) and pair-reared (n=18) juveniles. Differences in PC1 between juveniles in treatment groups for 1 vs. 5 weeks (A). Differences in PC4 between juveniles reared in groups vs. pairs (p=0.011) (B). Vector plot showing how candidate genes load on PC1 and PC4 (C). Percentages refer to the amount of variation explained by that component. Androgen receptor α (AR). Estrogen receptor α (ER). Glucocorticoid receptors (GR). Mineralocorticoid receptor (MR). Corticotropin-releasing factor (CRF).