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 the concomitant experiences, are translated into individual variation in brain and behavior. We investigate whether early-life social environments generate variation in behavior and neuroendocrine gene expression by rearing juvenile *Astatotilapia burtoni* cichlids in either social groups or pairs. This species is well studied for its nuanced, plastic social behavior in adulthood. We find that juvenile behavior and neuroendocrine function are both sensitive to early-life social effects. Behavior correlates across multiple assays (open field test, social cue investigation, and dominance behavior) to form a behavioral syndrome, and rearing environment significantly 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 of juveniles, we then measured neural expression for stress and sex hormone genes, because these signaling systems are known to translate environmental conditions into biological responses, are sensitive to early-life effects, and regulate adult social behavior. Rearing environment causes striking differences in neural gene co-expression networks. Specifically, gene expression was tightly correlated for 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.

Introduction

Ontogeny has long been recognized as essential to understanding behavior. As the fourth of Tinbergen's Four Questions, development reveals the proximate mechanisms by which genes interact with the environment during early life to change the 'machinery of behavior' (Stamps, 2003; Tinbergen, 1963) and affect fitness and health. The long-lasting, or even permanent, changes in behavior that occur via developmental plasticity, in response to current or predicted environmental conditions, can facilitate locally-adapted phenotypes (Kasumovic & Brooks, 2011; Lummaa & Clutton-Brock, 2002; Piersma & Drent, 2003; Snell-Rood, 2013; Stamps, 2003; Stearns, 1989; West-Eberhard, 1989). For example, in the presence of predators, resistant phenotypes can develop in some species (e.g., armor in *Daphnia*, accelerated growth rates in *Hyla*) (Gilbert, 2001). In particular, the developmental mechanisms that shape individual variation in social behavior via the underlying neuroendocrine mechanisms should be an important target for natural selection because of the direct consequences of social behavior for fitness and health (Bennett, Schneider, Tang, Arnold, & Wilson, 2006; Meyer-Lindenberg & Tost, 2012; Silk, 2007; Solomon-Lane, Pradhan, Willis, & Grober, 2015; Wilson, 1980).

Understanding the scope and consequences of developmental plasticity in social behavior is complex. The effects of plasticity can extend beyond a single behavior to affect an entire suite, and those behaviors are expressed within dynamic contexts that change over multiple timescales. For example, affected behaviors may remain correlated as a behavioral syndrome (or animal personality), constrained by early plasticity, such that behavior appears consistent across contexts (Bell, 2007; Conrad, Weinersmith, Brodin, Saltz, & Sih, 2011; Duckworth, 2010; Sih, Bell, & Johnson, 2004; Sih, Bell, & Ziemba, 2004; Snell-Rood, 2013; Stamps, 2003). Social stimuli are among the most important features 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 social environment beyond the parents is increasingly recognized for its role shaping offspring behavior and brain (Buist, Deković, & Prinzie, 2013; Creel, Dantzer, Goymann, & Rubenstein, 2013; Jonsson & Jonsson, 2014; Kasumovic & Brooks, 2011; Taborsky, 2016; White, 2010). For example, the presence of brood helpers, unrelated adult males, and multiple litters and mothers during early-life has long-term effects on social behavior in the Daffodil cichlid fish *Neolamprologus pulcher* (Arnold & Taborsky, 2010; Taborsky, Arnold, Junker, & Tschopp, 2012), brown-headed cowbirds (White, King, & West, 2002), and laboratory mice (Branchi et al., 2006; Branchi, Santarelli, D'Andrea, & Alleva, 2013; D'Andrea, Alleva, & Branchi, 2007), respectively. The social environment dictates the quantity and nature of social experiences and sensory cues perceived, which together influence neural function and behavior (Taborsky, 2016).

Neuroendocrine signaling is a primary mechanism by which environmental conditions and experience are translated into physiological responses (Crespi & Denver, 2005; Remage-Healey & Romero, 2000; Wingfield, Hegner, Dufty, Jr., & Ball, 1990). As important sources of individual variation in social behavior (e.g., season, sex, reproductive tactics), hormones also 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 & Curley, 2005; Francis, Caldji, Champagne, Plotsky, & Meaney, 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

93 release of corticotropin-releasing factor (CRF) from the hypothalamus, which signals to the
94 pituitary to release adrenocorticotropic hormone, which then signals to the adrenal glands to
95 release glucocorticoids (e.g., cortisol in fish) (Denver, 2009; Lowry & Moore, 2006).
96 Effects of early-life experiences on HPA/I axis function have been demonstrated in every
97 vertebrate lineage (e.g., Banerjee, Arterbery, Fergus, & Adkins-Regan, 2012; Champagne &
98 Curley, 2005; Crespi & Denver, 2005; Jonsson & Jonsson, 2014). For example, the effects on
99 social behavior and competence in the cichlid *N. pulcher* are caused by changes in neural
100 expression levels of CRF and glucocorticoid receptor, as well as receptor ratios (Taborsky,
101 Tschirren, Meunier, & Aubin-Horth, 2013). Stress axis mechanisms have also been implicated
102 for health effects documented in humans (e.g., Turecki & Meaney, 2016). Sex steroid hormones
103 (e.g., androgens, estrogens) also play a role mediating the long-term effects of early-life
104 experiences (Adkins-Regan, 2009; Brown & Spencer, 2013; Shepard, Michopoulos, Toufexis, &
105 Wilson, 2009) and regulating social behavior (Goodson, 2005; Newman & Newman, 1999). For
106 example, neural estrogen receptor expression is associated with variation in maternal behavior in
107 mother rats and offspring (Champagne & Meaney, 2007; Champagne, Weaver, Diorio, Sharma,
108 & Meaney, 2003), and pre- and early postnatal female guinea pigs exposed to social stress show
109 an upregulation of neural estrogen and androgen receptor levels, elevated testosterone, and
110 masculinized behavior (Kaiser, Kruijver, Swaab, & Sachser, 2003). Together, these
111 neuroendocrine systems interact to affect phenotype.
112 To investigate the effects of early-life social experience on behavior and the brain we
113 used the African cichlid, *Astatotilapia burtoni*, a model system in social neuroscience (Fernald &
114 Maruska, 2012; Hofmann, 2003). This highly social fish forms mixed-sex, hierarchical
115 communities comprised of females and males of dominant or subordinate status. Dominant

116 males are territorial, reproductively active, and colorful, while subordinate males shoal with
117 females, are reproductively suppressed, and drab in coloration. The dramatic plasticity exhibited
118 by this species is among the reasons it is a model system in social neuroscience. Male status is
119 socially-regulated, and individuals regularly transition between dominant and subordinate
120 phenotypes (Fernald & Maruska, 2012; Hofmann, 2003). Male reproductive maturation is also
121 socially regulated and affected by early-life social experience (Fraley & Fernald, 1982). *A.*
122 *burtoni* express a suite of social behaviors, as juveniles (Fernald & Hirata, 1979) and adults,
123 including aggression, affiliation, courtship, and cooperation (Fernald, 2012; Hofmann, 2003;
124 Weitekamp et al., 2017). Individual variation across adults has been well described, showing that
125 individuals vary not only in the categories of behavior they display (e.g., courtship in dominant
126 but not subordinate males), but also in their expression patterns (e.g., high vs. low aggression).
127 Substantial progress has been made towards understanding stress and sex steroid hormone
128 function in this species, including in the regulation of social behavior (Chen & Fernald, 2008;
129 Fox, White, Kao, & Fernald, 1997; Greenwood et al., 2003; Munchrath & Hofmann, 2010;
130 O'Connell & Hofmann, 2012b). For example, the distribution of all GRs (Greenwood et al.,
131 2003), ERs, and ARs (Munchrath & Hofmann, 2010) has been mapped in the adult *A. burtoni*
132 brain. Also, subordinate males have lower levels of whole brain CRF and GR2 (Chen & Fernald,
133 2008), higher cortisol, and lower testosterone than dominants (Fox et al., 1997; O'Connell &
134 Hofmann, 2012b), and the transcriptomic response to an ER antagonist is status-specific
135 (O'Connell & Hofmann, 2012b). The developmental origins of individual variation in behavior
136 and neuroendocrine function remain unknown.
137 In the present study, we conducted two experiments to test the hypothesis that the early-
138 life social environment generates variation in juvenile behavior through neuroendocrine gene

expression. We manipulated the early-life social environment, and consequently social experience, by rearing juveniles in either social groups or pairs. In the group condition, social experience implies interactions with more social partners, who also vary in size, sex, experience, and patterns of behavior. Interactions in groups can also involve more than two individuals, and it is possible to observe, and learn from, interactions of group members as a bystander. Although it has not been tested in juveniles, adults are capable of gaining important social information as a bystander (Desjardins, Hofmann, & Fernald, 2012; Desjardins, Klausner, & Fernald, 2010; Grosenick, Clement, & Fernald, 2007). In experimental pairs, juveniles occupy only one social role and form a relationship with just one other individual. Similar manipulations of early-life social complexity have been important for behavioral and neural development in other species (reviewed in Taborsky, 2016). We predicted that rearing environment would affect a suite of social behaviors across contexts, including social investigation, dominant, and subordinate behavior. In the brain, we predicted effects on whole brain gene expression of neuroendocrine systems that mediate early-life experiences. Related to the HPA/I axis, we measured glucocorticoid receptor 1a (GR1a), glucocorticoid receptor 1b (GR1b), glucocorticoid receptor 2 (GR2) (nomenclature from Maruska & Fernald, 2010), mineralocorticoid receptor (MR), and CRF. For sex steroid hormone signaling, we quantified androgen receptor (ARα) and estrogen receptor (ERα). By investigating these early-life effects in juveniles, we can identify important intermediary steps that inform how developmental plasticity may shape the adult phenotype.

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 in 100 L aquaria. Dominant males court gravid females that then lay eggs inside of 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. In nature (and under some laboratory conditions), following their initial release from the mother's mouth, juveniles remain close by for 2-4 weeks, seeking shelter in her mouth less often as they age. In the first two weeks, juveniles primarily school together, with overt social interactions beginning at 2-3 weeks old (Fernald & Hirata, 1979; Renn, Carleton, Magee, Nguyen, & Tanner, 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 social conditions (Fernald & Hirata, 1979; Fraley & Fernald, 1982).

In this study, juveniles were removed from the mother's mouth (Renn et al., 2009) 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 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.

Experimental rearing conditions (Experiments 1 & 2)

In Experiment 1, juveniles for the behavioral assays were reared in social groups of 16 fish (n=12 groups) or in pairs (n=9 pairs). Juveniles spent 58-73 days (average 65.76 ± 0.81; ~8-10 weeks) in these conditions until behavioral testing. In Experiment 2, a separate cohort of

juveniles, reared in groups of 16 fish (n=11 groups), pairs (n=10 pairs), or in isolation (n=8), was used for neural gene expression analyses. Isolation was included not as a social control, but because we expected it to impact gene expression in this highly social species. We opted to quantify behavior and gene expression in separate experiments in order to capture different time points. As the first study of this kind in this species, the timing of effects was unknown. For Experiment 1 (behavior), we allowed juveniles to develop in the experimental environments for an extended period of time, without yet reaching reproductive maturity. For experiment 2 (brain), we aimed to capture early changes (after 1 week, 5 weeks) in gene expression that might set individuals along different developmental trajectories. We cannot distinguish between the effects of chronological age from the treatment duration (e.g., 1 vs. 5 weeks) in this study.

For both Experiments, juveniles from multiple clutches of the same age and developmental stage (day 12-14 fry) were divided among treatment groups. Group-reared fish were housed in 35 L aquaria with three shards of terracotta pots for a shelter and/or territory. Pairs and isolated fish were housed in small aquaria (9" x 6" x 6") with one terracotta shard. The volume of water per fish was similar for the group (2.6 L) and paired (2.7 L) treatments. Juveniles were fed daily with Hikari plankton (Pentair Aquatic Eco-Systems, Cary, NC). The food was mixed in water, and a transfer pipette was used to deliver a set volume to each tank. Groups received eight times more food than pairs. Pairs and isolated fish received the same amount. All juveniles were maintained on a 12:12 light/dark cycle.

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

208 fish from the aquarium and selected the largest fish. Since size is a strong predicator of social
209 dominance (Alcazar, Hilliard, Becker, Bernaba, & Fernald, 2014), this individual was very likely
210 to have dominance experience, similar to the larger fish in the pair. A smaller fish was then
211 chosen such that the ratio of large-to-small fish standard length (SL, mm) was approximately
212 equal in the group and a pair from the same cohort of juveniles (same age). Standard length was
213 recorded for all focal fish. Behavior was observed in four sequential assays (Fig. 1). The tests
214 were always presented in the same order and conducted in small aquaria (9" x 6" x 6") without a
215 cover. For analysis, the aquaria were divided into 4 zones, delineated with permanent marker. In
216 the middle of each short side, a circle was drawn (28 mm diameter) to indicate the placement of
217 the scintillation vial (see below: social cue investigation assay). An arc 1 inch from the edge of
218 the circle was drawn to form a semicircle. One semicircle was designated the "territory" zone
219 and had a terracotta shard for a shelter and/or territory. The other semicircle was designated the
220 "investigate" zone. The "close" zone was between the territory zone and halfway along the long
221 side of the tank. The "far" zone was between the halfway mark and the investigate zone (see Fig
222 1). Video cameras recorded behavior from above so that all areas of the tank, except under the
223 terracotta pot, were visible. Behavior was analyzed using Solomon Coder (solomoncoder.com).
224 Open field test: The focal fish was transferred to the test aquarium with a hand net and
225 remained in the tank alone for 30 min. Movement around the tank was analyzed from minutes 20
226 to 30. We recorded the number of times a fish crossed into each zone (frequency) and the time
227 (s) spent in each zone. Social cue investigation: Novel juveniles were collected from a
228 community tank and placed into scintillation vials (20 mL). The top of the vial was covered with
229 parafilm with holes to allow water through. A vial containing one cue fish was placed into each
230 test aquarium (n=16 group-reared, n=13 pair-reared). Cue fish were 0-6.4 mm SL (average 3.37

± 0.27) smaller than their focal fish. An empty vial was used as a control (n=8 group-reared, n=5 pair-reared). The social cues were in the aquarium for 30 min. Movement around the tank (frequency and time in each zone) was analyzed from minutes 2 to 12.

Dominance behavior: The scintillation vials were removed from the aquaria and a novel smaller fish (by 1-6.4 mm SL, average 3.37 ± 0.25) was immediately added to each aquarium, freely swimming with the focal fish. The pair remained together for 30 minutes, and behavior was analyzed from minutes 2 to 12. Subordinate behavior: The small cue fish was removed from the aquaria and a novel, larger (by 2.4-12 mm SL, average 5.74 ± 0.34) fish was immediately added to each aquarium, freely swimming with the focal fish. The pair remained together for 30 minutes, and behavior was analyzed from minutes 2 to 12. In the dominance and subordinate behavior assays, we analyzed agonistic interactions between the pair. An approach was defined as one fish swimming directly towards any part of the other fish's body, within 3 body lengths. If the approached fish responded by moving away, in any direction, the behavior was recorded as a displacement for the initiator and a submission for the responder. From these measures, we calculated agonistic efficiency, or the proportion of approaches that led to a displacement (Solomon-Lane, Pradhan, Willis, & Grober, 2014) for focal and cue fish. The difference in agonistic efficiency between the focal and cue fish was used as a measure of agonistic asymmetry, which characterizes status relationships (Drews, 1993). We also recorded the frequency of entering and the time spent in the territory, for the focal fish, cue fish, and both together.

Experiment 2: Whole brain gene expression

Gene expression was analyzed from juveniles reared in groups (n=8), pairs (n=8), or

isolated (n=8) for 1 week, and from juveniles reared in groups (n=14) or pairs (n=10) for 5 weeks. Juveniles were rapidly decapitated, and then the brains were dissected out, flash frozen on dry ice, and stored at -80° C until processing. Gene expression was quantified using qPCR and previously validated primers (Chen & Fernald, 2008; Greenwood et al., 2003; O'Connell & Hofmann, 2012b) 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. We present the analyses for 18S.

Statistical analyses

All statistics were conducted using R Studio (version 1.0.143), and results were considered significant at the $p<0.05$ level. 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 treatment groups for fish size, time and frequency in each tank zone, and rates of agonistic behavior were conducted using t-tests. Mann-Whitney U tests were used for data that did not meet the assumptions of parametric statistics. Regression analysis was used to identify significant associations between the frequency of entering tank zones and the time spent in that zone, between SL and frequency and time in a zone, and between SL and agonistic behavior. Two-way ANOVAs were used to identify

277 significant effects of rearing environment, presence of the social cue, or an interaction, on the
278 frequency and time spent in each zone of the tank. We used Principal Components Analysis
279 (PCA) to identify how behaviors clustered across the four assays and for each assay individually.
280 T-tests were used to compare principal component scores between group- and pair reared
281 juveniles. Correlation analysis was used to identify significant associations among principal
282 components (PCs).
283 The effects of rearing environment (group, pair, isolated) and treatment duration (1 week,
284 5 weeks) on relative gene expression was analyzed separately using Kruskal-Wallis tests or one-
285 way ANOVA and Wilcoxon tests, respectively. A familywise p-value correction was used to
286 correct for the multiple comparisons. Even though these data did not meet the assumptions of
287 parametric statistics, we conducted a two-way ANOVA to get a tentative estimate for interaction
288 effects. This analysis, and visual inspection, do not suggest any interactions. We also used PCA
289 to identify how expression of the candidate genes clusters. T-tests, or Mann-Whitney U tests if
290 appropriate, were used to compare group- and pair-reared juveniles and expression following 1
291 vs. 5 weeks in treatment groups. Dunn's test was used for *post hoc* analysis of significant results.
292 Partial correlation networks were calculated using the "ppcor" package in R and visualized using
293 "qgraph." The nodes of the networks represent the gene. The edges are the partial correlation
294 coefficient, with thicker edges indicating stronger correlations. Only significant correlations are
295 shown. Mantel tests were used to test for pairwise differences between the gene expression
296 networks. A non-significant p-value (> 0.05) indicates that the partial correlation matrices are not
297 related.
298
299

Results

Experiment 1

Juvenile size

Group-reared juveniles (16.85 ± 0.32) were significantly larger than pair-reared juveniles (13.76 ± 0.40) (t-test: p=7.2e-07) after 8-10 weeks in their respective treatment condition. This size difference subsequently influenced the size of the fish selected to be the social cue for the social investigation assay, the small cue fish for the dominance behavior assay (same group of fish as the social cue), and the large cue fish for the subordinate behavior assay. The size difference (SL) between the focal fish and the social cue (t-test: p=0.0016), as well as the focal fish and the small cue fish (Mann-Whitney U Test: p=0.001), was significantly greater for group-reared juveniles. The size difference (SL) between the focal fish and the large cue fish was significantly greater for pair-reared juveniles (t-test: p=0.0025).

Open field test and social cue investigation

We first asked whether group- and pair-reared juveniles differed in the open field test. Importantly, juveniles of all treatment groups moved readily around the novel environment with minimal acclimation. We present the data for the frequency of visits to and time spent in each zone. There were no significant effects for the time spent in each zone ($p>0.05$). Group- and pair-reared juveniles spent similar amounts of time in each zone of the tank (Mann-Whitney U Tests: territory: p=0.19; close: p=0.53; far: p=0.34; investigate: p=0.69); however, the frequency of entering zones differed. Group-reared juveniles entered the territory (Mann-Whitney U Test: p=0.034), far (Mann-Whitney U Test: p=0049), and investigate zones (Mann-Whitney U Test:

p=0.049) significantly more frequently than pair-reared juveniles. There was no significant difference for the close zone (Mann-Whitney U Test: p=0.064). Linear regression analysis indicated a significant relationship between SL and the frequency of entering each zone (investigate: p=0.0036, r^2=0.17; far: p=0.0059, r^2=0.15; close: 0.017, r^2=0.11; territory: p=0.0028, r^2=0.18).

Next, we used a social cue investigation task to examine whether and how locomotor activity is affected by rearing environment and/or the presence of the social cue. 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=0.047), 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.

Similar to the open field test, SL was strongly correlated with the frequency of entering the close (p=0.0061, r^2=0.15) and territory zones (p= 0.0076, r^2=0.14), but there was no effect for entering the far zone (p=0.064). Unlike the open field test, there was no association for the investigate zone, where there was either a social cue or control vial (p=0.17). There were no associations between SL and time spent in any zone (investigate: p= 0.80, far: p=0.39; close: p=0.22, territory: p=0.89).

346 Dominant and subordinate behavior

347 To investigate the effect of rearing environment on social behavior, we dominant and
348 subordinate displays in these juveniles. Interestingly, rearing environment did not affect rates of
349 focal fish behavior. As the dominant fish, there were no differences in approaching (Mann-
350 Whitney U Test: p=0.20, 2 outliers removed) and displacing (Mann-Whitney U Test: p=0.12, 2
351 outliers removed) the small cue fish. As the subordinate, there were no differences in
352 approaching (Mann-Whitney U Test: p=0.85), displacing (Mann-Whitney U Test: p=0.62, 2
353 outliers removed), or submitting to (Mann-Whitney U Test: p=0.56) the large cue fish. In the
354 dominance assay, rearing environment did not affect agonistic efficiency for the focal fish (t-test:
355 p=0.41), small cue fish (Mann-Whitney U Test: p= 0.97), or the difference between the pair
356 (Mann-Whitney U Test: p=0.32). In the subordinate assay, although there was no difference in
357 agonistic efficiency for the focal fish (Mann-Whitney U Test: p=0.28) or the large cue fish
358 (Mann-Whitney U Test: p=0.061), the difference in agonistic efficiency was significantly higher
359 for pair-reared juveniles (t-test: p=0.022).

360 Focal fish SL was positively associated with approaching (p=0.035, r^2=0.089) and
361 displacing p=0.024, r^2=0.10) the subordinate fish; however, there was no association between
362 focal SL and approaching (p=0.57), displacing (p=0.37), or submitting (p=0.72) to the dominant
363 fish. In the dominance assay, focal fish SL was positively associated with the frequency of
364 entering the territory (p=0.022, r^2=0.11, 2 outliers removed), the frequency of the small cue fish
365 entering the territory (p= 0.011, r^2=0.14, 2 outliers removed), and the number of times both fish
366 were in the territory together (p=0.02, r^2=0.11, 2 outliers removed). There were no associations
367 between focal fish SL with the time the focal fish spent in the territory (p=0.055), the time the
368 small cue fish spent in the territory (p=0.56), or the time both spent together (p=0.97). During the

subordinate test, focal fish SL was not associated with the frequency or time in the territory, alone or with the large cue fish ($p>0.05$).

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 (t-test: $p=0.029$, 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. Measures of subordinate behavior, however, do not contribute. There were no significant treatment differences in higher order PCs except for PC6, which accounted for 5.0% of the variation in the data and was significantly higher in group-reared juveniles (t-test: $p= 4.082e-05$, Fig 2C). Note that focal fish SL loads most strongly on PC6 (data not shown).

To better understand how rearing environment affected behavior within the assays contributing to the treatment difference, we conducted PCAs for the open field test, social cue investigation, and dominance behavior assays separately. We expanded these analyses to include all of the measured variables, for the focal and cue fish. The open field test analysis included focal fish SL and the frequency of entering and time in each zone of the tank. The social cue

investigation included the same measures, as well as the SL of the cue fish. Finally, the dominance behavior analysis included SL of the focal fish and small cue fish, approaches and displacements of both fish, and the frequency of entering and time spent in the territory by either or both fish. For each analysis, PC1 differed significantly between group- and pair-reared juveniles: open field (Fig 3A, 43.4% variation, t-test: p=0.04), social cue investigation (Fig 3B, 37.2% variation, Wilcoxon: p = 0.0032), and dominance behavior (Fig 3C, 29.8% variation, Wilcoxon: p=0.025). The PC1s were also significantly and linearly correlated with each other (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; social cue x dominance: r^2=0.46, p= 4.97e-07). We found no significant differences for any higher order PCs in the three analyses.

For the open field test, all variables loaded on PC1 except time in the territory and investigate zones. For the social cue investigation, all variables loaded on PC1 except time in the territory, investigate, and close zones. Finally, for dominance behavior, the strongest loadings for PC1 include approaches and displacements by the focal and small cue fish, the frequency of the focal fish entering the territory, the time spent in the territory by the small cue fish, and the SL of both the focal and small cue fish.

Experiment 2

Neural gene expression patterns

Neuroendocrine signaling is a primary mechanism by which early-life experiences are translated into biological changes. To identify potential mediators of the behavioral effects we identified, we measured, in the brains of a separate cohort of juveniles, mRNA levels of genes involved in the stress axis and in sex steroid signaling. We compared relative expression across

415 rearing environments (isolation, pairs, groups) and time in rearing environment (1 week, 5
416 weeks). Overall, there was little significant variation in neural gene expression with regards to
417 either rearing environment or treatment duration ($p>0.05$), with the exception of an effect of
418 rearing environment on GR1a expression (Kruskal-Wallis H test: $p=0.0012$). *Post hoc* analysis
419 showed that expression was significantly higher in groups-reared juveniles than pair-reared
420 ($p=0.0015$) or isolated ($p=0.0015$) juveniles, which did not differ from each other ($p=0.39$).
421 Genes function within regulatory networks, rather than in isolation, and they can affect
422 each other's expression. Similarly, a common upstream regulator may control multiple
423 functional networks of genes. Because of their known effects on physiology and behavior, these
424 candidate genes are likely function in pathways that interact with each other. To quantify how
425 rearing environment affects gene co-expression, we calculated partial correlation networks (Fig
426 4). Partial correlations show the associations between gene pairs, independent of other
427 correlations in the network. Comparing the group and pair networks (Mantel test: $p=0.31$), the
428 group and isolate networks (Mantel test: $p=0.61$), and the pair and isolate networks (Mantel test:
429 $p=0.12$) revealed that there was no evidence that any of these networks were similar to any other.
430 To gain a more holistic understanding of how rearing environment and/or the time spent
431 in treatment groups affect neural gene expression variation, we used PCA. PC1 accounts for
432 69.1% of the variation in the data. While there were no differences in PC1 based on rearing
433 environment (Mann-Whitney U Test: $p=0.13$), there was a trend for differences based on time in
434 treatment groups (1 vs. 5 weeks) (Mann-Whitney U Test: $p=0.053$; Fig 5A). There were no
435 differences due to rearing environment for any higher order PCs except for PC4, which
436 accounted for 5.7% of the variation in the data and differed significantly according to rearing
437 environment (t-test: $p=0.011$; Fig 5B). Fig 5C shows how the different candidate genes load onto

PC1 vs. PC4.

Discussion

In the present study, we set out to test the hypothesis that early-life social experience shapes juvenile *A. burtoni* behavior and neural gene expression. After rearing juveniles in social groups of 16 fish or in pairs, we quantified behavior in multiple contexts and, in a separate cohort, neuroendocrine gene expression in whole brain. Our data clearly demonstrate that juvenile behavior and neuroendocrine function are both sensitive to early-life social effects. These effects were evident even with a relatively subtle manipulation in which fish reared in both environments could interact freely. Using a novel battery of four behavioral assays to gain a comprehensive understanding of behavior across contexts (Fig 1), we found that behavior across the open field test, social cue investigation, and dominance behavior assays was correlated and contributed to a significant difference between group- and pair-reared juveniles (Fig 2, Fig 3). To our knowledge, this is the first behavioral syndrome to be identified in *A. burtoni* at any developmental stage (Fig 3). Pair-reared juveniles may also behave more subordinately in the subordinate behavior assay, a critically important behavior for fish that are nearly mature but still subordinate to all adults.

In the brain, we found that rearing environment caused a dramatic change in the co-expression patterns of key neuroendocrine genes. Expression was tightly correlated for pair-reared juveniles, but not for juveniles reared in groups or isolation (Fig 5). The expression of glucocorticoid receptors (GR1a, GR1b, GR2) and AR, in particular, drive the significant differences between treatment groups (Fig 6), supporting the involvement of highly-conserved stress axis mechanisms (Crespi & Denver, 2005; Jonsson & Jonsson, 2014). Together, these

experiments provide an essential step towards understanding how developmental plasticity shapes the adult phenotype. Interestingly, despite the prominence of *A. burtoni* in social neuroscience (Fernald & Maruska, 2012; Hofmann, 2003), few studies have incorporated juveniles (e.g., Alvarado, Lenkov, Williams, & Fernald, 2015; Fernald & Hirata, 1979; Fraley & Fernald, 1982). Our research contributes to the growing literature demonstrating the importance of the early-life social environment, beyond parental interactions (Champagne & Curley, 2005; Taborsky, 2016).

Juvenile behavior forms a syndrome affected by rearing environment

We administered a battery of four assays to quantify a range of behaviors in distinct contexts (Fig 1), including an open field test that is 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 interactions within dominant and subordinate status contexts, which regularly occur in social communities (Hofmann, 2003). For the first time in *A. burtoni*, we discovered that behavior across the open field, social cue investigation, and dominance behavior assays forms a behavioral syndrome (Bell, 2007), indicated by the linear associations among the PC1s of the contributing assays (Fig 3). Syndromes are a population-level metric defined as the correlation between rank-order differences between individuals, across contexts and/or over time (Bell, 2007). We found that 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. The presence of a syndrome indicates consistency in patterns of individual behavior across contexts and/or over time (Bell, 2007; Sih, Bell, & Johnson, 2004; Sih, Bell, & Ziemba, 2004). Our data suggests

that how individuals move around in space is relevant to the social role they play. Interestingly, behavior from the subordinate assay does not to contribute to the treatment effect or syndrome, likely because subordinate focal individuals respond primarily to the dominant fish's behavior.

Behaviors may be correlated as a syndrome due to shared mechanisms, correlational selection, or early-life experiences that set individuals along developmentally plasticity trajectories (Bell, 2007; Ketterson & Nolan, Jr., 1999; Stamps, 2003), as may be the case for *A. burtoni* juveniles. We found that behavior for all juveniles was described by the same syndrome, and rearing environment dictated where an individual fell along the continuum of the syndrome (Fig 3D). The significant difference was caused by the restriction of pair-reared juveniles towards one end. In contrast, group-reared juveniles, which were reared in the more naturalistic context, are represented along the full range of variation. That there are group-reared juveniles that behaviorally resemble the pair-reared suggests there may be social environments within the group (Saltz, Geiger, Anderson, Johnson, & Marren, 2016) that share key elements with the paired experience. To identify the causal behavioral and/or sensory cues, it will be necessary to observe individuals within the rearing environment (Taborsky, 2016). Based on pilot observations that juveniles in groups and pairs interact at similar rates, on average, and engage in agonistic and affiliative behavior in similar proportion (Solomon-Lane, et al., unpublished data), we hypothesize that the complexity of interactions and/or the abundance of social sensory cues in groups cause the treatment differences (Taborsky, 2016, e.g., Arnold & Taborsky, 2010).

Activity and social interaction are common components of syndromes in other species, along with axes of boldness-shyness and/or proactive-reactive (Bell, 2007; Conrad et al., 2011; Groothuis & Carere, 2005; Koolhaas et al., 1999; Sih, Bell, & Ziemba, 2004; Verbeek, Drent, & Wiepkema, 1994). For example, in brown trout, larger juveniles were more active and aggressive

(Näslund & Johnsson, 2016), which closely parallels our findings. Similar activity-aggression syndromes are also found in a number of other fish species (reviewed in Conrad et al., 2011). For *A. burtoni* juveniles, activity and interaction may be causally related. First, active individuals may encounter conspecifics more frequently and initiate more interactions, as a result. Second, social interactions appear to serve a prosocial role for juveniles in that they increase the likelihood of future proximity and interaction, which in turn may result in increased shoaling and reduced predation risk. In the dominance behavior assay, the focal fish's approaches and displacements load in the same direction on PC1 as the subordinate cue's approaches and displacements. Correlation analysis (data not shown) confirms that the more one member of the pair initiates social interaction, the more the other member also initiates, potentially leading to more activity. Interestingly, this positive feedback loop is not present for adult aggression, suggesting that although juvenile social behavior appears similar to the adult form (Fernald & Hirata, 1979; Fraley & Fernald, 1982), there are life history-dependent differences.

Size is central to understanding activity, social interactions, and early-life effects. In the present study, group-reared juveniles were larger than pair-reared juveniles, 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 for juvenile *A. burtoni* is consistent with the adult research showing that growth is socially-regulated (Hofmann, Benson, & Fernald, 1999) and even small size differences affect social interactions (e.g., Alcazar et al., 2014; Weitekamp & Hofmann, 2017). However, size does not explain the total effect of the early-life social environment. First, the PCA of behavior across the four assays (Fig 2) shows that focal fish SL contributes to the treatment difference for PC1; however, many other variables

530 also load on PC1 in the same direction (Fig 2B). Second, focal fish SL is the strongest
531 contributing variable for PC6, which differs significantly between group- and pair-reared
532 juveniles and accounts for 5% of the variation in the data. While this proportion is important, that
533 PC1 explains 43.3% of the variation (Fig 2A) suggests treatment has a much larger overall
534 effect. Finally, the group-reared juveniles that overlap on the behavioral syndrome with pair-
535 reared juveniles (e.g., high PCA eigenvalues, Fig 3) were not the smallest individuals. This
536 suggests there are multiple early-life social environments that can slow growth—including in
537 pairs, in this study, and environments within the social group—but size, on its own, does not
538 determine behavioral phenotype. It is common in fish for social factors to influence physical
539 growth and/or reproductive maturation, which both may be decoupled from chronological age
540 (Fraley & Fernald, 1982; Hofmann et al., 1999; Jonsson & Jonsson, 2014).
541 Early-life social experiences can shift behavior in ways that ultimately affect fitness
542 (Smith & Blumstein, 2008), in part because social behavior has consequences for reproductive
543 success (e.g., Silk, 2007; Solomon-Lane, Pradhan, Willis, & Grober, 2015). Specifically,
544 experimentally increasing the frequency, diversity, or complexity of early-life social experiences
545 enhanced social skills (or similar) in a majority (64%) of studies (Taborsky, 2016). For example,
546 juvenile *N. pulcher* cichlids reared with brood helpers demonstrated more context-appropriate
547 behavior in assays for status establishment, integrating into novel social groups, and competing
548 over a resource (Arnold & Taborsky, 2010; Fischer, Bessert-Nettelbeck, Kotrschal, & Taborsky,
549 2015; Taborsky et al., 2012, 2013). We have no evidence of any advantage of group-reared over
550 pair-reared juveniles, but juveniles may fill the subordinate role differently (there were no
551 differences for dominance behavior). While nearly all focal fish successfully established
552 themselves as subordinate (88%), and there were no treatment differences in approaches or

553 displacements, there was a trend for pair-reared fish to submit more readily (measured as large
554 fish agonistic efficiency). There was also a significantly larger asymmetry in agonistic efficiency
555 for pair-reared juveniles. Status relationships are defined by asymmetrical agonistic displays
556 (Drews, 1993); therefore, pair-reared juveniles may behave more subordinately than group-
557 reared juveniles. Without behavior data for these individuals as adults or a measure of fitness,
558 however, it is not yet possible to determine whether one phenotype is more successful than
559 another (Pradhan, Solomon-Lane, & Grober, 2015).
560
561 Rearing environment affects patterns of neuroendocrine gene expression
562 Identifying the neuroendocrine mechanisms that mediate the effects of early-life
563 experience on behavior is important both for understanding the causes underlying the effects and
564 their consequences. We measured gene expression in whole brains from juveniles reared in
565 groups (1 week or 5 weeks), pairs (1 week or 5 weeks), or isolation (1 week). The candidate
566 stress and sex hormone genes were chosen because these systems translate environmental
567 conditions and experiences into biological responses (e.g., Crespi & Denver, 2005; Wingfield et
568 al., 1990), are sensitive to early-life effects (e.g., Champagne & Curley, 2005; Shepard et al.,
569 2009), and are involved in the regulation of social behavior (e.g., Adkins-Regan, 2009; Solomon-
570 Lane, Crespi, & Grober, 2013). Specifically, we focused on steroid hormone nuclear receptor
571 genes. These receptors are located in the cytoplasm, and when bound by their ligand, they
572 translocate to the nucleus, bind to hormone response elements, and regulate the transcription of
573 target genes with a diversity of physiological and behavioral roles (Rochette-Egly, 2005).
574 Overall, we found gene expression to be highly variable, especially among group-reared
575 juveniles. With the exception of GR1a, we found no differences when comparing the expression

of single genes across rearing environments or treatment durations (Fig 4). In adult *A. burtoni*, as well as other fish species (Li, Earley, Huang, & Hsu, 2014), gene expression is affected by social experience and status. For example, dominant males have higher expression of ARα, MR, GR1a, and GR2 in the preoptic area of the hypothalamus, whereas subordinate males have higher levels of GR1b (Korzan, Fernald, & Grone, 2014). Thus, understanding variation in the expression of specific genes likely requires social behavioral and contextual information more specific than rearing environment.

Neuroendocrine systems are dynamic and interact on multiple biological levels, including gene expression (e.g., Huffman et al., 2012; Korzan, Fernald, & Grone, 2014; O'Connell & Hofmann, 2012). Genes function within regulatory networks, and these candidates are likely to interact based on their co-localization in *A. burtoni* (e.g., Korzan et al., 2014; Maruska & Fernald, 2010), co-localization and correlation in other species (e.g., Meyer & Korz, 2013), and overlapping physiological effects (e.g., Crespi & Denver, 2005; Wingfield et al., 1990). Partial correlation analysis revealed striking differences in patterns of co-expression. Gene 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, AR shares five significant connections. The two sex steroid hormone genes (AR, ER) are also integrated with the stress axis genes, which form distinct smaller networks (CRF-GR1a-GR1b; GR2-MR). In contrast, group-reared juveniles have only one significant partial correlation between ER and GR1b, a connection that is not present in the pair-reared network (Fig 5B). There are no significant partial correlations for isolated juveniles. These networks suggest that neuroendocrine correlates of social behavior and the stress response function very differently in juveniles reared in different social environments. It remains to be tested whether and how these differences

599 manifest for behavior. Specific genes can be manipulated centrally via pharmacology to establish
600 causal relationships with gene co-expression and behavior (Solomon-Lane, Butler & Hofmann,
601 unpublished data).

602 From the partial correlation networks alone, it is challenging to identify whether specific
603 genes drive the differences in co-expression between fish from different rearing environments.
604 Principal component analysis revealed that PC4, which explains 5.7% of variation in the data,
605 was significantly different between group- and pair-reared juveniles (Fig 6B). HPA/I axis
606 signaling contributes strongly to the effect of the early-life social environment. All of the GRs, in
607 addition to AR, load on PC4 and contribute to the treatment effect (Fig 6C). Many teleosts,
608 including *A. burtoni*, have three glucocorticoid receptors: MR, GR1, and GR2. Receptor 1 has
609 splice variants 1a and 1b, which differ by a nine amino acid insertion in the DNA-binding
610 domain of 1b that reduces transcriptional response (Greenwood et al., 2003; Korzan et al., 2014).
611 Consistent with the distinct roles for the different receptors and splice variants (Greenwood et al.,
612 2003), GR1a and GR2 load in the opposite direction from GR1b and AR (Fig 6C), suggesting
613 their expression may be independently regulated (e.g., Fig 5). Treatment duration may also be a
614 major factor explaining variation in juvenile gene expression. There was a trend for PC1 to differ
615 between the 1 and 5 week treatments (Fig 6A), which suggests that gene expression changes in
616 important ways over development. Because all juveniles were put into treatment at the same age
617 and developmental stage, we cannot yet distinguish between treatment duration and age, or
618 identify critical periods for early-life effects.

619

620 <u>Integrating early-life effects on behavior and brain</u>

621 Our results show that HPA/I axis plays a central, and likely highly-conserved (Crespi &

622 Denver, 2005), role in responding to the early-life social environment in juvenile *A. burtoni*.
623 Changes in HPA/I axis function generally manifest as altered baseline levels of glucocorticoids,
624 a higher or lower glucocorticoid 'peak' in response to an acute stressor, and/or altered efficiency
625 of the negative feedback loop that returns the system to baseline. Negative feedback, in
626 particular, is regulated by neural GR expression and can be affected by early-life experience
627 (Champagne & Curley, 2005; Francis et al., 1999). This suggests either group- or pair-reared
628 juveniles, or both, may have altered negative feedback mechanisms via differential GR
629 expression, especially in brain regions homologous to the hippocampus and amygdala, which are
630 important in spatial cognition and emotional processing, respectively. Brain regions of overlap
631 for GRs (Greenwood et al., 2003; Korzan et al., 2014) and AR (Munchrath & Hofmann, 2010),
632 in particular, will also reveal areas that sensitive to early-life effects and provide possible links to
633 behavior based on their function. The social decision-making network (O'Connell & Hofmann,
634 2012a), which regulates social behavior, is well-established in adults, but has yet to be
635 investigated in juvenile *A. burtoni*. One hypothesis is that the effects on behavior and HPA/I axis
636 function will correlate in a behavioral syndrome called a coping style. Proactive copers tend to
637 be highly active, aggressive, actively avoid, and less responsive to stress (i.e., lower baseline
638 glucocorticoid levels, faster negative feedback). Reactive copers have higher rates of immobility
639 and lower rates of aggression (Koolhaas et al., 1999).
640
641 Future directions
642 Our work demonstrates that early-life social environments shape behavioral phenotype
643 and neuroendocrine gene expression in powerful ways for *A. burtoni* juveniles. It will be critical
644 to follow individual juveniles into adulthood in order to track changes in behavior over time and

measure consequences for social status and reproduction. Furthermore, at different developmental time points, changes in brain and behavior should be quantified in the same individuals to investigate the causal role of the neuroendocrine mechanisms. Acute and long-term pharmacological manipulations can also be used to establish causation. Finally, it will be critical to identify the specific cues within the social environments that cause differences across biological levels between group- and pair-reared juveniles. Across species, remarkably little is known about the behavioral mechanisms that shape the ontogeny of behavior (Taborsky, 2016). Of particular interest is the natural variation in social environments and experiences within the same social community that can generate individual phenotypic variation and impact behavior, growth, maturation, and reproductive success (Saltz et al., 2016). Together, this line of 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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Figures and legends

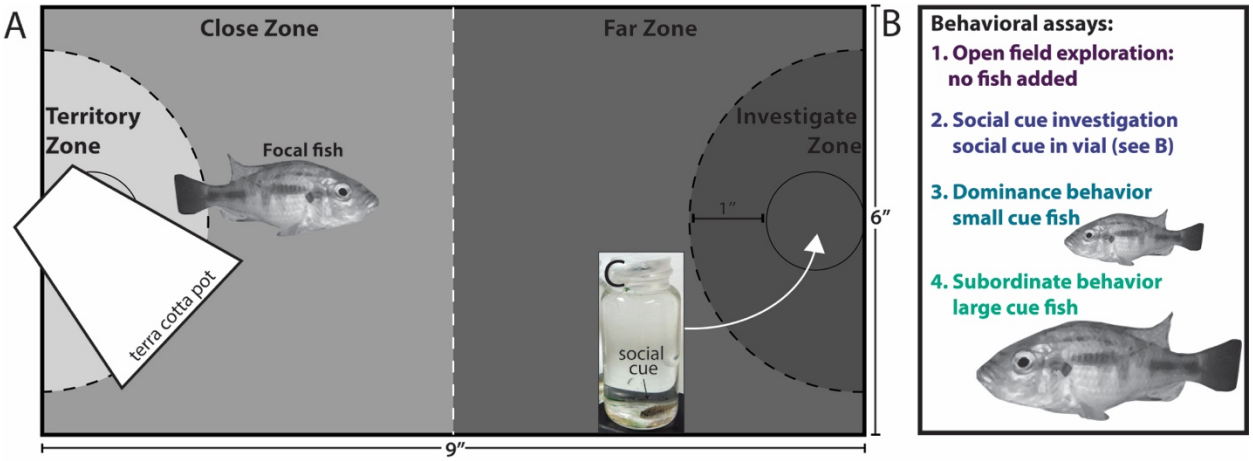
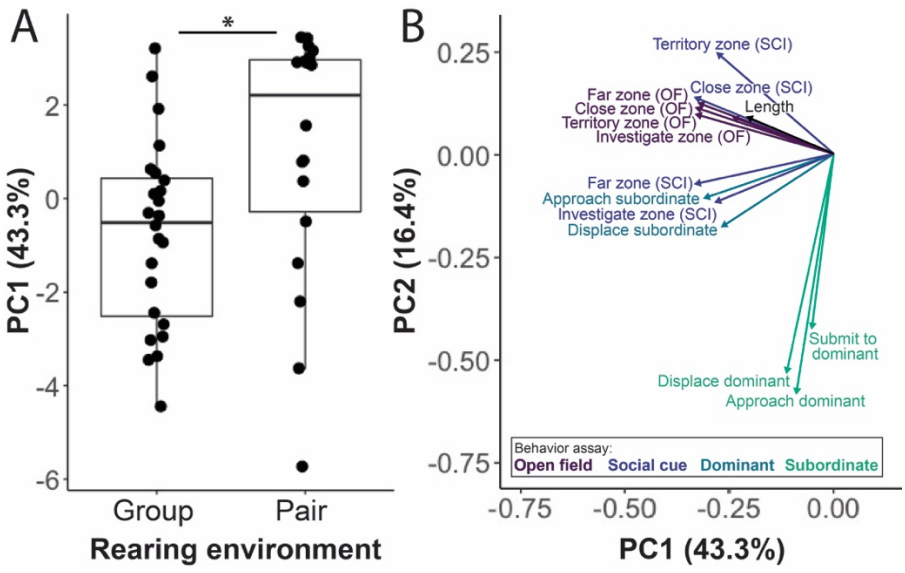


Figure 1: Experimental design for behavior assays. Juvenile behavior was observed in a novel experimental tank in four sequential assays administered in the same order, each lasting for 30 min. A terracotta shard served as a shelter and/or territory. The black lines (dotted, solid) were drawn on the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



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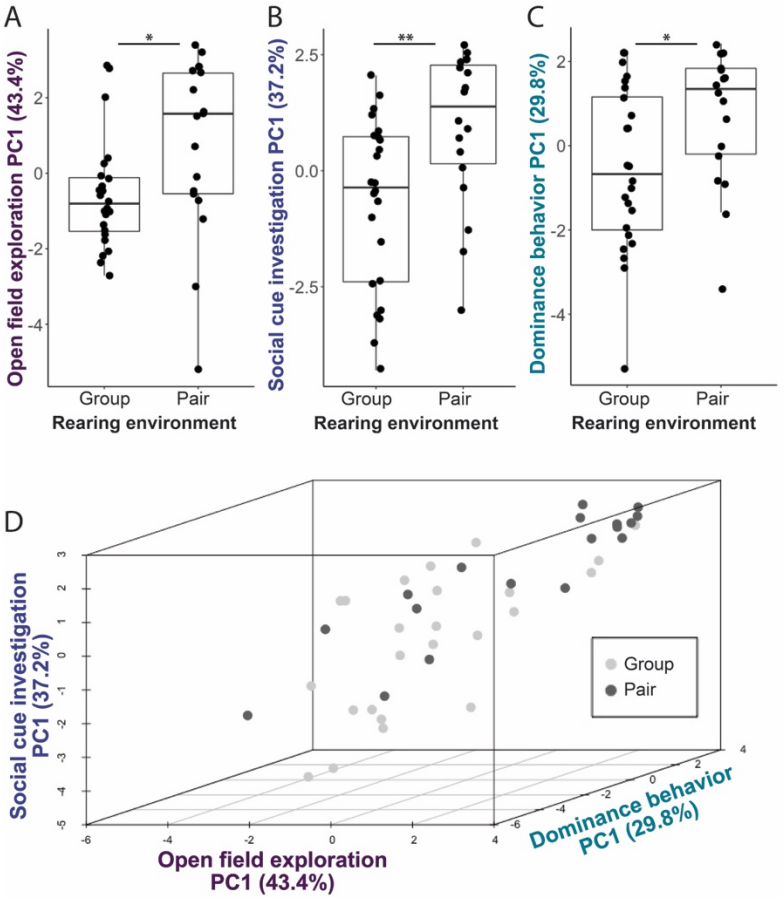
960 **Figure 2:** Principal component analysis (PCA) of focal fish behavior from all four behavioral
961 assays. PC1 accounts for 43.4% of the variation in the data and was significant higher in pair-
962 reared juveniles (p=0.029) (A). Vector plot showing the PCA variables that load on PC1 and
963 contribute to the treatment effect (B). Pair (n=18 individuals). Group (n=24 individuals). Social
964 cue investigation (SCI). Open field exploration (OF). *p<0.05.



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967 **Figure 3:** Principal component (PC) 1 from separate analyses performed for the open field (A),
968 social cue investigation (B), and dominance behavior (C) assays. Focal and non-focal fish
969 variables (behavior, size) were included. The correlation among PC1s for the open field, social
970 cue investigation, and dominance behavior is shown in a three-dimensional plot (D). Percentages
971 refer to the amount of variation explained by that component. Pair (n=18 individuals). Group
972 (n=24 individuals).

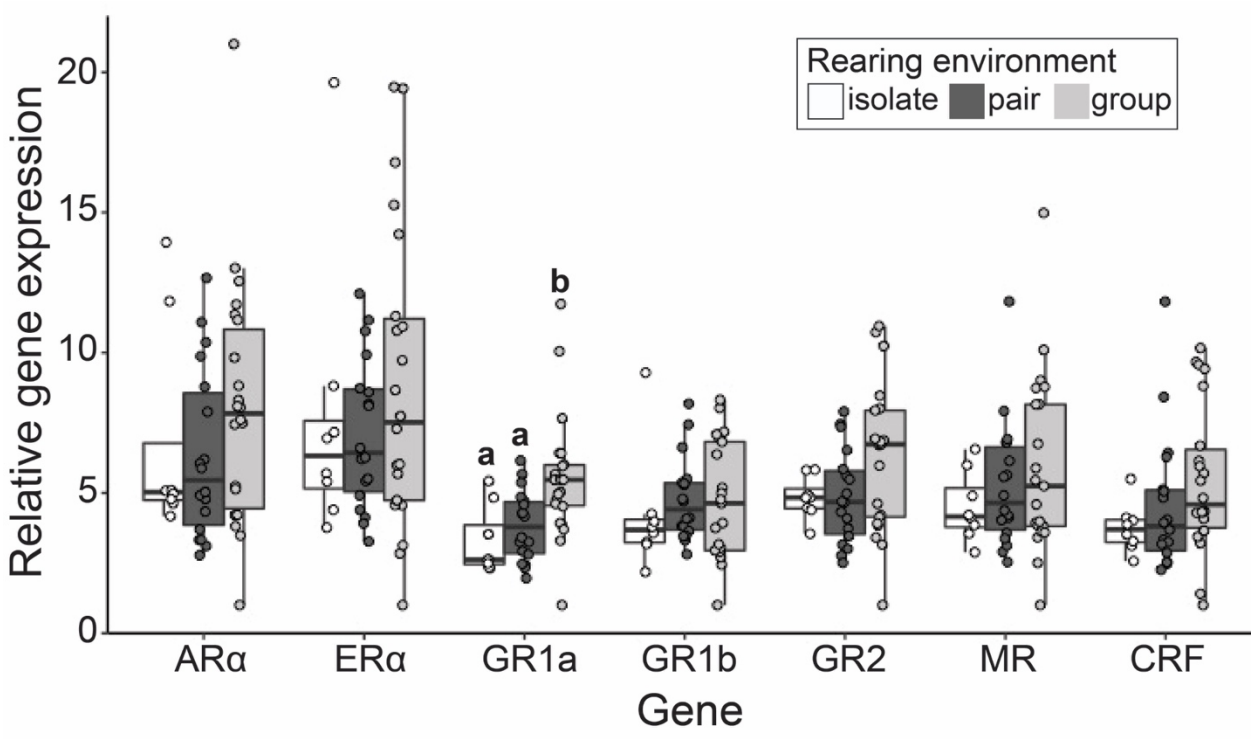


Figure 4: Relative gene expression calculated using ΔΔCT analysis (reference gene 18S) for juveniles reared in isolation (1 week), pairs (1 week or 5 weeks), and groups (1 week or 5 weeks). Time in treatment (1 vs. 5 weeks) did not affect relative gene expression; therefore, all individuals are shown together: isolates (n=8), pair (n=18), group (n=22). Androgen receptor α (ARα). Estrogen receptor α (ERα). Glucocorticoid receptors (GR). Mineralocorticoid receptor (MR). Corticotropin-releasing factor (CRF). Letters indicate significant *post hoc* differences ($p<0.05$).

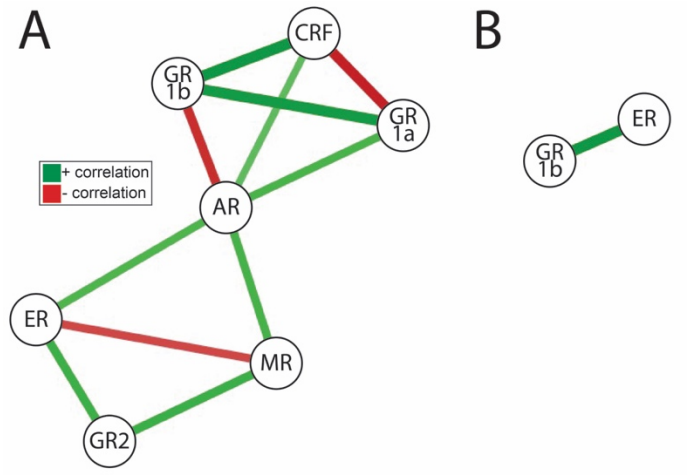
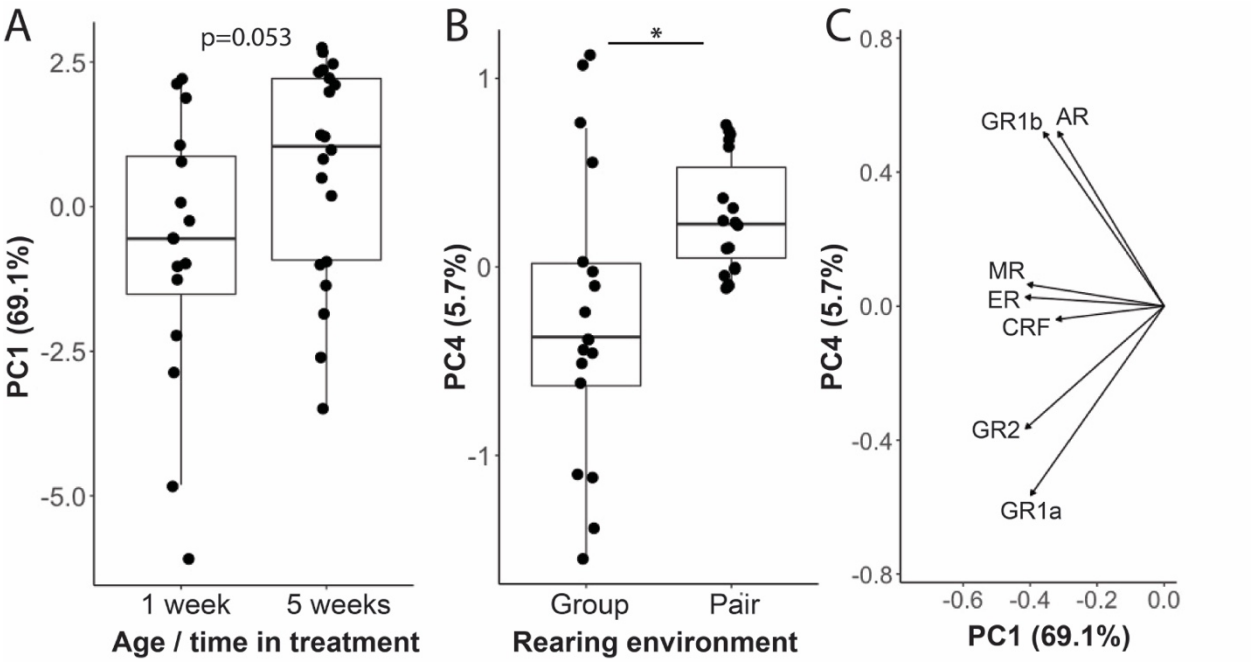


Figure 5: Partial correlation network of gene expression in pair-reared juveniles (n=18) (A) and group-reared juveniles (n=22) (B). Nodes are the candidate genes. Edges represent partial correlations between nodes. Only significant partial correlations are shown ($p<0.05$), and edge thickness indicates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



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993 **Figure 6:** Principal component analysis of relative expression of candidate genes in group-
994 (n=22) and pair-reared (n=18) juveniles. PC1 accounts for 69.1% of the variation in the data,
995 there was a trend for differences between juveniles in treatment groups for 1 vs. 5 weeks (A).
996 PC4 accounts for 5.7% of the variation in the data and was significantly higher in pair-reared
997 juveniles (p=0.011) (B). Vector plot showing how the genes examined load on PC1 and PC4,
998 which differ significantly with regards to treatment duration or rearing environment (C).
999 Androgen receptor α (AR). Estrogen receptor α (ER). Glucocorticoid receptors (GR).
1000 Mineralocorticoid receptor (MR). Corticotropin-releasing factor (CRF).