Multiple behavioral mechanisms shape social behavioral development in a highly social cichlid fish

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ABSTRACT

Early-life social experiences shape adult phenotype, yet the underlying behavioral mechanisms remain poorly understood. We manipulated early-life social experience in the highly social African cichlid fish *Astatotilapia burtoni* to investigate the effects on behavior and neuroendocrine stress axis function. Juveniles experienced different numbers of early-life social partners in stable pairs (1 partner), stable groups (6 fish; 5 partners), and socialized pairs (a novel fish was exchanged every 5 days; 5 partners). Treatments also differed in group size (groups vs. pairs) and stability (stable vs. socialized). We then measured behavior in multiple contexts and water-borne cortisol. We found effects of treatment on behavior across all assays: open field exploration, social cue investigation, dominant behavior, and subordinate behavior. Cortisol did not differ across treatments. Principle components (PC) analysis revealed robust co-variation of behavior across contexts, including with cortisol, to form behavioral syndromes sensitive to early-life social experience. PC1 (25.1%) differed by numbers of social partners: juveniles with more social partners were more active during the social cue investigation, spent less time in the territory, and were more interactive as dominants. Differences in PC5 (8.5%) were based on stability: socialized pairs were more dominant, spent less time in and around the territory, were more socially investigative, and had lower cortisol than stable groups or pairs. Behavior observations in the home tanks provided further insights into the behavioral mechanisms underlying these effects. These results contribute to our understanding of how early-life social experiences are accrued and exert strong, lasting effects on adult phenotype.

Key words: Cortisol; Early-life experience; Early-life social effects; Ontogeny; Hypothalamic-pituitary-adrenal axis

INTRODUCTION

Early-life social environments and experiences are potent drivers of developmental plasticity for social species and, as a result, can have strong, long-term effects on organismal phenotype (Bateson, 2001; Bateson et al., 2004; Kuijper & Johnstone, 2019; Taborsky, 2017; Weaver, 2009). Early-life social effects have been documented across vertebrate taxa (e.g., (Arnold & Taborsky, 2010; Bölting & von Engelhardt, 2017; Champagne & Curley, 2005; Moretz et al., 2007; Perkeybile & Bales, 2017; White et al., 2010), yet the specific attributes of early social environments and experiences that cause phenotypic changes are often unidentified (Kasumovic, 2013; Taborsky, 2016). Social interactions and stimuli can make up a substantial part of juvenile experience. Depending on the species and group structure, early social interactions can involve parents (maternal, paternal, or biparental care) (Champagne & Curley, 2005; McClelland et al., 2011; Perkeybile et al., 2013), parental helpers (Arnold & Taborsky, 2010; Taborsky et al., 2012), siblings (Branchi et al., 2013; Buist et al., 2013; D'Andrea et al., 2007; Monclús et al., 2012), peers (Ahloy Dallaire & Mason, 2017; Bölting & von Engelhardt, 2017; Förster & Cords, 2005; Moretz et al., 2007; Weinstein et al., 2014), and other members of the group (Bray, Murray, et al., 2021; Förster & Cords, 2005; Jin et al., 2015), as well as observations of others interacting (Clay & de Waal, 2013; Desjardins et al., 2012; Oliveira et al., 1998). Identifying the specific, proximate causes—the behavioral mechanisms—is critical to understanding how gene-by-environment interactions shape processes of developmental plasticity and behavioral developmental trajectories. Given the fitness and health consequences of social behavior (Bennett et al., 2006; Meyer-Lindenberg & Tost, 2012; Silk, 2007; Solomon-Lane et al., 2015; Wilson, 1980), the developmental origins of adult behavioral phenotype are particularly important to understand, including as a target for natural selection (CITATION).

69 Manipulating the early social environment is a common approach to studying early-life
70 social effects. For example, rearing animals in groups of different sizes and/or complexities often
71 has significant effects on development and phenotype (reviewed in (Taborsky, 2016). In ravens
72 (*Corvus corax*), family size affected social attentiveness (Gallego-Abenza et al., 2022); in zebra
73 finches (*Taeniopygia guttata*), rearing group size and age diversity of early-life flocks affected
74 courtship and aggressive behavior, as well as plumage development (Bölting & von Engelhardt,
75 2017); in mice, communal nesting affected social skills and neuroendocrine function, with
76 separate effects of maternal care and peer interactions (Branchi et al., 2009, 2013); and in
77 Daffodil cichlids (*Neolamprologus pulcher*), the presence of parents and brood helpers increased
78 social competence (Arnold & Taborsky, 2010; Taborsky et al., 2012). In general, larger social
79 groups are more complex than smaller groups, and early exposure to social complexity tends to
80 benefit social skills and competence (e.g., (Branchi et al., 2009, 2013; Fischer et al., 2015; White
81 et al., 2010). However, individuals accrue different social experiences, even within shared
82 environments. For example, mouse pups in mixed-age, communal nests interact with siblings at
83 varying rates and receive different levels of maternal care (Branchi et al., 2013); infant blue
84 monkeys (*Cercopithecus mitis stuhlmanni*) receive varying rates of allomaternal care, and from
85 different non-mothers in the group (Förster & Cords, 2005); immature male chimpanzees
86 socially associate with adult males at different rates (Bray, Feldblum, et al., 2021); and young
87 male long-tailed manakins occupy varied positions in the social network (McDonald, 2007). This
88 variation can have long-term effects on social behavior (e.g., (Branchi et al., 2013; Bray, Murray,
89 et al., 2021; McDonald, 2007). Therefore, to identify the behavioral mechanisms underlying
90 behavioral development, it is necessary to observe individuals in the rearing environment and

91 test mechanistic hypotheses directly by manipulating the quality and/or quantity of social
92 experiences (Kasumovic, 2013; Taborsky, 2016).
93 Early social experiences exert long-term effects on organismal phenotype through
94 persistent changes in underlying neural and neuroendocrine mechanisms (e.g., (Antunes et al.,
95 2021; Branchi et al., 2013; Champagne & Curley, 2005; McClelland et al., 2011). The
96 neuroendocrine stress axis, or hypothalamic-pituitary-adrenal (interrenal in fish; HPA/I) axis, is a
97 highly-conserved mechanism underlying early-life effects, including social effects (Banerjee et
98 al., 2012; Champagne & Curley, 2005; Crespi & Denver, 2005; Ensminger et al., 2018; Francis
99 et al., 1999; Jonsson & Jonsson, 2014; McClelland et al., 2011; Taborsky, 2016). The HPA/I axis
100 translates environmental conditions and experiences into coordinated physiological and
101 behavioral responses through a neuroendocrine cascade that initiates in response to an
102 environmental stressor. Detection of a stressor, which can include any external condition that
103 disrupts or threatens to disrupt homeostasis, leads to the release of corticotropin-releasing factor
104 (CRF) from the hypothalamus. The pituitary responds to CRF with the release of
105 adrenocorticotropic (ACTH) hormone, which signals for the adrenal (or interrenal) glands to
106 release glucocorticoids (cortisol in fishes) into circulation (Denver, 2009; Lowry & Moore, 2006;
107 Wendelaar Bonga, 1997). Early-life social experiences shape HPA/I axis function in multiple
108 ways (Spencer, 2017; Turecki & Meaney, 2016). For example, peer-reared rhesus macaques
109 (*Macaca mulatta*) had higher baseline and stress-induced levels of ACTH and cortisol, and the
110 HPA axis was more reactive, compared to maternal-reared juveniles (Stevens et al., 2009).
111 Similarly, zebra finch chicks reared with fathers only had hyperresponsive HPA axes, along with
112 altered levels of neural glucocorticoid (GR) and mineralocorticoid receptor (MR) expression,
113 compared to biparental rearing (Banerjee et al., 2012). In *N. pulcher* cichlids, rearing with brood

114 helpers altered neural *crf* and *gr* expression, as well as the ratio of *mr* to *gr1* (Taborsky et al.,
115 2013). The HPA/I axis is also an important source of individual variation in social behavior
116 (Boogert et al., 2014; Dettmer et al., 2017; Farine et al., 2015; Freeman et al., 2021; Pryce et al.,
117 2011; Reyes-Contreras et al., 2019; Sih, 2011) and health (e.g., (Turecki & Meaney, 2016).
118 In this study, we used Burton's Mouthbrooder (*Astatotilapia burtoni*), a highly social
119 cichlid fish and model system in social neuroscience (Fernald & Maruska, 2012; Hofmann,
120 2003; Stevenson et al., 2018), to investigate the behavioral mechanisms of early-life social
121 effects and the consequences for behavior and HPI axis function. The vast majority of research
122 on this species has focused on adults, which form mixed-sex, hierarchical social communities.
123 Dominant males are colorful, territorial, and reproductively active. In contrast, subordinate males
124 are drab in coloration, shoal with females, and are reproductively suppressed. Social status is
125 socially-regulated, and individuals regularly transition between dominant and subordinate
126 positions (Fernald & Maruska, 2012; Hofmann, 2003). Juveniles also form status relationships
127 (Solomon-Lane et al., 2022), and both juveniles and adults express a suite of highly-conserved
128 social behaviors, such as aggression, affiliation, courtship, and cooperation (Fernald & Hirata,
129 1979; Fernald & Maruska, 2012; Solomon-Lane et al., 2022; Weitekamp & Hofmann, 2017).
130 Although sex and social status have strong effects on adult behavior, individual variation
131 persists, including in the frequency or quality of behavioral expression, such as aggression,
132 territoriality, courtship, reproductive behavior, and maternal behavior (Alward et al., 2021;
133 Friesen et al., 2022; Fulmer et al., 2017; M. R. Kidd et al., 2013; Maruska, Becker, et al., 2013;
134 Renn et al., 2009); tenure in a dominant vs. subordinate role (Hofmann et al., 1999); female mate
135 choice (M. R. Kidd et al., 2013); social learning (Rodriguez-Santiago et al., 2020); and cognition
136 (Wallace & Hofmann, 2021). HPI axis signaling also varies among adults (Alcazar et al., 2016;

137 Chen & Fernald, 2008; Clement et al., 2005; Dijkstra et al., 2017; Greenwood et al., 2003;
138 Korzan et al., 2014; Maruska et al., 2022) and is highly responsive to social context, such as
139 social status and changes in status (Carpenter et al., 2014; Chen & Fernald, 2008; Clement et al.,
140 2005; Fox et al., 1997; Huffman et al., 2015; Korzan et al., 2014; Maruska et al., 2022; Maruska,
141 Zhang, et al., 2013; Parikh et al., 2006), group social dynamics (Maguire et al., 2021), social
142 habituation (Weitekamp & Hofmann, 2017), and in response to an intruder (Alcazar et al., 2016;
143 Weitekamp et al., 2017).

144 Developmental plasticity may be an important source of individual variation in behavior
145 and HPI axis function among adults (Fernald & Hirata, 1979; Fraley & Fernald, 1982; Solomon-
146 Lane & Hofmann, 2019). We have previously demonstrated early-life social effects in juvenile
147 *A. burtoni* in response to early-life social group size. Juveniles reared in social groups (16 fish)
148 developed to be more active, more socially interactive, and less submissive in a subordinate role
149 compared to pair-reared juveniles. Whole brain gene expression related to HPI axis function was
150 also altered, including elevated GR1a expression in group-reared animals and tight co-expression
151 of candidate genes (including GR1a, GR1b, GR2, MR, and CRF) in pair-reared, but not group-
152 reared or isolated, juveniles (Solomon-Lane & Hofmann, 2019). Juveniles in this study were not
153 observed in the rearing environment, and there are multiple, possible behavioral mechanisms
154 responsible for these effects (Solomon-Lane & Hofmann, 2019; Taborsky, 2016). Individuals
155 reared in larger and/or more complex groups may interact socially at higher rates, experience a
156 greater diversity of types of social interaction (e.g., affiliative, aggressive, cooperative, etc.), gain
157 experience in multiple social roles (e.g., dominant and subordinate, younger and older) (Chase et
158 al., 2022; Solomon-Lane et al., 2022; Williamson et al., 2016), have more social partners, have
159 social partners of varied identities / traits (e.g., age, body size, life history stage diversity)

160 (Arnold & Taborsky, 2010; Bölting & von Engelhardt, 2017; Branchi & Alleva, 2006; Taborsky
161 et al., 2012; White et al., 2010), experience interactions involving more actors simultaneously
162 (i.e., 3-way, or more, interactions), and learn indirectly by watching others (e.g., (Desjardins et
163 al., 2012; Oliveira et al., 1998). For juvenile *A. burtoni*, group size and the relative body sizes of
164 individuals affects social behavior and group structure. For example, pairs and triads containing
165 same-sized fish are less hierarchical (Solomon-Lane et al., 2022).

166 In this study, we tested the hypothesis that the number of social partners experienced by
167 juvenile *A. burtoni* during early-life is a behavioral mechanism that shapes behavioral phenotype
168 and HPI axis signaling. The number of social partners is one clear way that groups and pairs
169 differ (Solomon-Lane & Hofmann, 2019). There is also evidence from other species that this
170 may be a meaningful attribute of social experience that varies across individuals. Assortative
171 social interactions have been observed in a variety of species. For example, more socially
172 interactive juvenile yellow-bellied marmots (*Marmota flaviventris*) had more novel social
173 partners (Monclús et al., 2012); juvenile male geladas (*Theropithecus gelada*) had more novel
174 play partners than juvenile females (Barale et al., 2015); there is variation in the number of
175 friendships initiated and reciprocated by juvenile rhesus macaques (Weinstein et al., 2014); and
176 bold three-spined sticklebacks (*Gasterosteus aculeatus*) had a larger number of social contacts
177 compared to shy fish (Pike et al., 2008). For juvenile *A. burtoni* in pairs and triads, individuals
178 are not equally likely to initiate interactions (Solomon-Lane et al., 2022), suggesting that in
179 larger, more naturalistic groups, social network connectivity may vary considerably, as it does in
180 adults (Maguire et al., 2021). Direct manipulations of social partner number can also affect social
181 behavioral development. For example, juvenile male brown-headed cowbirds reared in dynamic
182 flocks outcompeted males reared in stable flocks of the same size (White et al., 2010).

183 To manipulate the number of social partners, we reared juveniles in stable pairs (2 fish,
184 one partner each), in stable groups (6 fish, 5 partners each), or in socialized pairs, in which one
185 member of the pair was changed 5 times over the course of the 26 day experiment (2 fish at a
186 time in the pair, 5 total partners each) (Figure 1). This design also allows us to compare the
187 effects of group size (pair vs. group) and social stability (stable vs. socialized). We observed
188 social interactions in the different rearing environments to quantify social experience, and then
189 we tested the effects on individual social behavior in a series of behavior assays. We
190 hypothesized that the number of social partners would be the strongest influence on behavioral
191 phenotype. If the number of early-life social partners was an important behavioral mechanism
192 underlying development in Solomon-Lane & Hofmann (2019), then in this study, we predict that
193 juveniles from the stable groups and socialized pairs will be more active and socially interactive
194 in novel social context, as well as less submissive in a subordinate role. To test the hypothesis
195 that early-life social experience affects HPI axis signaling, we collected water-borne cortisol
196 following the individual behavior assays. By investigating specific behavioral mechanisms
197 underlying behavioral development and neuroendocrine function, this research can provide
198 insights into processes of developmental plasticity and uncover the origins of adult phenotype.
199

200 **METHODS**

201 *Animals*

202 The juveniles used in this experiment were bred in the laboratory. The breeding adult *A.*
203 *burtoni* are a laboratory population that descended ~65-70 generations from a wild caught stock
204 from Lake Tanganyika (Fernald & Hirata, 1977). In the naturalistic, mixed-sex adult breeding
205 communities, there are dominant males that are territorial, reproductively active and colorful, as

206 well as subordinate males that shoal with the females, are reproductively suppressed, and drab in
207 color. Individual males may transition regularly between statuses, depending on the social
208 context (Fernald & Maruska, 2012; Hofmann, 2003). Females are courted by dominant males
209 and lay their eggs in his territory. She then immediately scoops up the eggs into her mouth, and
210 then male fertilizes them. The developing larvae are then brooded in the female's buccal cavity
211 for ~14 days. In the wild, and under some laboratory conditions, mothers guard their offspring
212 for 10 days or more after initially releasing the free-swimming fry from her mouth (Renn et al.,
213 2009).

214 In this study, 86 fry were removed from the buccal cavities of seven mothers at
215 approximately 5-7 days post-fertilization. Individual broods were next placed in shallow water in
216 a petri dish, and a digital image was taken with a ruler for scale. ImageJ was used to measure
217 standard length (SL, mm) (Schneider et al., 2012), from the tip of the jaw to the caudal peduncle.
218 Broods ranged in size from 4-21 fry (mean: 12.43 ± 2.84 fry, median: 15), and fry size ranged
219 from 4.59-8.13 mm SL (mean: 5.96 ± 0.095 mm, median: 5.81 mm). See Supplemental Table 1
220 for mean, median, minimum, and maximum SL of fry per brood. Fry from all of the broods were
221 then placed briefly (~30 min) in a common bucket to intermix. We used a hand net to remove
222 individuals from the bucket and haphazardly assigned them to different home tanks and
223 treatment groups, including stable groups, socialized pairs, and stable pairs (see below for
224 descriptions). We selected fry at this early developmental stage before overt social interactions
225 occur (Fraley & Fernald, 1982) so that even during the experimental set up, when fry were
226 briefly placed together in the same bucket, individuals would not gain any experience with social
227 interactions. During this time, they were briefly exposed to social sensory cues (visual,
228 chemosensory, tactile). In their experimental tanks, fry were allowed to mature for 7 days, when

229 visual inspection confirmed their yolks had been fully absorbed and they were mobile.
230 Methylene blue was added to the water to reduce the incidence of fungal infection during this
231 time. The experimental timeline started after the 7-day developmental period. The sex ratio of
232 fish in this experiment is not known because sex cannot be determined anatomically until
233 reproductive maturation. The sex ratio of *A. burtoni* broods is approximately 1:1 (Heule et al.,
234 2014). All research was done in compliance with the Institutional Animal Care and use
235 Committee in the Keck Science Department of Scripps, Pitzer, and Claremont McKenna
236 Colleges (IACUC Protocol # 19-001).
237
238 *Early-life social experiences*
239 Fry were reared for 26 days in one of three conditions to manipulate early-life social
240 experience: stable groups of 6 fish (n=6 groups), stable pairs of 2 fish (n=7 pairs), and socialized
241 pairs (n=8 pairs) (Figure 1). These treatment groups were designed to manipulate the number of
242 early-life social partners. In the stable groups, every individual had 5 social partners. In the stable
243 pairs, every individual had 1 social partner. In the socialized pairs, one individual was removed
244 from the tank using a hand net every 5 days and was replaced with a novel partner. The fish that
245 was removed was also transferred to another socialized pair tank with a novel partner. After a
246 total of 5 transfers, all of the fish in the socialized pairs were exposed to a total of 5 social
247 partners, the same number as fish in the stable groups. However, the size of the social group for
248 socialized pairs was always 2 fish, the same as in the stable pairs. As a control, on the day fish in
249 the socialized pairs were transferred, we used a hand net to remove one fish from each of the
250 stable pairs and stable groups. The fish was immediately returned to the same home tank so that
251 group membership in the stable pairs and stable groups was consistent.

Stable and socialized pairs were housed in small acrylic aquaria (23 × 15 × 15 cm), and the stable groups were housed in larger acrylic aquaria (30 x 20 x 17 cm). One, small terracotta pot (5 cm at the widest diameter) was placed in each aquarium, and an air stone was used to keep the water oxygenated. There was no substrate on the bottom of the aquaria. White plastic dividers were placed to prevent visual contact between aquaria. The fish were maintained on a 12:12 light:dark schedule at 24°C, and they were fed Hikari Middle Larval Stage Plankton (Pentair Aquatic Eco-Systems, Cary, NC) once a day. We mixed 0.015 g of fish food in 200 mL of water. The mixture was actively stirred, and a transfer pipette was used to add 1 mL to the pairs and 3 mL to the groups.

Tagging individual fish

To track individuals as they developed over the course of the experiment, we tagged each fish, from all treatments, following the first rotation in the socialized pairs. We waited until this time so the fish would be slightly bigger, as their small size is a challenge for existing tagging methods (Hill & Grossman, 1987; Lotrich & Meredith, 1974; Solomon-Lane & Hofmann, 2018; Thresher & Gronell, 1978). We first lightly anesthetized fry in 0.03 g MS-222 / L aquarium water (buffered with sodium bicarbonate to pH 7.0 –7.5). They were then removed from the water and placed on a wet paper towel. We placed a small amount of Alcian Blue (Fisher Scientific, Pittsburgh, PA) powder onto the dorsal muscle of the fish and perforated the surface of the fish using a pulled capillary tube micropipette. This method of tattooing led the ink to seep into the tissue of the fish. We used different tag locations (left or right; anterior, middle, or posterior regions) on the dorsal muscle to differentiate individuals in the same tank. In the socialized pairs, tagging was sufficient in most tanks to transfer the same individual each time.

275 The persistence of the tag varied, and in the best cases, it remained visible for 3 weeks. If the
276 tags were not visible in the socialized pair, the smaller individual was transferred.
277
278 *Survival and number of early-life social partners*
279 The tagging procedure had an 85% survival rate. For fish that did not survive tagging, or
280 if a fish died at another point in the experiment, it was replaced with a fish of similar size and
281 age. The replacement fish were not included in the individual behavior assays conducted at the
282 end of the experimental rearing period (see below). We tracked the actual number of social
283 partners for each experimental tank. All fish in the socialized pairs had exactly 5 social partners.
284 Stable group fish had an average of 6.43 ± 0.14 social partners. Stable pair fish had an average of
285 1.2 ± 0.13 social partners. In our analyses, we consider the treatment groups as categorical,
286 although there is limited variation in the number of social partners (Supplemental Figure 1).
287
288 *Behavioral Observations*
289 Home tanks: We recorded social behavior in the home tanks 2 times during the rearing period.
290 Cameras (Warrior 4.0, Security Camera Warehouse, Asheville, NC) were positioned above the
291 tanks, and with the tank lids removed, all areas of the tank were visible except under the
292 terracotta pot. Using Solomon Coder (www.solomoncoder.com), 10 minutes of video was scored
293 for approaches and displacements. Approaches were counted anytime one fish swam directly
294 towards any part of another fish, within 3 body lengths. If the approached fish swam away in any
295 direction, it was scored as a displacement. Because the identification tags were not visible on the
296 video, we counted the total number of social interactions (approaches, displacements) for the
297 group or pair. We also quantified the number of times a fish entered the terracotta pot territory,

298 defined as at least 50% of a fish's body crossing into / under the pot. Exiting the territory was
299 highly correlated with entering the territory ($p<0.0001$, $r^2=0.97$); therefore, we only present data
300 for entering. To measure social grouping, we captured screenshots from the video every 30 s for
301 a total of 20 time points. Distance was measured between every dyad, from a focal point in the
302 center of the head, using ImageJ (Schneider et al., 2012). The researchers scoring behavior were
303 unaware of the treatment of the pairs (socialized vs. stable), but the stable groups were easily
304 recognizable. For behavior and distance, we analyzed the raw values (behaviors per minute and
305 distance), as well as scaled values to account for differences in group size (behaviors per minute
306 divided by 2 for the pairs and divided by 6 for the groups) and tank size (distance divided by the
307 hypotenuse of the tank) among treatments.
308
309 Individual Behavior Assays: After the 26-day rearing period (on days 27 and 28), we measured
310 individual behavior in a series of assays, including for open field exploration, social cue
311 investigation, dominance behavior, and subordinate behavior. Every fish in the socialized (n=9)
312 and stable pairs (n=10) was included in the individual assays. Three of the 6 fish from each
313 stable group was selected randomly to be included in the individual assays using a random
314 number generator (n=14). The procedures have been described previously in detail (Solomon-
315 Lane & Hofmann, 2019). Briefly, these assays (or similar) are used across species and assess
316 different elements of behavioral phenotype, including behaviors affected by early-life social
317 experience (Solomon-Lane & Hofmann, 2019). The open field exploration is used to assess
318 locomotion and anxiety-like behaviors (e.g., (Cachat et al., 2010; Prut & Belzung, 2003), and the
319 social cue investigation is used as a measure of social motivation or preference (e.g., (Bonuti &
320 Morato, 2018; Moy et al., 2004). The dominant and subordinate behavior assays are used to

321 assess how the focal fish behaves when in the position to be dominant or subordinate. In social
322 groups, including for juvenile *A. burtoni* (Solomon-Lane et al., 2022), most individuals will be
323 both subordinate to some group members and dominant over others at a given time (e.g., (Chase
324 et al., 2022). Social status also changes over a lifetime (Fernald & Maruska, 2012; Hofmann,
325 2003).

326 The behavioral assays took place in novel experimental aquaria, which were the same
327 size as the pairs' home tanks (3.27 L, 23 × 15 × 15 cm). Cameras recorded behavior from above,
328 and all areas of the tank were visible except under the terracotta pot. A permanent marker was
329 used to delineate different zones (Figure 1B): the territory zone contained a small terracotta pot,
330 close zone, far zone, and investigate zone, where the social cue was placed for the social cue
331 investigation assay. Each assay lasted for 30 min. The focal fish was alone in the tank for the
332 open field exploration (minutes 20-30 were analyzed). For the social cue investigation, a novel
333 cue fish inside of a 20 mL glass vial was placed in the investigate zone (see placement in Figure
334 1B, minutes 2-12 were analyzed). In the open field and social cue assays, we recorded the
335 number of times a fish entered a zone, and how long it spent there. After removing the vial and
336 cue fish, a free-swimming, novel fish that was smaller than the focal was added to the tank to
337 assess dominant behavior (minutes 2-12 were analyzed). After removing the small fish, a free-
338 swimming novel fish larger than the focal was added to assess subordinate behavior (minutes 2-
339 12 were analyzed). In the dominant and subordinate behavior assays, we scored approaches and
340 displacements. The researchers scoring behavior were unaware of the treatment of the focal fish.

341 From the approaches and displacements in the dominant and subordinate behavior assays,
342 we calculated two additional social measures using the *compete* package in R (Curley, 2016).
343 First, we calculated an adjusted version of David's Score, which is a dominance index. Rather

344 than categorizing all social interactions as a win or loss, we incorporated both approaches and
345 displacements, such that the agonistic outcome of individual i in interaction with another
346 individual j is the number of times that i displaces j, divided by the totalnumber of interactions
347 (approaches) between i and j (i.e., i displaces j / (i approaches j + j approaches i)). The rest of the
348 calculations were done as described in (Gammell et al., 2003). See Supplemental Information for
349 (Solomon-Lane et al., 2022) for the R code. Second, we calculated directional consistency index
350 (DCI) as a measure of whether patterns of approaching in the dyads is reciprocal, from perfectly
351 reciprocal (0) to unidirectional (1). We also used the *compete* package to run a randomization
352 test to determine if the directionality was significantly more asymmetrical (unidirectional) than
353 expected by chance (Leiva et al., 2008).
354
355 *Water-borne cortisol*
356 Following the individual behavior assays, we collected water-borne hormone samples.
357 This is a non-invasive method of quantifying steroid hormones, and values strongly correlate
358 with circulating hormone levels (C. E. Kidd et al., 2010). We chose cortisol, as HPI axis function
359 is often affected by early-life experience (e.g., (Champagne & Curley, 2005; Taborsky et al.,
360 2013) and cortisol can correlate with behavior and behavioral syndromes (e.g., (Koolhaas et al.,
361 1999). Focal fish were placed individually in clean glass vials filled with 15 mL of clean
362 aquarium water for 2 hrs. Visual barriers were placed around the vials to limit disturbances to the
363 fish during the collection. After two hours, fish were removed from the vial using a hand net
364 rinsed in clean aquarium water, and samples were frozen at 4°C until processing. Hormones were
365 extracted from the water samples using 3 cc Sep-Pak Vac C18 columns (Water Associates,
366 Milford, MA). Columns were first primed by passing 2 mL methanol through the columns twice,

367 being careful not to let the columns run dry. This was followed by 2 mL ultrapure water through
368 the columns twice. The thawed samples were then passed through the columns from the sample
369 vials using the vacuum pump. Ultrapure water (2 mL twice) was then passed through the
370 columns again, followed by 5 min of the vacuum to dry the columns. Finally, the hormone
371 samples were eluted into 13 x 100 borosilicate test tubes by passing 2 mL methanol through the
372 columns twice. The vacuum was used again to try the columns. The sample in methanol was then
373 evaporated under a gentle stream of nitrogen at 37°C and resuspended to a volume of 200 µL per
374 sample (5% EtOH and 95% ELISA buffer). Resuspended samples were shaken on a multitube
375 vortex for 1 hr before being stored at -20°C. Before measuring cortisol, samples were thawed
376 and shaken again on the multitube vortex for 1 hr. ELISAs were completed according to the
377 supplier's instructions (Cayman Chemical, Ann Arbor, Michigan).
378 Cortisol data are presented here as pg hormone per mL sample volume per hr sample
379 collection per g body weight. During hormone collection, fish excrete hormones into the water
380 through their gills, urine, and feces. The gills of larger fish have a larger surface area, thus body
381 size can influence hormone levels. Fish size was measured following the collection of water-
382 borne hormones. Mass was measured on an analytical balance, and SL was measured using
383 ImageJ (Schneider et al., 2012) from digital images with a rule for scale.
384
385 *Statistical Analyses*
386 All statistical analyses were conducted using R Studio (R version 4.2.1) (RStudio Team,
387 2022). Results were considered significant at the $p<0.05$ level, and averages ± standard error of
388 the mean are included in the text. The box of the box and whisker plots show the median and the
389 first and third quartiles. The whiskers extend to the largest and smallest observations within or

390 equal to 1.5 times the interquartile range. To test for the effects of early-life social experience,
391 we compared body size and condition; behavior in the home tank rearing environments (total
392 behaviors per minute and behaviors per fish per minute); spatial distancing in the home tank
393 rearing environments (raw distance and scaled to account for the different sized aquaria for pairs
394 vs. groups); behavior in the open field exploration and social cue investigation (frequency
395 entering, and time in, the territory, close, far, and investigate zones); social behavior
396 (approaches, displacements, approaches received, submissions) and status (David's score, DCI)
397 in the dominant and subordinate assays; cortisol; and principal components (see below, PCs 1-5)
398 across fish reared in the stable groups, socialized pairs, or stable pairs. For data that met the
399 assumptions of parametric statistics, we used one-way ANOVAs. The cortisol data were log
400 transformed first to meet the assumption of normal distribution. *Post hoc* analysis of significant
401 ANOVA results was calculated using Tukey HSD tests. For data that did not meet the
402 assumptions of parametric statistics, we used non-parametric Kruskal-Wallis tests, and Dunn's
403 tests were used for *post hoc* analysis of significant results. Eta squared is reported for the effect
404 size of both the one-way ANVOAs and Kruskal-Wallis tests (small effect: $0 < \eta 2 < 0.01$;
405 moderate: $0.01 < \eta 2 < 0.06$; large: $0.06 < \eta 2$). We used Principal Components Analysis (PCA) to
406 identify how behavior in the open field exploration, social cue investigation, dominance behavior
407 assay, and subordinate behavior assay, and cortisol clustered.
408
409 **RESULTS**
410 *Body size and condition*
411 To determine whether rearing experience in a stable group, socialized pair, or stable pair
412 affected body size and condition, we compared SL, mass, and condition index (Fulton's K),

413 across treatment groups. We found that mass differed significantly across treatment groups
414 (Kruskal-Wallis: $\chi^2(2)=6.39$, p=0.041, $\eta^2=0.15$; Supplemental Figure 2A). *Post hoc* analysis
415 showed juveniles reared in stable pairs were significantly heavier than those from stable groups
416 (Dunn, Bonferroni correction: p=0.036). There were no significant differences between stable
417 groups and socialized pairs (p=0.63) or between stable pairs and socialized pairs (p=0.82). There
418 were no significant treatment differences in SL (ANOVA: $F_{2,30}=2.15$, p=0.14; Supplemental
419 Figure 2B). Because we found an effect of rearing experience on mass, but not SL, we also asked
420 whether condition index was affected by treatment. We found no significant treatment
421 differences in condition index (ANOVA: $F_{2,30}=0.45$, p=0.62, Supplemental Figure 2C). Mass
422 was also positively correlated with water-borne cortisol levels (p=0.017, $r^2=0.15$, Supplemental
423 Figure 2D); therefore, we corrected for body size in the cortisol analyses below.
424
425 *Home tank social behavior*
426 We observed juveniles in the stable groups, socialized pairs, and stable pairs to
427 understand the differences in social experience and environment among the rearing treatments.
428 Unsurprisingly, there were significantly more approaches and displacements in groups compared
429 to pairs, and there were significantly more entrances to the territory in groups compared to stable
430 pairs (Figure 2A). There were no differences in efficiency (Figure 2B). There were also no
431 differences in rates of behavioral interactions per fish. Mean dyad distance was significantly
432 larger in groups than pairs (Figure 2C), but interestingly, the minimum dyad distance was
433 significantly smaller in groups than socialized pairs (Figure 2D). There were no treatment
434 differences in mean dyad distance when scaled for tank size. Statistics are reported in Table 1
435 and Supplemental Table 2.

436

437 *Open field exploration and social cue investigation*

438 For the open field exploration and social cue investigation, we compared across treatment
439 groups for the frequency of entering each zone of the tank, as well as time spent in each zone.
440 Statistics are reported in Table 2 (Supplemental Figure 3). Overall, we found for the open field
441 exploration that fish from the socialized pairs entered the territory zone significantly more
442 frequently than stable pair fish, and fish from the stable groups spent significantly more time in
443 the far zone than stable pair fish. In the social cue investigation, we found that social group fish
444 entered the far zone significantly more frequently and spent significantly more time in the
445 territory and far zones, than fish reared in stable pairs.

446

447 *Dominance and Subordinate Assays*

448 To determine whether rearing experience affected social behavior and status, we
449 quantified patterns of social interaction between the focal fish and a novel social partner (cue
450 fish). Because juvenile (and adult, (Alward et al., 2021) a size advantage benefits dominance
451 (Solomon-Lane et al., 2022), in pairing the focal fish with a smaller cue fish (dominance assay),
452 the focal fish has the opportunity to express social behaviors as the dominant. In pairing the focal
453 fish with a larger cue fish (subordinate assay), the focal fish can express social behaviors as the
454 subordinate. We found significant differences in social behavior and status in both the
455 dominance and subordinate assays (statistics reported in Table 3, Figure 3). In the dominance
456 assay, socialized pair fish approached and displaced significantly more than stable pair fish, and
457 their David's Scores were significantly higher than both stable pair and stable group fish. In the
458 subordinate assay, socialized pair fish received significantly more approaches than stable pair

459 fish from the larger cue fish with which they were paired. Socialized pairs with their cue fish also
460 had significantly lower directional consistency than stable group or stable pair fish.
461
462 *Cortisol*
463 Following the behavior assays, we collected water-borne cortisol to determine whether
464 hormone levels were affected by rearing experience and/or associated with behavioral phenotype
465 (see PCA below). We found there were no significant differences in cortisol across treatments
466 (ANOVA, log transform: $F_{2,29}$=2.30, p=0.12, Figure 4).
467
468 *Integrative analysis of behavior, social status, and cortisol*
469 Given the treatment differences we identified across the open field exploration, social cue
470 investigation, dominance assay, and subordinate assay, we next used PCA to integrate across
471 assays. This allows us to identify subsets of factors that together contribute to juvenile phenotype
472 and test if suites of factors (principal components, PCs) differ significantly across treatment
473 groups. To determine which variables to include from the open field exploration and social cue
474 investigation (time and frequency in the territory, close, far, and investigate zones), we ran an
475 initial PCA with just these variables. Examining the vector plot for PC1 with PC2 (Supplemental
476 Figure 4), we found that nearly all of the open field exploration measures were strongly aligned
477 with the same measure in the social cue investigation (i.e., the vectors for open field and social
478 cue time in the territory zone are identical). The exceptions were for time spent in the far and
479 investigate zones. Therefore, to avoid unnecessary replication, we chose to include all of the
480 variables from the social cue investigation, in addition to time spent in the far and investigate
481 zones during the open field exploration. From the dominant and subordinate assays, we included

482 approaches, displacements, approaches received, submissions, and David's score. Finally, we
483 included cortisol.

484 Here, we focused on the first 5 PCs, which together explain 76.6% of the variation in the
485 data (Figure 5A). We found significant effects of rearing experience on PC1 (25.1%) and PC5
486 (8.48%). For PC1, stable pairs were significantly higher than stable groups, and there was a trend
487 for stable pairs to be higher than socialized pairs (p=0.063) (Figure 5B). Behaviors from the
488 dominance behavior assay and the social cue investigation assay loaded strongly on PC1.
489 Approaches and displacements as a dominant; time in, and frequency entering, the far and
490 investigate zones during the social cue investigation; and frequency entering the close zone
491 during the social cue investigation loaded strongly in the same direction. Time spent in the
492 territory zone during the social cue investigation loaded strongly in the opposite direction. For
493 PC5, socialized pairs were significantly higher than both stable groups and stable pairs (Figure
494 5C). David's Score from the dominance assay and time in the investigate zone during the social
495 cue investigation loaded strongly together in the same direction. In the opposite direction,
496 submissions as a dominant; cortisol; time and frequency in the close zone during the social cue
497 investigation; and the frequency entering the territory zone during the social cue investigation
498 loaded strongly together. Statistics for the PC1-PC5 treatment comparisons are in Table 4 (also
499 see Supplemental Figure 5).

500

501 **DISCUSSION**

502 We investigated the effects of early-life social experience on social behavior and
503 neuroendocrine stress axis function in juvenile *A. burtoni*. We tested the hypothesis that the
504 number of novel social partners experienced during early-life is a behavioral mechanism driving

variation in behavioral development. In manipulating the number of social partners, our experimental treatment groups—stable groups, socialized pairs, and stable pairs—also varied in social group size (pairs vs. group) and group stability (socialized vs. stable). We present strong evidence for early-life social effects on juvenile social behavior, consistent with previous studies in this species (Fernald & Hirata, 1979; Fraley & Fernald, 1982; Solomon-Lane & Hofmann, 2019). In particular, our results support two behavioral mechanisms: the number of social partners and social stability. Although early-life social effects are widely documented across social species, the specific attributes of social experience that affect the mechanisms and trajectory of developmental plasticity are not typically identified (Kasumovic, 2013; Taborsky, 2016). The social interactions and social spacing we observed in the home tanks also provide insights into how social environments vary within and across treatments and may shape the experiences individuals accrue during development. Causation at this level is needed because it is these environmental elements that interact with genes dynamically over development to influence plasticity and the emergence of adult phenotype (Kasumovic, 2013; Taborsky, 2016, 2017). Overall, it is likely that multiple behavioral mechanisms contribute and interact to shape behavioral development (e.g., (Branchi et al., 2013).

We found that manipulating early-life social experience affected behavior across multiple contexts, including the open field exploration, social cue investigation, dominance behavior assay, and subordinate behavior assay. Principal components analysis revealed that these effects were correlated across contexts. Principal component 1 (25.1%) and PC5 (8.5%), which both differed significantly across treatment groups, had behaviors from multiple assays that loaded strongly. These results support our previous finding that juvenile *A. burtoni* behavior can form a syndrome, and an individual's position along the syndrome continuum is sensitive to early-life

528 social experience (Solomon-Lane & Hofmann, 2019). A syndrome is a population-level measure
529 in which rank-order differences between individuals are correlated across contexts and/or over
530 time (Bell, 2007). Behavioral syndromes have been identified across species and can indicate
531 consistency in individual behavior across contexts and/or over time (Bell, 2007; Sih, Bell, &
532 Johnson, 2004; Sih, Bell, Johnson, et al., 2004). The PC1 syndrome included approaches and
533 displacements in the dominance assay loading strongly in the same direction with time in and
534 frequency entering the investigate and far zones, and frequency entering the close zone, during
535 the social cue investigation. Time in the territory zone during the social cue investigation loaded
536 in the opposite direction. This is syndrome is highly similar to the one we identified after rearing
537 juvenile *A. burtoni* in pairs or social groups of 16 fish (Solomon-Lane & Hofmann, 2019).
538 The treatment differences in PC1 are most consistent with an effect of the number of
539 social partners. Stable pairs had significantly higher PC1 values compared to stable groups, and
540 there was a strong trend to be higher than socialized pairs ($p=0.063$). There were no differences
541 between stable groups and socialized pairs. Juveniles that experienced more social partners
542 during early-life were more active during the social cue investigation, spent less time in the
543 territory in the presence of a social cue, and were more socially interactive in a dominant social
544 role. The direction of this effect is also the same as in our previous study: group-reared juveniles
545 were more active and socially interactive than pair-reared juveniles (Solomon-Lane & Hofmann,
546 2019). This suggests that social experiences resulting from more novel partners may be an
547 important behavioral mechanism underlying the effect of group size. Syndromes involving
548 activity and social interaction are common across species (e.g., (Conrad et al., 2011; Näslund &
549 Johnsson, 2016) and may also be related to bold-shy and proactive-reactive behaviors (Bell,

550 2007; Conrad et al., 2011; Groothuis & Carere, 2005; Koolhaas et al., 1999; Sih, Bell, Johnson,
551 et al., 2004).

552 It is not yet known how variable the number of social partners is for juvenile *A. burtoni* in
553 the wild or in larger, more naturalistic laboratory social groups. Evidence from a diversity of
554 other species suggests there can be considerable individual variation. In many species, siblings
555 (and parents) are the most proximate—and sometimes the only—early-life social contacts. The
556 number of offspring produced can vary both among individuals and within individuals across
557 reproductive bouts / over time. Within a group or population, individuals can also vary in the size
558 and makeup of their social network or niche (Barale et al., 2015; Beirão-Campos et al., 2016;
559 Branchi et al., 2013; Croft et al., 2005; Förster & Cords, 2005; Maguire et al., 2021; Monclús et
560 al., 2012; Pike et al., 2008; Saltz et al., 2016; Weinstein et al., 2014; Weinstein & Capitanio,
561 2008, 2008). For juveniles, this can have long-term, phenotypic effects (Branchi et al., 2013;
562 Monclús et al., 2012; Weinstein et al., 2014; Weinstein & Capitanio, 2008). Socialization
563 strategies are also used with animals valuable to humans, such as working dogs (Gfrerer et al.,
564 2018), family dogs (Howell et al., 2015), and livestock like piglets (Morgan et al., 2014; Salazar
565 et al., 2018). Although many studies have manipulated the number of early-life social partners as
566 a consequence of group size, group size could exert independent or interacting effects on
567 phenotype. In two studies that also controlled for group size, brown-headed cowbirds (*Molothrus*
568 *ater*) were housed in stable or dynamic flocks, in which flock members were exchanged multiple
569 times with novel birds. Dynamic flock males had more variable social networks over time, larger
570 signing networks, and outcompeted stable flock males in mating opportunities (White et al.,
571 2010). When housing conditions were later reversed, the new dynamic flock males still had
572 higher reproductive success, which was achieved via changes in social strategy (Gersick et al.,

573 2012). For juvenile *A. burtoni* in pairs and triads, individuals are not equally likely to initiate
574 interactions. Both group size and relative body size influenced social group structure (Solomon-
575 Lane et al., 2022). This suggests that, like adults (Maguire et al., 2021), social network position
576 and connectivity may vary considerably across individuals, with consequences for
577 developmental plasticity and behavioral development.

578 The treatment differences in PC5 (8.5%) support an effect of early-life social stability.
579 Socialized pairs had significantly higher PC5 values than stable groups and stable pairs, which
580 were not different from each other. David's score in the dominance behavior assay loaded in the
581 same direction as time in the investigate zone during the social cue investigation. Submissions in
582 the dominance behavior assay, frequency entering the territory zone in the social cue
583 investigation, time in and frequency entering the close zone in the social cue investigation, and
584 water-borne cortisol loaded together in the opposite direction. Socialized pairs were more
585 dominant in the dominance behavior assay, spent less time in and near the territory zone, spent
586 more time in the investigate zone, and had lower cortisol levels than stable groups or pairs. This
587 suite of behaviors for PC5 shares multiple similarities with the PC1 syndrome. Dominance
588 behavior is represented as high rates of approaching and displacing on PC1 and as the opposing
589 loadings of David's score (indication of dominant status) and submissions (indication of
590 subordinate status) on PC5. Dominant juvenile *A. burtoni* approach and displace at significantly
591 higher rates than subordinates (Solomon-Lane et al., 2022). For PC5, being in or near the
592 territory zone, with less time in the investigate zone, was associated with low status when given
593 the opportunity to be dominant. This is mirrored on PC1 by the negative association between
594 being in the territory zone and approaching and displacing in the dominance behavior assay. The
595 relative dominance of juveniles from the socialized pairs is also consistent with having

596 significantly lower directional consistency (i.e., more agonistically symmetrical) in the
597 subordinate behavior assay. In our previous study, group-reared juveniles were less submissive
598 in a subordinate role than pair-reared juveniles (Solomon-Lane & Hofmann, 2019). It is possible
599 this was not the case for stable group juveniles in this study due to group size differences (6 fish
600 vs. 16 fish).

601 The most striking difference for PC5 and the effect of social stability is the involvement
602 of cortisol. We found no treatment differences in water-borne cortisol levels, and PC5 was the
603 only PC on which cortisol loaded strongly. That high cortisol was associated with these
604 variables—lower social status, less exploratory behavior, and more time in the territory away
605 from a novel cue—is consistent with numerous other studies. For example, HPA/I axis function
606 is correlated with social status in many hierarchical species, and elevated cortisol is a
607 consequence of subordinate in approximately half of species, depending on how status is
608 established and maintained (Creel, 2001). In adult male *A. burtoni*, the relationship between
609 status and HPI axis function varies across contexts, but can be elevated in subordinates (Maruska
610 et al., 2022). In juveniles, we have previously found that group-rearing increases the expression
611 of whole brain glucocorticoid receptor (GR) 1a, which may increase negative feedback
612 efficiency and lower cortisol (Solomon-Lane & Hofmann, 2019). Higher GR1a and GR1b
613 expression, and lower GR2 and mineralocorticoid receptor (MR) expression, is also associated
614 with higher dominance status (Solomon-Lane et al., 2022). Together, these findings are
615 consistent with the lower cortisol levels and higher social status we see in socialized pairs.
616 Coping styles are an example of an integrative phenotype in which a behavioral syndrome aligns
617 with stress physiology. Proactive copers are more active, aggressive, and less responsive to
618 stress, including lower baseline glucocorticoid levels and more efficient negative feedback

619 mechanisms than reactive copers. Coping styles are sensitive to early-life social effects and have
620 been observed across species (Koolhaas et al., 1999). Our data suggest that juveniles reared in
621 socialized pairs, and group-reared juveniles in our previous study (Solomon-Lane & Hofmann,
622 2019), may be proactive copers, a hypothesis that should be tested directly. Adult *A. burtoni* do
623 not appear to form coping styles (Butler et al., 2018). Early-life social stability is known to affect
624 HPA/I axis function across species and affect behaviors related to status, activity, boldness-
625 shyness, and neophobia, which are related to the behavioral variables loading on PC5. In these
626 cases, however, instability tends to negatively impacts statu and activity and enhance shyness
627 and neophobia. This is the opposite pattern to our data. An alternative explanation is that
628 familiarity shaped behavior in the assays. The dominance and subordinate behavior assays were
629 in a novel tank with a novel conspecific. This is highly similar to the way we socialized the pairs.
630 Familiarity could lead socialized pairs to appear more active and competent in the assays. A
631 future study can directly test these potential behavioral mechanisms of stability and familiarity.
632 In either case, social experience has an important role shaping phenotype.
633 We observed fish in their rearing environments to gain insights into the specific social
634 experiences and social sensory cues, or proximate behavioral mechanisms, responsible for the
635 early-life social effects we observed (Taborsky, 2016). It is logical that with more individuals,
636 rates of behavior were higher in the stable groups compared to the pairs. Rates of behavior per
637 fish did not differ across treatment groups, and there were no differences in total agonistic
638 efficiency. These data confirm that the group social environment has more opportunities for
639 social experiences, which can include direct involvement in an interaction and observations of
640 others interacting (Desjardins et al., 2012; Oliveira et al., 1998). The mean distance between
641 dyads was also significantly larger in stable groups than the pairs, and, interestingly, the smallest

642 dyad distance was significantly smaller in groups than social pairs. There were no differences
643 across treatments when scaled for the size of the tank. We were unable to identify and track
644 individuals because the ink tags were not visible on video. As a result, we could not quantify
645 individual social experience, social status, social network position, or spatial position, which we
646 hypothesize are causally related to behavioral development and phenotype (Kasumovic, 2013;
647 Taborsky, 2016). The evidence that social interactions are assortative within groups is
648 overwhelming across species (e.g., (Chase et al., 2022; Croft et al., 2005, 2009; Pike et al., 2008;
649 Williamson et al., 2016), and juvenile *A. burtoni* form social status relationships and nuanced
650 social structures (Solomon-Lane et al., 2022). Therefore, we expect social status experience and
651 the degree of agonistic asymmetry across social partners to be particularly important for
652 behavioral development. In addition to spatial proximity potentially being an indicator of social
653 relationships, it may also affect social sensory cue perception and communication, for example,
654 via mechanosensory and chemosensory cues in adult *A. burtoni* (Butler & Maruska, 2016;
655 Nikonov et al., 2017).

656 Overall, our work demonstrates that multiple behavioral mechanisms—the number of
657 early-life social partners and social stability—affect juvenile *A. burtoni* development and
658 phenotype, including integrated behavioral and neuroendocrine characteristics. In addition to
659 manipulating social experience directly, we observed juveniles in their rearing environments,
660 which are necessary steps towards understanding the social experiences accrued during
661 development and the mechanisms by which early experiences exert long-term and
662 disproportionately strong effects on adult phenotype (Buist et al., 2013; Jonsson & Jonsson,
663 2014; Kasumovic, 2013; Taborsky, 2016, 2017). Although the simplistic social contexts we used
664 in this study are unlikely characteristic of larger, more complex groups found in nature (Chase et

al., 2003), we expect that these behavioral mechanisms, and potentially others—acting additively or synergistically—will also be influential in more naturalistic contexts. Testing the hypotheses we generated here will be key to uncovering the behavioral mechanisms of developmental plasticity and behavioral development, including a role for neuroendocrine stress axis function, in *A. burtoni* and other social species.

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1098 **TABLES**

1099 **Table 1:** Treatment differences in social behavior and distances between dyads in the stable

1100 group, socialized pair, and stable pair home tanks.

Behavior / measure	DF	Test Statistic	p-value	Effect size	*Post hoc* / direction of effect
Approaches	**2, 38**	**F=11.46**	**0.00013**	**0.38**	**Stable group > social. pair: p<0.0001** **Stable group > stable pair: p=0.0061** Social. pair vs. stable pair: p=0.41
Displacements	**2, 37**	**F=11.37**	**0.00014**	**0.38**	**Stable group > social. pair: p<0.0001** **Stable group > stable pair: p=0.02** Social. pair vs. stable pair: p=0.21
Efficiency	2	χ^2=1.06	0.59		
Into territory	**2**	**χ^2=10.81**	**0.0045**	**0.23**	**Stable group > stable pair: p=0.0037** Stable group > social. pair: p=0.071 Social. pair vs. stable pair: p=0.75
Mean dyad distance	**2, 46**	**F=13.62**	**<0.0001**	**0.37**	**Stable group > stable pair: p<0.0001** **Stable group > social. pair: p<0.0001** Social. pair vs. stable pair: p=0.92
Minimum dyad distance	**2**	**χ^2=7.49**	**0.0025**	**0.12**	**Stable group > social. pair: p=0.021** Stable group > stable pair: p=0.085 Social. pair vs. stable pair: p=1.0

1101 Results of one-way ANOVAs (F) or nonparametric Kruskal-Wallis tests (χ^2). Eta-squared is

1102 reported for effect sizes. Significant results in bold. Tukey HSD tests were used for *post hoc*

1103 analysis of significant ANOVA results. Dunn's tests were used for *post hoc* analysis of

1104 significant Kruskal-Wallis results.

1105 **Table 2:** Treatment differences in the frequency entering and time spent in tank zones during the

1106 open field exploration and social cue investigation.

Tank Zones / measure	DF	Test Statistic	p-value	Effect size	*Post hoc* / direction of effect
Open Field Exploration					
Territory (Frequency)	**2,30**	**F=3.70**	**0.037**	**0.2**	**Social. pair > stable pair: p=0.037** Social. pair vs. stable group: p=0.1 Stable group vs. stable pair: p=0.77
Close (Frequency)	2,30	F=3.30	0.051		
Far (Frequency)	2	χ^2=4.97	0.083		
Investigate (Frequency)	2	χ^2=3.51	0.17		
Territory (Time)	2	χ^2=4.34	0.11		
Close (Time)	2	χ^2=0.34	0.84		
Far (Time)	**2**	**χ^2=7.47**	**0.024**	**0.18**	**Stable group > stable pair: p=0.032** Stable group vs. social. pair: 0.16 Social. pair vs. stable pair: p=1.0
Investigate (Time)	2	χ^2=2.13	0.35		
Social Cue Investigation					
Territory (Frequency)	2	χ^2=0.76	0.69		
Close (Frequency)	2	χ^2=1.51	0.47		
Far (Frequency)	**2**	**χ^2=6.60**	**0.037**	**0.15**	**Stable group > stable pair: p=0.036** Stable group vs. social. pair: 0.40 Social. pair vs. stable pair: p=1.0
Investigate (Frequency)	2	χ^2=4.49	0.11		
Territory (Time)	**2**	**χ^2=6.36**	**0.042**	**0.15**	**Stable pair > stable group: p=0.042** Stable group vs. social. pair: 0.39 Social. pair vs. stable pair: p=1.0
Close (Time)	2	χ^2=2.19	0.33		
Far (Time)	**2**	**χ^2=7.93**	**0.019**	**0.20**	**Stable group > stable pair: p=0.019** Stable group vs. social. pair: 0.25 Social. pair vs. stable pair: p=1.0
Investigate (Time)	2	χ^2=5.00	0.082		

1107 Results of one-way ANOVAs (F) or nonparametric Kruskal-Wallis tests (χ^2). Eta-squared is

1108 reported for effect sizes. Significant results in bold. Tukey HSD tests were used for *post hoc*

1109 analysis of significant ANOVA results. Dunn's tests were used for *post hoc* analysis of

1110 significant Kruskal-Wallis results.

1111 **Table 3:** Treatment differences in social behavior and status in the dominant and subordinate

1112 behavior assays.

Assay / Behavior	DF	χ^2	p-value	Effect size	*Post hoc* / direction of effect
Dominant Behavior Assay					
Approaches	**2**	**8.71**	**0.013**	**0.22**	**Social. pair > stable pair: p=0.01** Social. pair vs. stable group: p=0.16 Stable group vs. stable pair: p=0.57
Displacements	**2**	**7.62**	**0.022**	**0.19**	**Social. pair > stable pair: p=0.02** Social. pair vs. stable group: p=0.16 Stable group vs. stable pair: p=0.85
Approaches Received	2	5.44	0.066		
Submissions	2	4.63	0.099		
David's Score	**2**	**8.48**	**0.014**	**0.22**	**Social. pair > stable pair: p=0.043** **Social. pair > stable group: p=0.022** Stable group vs. stable pair: p=1.0
Directional consistency (App.)	2	3.89	0.14		
Subordinate Behavior Assay					
Approaches	2	5.23	0.073		
Displacements	2	2.69	0.26		
Approaches Received	**2**	**6.13**	**0.047**	**0.14**	**Social. pair > stable pair: p=0.042** Social. pair vs. stable group: p=0.73 Stable group vs. stable pair: p=0.38
Submissions	2	4.05	0.13		
Focal efficiency	2	1.38	0.50		
Directional consistency (App.)	**2**	**13.22**	**0.0014**	**0.37**	**Social. pair < stable pair: p=0.0038** **Social. pair < stable group: p=0.004** Stable group vs. stable pair: p=1.0

1113 Results of Kruskal-Wallis tests (χ^2). Eta-squared is reported for effect sizes. Significant results in

1114 bold. Dunn's tests were used for *post hoc* analysis of significant results.

1115 **Table 4:** Treatment differences for principal components (PC) 1-5.

Principal component (% variation)	DF	Test Statistic	p-value	Effect size	*Post hoc* / direction of effect
PC1 (25.1%)	2,28	**F=4.19**	**0.026**	**0.23**	**Stable pair > stable group: p=0.032** Stable pair > social. pair: p=0.063 Stable group vs. social. pair: p=1.0
PC2 (18.2%)	2,28	F=1.82	0.18		
PC3 (14.3%)	2,28	F=0.97	0.39		
PC4 (10.5%)	2	χ^2=2.69	0.26		
PC5 (8.5%)	**2,28**	**F=6.62**	**0.0044**	**0.32**	**Social. pair > stable group: p=0.0036** **Social. pair > stable pair: p=0.036** Stable group vs. stable pair: p=0.75

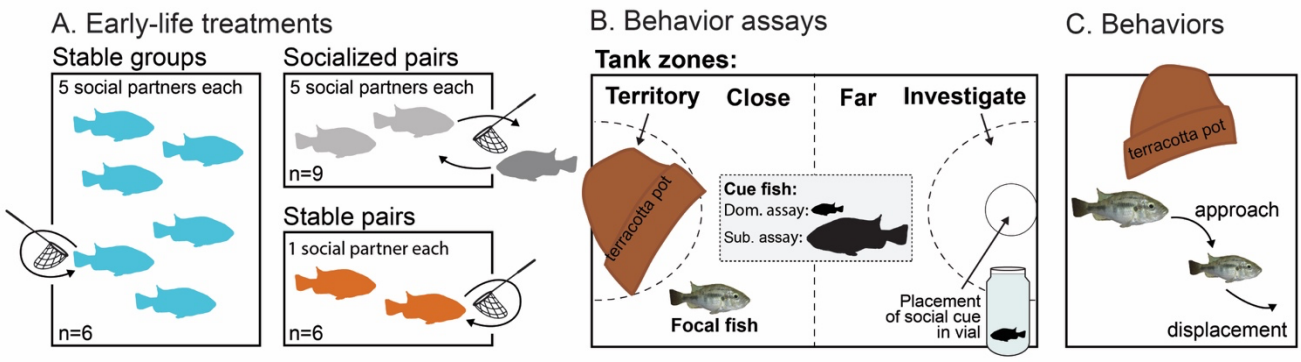
1116 Results of one-way ANOVAs (F) or nonparametric Kruskal-Wallis tests (χ^2) for PC1-PC5.

1117 Percentages indicate the variance explained. Eta-squared is reported for effect sizes. Significant

1118 results in bold. Tukey HSD tests were used for *post hoc* analysis of significant ANOVA results.

1119 Dunn's tests were used for *post hoc* analysis of significant Kruskal-Wallis results.

1120 **FIGURES**



1121 **Figure 1:** A) Juvenile fish were reared in stable groups (n=6, 6 fish each), stable pairs (n=6, 2
1122 fish each), or socialized pairs (n=9, 2 fish each). Every 5 days in the socialized pairs, one fish of
1123 the pair was removed, and a novel juvenile was introduced. The novel juvenile came from a
1124 different socialized pair, and the removed juvenile became the novel partner for a socialized pair.
1125 This was repeated 5 times so that socialized fish had a total of 5 social partners – equal to the
1126 stable group fish. One fish per stable group and pair was also removed from their tank with a
1127 hand net as a control. This fish was immediately returned to its home tank. B) After 26 days in
1128 these rearing environments, individual juvenile behavior was quantified in a novel experimental
1129 tank. The tank contained a terracotta pot shard, and black lines (drawn in permanent marker)
1130 divided the tank into four zones: territory, close, far, and investigate. In the open field
1131 exploration, the focal fish was alone in the tank. In the social cue investigation, a small juvenile
1132 cue fish inside of a scintillation vial was placed in the circle in the investigate zone. In the
1133 dominance assay, a freely-swimming novel cue fish (smaller than the focal) was added to the
1134 tank. In the subordinate assay, a freely-swimming novel cue fish (larger than the focal) was
1135 added to the tank. C) In the dominance and subordinate assay, we quantified the number of
1136 approaches, where one fish swims within three body lengths directly towards any part of another
1137 fish. If the approached fish swam away in any direction, it was counted as a displacement.

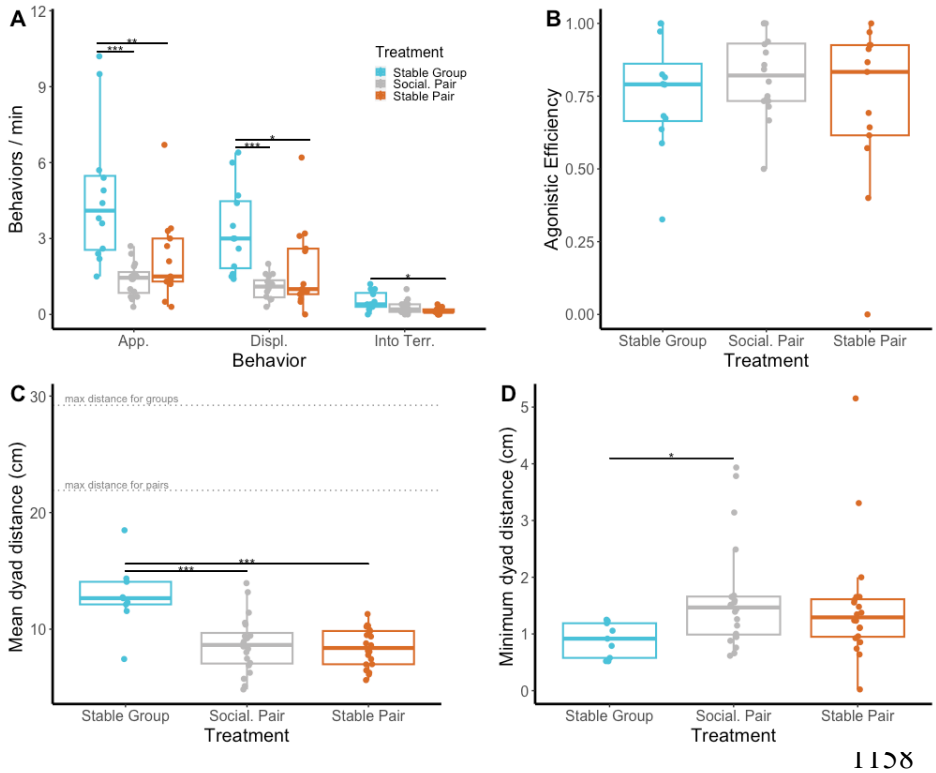


Figure 2: Social behavior and distances in the stable groups, socialized pairs, and stable pairs. A) Frequency of social approaches, displacements, and times entering the territory / terracotta pot in the rearing environments. B) Total agonistic efficiency (total displacements / total approaches). C) Distance between all dyads of fish was measured twice for each tank (20 time points each, 40 total time points). Mean distance between dyads was then calculated per tank (each data point is the mean for one tank). The maximum distance lines are the lengths of the diagonal along the bottom of the tanks. D) The minimum distance recorded between a dyad across all time points. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

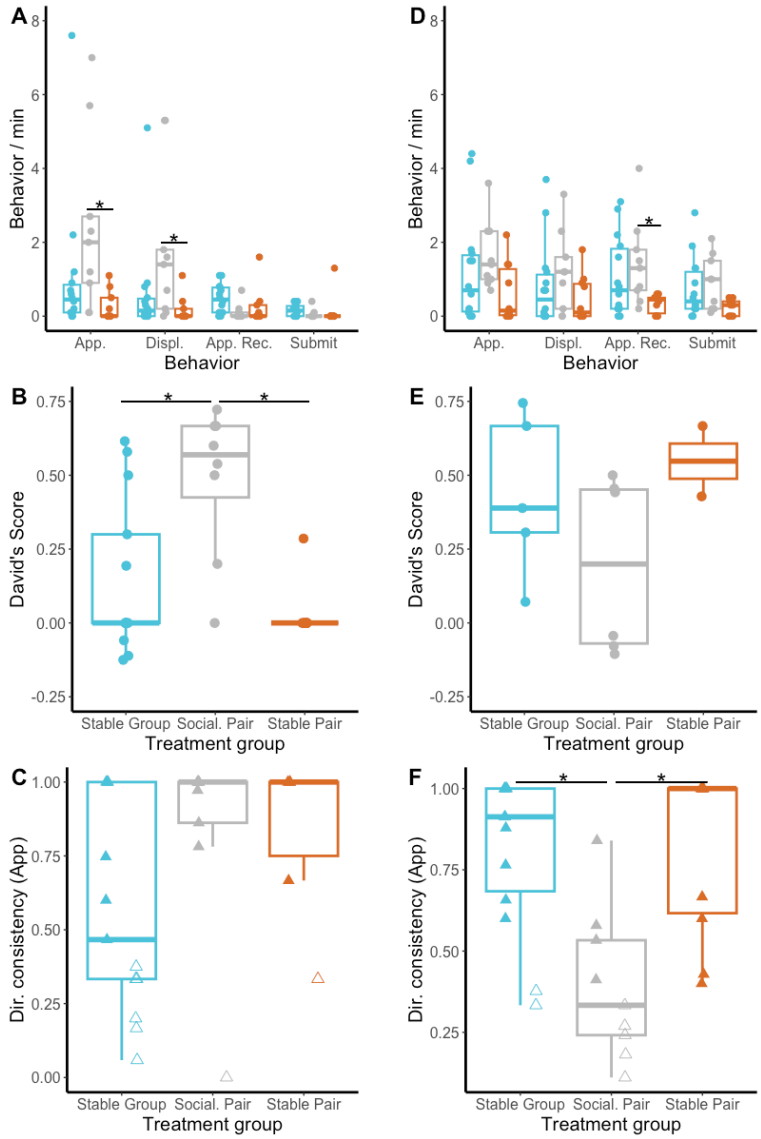


Figure 3: Social measures from the dominant (A-C) and subordinate (D-F) behavior assays. A) Social approaches (App.), displacements (Displ.), approaches received (App. Rec.), and submissions (Submit) in the dominance behavior assay. B) Focal fish David's score in the dominance behavior assay. C) Directional consistency (calculated based on approaches) in the dominance behavior assay. Filled triangles indicate directionality was significantly greater than zero. Empty triangles indicate pairs were not significantly directional. D) Social behavior in the subordinate behavior assay. B) Focal fish David's score in the subordinate behavior assay. C) Directional consistency (calculated based on approaches) in the subordinate behavior assay. *p<0.05.

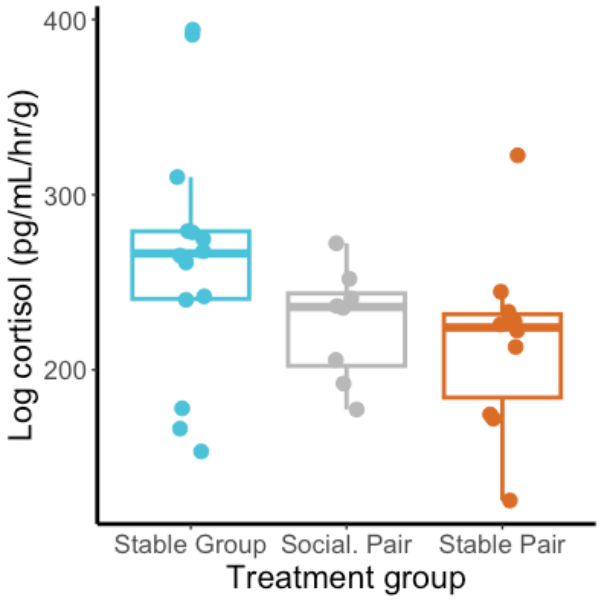


Figure 4: Focal fish water-borne cortisol (pg/mL/hr), corrected for body mass (g). Log transformed data are shown.

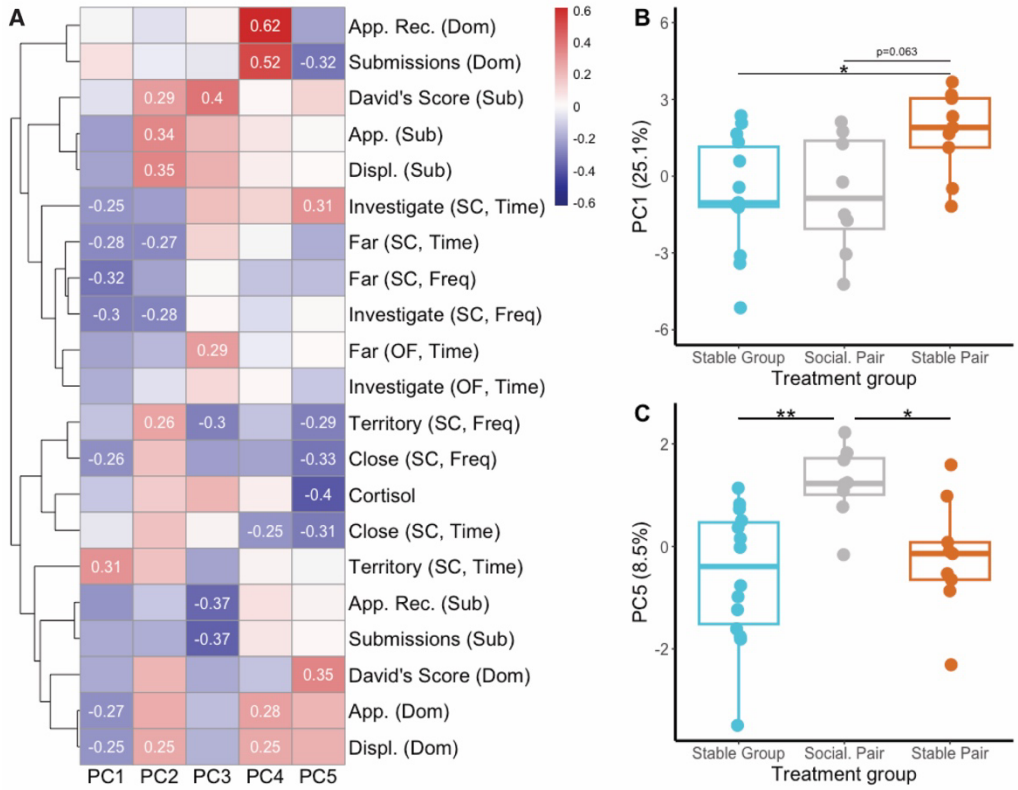


Figure 5: Principal components analysis (PCA) of cortisol and behavior—including open field (OF) time in the far and investigate zones; social cue (SC) time in and frequency entering the territory, close, far, and investigate zones; and dominant and subordinate behavior assay approaches (App.), displacements (Displ.), approaches received (App. Rec.), submissions, and David's Score. A) A heatmap of eigenvalues showing the PCA variables that load on PC1 (25.1%), PC2 (18.2%), PC3 (14.3%), PC4 (10.5%), and PC5 (8.5%). Numerical values are shown for variables stronger than ±0.25. Rows are hierarchically clustered. B) Treatment differences in PC1. C) Treatment differences in PC5. Percentages refer to the amount of variance explained by that PC. *p<0.05. **p<0.01.