Multiple behavioural mechanisms shape development in a highly social cichlid fish

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ABSTRACT

Early-life social experiences shape adult phenotype, yet the underlying behavioural mechanisms remain poorly understood. We manipulated early-life social experience in the highly social African cichlid fish *Astatotilapia burtoni* to investigate the effects on behaviour 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 differed in group size (groups vs. pairs) and stability (stable vs. socialized). We then measured behaviour in multiple contexts and collected water-borne cortisol. We found effects of treatment on behaviour across all assays: open field exploration, social cue investigation, dominant behaviour, and subordinate behaviour. Cortisol did not differ across treatments. Principal components (PC) analysis revealed robust co-variation of behaviour across contexts, including with cortisol, to form behavioural 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. Behaviour observations in the home tanks provided further insights into the behavioural 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: Behavioural syndrome; 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 (Kohn, 2019). 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 behavioural mechanisms—is critical to understanding how gene-by-environment interactions shape processes of developmental plasticity and behavioural developmental trajectories. Given the fitness and health consequences of social behaviour (Bennett et al., 2006; Meyer-Lindenberg & Tost, 2012; Silk, 2007; Solomon-Lane et al., 2015; Wilson, 1980), the developmental origins of adult behavioural phenotype are particularly important to understand, including as a target for natural selection.

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

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

115 compared to biparental rearing (Banerjee et al., 2012). In *N. pulcher* cichlids, rearing with or
116 without parents affected *gr1* expression in the telencephalon (Nyman et al., 2018), and rearing
117 with or without brood helpers altered neural *crf* and *gr* expression, as well as the ratio of *mr* to
118 *gr1* (Taborsky et al., 2013). The HPA/I axis is also an important source of individual variation in
119 social behaviour (Boogert et al., 2014; Dettmer et al., 2017; Farine et al., 2015; Freeman et al.,
120 2021; Pryce et al., 2011; Reyes-Contreras et al., 2019; Sih, 2011) and health (e.g., Turecki &
121 Meaney, 2016).

122 In this study, we used Burton's Mouthbrooder (*Astatotilapia burtoni*), a highly social
123 cichlid fish and model system in social neuroscience (Fernald & Maruska, 2012; Hofmann,
124 2003; Stevenson et al., 2018), to investigate the behavioural mechanisms through which early-
125 life social experiences affect behaviour and HPI axis function. The vast majority of research on
126 this species has focused on adults, which form mixed-sex, hierarchical social communities.
127 Dominant males are colourful, territorial, and reproductively active. In contrast, subordinate
128 males are drab in coloration, shoal with females, and are reproductively suppressed. Social status
129 is socially-regulated, and males regularly transition between dominant and subordinate positions
130 (Fernald & Maruska, 2012; Hofmann, 2003). Juveniles also form status relationships (Solomon-
131 Lane et al., 2022), and both juveniles and adults express a suite of highly-conserved social
132 behaviours (Fernald & Hirata, 1979; Fernald & Maruska, 2012; Solomon-Lane et al., 2022;
133 Weitekamp & Hofmann, 2017). Although sex and social status have strong effects on adult
134 behaviour, individual variation persists, including in the frequency and quality of behavioural
135 expression, such as aggression, territoriality, courtship, cooperation, reproductive behaviour, and
136 maternal behaviour (Alward et al., 2021; Friesen et al., 2022; Fulmer et al., 2017; Kidd et al.,
137 2013; Maruska, Becker, et al., 2013; Renn et al., 2009; Weitekamp & Hofmann, 2017); tenure in

138 a dominant vs. subordinate role (Hofmann et al., 1999); female mate choice (Kidd et al., 2013);
139 social learning (Rodriguez-Santiago et al., 2020); and cognition (Wallace & Hofmann, 2021).
140 HPI axis function also varies among adults (Alcazar et al., 2016; Chen & Fernald, 2008; Clement
141 et al., 2005; Dijkstra et al., 2017; Greenwood et al., 2003; Korzan et al., 2014; Maruska et al.,
142 2022) and is highly responsive to social context, such as social status and changes in status
143 (Carpenter et al., 2014; Chen & Fernald, 2008; Clement et al., 2005; Fox et al., 1997; Huffman et
144 al., 2015; Korzan et al., 2014; Maruska et al., 2022; Maruska, Zhang, et al., 2013; Parikh et al.,
145 2006), group social dynamics (Maguire et al., 2021), social habituation (Weitekamp & Hofmann,
146 2017), and in response to an intruder (Alcazar et al., 2016; Weitekamp et al., 2017).
147 Developmental plasticity may be an important source of individual variation in behaviour
148 and HPI axis function among adults (Fernald & Hirata, 1979; Fraley & Fernald, 1982; Solomon-
149 Lane & Hofmann, 2019). We have previously demonstrated early-life social effects in juvenile
150 *A. burtoni* in response to early-life social group size. Juveniles reared in social groups (16 fish)
151 developed to be more active, more socially interactive, and less submissive in a subordinate role
152 compared to pair-reared juveniles. Whole brain gene expression related to HPI axis function was
153 also altered, including elevated GR1a expression in group-reared animals and tight co-expression
154 of candidate genes (including GR1a, GR1b, GR2, MR, and CRF) in pair-reared, but not group-
155 reared or isolated, juveniles (Solomon-Lane & Hofmann, 2019). Juveniles in the previous study
156 were not observed in the rearing environment, and there are multiple, possible behavioural
157 mechanisms responsible for these effects (Solomon-Lane & Hofmann, 2019; Taborsky, 2016).
158 Individuals reared in larger and/or more complex groups may interact socially at higher rates,
159 experience a greater diversity of types of social interaction (e.g., affiliative, aggressive,
160 cooperative, etc.), gain experience in multiple social roles (e.g., dominant and subordinate,

161 younger and older) (Chase et al., 2022; Solomon-Lane et al., 2022; Williamson et al., 2016),
162 have more social partners, have social partners of varied identities / traits (e.g., age, body size,
163 life history stage diversity) (Arnold & Taborsky, 2010; Bölting & von Engelhardt, 2017; Branchi
164 & Alleva, 2006; Taborsky et al., 2012; White et al., 2010), experience interactions involving
165 more actors simultaneously (i.e., 3-way, or more, interactions), and learn indirectly by watching
166 others (e.g., Desjardins et al., 2012; Oliveira et al., 1998)). For juvenile *A. burtoni*, group size
167 and the relative body sizes of individuals affects social behaviour and group structure. For
168 example, pairs and triads containing same-sized fish are less hierarchical (Solomon-Lane et al.,
169 2022).

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

184 adults (Maguire et al., 2021). Direct manipulations of social partner numbers can also affect
185 social behavioural development. For example, juvenile male brown-headed cowbirds reared in
186 dynamic flocks outcompeted males reared in stable flocks of the same size (White et al., 2010).
187 To manipulate the number of social partners, we reared juveniles in stable pairs (2 fish, 1
188 partner each), in stable groups (6 fish, 5 partners each), or in socialized pairs, in which one
189 member of the pair was changed 5 times over the course of the ~1 month experiment (2 fish at a
190 time in the pair, 5 total partners each) (Figure 1). This design also allowed us to compare the
191 effects of group size (pair vs. group) and social stability (stable vs. socialized). We observed
192 social interactions in the different rearing environments to quantify social experience and then
193 tested the effects on individual social behaviour through a series of behaviour assays. We
194 hypothesized that the number of social partners would be the strongest influence on behavioural
195 phenotype. If the number of early-life social partners was an important behavioural mechanism
196 underlying group- vs. pair-reared development in Solomon-Lane & Hofmann (2019), then in this
197 study, we predict that juveniles from the stable groups and socialized pairs will be more active
198 and socially interactive in novel social context, as well as less submissive in a subordinate role.
199 To test the hypothesis that early-life social experience affects HPI axis signalling, we collected
200 water-borne cortisol following the individual behaviour assays. By investigating specific
201 behavioural mechanisms underlying behavioural development and neuroendocrine function, this
202 research can provide insights into developmental plasticity processes and uncover the origins of
203 adult phenotype.
204
205 **METHODS**
206 *Animals*

207 The juvenile *A. burtoni* used in this experiment were bred in the laboratory. The breeding
208 adults are a laboratory population that descended ~65-70 generations from a wild caught stock
209 from Lake Tanganyika (Fernald & Hirata, 1977). The adults are housed in naturalistic, mixed-
210 sex breeding communities. Dominant males court females to lay eggs in his territory, after which
211 she immediately scoops up the eggs into her mouth, where the male fertilizes them. The
212 developing larvae are brooded in the female's buccal cavity for ~14 days before being released.
213 In the wild, and under some laboratory conditions, females display maternal behaviour by
214 guarding their offspring for 10 days or more after initially releasing the free-swimming fry from
215 her mouth (Renn et al., 2009).
216 In this study, 86 fry were removed from the buccal cavities of 7 mothers approximately
217 5-7 days after fertilization. We selected fry at this early developmental stage before overt social
218 interactions occurred (Fraley & Fernald, 1982) to ensure that the scope of our experiment
219 captured meaningful social experience as early as possible during development. Individual
220 broods were then placed in shallow water in a petri dish, and a digital image was taken with a
221 ruler for scale. ImageJ was used to measure standard length (SL, mm) (Schneider et al., 2012),
222 from the tip of the jaw to the caudal peduncle. Broods ranged in size from 4-21 fry (mean: 12.43
223 ± 2.84 fry, median: 15), and fry size ranged from 4.59-8.13 mm SL (mean: 5.96 ± 0.095 mm,
224 median: 5.81 mm). See Supplemental Table 1 for mean, median, minimum, and maximum SL of
225 fry per brood. All 7 broods were then placed briefly (~30 min) in a common bucket to intermix.
226 Given their developmental stage, social interactions were highly unlikely to occur, but visual,
227 chemosensory, and tactile social sensory cues were likely exchanged. We used a hand net to
228 remove individuals from the bucket and haphazardly assigned them to different home tanks and
229 treatment groups (stable groups, socialized pairs, or stable pairs; see below for descriptions).

230 Methylene blue was added to the water to reduce the incidence of fungal infection during this
231 time. After 7 days, we confirmed visually that the fry were all mobile and their yolks had been
232 fully absorbed. The sex ratio of fish in this experiment is not known because sex cannot be
233 determined anatomically until reproductive maturation. The sex ratio of *A. burtoni* broods (a
234 laboratory population descended from wild-caught fish) is approximately 1:1 (Heule et al.,
235 2014).
236
237 *Early-life social experiences*
238 Fry were reared for 32 or 35 days (depending on the behavioural test day, see below) in
239 one of three conditions to manipulate early-life social experience: stable groups of 6 fish (n=6
240 groups), stable pairs of 2 fish (n=7 pairs), and socialized pairs of 2 fish (n=8 pairs) (Figure 1).
241 These treatment groups were designed to manipulate the number of early-life social partners. In
242 the stable groups, every individual had 5 social partners. In the stable pairs, every individual had
243 1 social partner. In the socialized pairs, 1 individual was removed from the tank using a hand net
244 every 5 days and was replaced with a novel partner. The fish that was removed was transferred to
245 another socialized pair tank with a novel partner. The social exchanges began on experimental
246 day 12 (all fry were sufficiently mature on day 7, followed by 5 days with the first social
247 partner). After a total of 4 exchanges, each of the fish in the socialized pairs were exposed to a
248 total of 5 social partners, the same number of partners that each individual experienced in the
249 stable groups. However, the size of the social group for socialized pairs was always 2 fish, the
250 same as in the stable pairs. As a control, on the day that fish in the socialized pairs were
251 transferred, we used a hand net to remove one fish from each of the stable pairs and stable

252 groups. That fish was immediately returned to the same home tank so that group membership in
253 the stable pairs and stable groups was consistent.
254 Stable and socialized pairs were housed in small acrylic tanks (23 × 15 × 15 cm), while
255 the stable groups were housed in larger acrylic tanks (30 x 20 x 17 cm). One small terracotta pot
256 (5 cm at the widest diameter) was placed in each tank, and an air stone was used to keep the
257 water oxygenated. There was no substrate on the bottom of the tank. White plastic dividers were
258 placed outside of the tanks to prevent visual contact between tanks. The fish were maintained on
259 a 12:12 light:dark schedule at 24°C, and they were fed Hikari Middle Larval Stage Plankton
260 (Pentair Aquatic Eco-Systems, Cary, NC) once a day. We mixed 0.015 g of fish food in 200 mL
261 of water. The mixture was actively stirred, and a transfer pipette was used to add 1 mL to the
262 pairs and 3 mL to the groups.
263
264 *Tagging individual fish*
265 To track individuals as they developed over the course of the experiment, we tagged
266 every fish following the first rotation in the socialized pairs. We waited until this time so the fish
267 would be slightly bigger, as their very small size is a challenge for existing tagging methods
268 (Lotrich & Meredith, 1974; Solomon-Lane & Hofmann, 2018; Thresher & Gronell, 1978). We
269 first lightly anesthetized each fry in 0.03 g MS-222 / L aquarium water (buffered with sodium
270 bicarbonate to pH 7.0 –7.5). They were then removed from the water and placed on a wet paper
271 towel. We placed a small amount of Alcian Blue (Fisher Scientific, Pittsburgh, PA) powder onto
272 the dorsal muscle of the fish and perforated the surface of the fish using a pulled capillary tube
273 micropipette. This method of tattooing allowed the ink to seep into the tissue of the fish. We
274 used different tag locations (left or right; anterior, middle, or posterior regions) on the dorsal

275 muscle to differentiate individuals in the same tank. In the socialized pairs, tagging was
276 sufficient in most tanks to identify and move the same individual each time. The persistence of
277 the tag varied, and in the best cases, it remained visible for 3 weeks. If the tags were not visible
278 in the socialized pair, the smaller individual was moved.
279
280 *Survival and number of early-life social partners*
281 The tagging procedure had an 85% survival rate. Fish that did not survive tagging or that
282 died at another point in the experiment were replaced with a fish of similar size and age, selected
283 from a community tank. The replacement fish were not included in the individual behaviour
284 assays conducted at the end of the experimental rearing period (see below). We tracked the exact
285 number of social partners for each experimental tank. All fish in the socialized pairs had 5 social
286 partners. Stable group fish had an average of 6.43 ± 0.14 social partners. Stable pair fish had an
287 average of 1.2 ± 0.13 social partners. In our analyses, we consider the treatment groups as
288 categorical, although there is limited variation in the number of social partners (Supplemental
289 Figure 1).
290
291 *Behavioural Observations*
292 Home tanks: We recorded social behaviour in the home tanks twice during the rearing period, on
293 days 20 and 25 of the experiment, at the same time of day. Cameras (Warrior 4.0, Security
294 Camera Warehouse, Asheville, NC) were positioned above the tanks, and with the tank lids
295 removed, all areas of the tank were visible except under the terracotta pot. Using Solomon Coder
296 (www.solomoncoder.com), 10 minutes of video was scored for approaches and displacements.
297 Approaches were counted anytime one fish swam directly towards any part of another fish,

298 within 3 body lengths. Approaches can range from affiliative to aggressive, and the response of
299 the approached fish provides insight into the degree of agonism. If the approached fish swam
300 away in any direction, it was scored as a displacement, which is an indication that the approach
301 was aggressive. Because the identification tags were not visible on the video, we counted the
302 total number of social interactions (approaches, displacements) for the group or pair. We also
303 quantified the number of times a fish entered the terracotta pot territory, defined as at least 50%
304 of a fish's body crossing into / under the pot. Exiting the territory was highly correlated with
305 entering the territory ($p<0.0001$, $r^2=0.97$); therefore, we only present data for entering. To
306 measure social grouping, we captured screenshots from the video every 30 s for a total of 20 time
307 points. Distance was measured between every dyad, from a focal point in the centre of the head,
308 using ImageJ (Schneider et al., 2012). The researchers scoring these behaviours were unaware of
309 the treatment of the pairs (socialized vs. stable), but the stable groups were easily recognizable.
310 For behaviour and distance, we analysed the raw values (behaviours per minute and distance), as
311 well as scaled these values to account for differences in group size (behaviours per minute
312 divided by 2 for the pairs and divided by 6 for the groups) and tank size (distance divided by the
313 hypotenuse of the tank) among treatments.
314
315 Individual Behaviour Assays: After the 32 or 35-day rearing period, we measured individual
316 behaviour in a series of assays, including open field exploration, social cue investigation,
317 dominant behaviour, and subordinate behaviour. Every fish in the socialized (final sample size:
318 n=9) and stable pairs (final sample size: n=10) was included in the individual assays. Three of
319 the 6 fish from each stable group were selected randomly to be included in the individual assays
320 using a random number generator (final sample size: n=14). The procedures have been described

321 previously in detail (Solomon-Lane & Hofmann, 2019). Briefly, these assays (or similar) are
322 used across species to assess different elements of behavioural phenotype, including behaviours
323 affected by early-life social experience (Sih, 2011; Sih et al., 2015; Solomon-Lane & Hofmann,
324 2019; Taborsky, 2016). The open field exploration is used to assess locomotion and anxiety-like
325 behaviours (e.g., Cachat et al., 2010; Prut & Belzung, 2003). The social cue investigation is used
326 as a measure of social motivation or preference (e.g., Bonuti & Morato, 2018; Moy et al., 2004).
327 The dominant and subordinate behaviour assays are used to assess how the focal fish behaves
328 when in the position to be dominant or subordinate. In social groups, including for juvenile *A.*
329 *burtoni* (Solomon-Lane et al., 2022), most individuals will be subordinate to some group
330 members and dominant over others at a given time (e.g., Chase et al., 2022). Social status also
331 changes over a lifetime (Fernald & Maruska, 2012; Hofmann, 2003).

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

removing the small fish, a free-swimming novel fish larger than the focal was added to assess subordinate behaviour (minutes 2-12 were analysed). In the dominant and subordinate behaviour assays, we scored approaches and displacements. The researchers scoring these behaviours were unaware of the treatment of the focal fish.

From the approaches and displacements in the dominant and subordinate behaviour assays, we calculated two additional social measures using the *compete* package in R (Curley, 2016). First, we calculated an adjusted version of David's Score, which is a dominance index. Rather than categorizing all social interactions as a one-dimensional "win" or "loss," we incorporated both approaches and displacements, such that the agonistic outcome of individual i in interaction with another individual j is the number of times that i displaces j, divided by the total number of interactions (approaches) between i and j (i.e., i displaces j / (i approaches j + j approaches i)). The rest of the calculations were done as described in Gammell et al. (2003). See the Supplemental Information in Solomon-Lane et al. (2022) for the R code. Second, we calculated directional consistency index (DCI) to assess whether patterns of approaching in the dyads are reciprocal, from perfectly reciprocal (0) to unidirectional (1). We also used the *compete* package to run a randomization test to determine if the directionality was significantly more asymmetrical (unidirectional) than expected by chance (Leiva et al., 2008).

Water-borne cortisol

Following the individual behaviour assays, we collected water-borne hormone samples. This is a non-invasive method of quantifying steroid hormones, and values strongly correlate with circulating hormone levels (Kidd et al., 2010). We chose cortisol, as HPI axis function is often affected by early-life experience (e.g., Champagne & Curley, 2005; Taborsky et al., 2013)

367 and cortisol can correlate with behaviour and behavioural syndromes (e.g., Koolhaas et al.,
368 1999). Focal fish were placed individually in clean glass vials filled with 15 mL of clean
369 aquarium water for 2 hrs. Visual barriers were placed around the vials to limit disturbances to the
370 fish during the collection. After two hours, fish were removed from the vial using a hand net
371 rinsed in clean aquarium water, and samples were frozen at 4°C until processing. Hormones were
372 extracted from the water samples using 3 cc Sep-Pak Vac C18 columns (Water Associates,
373 Milford, MA). Columns were first primed by passing 2 mL methanol through the columns twice,
374 being careful not to let the columns run dry. This was followed by running 2 mL ultrapure water
375 through the columns twice. The thawed samples were then passed through the columns from the
376 sample vials using the vacuum pump. Ultrapure water (2 mL twice) was then passed through the
377 columns again, followed by 5 min of the vacuum to dry the columns. Finally, the hormone
378 samples were eluted into 13 x 100 borosilicate test tubes by passing 2 mL methanol through the
379 columns twice. The vacuum was used again to dry the columns. The sample in methanol was
380 then evaporated under a gentle stream of nitrogen at 37°C and resuspended to a volume of 200
381 µL per sample (5% EtOH and 95% ELISA buffer). Resuspended samples were shaken on a
382 multitube vortex for 1 hr before being stored at -20°C. Before measuring cortisol, samples were
383 thawed and shaken again on the multitube vortex for 1 hr. ELISAs were completed according to
384 the supplier's instructions (Cayman Chemical, Ann Arbor, Michigan).
385 Cortisol data are presented here as pg hormone per mL sample volume per hr sample
386 collection per g body weight. During hormone collection, fish excrete hormones into the water
387 through their gills, urine, and faeces. The gills of larger fish have a larger surface area compared
388 to smaller fish, thus body size can influence water-borne hormone levels. Fish size was measured
389 following the collection of water-borne hormones. Mass was measured on an analytical balance,

390 and SL was measured using ImageJ (Schneider et al., 2012) from digital images with a rule for
391 scale. We calculated the condition index as Fulton's *K*, which estimates expected mass as SL^3.
392 We used the mass and SL of 400 laboratory *A. burtoni* across a range of ages and sizes to
393 empirically calculate expected mass as: $mass=0.00002*SL^{3.02}$ (Stevenson & Woods, 2006).
394
395 *Ethical Note*
396 All research was done in compliance with the Institutional Animal Care and use Committee
397 (IACUC Protocol # 19-001). We used non-invasive approaches when possible, such as collecting
398 water-borne hormones, and we used a tested and effective anaesthetic (MS-222) for tagging to
399 minimize stress and pain. We took steps to minimize stress throughout the experiment, including
400 limiting handling time to less than 2 min when measuring size or transferring between tanks. A
401 total of 76 juvenile *A. burtoni* were included in this study, and fish were returned to community
402 tanks at the end of the experiment.
403
404 *Statistical Analyses*
405 All statistical analyses were conducted using R Studio (R version 4.2.1) (RStudio Team,
406 2022). Results were considered significant at the $p<0.05$ level, and averages ± standard error of
407 the mean are included in the text. The boxes of the box and whisker plots show the median and
408 the first and third quartiles. The whiskers extend to the largest and smallest observations within
409 or equal to 1.5 times the interquartile range. To test for the effects of early-life social experience,
410 we compared body size and condition index; behaviour in the home tank rearing environments
411 (total behaviours per minute and behaviours per fish per minute); spatial distancing in the home
412 tank rearing environments (raw distance and scaled to account for the different sized aquaria for

413 pairs vs. groups); behaviour in the open field exploration and social cue investigation (frequency
414 entering and time in the territory, close, far, and investigate zones); social behaviour
415 (approaches, displacements, approaches received, submissions) and status (David's score, DCI)
416 in the dominant and subordinate assays; cortisol; and principal components (see below, PCs 1-5)
417 across fish reared in the stable groups, socialized pairs, or stable pairs. For data that met the
418 assumptions of parametric statistics, we used one-way ANOVAs. The cortisol data were log
419 transformed first to meet the assumption of normal distribution. *Post hoc* analysis of significant
420 ANOVA results was calculated using Tukey HSD tests. For data that did not meet the
421 assumptions of parametric statistics, we used non-parametric Kruskal-Wallis tests, and Dunn's
422 tests with Bonferroni correction were used for *post hoc* analysis of significant results. Eta
423 squared is reported for the effect size of both the one-way ANVOAs and Kruskal-Wallis tests
424 (small effect: $0 < \eta 2 < 0.01$; moderate: $0.01 < \eta 2 < 0.06$; large: $0.06 < \eta 2$). We used Principal
425 Components Analysis (PCA) to identify how behaviour in the open field exploration, social cue
426 investigation, dominance behaviour assay, subordinate behaviour assay, and cortisol clustered.
427

428 **RESULTS**

429 *Body size and condition*

430 To determine whether early-life experience in a stable group, socialized pair, or stable
431 pair affected body size and condition, we compared SL, mass, and condition index across
432 treatment groups. We found that mass differed significantly across treatment groups (χ^2_2=6.39,
433 p=0.041, η^2=0.15; Supplemental Figure 2A). *Post hoc* analysis showed juveniles reared in stable
434 pairs were significantly heavier than those from stable groups (p=0.036). There were no
435 significant differences in mass between stable groups and socialized pairs (p=0.63) or between

436 stable pairs and socialized pairs (p=0.82). There were no significant treatment differences in SL
437 ($F_{2,30}$=2.15, p=0.14; Supplemental Figure 2B). Because we found an effect of rearing experience
438 on mass, but not SL, we also asked whether condition index was affected by treatment. We
439 found no significant treatment differences in condition index ($F_{2,30}$=0.45, p=0.62, Supplemental
440 Figure 2C). Mass was also positively correlated with water-borne cortisol levels (p=0.017,
441 r^2=0.15, Supplemental Figure 2D); therefore, we corrected for body size in the cortisol analyses
442 below.
443
444 *Home tank social behaviour*
445 We observed juveniles in the stable groups, socialized pairs, and stable pairs to
446 understand the differences in social experience and environment among the rearing treatments.
447 Unsurprisingly, there were significantly more approaches and displacements in groups compared
448 to pairs (both stable and socialized), and there were significantly more entrances to the territory
449 in groups compared to stable pairs (Figure 2A). There were no differences in efficiency (Figure
450 2B). There were also no differences in rates of behavioural interactions per fish. Mean dyad
451 distance was significantly larger in groups than pairs (Figure 2C), but interestingly, the minimum
452 dyad distance was significantly smaller in groups than socialized pairs (Figure 2D). There were
453 no treatment differences in mean dyad distance when scaled for tank size. Statistics are reported
454 in Table 1 and Supplemental Table 2.
455
456 *Open field exploration and social cue investigation*
457 For the open field exploration and social cue investigation, we compared across treatment
458 groups for the frequency of entering each zone of the tank, as well as time spent in each zone.

Statistics are reported in Table 2 (Supplemental Figure 3). Overall, we found for the open field exploration that fish from the socialized pairs entered the territory zone significantly more frequently than stable pair fish, and fish from the stable groups spent significantly more time in the far zone than stable pair fish. In the social cue investigation, we found that social group fish entered the far zone significantly more frequently and spent significantly more time in the territory and far zones, than fish reared in stable pairs.

Dominance and Subordinate Assays

To determine whether rearing experience affected social behaviour and status, we quantified patterns of social interaction between the focal fish and a novel social partner (cue fish). Because relative physical size often affects dominant and subordinate social dynamics for juveniles (Solomon-Lane et al., 2022), pairing the focal fish with a smaller fish (dominance assay) presented the focal fish with the opportunity to express social behaviours as the dominant. In pairing the focal fish with a larger fish (subordinate assay), the focal fish can express social behaviours as the subordinate. We found significant differences in social behaviour and status in both the dominance and subordinate assays (statistics reported in Table 3, Figure 3). In the dominance assay, socialized pair fish approached and displaced significantly more than stable pair fish, and their David's Scores were significantly higher than both stable pair and stable group fish. In the subordinate assay, socialized pair fish received significantly more approaches than stable pair fish from the larger cue fish with which they were paired. Socialized pairs with their cue fish also had significantly lower directional consistency than stable group or stable pair fish.

482 *Cortisol*

483 Following the behaviour assays, we collected water-borne cortisol to determine whether
484 hormone levels were affected by rearing experience and/or associated with behavioural
485 phenotype (see PCA below). We found there were no significant differences in cortisol across
486 treatments ($F_{2,29}$=2.30, p=0.12, Figure 4).

487

488 *Integrative analysis of behaviour, social status, and cortisol*

489 Given the treatment differences we identified across the open field exploration, social cue
490 investigation, dominance assay, and subordinate assay, we next used PCA to integrate across
491 assays. This allowed us to identify subsets of factors that together contribute to juvenile
492 phenotype and test if suites of factors—principal components (PCs)—differed significantly
493 across treatment groups. To determine which variables to include from the open field exploration
494 and social cue investigation (time and frequency in the territory, close, far, and investigate
495 zones), we ran an initial PCA with just these variables. Examining the vector plot for PC1 with
496 PC2 (Supplemental Figure 4), we found that nearly all of the open field exploration measures
497 were strongly aligned with the same measure in the social cue investigation (i.e., the vectors for
498 open field and social cue time in the territory zone are identical). The exceptions were for time
499 spent in the far and investigate zones. Therefore, to avoid unnecessary replication, we chose to
500 include all of the variables from the social cue investigation, in addition to time spent in the far
501 and investigate zones during the open field exploration. From the dominant and subordinate
502 assays, we included approaches, displacements, approaches received, submissions, and David's
503 score. Finally, we included cortisol.

504 Here, we focused on the first 5 PCs, which together explain 76.6% of the variation in the
505 data (Figure 5A). We found significant effects of rearing experience on PC1 (25.1%) and PC5
506 (8.48%). For PC1, stable pairs were significantly higher than stable groups, and there was a trend
507 for stable pairs to be higher than socialized pairs (p=0.063) (Figure 5B). Behaviours from the
508 dominant behaviour assay and the social cue investigation assay loaded strongly on PC1.
509 Approaches and displacements when in a dominant social role; time in and frequency entering
510 the far and investigate zones during the social cue investigation; and frequency entering the close
511 zone during the social cue investigation loaded strongly in the same direction. Time spent in the
512 territory zone during the social cue investigation loaded strongly in the opposite direction. For
513 PC5, socialized pairs were significantly higher than both stable groups and stable pairs (Figure
514 5C). David's Score from the dominance assay and time in the investigate zone during the social
515 cue investigation loaded strongly together in the same direction. In the opposite direction,
516 submissions as a dominant; cortisol; time and frequency in the close zone during the social cue
517 investigation; and the frequency entering the territory zone during the social cue investigation
518 loaded strongly together. Statistics for the PC1-PC5 treatment comparisons are in Table 4 (also
519 see Supplemental Figure 5).
520
521 **DISCUSSION**
522 We investigated the effects of early-life social experience, in the first month of life, on
523 social behaviour and neuroendocrine stress axis function in juvenile *A. burtoni*. We tested the
524 hypothesis that the number of novel social partners experienced during early-life is a behavioural
525 mechanism driving variation in development. In manipulating the number of social partners, our
526 experimental treatment groups—stable groups, socialized pairs, and stable pairs—also varied in

527 social group size (pairs vs. group) and group stability (socialized vs. stable). We present strong
528 evidence for early-life social effects on juvenile social behaviour, consistent with previous
529 studies in this species (Fernald & Hirata, 1979; Fraley & Fernald, 1982; Solomon-Lane &
530 Hofmann, 2019). In particular, our results support two behavioural mechanisms: the number of
531 social partners and social stability. Although early-life social effects are widely documented
532 across social species, the specific attributes of social experience that affect the mechanisms and
533 trajectory of developmental plasticity are not typically identified (Kasumovic, 2013; Taborsky,
534 2016). The social interactions and social spacing we observed in the home tanks also provide
535 insights into how social environments vary within and across treatments and may shape the
536 experiences individuals accrue during development. Causation at this level is needed because it
537 is these environmental elements that interact with genes dynamically over development to
538 influence plasticity and the emergence of adult phenotype (Kasumovic, 2013; Taborsky, 2016,
539 2017). Overall, it is likely that multiple behavioural mechanisms contribute and interact to shape
540 development (e.g., Branchi et al., 2013) and adult phenotype.

541 We found that manipulating early-life social experience affected behaviour across
542 multiple contexts, including the open field exploration, social cue investigation, dominant
543 behaviour assay, and subordinate behaviour assay. Principal components analysis revealed that
544 these effects were correlated across contexts. Principal component 1 (25.1%) and PC5 (8.5%),
545 which both differed significantly across treatment groups, had behaviours from multiple assays
546 that loaded strongly. These results support our previous finding that juvenile *A. burtoni*
547 behaviour can form a syndrome, and an individual's position along the syndrome continuum is
548 sensitive to early-life social experience (Solomon-Lane & Hofmann, 2019). A syndrome is a
549 population-level measure in which rank-order differences between individuals are correlated

550 across contexts and/or over time (Bell, 2007). Behavioural syndromes have been identified
551 across species and can indicate consistency in individual behaviour across contexts and/or over
552 time (Bell, 2007; Sih, Bell, & Johnson, 2004; Sih, Bell, Johnson, et al., 2004). The PC1
553 syndrome included approaches and displacements in the dominance assay loading strongly in the
554 same direction with time in and frequency entering the investigate and far zones, and frequency
555 entering the close zone, during the social cue investigation. Time in the territory zone during the
556 social cue investigation loaded in the opposite direction. This syndrome is highly similar to the
557 one we identified after rearing juvenile *A. burtoni* in pairs or social groups of 16 fish (Solomon-
558 Lane & Hofmann, 2019).

559 The treatment differences in PC1 are most consistent with an effect of the number of
560 social partners. Stable pairs had significantly higher PC1 values compared to stable groups, and
561 there was a strong trend to be higher than socialized pairs ($p=0.063$). There were no differences
562 between stable groups and socialized pairs. Juveniles that experienced more social partners
563 during early-life were more active during the social cue investigation, spent less time in the
564 territory in the presence of a social cue, and were more socially interactive in a dominant social
565 role. The direction of this effect is also the same as in our previous study: group-reared juveniles
566 were more active and socially interactive than pair-reared juveniles (Solomon-Lane & Hofmann,
567 2019). This suggests that social experiences resulting from more novel partners may be an
568 important behavioural mechanism underlying the effect of group size. Syndromes involving
569 activity and social interaction are common across species (e.g., Conrad et al., 2011; Näslund &
570 Johnsson, 2016) and may also be related to bold-shy and proactive-reactive behaviours (Bell,
571 2007; Conrad et al., 2011; Groothuis & Carere, 2005; Koolhaas et al., 1999; Sih, Bell, Johnson,
572 et al., 2004).

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

596 Lane et al., 2022). This suggests that, like adults (Maguire et al., 2021), social network position
597 and connectivity may vary considerably across individuals, with consequences for
598 developmental plasticity and behavioural development.

599 The treatment differences in PC5 (8.5%) support an effect of early-life social stability.
600 Socialized pairs had significantly higher PC5 values than stable groups and stable pairs, which
601 were not different from each other. David's score in the dominance behaviour assay loaded in the
602 same direction as time in the investigate zone during the social cue investigation. Submissions in
603 the dominance behaviour assay, frequency entering the territory zone in the social cue
604 investigation, time in and frequency entering the close zone in the social cue investigation, and
605 water-borne cortisol loaded together in the opposite direction. Socialized pairs were more
606 dominant in the dominance behaviour assay, spent less time in and near the territory zone, spent
607 more time in the investigate zone, and had lower cortisol levels than stable groups or pairs. This
608 suite of behaviours for PC5 shares multiple similarities with the PC1 syndrome, suggesting these
609 syndromes may not be independent of each other. Dominance behaviour is represented as high
610 rates of approaching and displacing on PC1 and as the opposing loadings of David's score
611 (indication of dominant status) and submissions (indication of subordinate status) on PC5.
612 Dominant juvenile *A. burtoni* approach and displace at significantly higher rates than
613 subordinates (Solomon-Lane et al., 2022). For PC5, being in or near the territory zone, with less
614 time in the investigate zone, was associated with low status when given the opportunity to be
615 dominant. This is mirrored on PC1 by the negative association between being in the territory
616 zone and approaching and displacing in the dominance behaviour assay. The relative dominance
617 of juveniles from the socialized pairs is also consistent with having significantly lower
618 directional consistency (i.e., more agonistically symmetrical) in the subordinate behaviour assay.

619 In our previous study, group-reared juveniles were less submissive in a subordinate role than
620 pair-reared juveniles (Solomon-Lane & Hofmann, 2019). It is possible this was not the case for
621 stable group juveniles in this study due to group size differences (6 fish vs. 16 fish).

622 The most striking difference for PC5 is the involvement of cortisol. We found no
623 treatment differences in water-borne cortisol levels, and PC5 was the only PC on which cortisol
624 loaded strongly. Higher cortisol was associated with lower social status, less exploratory
625 behaviour, and more time in the territory away from the novel cue fish. This suite of behaviours
626 with cortisol resembles previously identified syndromes (Réale et al., 2007), such as the pace-of-
627 life syndrome (Careau & Garland, 2012) and coping styles (Koolhaas et al., 1999). A "fast"
628 pace-of-life is associated with increased activity, exploration, boldness, and aggressiveness,
629 along with one or more traits from the slow-fast metabolic continuum (Careau & Garland, 2012).
630 Coping styles are an integrative phenotype in which a behavioural syndrome aligns with stress
631 physiology (Koolhaas et al., 1999). Coping styles have been observed across species (Alfonso et
632 al., 2019; Conrad et al., 2011; Øverli et al., 2007) and are sensitive to early-life effects (Sih,
633 2011). Behaviourally, proactive copers are more active, aggressive, and bold compared to
634 reactive copers. In response to stress, proactive copers have higher sympathetic reactivity and
635 lower HPA/I activity (Koolhaas et al., 1999). Whether socialized pairs (this study) or group-
636 reared juveniles (Solomon-Lane & Hofmann, 2019) exhibit a fast-pace-life or are proactive
637 copers are hypotheses that should be tested directly. The association between high cortisol and
638 low status is consistent with previous studies of juvenile *A. burtoni*, which showed higher whole
639 brain GR1a and GR1b expression, and lower GR2 and MR expression, in fish with higher
640 dominance scores (Solomon-Lane et al., 2022). Efficient negative feedback may be mediated, in
641 part, by GR1 expression, leading to lower cortisol levels (Solomon-Lane & Hofmann 2019). The

642 relationship between adult *A. burtoni* status and cortisol varies across studies and can be elevated
643 in subordinates (Maruska et al., 2022). Adult *A. burtoni* do not appear to form coping styles
644 (Butler et al., 2018), and this suite of behaviour is not always correlated with stress
645 responsiveness across species (e.g., Thomson et al., 2011). Overall, early-life exposure to social
646 complexity tends to benefit social competence and skills, whereas social instability tends to have
647 lasting, negative phenotypic effects, such as elevated HPA/I axis activity, weight loss, elevated
648 aggression, and decreased activity (Kohn, 2019). This suggests that exchanging members of the
649 pair in the socialized treatment may not have been perceived as instability, potentially because of
650 the predictable schedule (Kohn, 2019). An alternative explanation is that familiarity played a role
651 in the treatment differences between socialized pairs vs. stable groups and pairs. The dominant
652 and subordinate assays were highly similar to the way we socialized the pairs, and familiarity
653 with the assay could lead to appearing more active, interactive, and dominant. Future studies can
654 test these potential behavioural mechanisms directly.

655 We observed fish in their rearing environments to gain insights into the specific social
656 experiences and social sensory cues, or proximate behavioural mechanisms, responsible for
657 early-life social effects (Taborsky, 2016). Unsurprisingly rates of behaviour were higher in the
658 stable groups compared to the pairs. Rates of behaviour per fish did not differ across treatment
659 groups, and there were no differences in total agonistic efficiency. These data confirm that a
660 group (compared to pair) social environment has more opportunities for social experiences, such
661 as direct involvement in an interaction and observations of others interacting (Desjardins et al.,
662 2012; Oliveira et al., 1998). The mean distance between dyads was also significantly larger in
663 stable groups than the pairs, and although the tank was larger for groups, the smallest dyad
664 distance was significantly smaller in groups than social pairs. There were no differences across

treatments when scaled for the size of the tank. We were unable to identify and track individuals because the ink tags were not visible on video. As a result, we could not quantify individual social experience, social status, social network position, or spatial position, which we hypothesize are causally related to behavioural development and phenotype (Kasumovic, 2013; Taborsky, 2016). The evidence that social interactions are assortative within groups is overwhelming across species (e.g., Chase et al., 2022; Croft et al., 2005, 2009; Pike et al., 2008; Williamson et al., 2016), and juvenile *A. burtoni* form social status relationships and nuanced social structures (Solomon-Lane et al., 2022). Therefore, we expect social status experience and the degree of agonistic asymmetry across social partners to be particularly important for behavioural development. In addition to its role as an indicator of social relationships, spatial proximity may also affect social sensory cue perception and communication, for example, via mechanosensory and chemosensory cues (e.g., in adult *A. burtoni*, Butler & Maruska, 2016; Nikonov et al., 2017) that could be stronger for fish in closer proximity.

Overall, our work demonstrates that multiple behavioural mechanisms—the number of early-life social partners and social stability or familiarity—affect juvenile *A. burtoni* development and phenotype, including integrated behavioural and neuroendocrine traits. In addition to manipulating social experience directly, we observed juveniles in their rearing environments, which are necessary steps towards understanding the social experiences accrued during development and the mechanisms by which early experiences exert long-term and disproportionately strong effects on adult phenotype (Buist et al., 2013; Jonsson & Jonsson, 2014; Kasumovic, 2013; Taborsky, 2016, 2017). Although the simplistic social contexts we used in this study are unlikely to reflect the dynamics of f larger, more complex groups found in nature (Chase et al., 2003), we expect that these behavioural mechanisms, and potentially

688 others—acting additively or synergistically—will also be influential in more naturalistic
689 contexts. Testing the hypotheses we generated here will be key to uncovering the behavioural
690 mechanisms of developmental plasticity and phenotype, as well as the role of neuroendocrine
691 stress axis function, in *A. burtoni* and other social species.
692

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699

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701

702 **DATA AVAILABILITY:** Data will be made available in an online repository (Dryad).

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TABLES

Table 1: Treatment differences in social behaviour and distances between dyads in the stable group, socialized pair, and stable pair home tanks.

Behaviour / 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

Results of one-way ANOVAs (F) or nonparametric Kruskal-Wallis tests (χ^2). Eta-squared is reported for effect sizes. Significant results in bold. Tukey HSD tests were used for *post hoc* analysis of significant ANOVA results. Dunn's tests were used for *post hoc* analysis of significant Kruskal-Wallis results.

1162 **Table 2:** Treatment differences in the frequency entering and time spent in tank zones during the

1163 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		

1164 Results of one-way ANOVAs (F) or nonparametric Kruskal-Wallis tests (χ^2). Eta-squared is

1165 reported for effect sizes. Significant results in bold. Tukey HSD tests were used for *post hoc*

1166 analysis of significant ANOVA results. Dunn's tests were used for *post hoc* analysis of

1167 significant Kruskal-Wallis results.

1168 **Table 3:** Treatment differences in social behaviour and status in the dominant and subordinate

1169 behaviour assays.

Assay / Behaviour	DF	χ^2	p-value	Effect size	*Post hoc* / direction of effect
Dominant Behaviour 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 Behaviour 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

1170 Results of Kruskal-Wallis tests (χ^2). Eta-squared is reported for effect sizes. Significant results in

1171 bold. Dunn's tests were used for *post hoc* analysis of significant results.

1172 **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

1173 Results of one-way ANOVAs (F) or nonparametric Kruskal-Wallis tests (χ^2) for PC1-PC5.

1174 Percentages indicate the variance explained. Eta-squared is reported for effect sizes. Significant

1175 results in bold. Tukey HSD tests were used for *post hoc* analysis of significant ANOVA results.

1176 Dunn's tests were used for *post hoc* analysis of significant Kruskal-Wallis results.

FIGURES

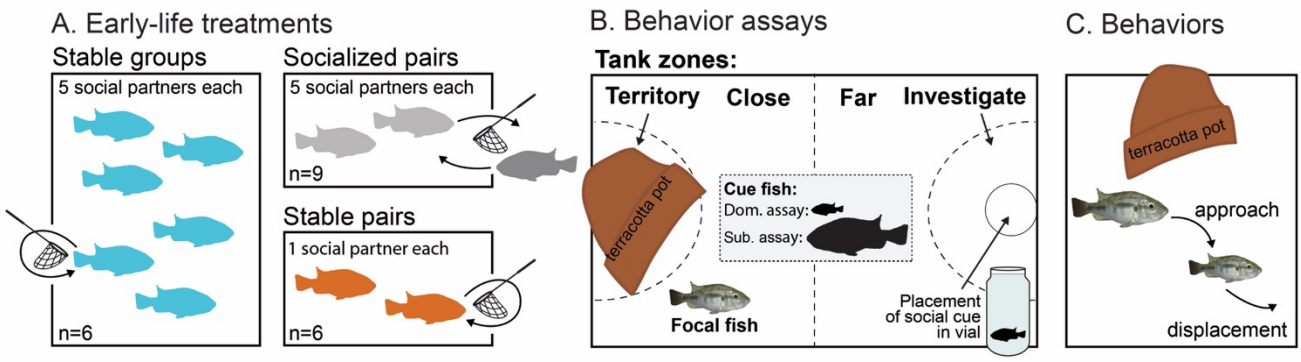

Figure 1: A) Juvenile fish were reared in stable groups (n=6, 6 fish each), stable pairs (n=6, 2 fish each), or socialized pairs (n=9, 2 fish each). Every 5 days in the socialized pairs, one fish of the pair was removed, and a novel juvenile was introduced. The novel juvenile came from a different socialized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

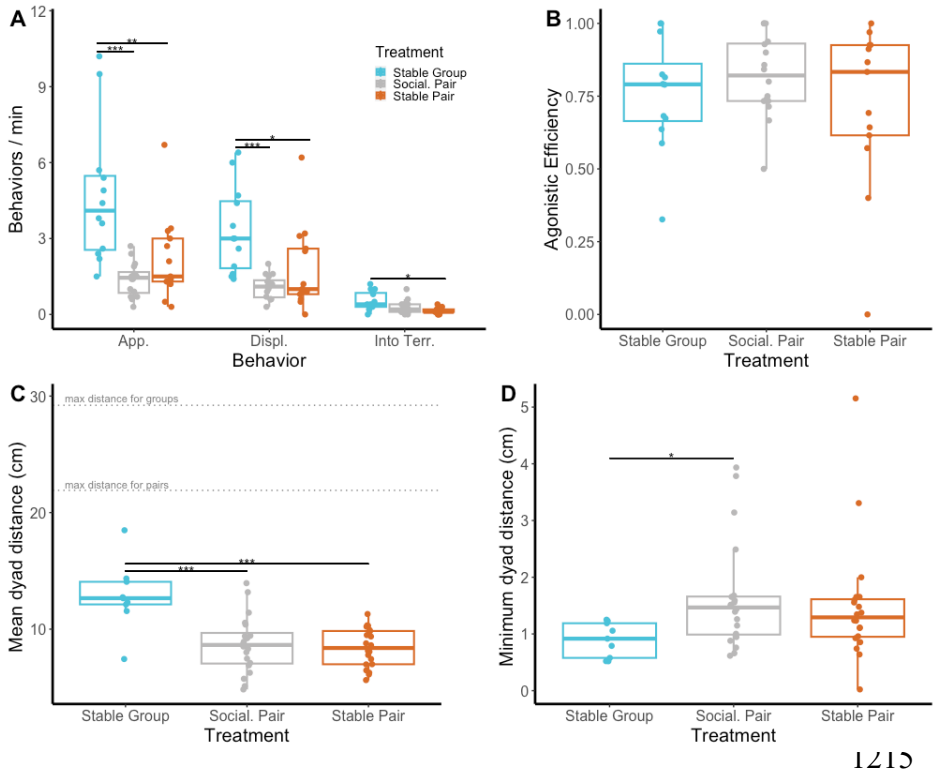

Figure 2: Social behaviour and distances in the stable groups, socialized pairs, and stable pairs. A) Frequency of social approaches, displacements, and times entering the territory / terracotta pot in the rearing environments. B) Total agonistic efficiency (total displacements / total approaches). C) 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. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

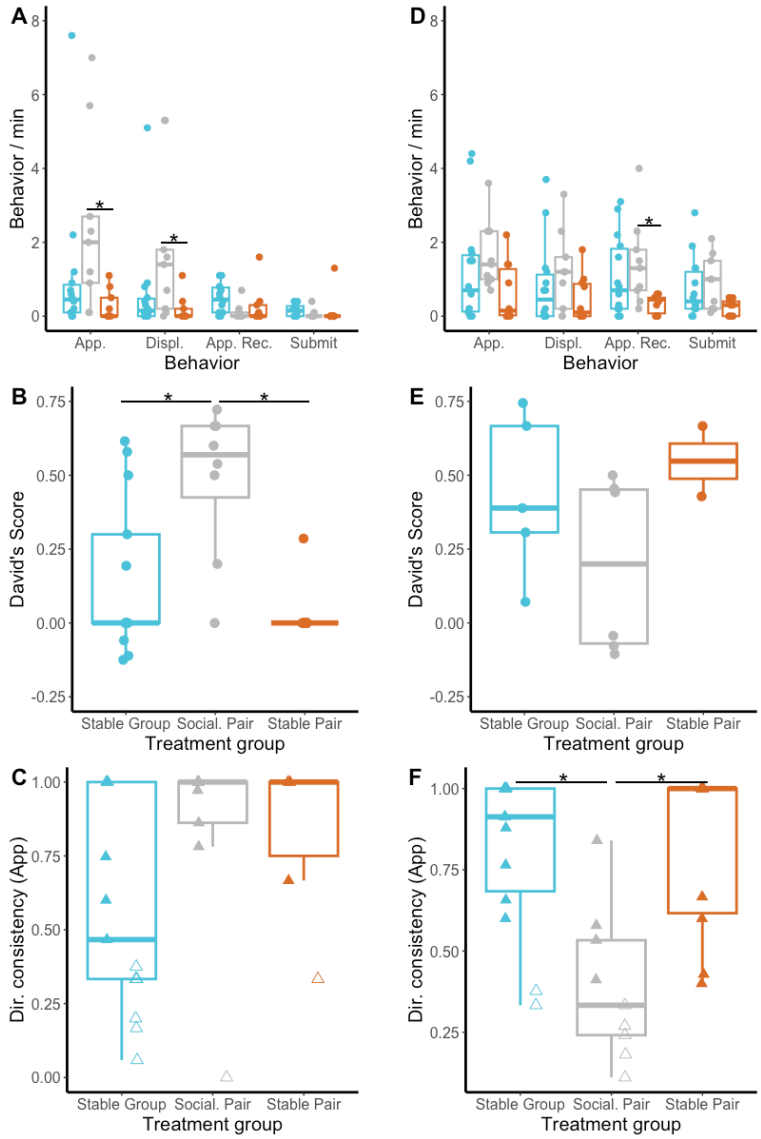

Figure 3: Social measures from the dominant (A-C) and subordinate (D-F) behaviour assays. A) Social approaches (App.), displacements (Displ.), approaches received (App. Rec.), and submissions (Submit) in the dominance behaviour assay. B) Focal fish David's score in the dominance behaviour assay. C) Directional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

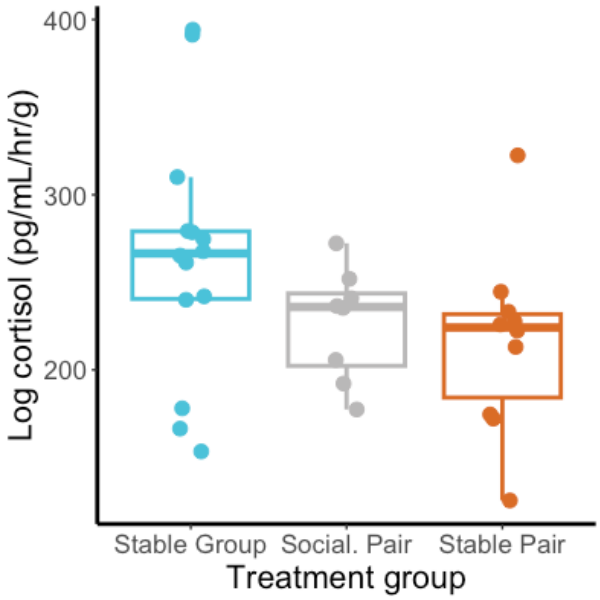

Figure 4: Focal fish water-borne cortisol (pg/mL/hr), corrected for body mass (g). Log transformed data are shown.

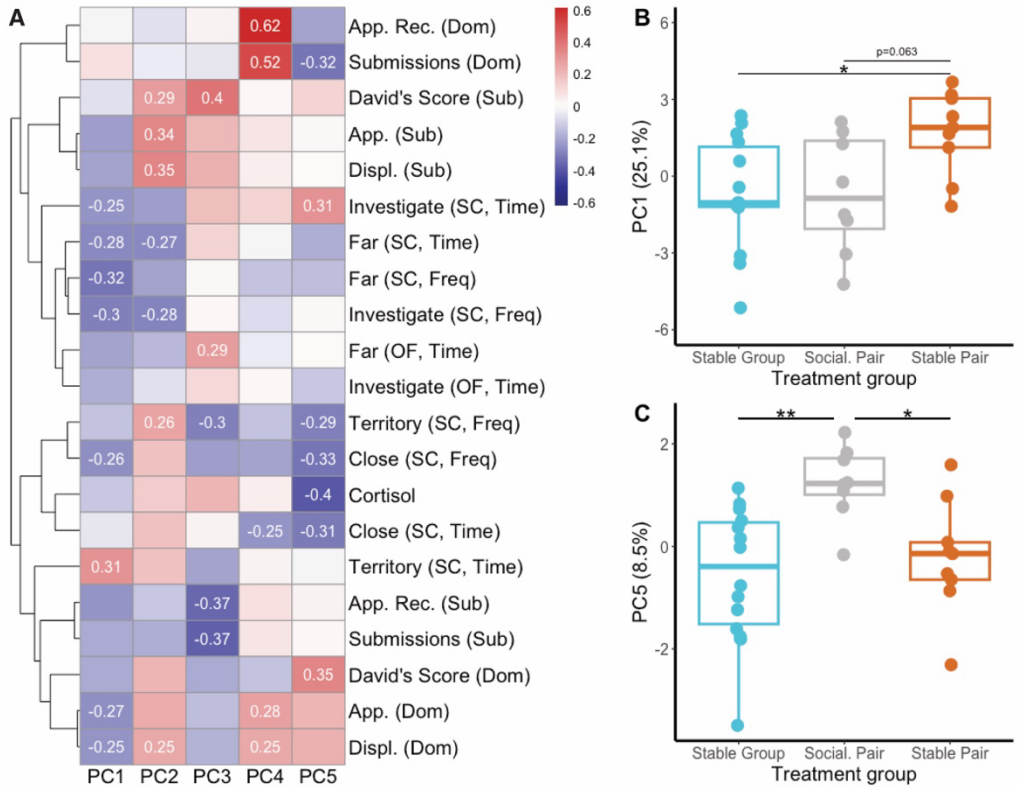

Figure 5: Principal components analysis (PCA) of cortisol and behaviour—including open field (OF) time in the far and investigate zones; social cue (SC) time in and frequency entering the territory, close, far, and investigate zones; and dominant and subordinate behaviour assay approaches (App.), displacements (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.