1   # Cascading indirect genetic effects in a clonal vertebrate

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11   **Short title:** Cascading IGE in Amazon mollies

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13   *Keywords*: exploratory behavior, gene x environment interaction, gynogens, *Poecilia formosa*,

14   social genetic effects, stress behavior

15

16   **Author contributions**

17   This study was conceived by AMM and KAH, data collected by AMM and CR, analyzed the data

18   by KAH, AMM and DB, and the manuscript was written by AMM and KAH.

19

20   **Data archiving**

21   All data will be archived in Figshare upon acceptance.

22

23   **Abstract**

24   Understanding how individual differences among organisms arise and how their effects

25   propagate through groups of interacting individuals are fundamental questions in biology.

26   Individual differences can arise from genetically-based variation in the conspecifics with which

27   an individual interacts, and these effects might then be propagated to other individuals. Using a

28   clonal species, the Amazon molly (*Poecilia formosa*), we test the hypothesis that such indirect

29   genetic effects (IGE) propagate beyond individuals that experience them firsthand. We tested

30   this hypothesis by exposing genetically identical Amazon mollies to social partners of different

31   genotypes, and then moving these individuals to new social groups in which they were the only

32   member to have experienced the IGE. We found that genetically different social environments

33   induced different levels of aggression experienced by the focal animals, and that these

34   genetically-based social effects carried over into new social groups to influence the behavior of

35   individuals that did not directly experience the previous social environments. Our data reveal

36   that IGE can cascade beyond the individuals that directly experience them to influence

37   phenotypes even when there is no genetically-based variation present within interacting groups.

38   Theoretical and empirical expansion of the quantitative genetic framework developed for IGE to

39   include cascading and other types of carry-over effects will improve understanding of social

40   behavior and its evolution.

41

42   **1. Introduction**

43   The environment an individual experiences includes its interactions with conspecifics,

44   and individual differences have long been known to influence these interactions [1, 2].

45   Understanding how these individual differences arise and how their effects propagate through

46   groups are fundamental questions in biology [2-4]. One cause of both individual variation and

47   propagation of effects in groups are indirect genetic effects (IGE) [4-7]. IGE arise when individual

48   phenotypes are influenced by genetically-based phenotypic differences in conspecific partners,

49   and they have been documented to affect behavioral, life history, and morphological traits in a

50   wide variety of taxa [e.g., 3, 8-17]. For example, behavior and body condition of mosquitofish is

51   influenced by genetically-based differences in body color of their social partners [18, 19], and

52   behavioral, physiological, and morphological traits in laboratory mice are influenced by

53   genotypes of their social partners [20]. Much of the empirical IGE literature focuses on how

54   partner genetic variation influences phenotypes of focal individuals. While understanding these

55   dyadic interactions is important, much less is known about IGE on group-level characteristics or

56   the degree to which IGE can propagate to affect phenotypes of individuals that do not

57   experience them firsthand. Because IGE can profoundly affect phenotypes, fitness, and the rate

58   and direction of evolution [21-23], understanding possible cascading or carry-over effects within

59   groups is necessary to understand phenotypic variation and evolution.

60   There have been two studies, to our knowledge, that have investigated IGE beyond those

61   caused by dyadic interactions. The first used fruit flies (*Drosophila melanogaster*), to measure

62   first-order IGE on male aggressive behavior (i.e., how the genotype of stimulus individuals

63   influences the phenotypes of individuals with which they interact) and second-order IGE (effects

64   of the stimulus genotypes on the interaction between two other members of the group) [24]. In

65   that study, Saltz showed that differences in aggressive behavior between two stimulus

66   genotypes had first-order effects on aggressive behavior in their partners and had second-order

67   effects on aggressive interactions between other group members. The second study, also using

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68   *D. melanogaster*, reported that the genotype of stimulus individuals influenced emergent,

69   group-level behavior of focal individuals [25]. Specifically, Anderson et al. reported that, if

70   individuals of the stimulus genotype were closer to one another, on average, then the focal

71   individuals were also more cohesive, and interactions between stimulus and focal individuals

72   were less frequent.

73   Together, these two experiments indicate that IGE can extend beyond the direct effects

74   of one individual on another. However, it remains unknown whether IGE previously experienced

75   by one or a few group members can influence phenotypes of individuals that were never

76   exposed to the IGE. That is, can IGE "cascade" beyond individuals that experience them

77   firsthand? Previous work in animals indicates that individual group members can influence

78   group behavior [1, 26-29]. However, this literature has generally not focused on prior social

79   experience as a factor that generates differences between influential group members [but see

80   30-32]; moreover, we know of no studies that implicate IGE as a cause of such differences. It is

81   challenging to measure prior influence of IGE because it is difficult to replicate group genotypic

82   composition and genetically-based differences in social environment in sexually-reproducing

83   species. Nevertheless, many organisms exhibit either dispersal or fission-fusion social structure,

84   so understanding IGE caused by prior social environments is critical to understanding the

85   evolution of social behaviors.

86   Naturally clonally-reproducing organisms provide an opportunity to measure these

87   effects outside of model species and without inbreeding or complex breeding designs. The

88   Amazon molly (*Poecilia formosa*) is a gynogenetic, all-female species [33] that arose from a

89   single hybridization event between a male sailfin molly (*Poecilia latipinna*) and a female Atlantic

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90   molly (*Poecilia mexicana*) about 100,000 generations ago [34, 35]. Although reproduction is

91   clonal, females require sperm from a male of one of the ancestral species (sailfin or Atlantic

92   molly) to initiate embryogenesis of unreduced ova [36]. Many distinct clonal lineages arose from

93   the original diploid lineage through mutation or complete and/or partial incorporation of

94   paternal genetic material, which can be stable and transmitted to subsequent generations [36,

95   37]. This accumulation of genetic diversity in a gynogenetic species produces groups in which

96   social interactions occur on multiple levels: within-clone interactions, among-clone interactions,

97   and interspecies interactions between Amazons and their sexual hosts. While the interactions

98   between Amazons and their hosts have been the focus of many investigations over the past

99   forty years [e.g., 33, 38-41], little attention has focused on the social interactions within [42] and

100   among the different clonal lineages; however, previous research does suggest that clonal

101   lineages vary in the social behaviors [43].

102   In natural populations, the number of clonal lineages that co-occur can vary dramatically

103   from a single lineage to more than a dozen [44-46]. Therefore, the degree of competition and

104   the frequency with which females encounter conspecifics of different lineages can vary greatly

105   across time and space. One of the first studies to investigate social behaviors among different

106   clones reported that females could distinguish between lineages, associate preferentially with

107   fish of their own lineage, and were more aggressive toward unrelated clones [43]. Other studies

108   have reported that different features of the social environment can influence social behavior,

109   especially aggression, within and among clonal lineages, including early dominance interactions

110   [47] and the degree of familiarity among individuals [42, 48]. These data suggest that individual

111   behavior depends in part on the clonal composition of the social environment; that is, IGE likely

112   regulate phenotypic variation and social dynamics in natural populations.

113   We used clonal variation in Amazon mollies to test the hypothesis that IGE propagate

114   beyond individuals that experience them firsthand. This hypothesis predicts that variation in

115   behavior generated by IGE in a previous social environment will influence the behavior of naïve

116   individuals when an animal with this prior experience joins their group. To distinguish this effect

117   from first- and second-order IGE, we use the term 'cascading IGE'. Based on extensive literature

118   indicating that individual differences in behavior affects group-emergent phenotypes [reviewed

119   by 1], we also predicted that cascading IGE will influence group-emergent behavior in these fish.

120   We tested these predictions by exposing genetically identical Amazon mollies to social partners

121   of different genotypes and then moving these individuals to new social groups in which they

122   were the only member to have experienced IGE. This experimental paradigm simulates the

123   fission-fusion dynamics often observed in poeciliid fishes in the natural environment [49-51].

124

125   **2. Material and methods**

126   *(a) Study Specimens*

127   Three distinct clonal lineages were used in this study, each descended from individuals

128   collected from the Río Purificacíon in Nuevo Padilla, Mexico (24°4'42.85''N, 99°7'21.76''W), and

129   maintained in a greenhouse at the Mission Road Research Facility of Florida State University.

130   Both Clone 1 (Schartl) and Clone 2 (AMM#11) are diploid with microchromosomes, although the

131   microchromosomes are distinctly different between the two lineages [43, 52]. The focal clone

132   (3N) is a triploid without any microchromosomes, this clonal lineage was chosen at random to

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133   be the focal clone. Details concerning fish care can be found in the electronic supplementary

134   materials, Methods.

135

136   *(b) Long-term social environments*

137   Focal females were placed into 18.9L aquaria in one of three different long-term social

138   environments: (1) 1 focal female + 2 sister clones; (2) 1 focal female + 2 females from a Clone 1;

139   and (3) 1 focal female + 2 females from Clone 2. That is, each aquarium contained 1 focal fish

140   and 2 "social partner" fish. Females placed into the Monoclonal social environment were

141   unfamiliar with each other as they originated from different rearing and recovery tanks prior to

142   the start of the experiment. The partner fish genotypes, but not the genotype of focal fish,

143   differed among treatments. Each social-environment treatment was replicated 12 times for a

144   total of 36 experimental tanks. Experimental tanks were set up using a randomized complete

145   block design (one replicate of each treatment per block) over the course of two weeks (6 blocks

146   set up per week) until all 12 blocks were complete. All females ranged between 27 and 38 mm in

147   body length with a maximum size difference among females within each social environment of 4

148   mm to reduce the influence of body size on aggression [48].

149   To characterize differences in the social environment induced by the three different

150   social treatments, we measured social interactions in the experimental tanks at 9 different times

151   over the course of the experiment: 10 min after placing the focal fish in the social environment

152   (week 0), weekly for the first four weeks thereafter (weeks 1-4), and then biweekly until a total of

153   12 weeks of exposure (weeks 6, 8, 10, and 12). Behavior measured at week 0 represents a

154   baseline because females had no prior exposure to experimental social environments at this

155   time point. Social behavior in the experimental tanks consisted mainly of aggressive interactions

156   (bites, tail beats, and chasing); few affiliative or neutral behaviors (e.g., swimming in the same

157   direction or foraging simultaneously within 2 body lengths) were observed outside an

158   aggressive context (e.g., proceeding or following biting, chasing or tail beating). We counted the

159   number of bites and tail beats performed and the total time spent performing these behaviors

160   and chasing other females. Tail beats were rarer than bites, and the distribution was zero-

161   inflated. We therefore summed the total number of bites and tail beats observed, and separately

162   summed the total time spent in these aggressive interactions to produce two overall measures

163   of aggression: total number of aggressive acts and total time spent in aggression. Both

164   measures were log-transformed before analysis, after adding 1 to account for zero values. These

165   assays were recorded by a live observer blind to the treatments for a duration of 10 minutes.

166

167   *(c) Naïve-group tests*

168   Each focal female was introduced to a pair of novel ('naïve') social partners three times

169   over the course of the experiment (at 0, 4, and 12 weeks). A different pair of naïve social partners

170   was used at each of these trials, and those partner fish were not used with any other focal

171   female. We measured the average behavior of these naïve-groups before exposing focal fish to

172   genetically different long-term social environments (week 0) and after 4- and 12-weeks of

173   exposure (see Figure 1). To do so, individual focal females were removed from their rearing tank

174   (at week 0) or their long-term social environment tank (at weeks 4 and 12) and placed in a

175   "naïve-group" test chamber with two unfamiliar females from the same clonal lineage as the

176   focal fish, size matched to the focal fish (± 4mm), and in the same reproductive state. These

177   novel fish were drawn from monoclonal, non-breeding rearing tanks similar to those from which

178   focal and stimulus females originated and were, therefore, not exposed to the experimental

179   social environments experienced by the focal females. After we introduced the focal fish into the

180   naïve-group test chamber, we video recorded all three fish for 10 minutes, after which the focal

181   female was removed and placed back into her experimental social environment (Figure 1).

182   Because we were interested in emergent (group-level) behavior of the naïve groups, the

183   naïve-group test chamber was an open field, circular tank (55.9 cm diameter), with half the

184   bottom and corresponding sides painted white and the other half grey. In the center of the

185   frame, a camera (JVC Everio 1920x1080 HD video camcorder) was suspended 1.1 m above the

186   tank. All videos were 6 minutes long and were analyzed by a blind observer using EthoVision XT

187   (Noldus, v14). For more information regarding video recording, editing, and EthoVision, see the

188   electronic supplementary materials, Methods.

189   Although fish could be individually tracked, the focal individual could not be

190   distinguished from the novel partner fish on the videos; therefore, we did not calculate separate

191   metrics for focal and novel partner fish. We interpret behaviors as reflecting stress-related

192   behavior or tendency to be exploratory. More stressed individuals are less active, travel shorter

193   distances at lower velocity, spend more time frozen and in the grey zone (negative phototaxis),

194   and are closer together; less stressed individuals tend to be more exploratory and cover more

195   distance, move at higher velocity, enter zones more frequently, spend more time in the white

196   zone and less time frozen, and have more distance between individuals [53, 54]. We also

197   gathered baseline data on these behaviors by following the same procedure at the start of the

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198   experiment, before the focal fish had experienced the experimental social treatments (week 0;

199   Figure 1: Pre-exposure).

200

201   *(d) Ethics*

202   This research was approved by the Institutional Animal Care and Use Committee of

203   Florida State University (1704 and 201900038).

204

205   *(e) Analyses*

206   There were no significant differences in size (SL) among focal females in different social

207   treatment groups, nor treatment-associated differences in size among the social partner fish

208   used in the long-term and naïve-group trials (electronic supplemental material, Table S1).

209   Nevertheless, we included SL of focal and partner fish as covariates in subsequent analyses

210   because there was a non-significant trend for Clone 1 and Clone 2 social partner fish to differ in

211   SL (electronic supplemental material, Table S2).

212

213   *(e.1) Long-term social environment groups.*

214   We assessed the correlation structure of the two measurements of aggression to determine if

215   they could be adequately represented by principal components (PC), and then used the first PC

216   from this analysis as our measure of aggression (see Results). To determine if aggression was

217   influenced by social treatment group, we used this PC1 score as the dependent variable in linear

218   mixed models. In addition to the social treatment group, initial models included fixed effects of

219   exposure time (weeks), treatment-by-time interaction, the baseline (week 0) measure of

10

220   aggression PC1, and focal female standard length (log-transformed. A random group ID effect

221   was used to account for repeated measures on groups (at weeks 4 and 12). Except where noted,

222   this and other analyses of linear mixed models were conducted using SAS *Proc Glimmix* in SAS v.

223   9.4 [55] with a Gaussian error distribution and an identity link function. Post hoc comparisons of

224   treatment group means were conducted using the simulation method of [56], as implemented

225   by using the *adjust=simulate* option. See the electronic supplementary materials, Methods for

226   more details on statistical models.

227

228

229   *(e.2) Naïve-group tests.*

230   To determine the extent to which the presence of the focal individual influenced

231   behavior in the naïve-groups, and thus to measure cascading IGE, we calculated two kinds of

232   metrics: those that described average behavior of the 3 members of the group, and those that

233   described individual behavior of fish within the group. For both analyses, we summarized six of

234   the movement variables (distance traveled (cm), velocity (cm/s^2), frequency entering white zone

235   (count), duration in white zone (s), latency to enter white zone (s), time spent immobile (s;

236   freezing behavior) with PC scores (see below). Distance between individuals (cm; shoaling

237   distance) was analyzed separately (see below).

238   *Average behavior of naïve-groups.* We first assessed the correlation structure of the 7

239   behaviors to determine if they could be adequately represented by principal components. The

240   six behaviors that described movement or physical position in the enclosure were all moderately

241   to highly correlated with one another ($0.4 < |r| < 1.0$), but they were not correlated with the

242   average shoaling distance between fish (all |r| < 0.2) (electronic supplementary material, Figure

243   S1A), indicating that a PCA should include the 6 movement/position variables, but that shoaling

244   distance should be analyzed separately. We used the first PC from this analysis as our measure

245   of the movement and position of fish (see Results), and we used the log-transformed average

246   shoaling distance as a measure of a group cohesion, since it arises from the relative positions of

247   all three members of the group.

248   To determine if these two measures of naïve-group behavior were differentially affected

249   by the social environment experienced by a single member of the group, we used the values at

250   weeks 4 and 12 as the dependent variable in linear mixed models. Neither the size-related fixed

251   effects nor the measures of aggression approached significance in the initial models (electronic

252   supplemental material, Table S3B and C, Methods), so only treatment and exposure time (and

253   their interaction) were retained in the final models. A random effect with focal female ID as the

254   subject was used to account for the different naïve-group trials in which each focal female was

255   used. Post hoc comparisons of group means were conducted as described above. We calculated

256   the proportion of variance explained by the long-term social environment using the method of

257   Jaeger et al. [57].

258   Since treatment groups varied in aggression (see Results), any overall association

259   between aggression experienced in the long-term social environment and the behavior of naïve

260   groups could have been obscured by the treatment effect in the models described above. To

261   assess the overall relationship between aggression in the long-term environment and naïve

262   group behavior, we therefore fit models for exploratory PC1 and shoaling identical to those

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263   described above, but with the only predictor variable being cumulative aggression in the long-

264   term environment.

265   Focal females were tested in naïve groups once at baseline and twice more after 4 and

266   12 weeks of exposure to their long-term social groups. To assess the consistency of behavior of

267   the naïve groups that contained the same focal female after 4 and 12 weeks of experience in

268   their long-term social environments, we calculated the Pearson's correlation between PC1 scores

269   (or shoaling) at the two time periods [58]; we calculated 95% confidence limits using the z-

270   transformation method.

271   *Behavior of individuals in naïve-groups.* The main purpose of this analysis was to

272   determine if differences in the average behavior among naïve groups was attributable to all

273   members of a group behaving similarly or to specific individuals within the group. For example,

274   if the behavior of the three females within a group was very similar, then average differences

275   among groups reflect the behavior of all group members. Alternately, if individuals within

276   groups behaved differently from one another, then between-group differences could have been

277   driven by the divergent behavior of a single group member. The former, but not the latter,

278   would support cascading IGE because it would indicate that non-focal behavior was influenced

279   by the prior social experience of the focal fish. Our primary measure of similarity of the behavior

280   of individuals within naïve-groups was the ICC.

281   We first investigated the correlation structure of the same 8 behaviors described above

282   but measured on individuals rather than the mean of the 3 fish in a group. As in the group-

283   average data, the position/movement variables were moderately to highly correlated with each

284   other, but not with shoaling distance (electronic supplementary material, Figure S1B). We

13

285   therefore summarized the movement/position behavior of individual fish using the first PC of

286   the 6 movement/position metrics (Table 1). We then calculated the ICC of the individual

287   exploratory behavior scores using a linear mixed model with a random effect corresponding to

288   group ID. The ICC was estimated as the ratio of among-group variance to the total variance and

289   confidence intervals were determined using parametric bootstrap estimates [58].

290

291   **3.   Results**

292   *(a) Summary measures of aggression and movement explain most of the variation*

293   The two measures of aggression (number of acts and time spent) were highly correlated

294   (R^2=0.803, p<0.0001), with the first PC explaining 96.9% of the total variation. For the average

295   behaviors of the naïve-groups, the first PC summarizing the 6 movement/position variables

296   explained 75.79% of the total variation, and it was the only PC with an eigenvalue >1 (Table 1).

297   Behaviors associated with exploration loaded positively on PC1 (distance, velocity, and duration

298   in the white zone, and frequency entering white zones), while behaviors associated with stress

299   loaded negatively on PC1 (freezing, latency to enter white zone, Table 1). We therefore

300   considered positive values of PC1 to indicate a tendency to explore, and negative values to

301   indicate lack of exploration or stress-like behaviors.

302

303   *(b) Long-term social environments differ in aggressive behavior*

304   Long-term social groups in which the focal fish was housed with two females of her own

305   clonal lineage exhibited more aggression than groups where the social partners were Clone 1 or

306   Clone 2 fish (Figure 2A, Table 2A, effect estimates provided in electronic supplementary material,

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307   Figure S2 and Table S4). On average, fish in the Monoclonal environment performed 60% more

308   aggressive acts than fish in the Clone 1 environment (14.45±1.49 vs. 9.04±1.27 acts per 10-

309   minute observation bout, respectively; fish in the Clone 2 environment performed 11.04±1.18

310   aggressive acts per bout, on average). Post hoc tests indicated that the Monoclonal social

311   environment elicited significantly more aggression than the Clone 1 environment ($t_{256.1}=3.93$,

312   p<0.001), but no other contrasts were significant after adjustment for multiple tests (Monoclonal

313   vs Clone 2: $t_{248.8}=2.15$, p=0.087; Clonal 1 vs Clone 2: $t_{247.1}=-2.04$, p=0.114).

314

315   *(c) Genetic differences in prior social experience for one group member affected average*

316   *behavior of the group.*

317   The long-term social environment experienced by a single focal fish affected the average

318   exploratory behavior of the group when paired with otherwise naïve individuals (Table 2B, Figure

319   2B, and electronic supplementary material, Figure S3, effect estimates in electronic

320   supplementary material, Table S5). Indeed, the social environment of the focal fish explained

321   43.1% of the total variation in the exploratory/stress PC1 scores. Groups in which the focal

322   individual experienced the Monoclonal long-term social environment exhibited more stress-

323   related behavior (negative values on exploratory PC1) than groups in which the focal individual

324   experienced Clone 1 or Clone 2 social environments (post-hoc tests: Monoclonal vs Clone 1,

325    $t_{17.58}=-2.59$, p=0.044; Monoclonal vs Clone 2, $t_{21.88}=-3.12$, p=0.015). Naïve-groups in which the

326   focal fish had experienced social environments containing Clone 1 and Clone 2 did not differ

327   significantly from each other after correction for multiple tests ($t_{16.81}=-1.31$, p=0.405). This result

328   is particularly striking because all focal and stimulus fish in this assay were members of a single

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329   clonal linage and, therefore, genetically identical. Surprisingly, the long-term social environment

330   of focal animals not only affected the average behavior of naïve groups, it also affected the

331   variance in behavior (i.e., the variance structure differed significantly between treatments;

332   electronic supplementary material, Table S6).

333   Cumulative aggression was linearly related to exploratory/stress behaviors of the naïve-

334   group trials in a model in which aggression was the only predictor (Table 2D). The more

335   aggression a female experienced in her long-term social environment, the less likely she was to

336   exhibit positive, exploratory behaviors and more likely to exhibit stress-like behaviors (β=-

337   0.401±0.172). This result suggests that aggression might be a phenotype that influenced focal

338   females and thus, the exploratory stress behaviors exhibited in the naïve-group trials.

339   The long-term social environment also affected the consistency in behavior over time of

340   the naïve groups were (electronic supplementary material, Table S7). Specifically, the naïve

341   groups with focal females originating from the Monoclonal social environment showed high

342   consistency of exploratory behavior across weeks 4 and 12 (r=0.732; figure 3A), whereas naïve

343   groups with focal females from the Clone 2 social environment exhibited much lower

344   consistency (r=0.280), and groups with focal females from the Clone 1 long-term social

345   environment exhibited a negative correlation between behaviors across time periods (r=-0.759).

346   The mean shoaling distance in the naïve-groups was unaffected by the social

347   environment experienced by the focal fish or duration of exposure (Table 2C, effect estimates for

348   fixed effects in electronic supplementary material, table S8, Figure S4). There was a trend for

349   different treatment groups to behave differently over time, but the interaction term did not

350   reach significance (table 2C, electronic supplementary material, Figure S5). Cumulative

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351   aggression did not significantly predict shoaling distance (Table 2E; β=-0.003±0.047). Among-

352   group variance was unaffected by treatment or exposure time (electronic supplementary

353   material, Table S6), and group-level consistency was low for shoaling behavior (r=0.142;

354   electronic supplementary material, Table S7).

355

356   *(d) Individuals within naïve groups behave very similarly.*

357   Focal and stimulus fish within the naïve groups were unfamiliar with one another and

358   had different social experiences prior to the trials. Focal fish were drawn from the long-term

359   social environments, whereas stimulus fish were all genetically identical, all of similar age and

360   size, and all had similar prior social experience that differed substantially from that of the focal

361   fish. Moreover, there was substantial variation in behavior across different trials, as indicated by

362   the significant effects of long-term social environment described above. Nevertheless, the three

363   individuals in a given naïve-group trial behaved in a remarkably similar manner. Figure 3C shows

364   representative tracking data for 3 different trios from the naïve-group tests (see electronic

365   supplementary material, Figure S6 for 12 additional representations). The striking visual

366   similarity of tracking patterns within a given trial is reflected in the high ICC estimate for

367   individual exploratory behavior (ICC=0.831 overall, values for each treatment group along with

368   95% CI and variance -component estimates used to calculate ICC reported in Figure 3B and

369   electronic supplementary material, Table S9). That is, less than 17% of the total variation in

370   behavior occurred among the three females within a given naïve-group trial, despite the

371   substantial differences in behavior among trials that is evident in Figure 3 and electronic

17

372   supplementary material, Figure S6. This high ICC value indicates that all three individuals within

373   a given trial exhibited highly similar behavior, despite their different prior experience.

374

375   **Discussion**

376   Elucidating the heritable causes of individual and group-emergent phenotypes is

377   necessary to understand the evolution of social traits and other interacting phenotypes. Here,

378   we demonstrate that phenotypic effects of genetically different social environments (IGE) carry

379   over to a novel social environment to influence the behavior of individuals that did not

380   experience IGE. This cascading effect is distinct from 'second-order' IGE [24, 25], in which the

381   presence of genetically different individuals influences interactions between other members of

382   the same group. Our results expand the scope of IGE by demonstrating that they can influence

383   phenotypes even when there is no genetically-based variation present within groups. Given the

384   prevalence of dispersal and fission-fusion group structure, there is substantial opportunity for

385   cascading IGE in nature.

386   The cascading IGE we observed was associated with different levels of aggression that

387   focal fish experienced in the long-term social environments. Somewhat surprisingly, it was the

388   social environment containing fish of the same clone as the focal animals that exhibited the

389   most aggression (and the naïve groups containing these focal fish exhibited the most stress

390   behaviors). Previous studies found that Amazon mollies exhibited less aggression towards sister

391   clones when compared to non-sister clones [43, 57]. However, a different focal clonal lineage

392   was used in those studies, suggesting that responses to sister and non-sister clones (and,

393   therefore, first-order and cascading IGE) vary across genotypes. Length of initial exposure may

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394 also play an important role, as in the former two studies, exposure time was short (only 10 mins

395 or so); whereas another study using 30-days of exposure showed that familiarity influenced

396 aggression within a clonal lineage [42]. Thus, the monoclonal environment fish may have found

397 their naïve groups more 'familiar' and therefore exhibited more aggression. Furthermore, this

398 kind of interaction between the direct effect of an individual's genotype and IGE can produce

399 frequency-dependent and other forms of balancing selection that can maintain, or rapidly erode

400 genetic variation [19, 58]. The possibility that similar effects could arise from the interaction of

401 direct genetic variance and cascading IGE warrants future empirical and theoretical investigation.

402 In this experiment, it is possible that the cascade of IGE that we observed occurred

403 because focal females in Clone 1 and Clone 2 treatments experienced a genetic change in the

404 social environment when they moved into the naïve-groups, but focal fish from the Monoclonal

405 treatment did not. If this were the primary cause of cascading IGE, we would expect a significant

406 difference in cascading effects between the Monoclonal treatment and both Clone 1 and Clone

407 2 (which we do find), but not between Clone 1 and Clone 2 (for which we found only a non-

408 significant trend). Clone 1 and Clone 2 treatment did significantly differ in variance among naïve

409 groups in the two treatments and in consistency of naïve-group behavior at 4 and 12 weeks .

410 These differences in variance and consistency of behavior of naïve groups with focal females

411 from Clone 1 versus Clone 2 suggest that sister-clone recognition was not the only cause of

412 cascading IGE in this experiment, and points to differences in phenotypic variance as an under-

413 explored consequence of IGE in general. In any case, our data support the hypothesis that

414 genetically identical fish (the naïve partners) behave differently depending on genetic variation

415 in the prior social environment experienced by another member of the group (the focal female).

19

416   Whether cascading IGE depend on the degree of genetic similarity between past and current

417   social partners should be a focus of future research. We also note that movement among

418   groups that differ in genetic similarity to the migrating individual is likely to occur in species

419   such as Amazon mollies that exhibit spatial population-genetic structure [35].

420   We detected no effects of exposure time within the long-term social environments on

421   aggression in those environments or on cascading IGE in the naïve groups. Time-course effects

422   on first-order IGE have been found in a related poeciliid, the eastern mosquitofish [18, 19], and

423   increased exposure time led to higher aggression in previous studies of Amazon mollies [42, 48].

424   However, the time course effects of IGE reported in mosquitofish occurred during maturation,

425   whereas the fish in our experiment were fully mature at the start of the study. The two studies

426   that reported exposure-time effects on aggression in Amazon mollies maintained the animals at

427   considerably higher density than that used in our experiment (48: 1.9 L / fish; 42: 4 L / fish; the

428   present study: 6.3 L / fish), suggesting that exposure-time effects could be density-dependent.

429   The relatively low density in our long-term social environments might also account for

430   lack of treatment or cascading effects on shoaling distance, despite strong effects on

431   exploratory behavior. Anderson et al. [25] found that second-order IGE influenced social

432   cohesion in *D. melanogaster*, and the extensive literature on leadership in social organisms

433   indicates that differences among individual group members can substantially influence group-

434   emergent behaviors such as shoaling [61, reviewed in 1]. We therefore expected that cascading

435   IGE would be an important source of individual variation that generates group-emergent

436   phenotypes [62, 63]. In our experiment, groups consisted of only 3 individuals in a small

437   enclosure, which might limit the tendency of these fish to shoal. Experiments that use larger

20

438   groups and enclosures that allow more flexibility in fission-fusion dynamics should be deployed

439   to determine the extent to which cascading IGE influence group-emergent phenotypes.

440   In summary, we found that IGE propagate beyond individuals that directly experience

441   them in Amazon mollies and possibly in many group-living species. These cascading IGE are a

442   potentially important cause of individual differences that can lead to the emergence of leaders

443   and followers, shoaling, swarming, and other group-emergent phenotypes. Theoretical and

444   empirical expansion of the quantitative genetic framework developed for IGE to include

445   cascading or other types of carry-over effects will facilitate understanding of phenotypic

446   variation and its evolution.

447

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Acknowledgements

612 **Table 1.** PC loadings for PC1 on group-averaged and individual-level exploratory/stress

613 behaviors.

614

Level	Model	Measurement	PC1 loading
Group-averaged	PC1 exploratory/stress	Total distance traveled	0.951
		Velocity	0.951
		Frequency entering white zone	0.956
		Duration in white zone	0.686
		Latency to enter white zone	-0.739
		Time spent frozen in place	-0.899
Individual-level	PC1 exploratory/stress	Total distance traveled	0.949
		Velocity	0.948
		Frequency entering white zone	0.937
		Duration in white zone	0.642
		Latency to enter white zone	-0.702
		Time spent frozen in place	-0.897

615

616 **Table 2:** Proportion of variance explained, test statistic and p-value for statistical models of

617 aggression PC1 (**A.**), exploratory/stress behaviors (PC1) (**B.**), shoaling behaviors (**C.**), the effects of

618 cumulative aggression on exploratory/stress behaviors (PC1) (**D.**), and the effects of cumulative

619 aggression on shoaling behaviors (**E.**).

Model	Effect	Proportion of variance explained	Statistic	P-value
A. Aggressive behavior in long-term social treatments (PC1)				
	Focal female standard length	$R^2 = 0.015$	$F_{1,208.1} = 3.26$	0.072
	Social environment	$R^2 = 0.058$	$\mathbf{F_{2,250.3} = 7.70}$	**<0.001**
	Exposure time	$R^2 = 0.039$	$F_{7,221.2} = 1.30$	0.253
	Social environment*Time	$R^2 = 0.040$	$F_{14,232.6} = 0.69$	0.788
B. Exploratory/stress behavior in naïve-group trials (PC1)				
	Social environment	$R^2 = 0.431$	$\mathbf{F_{2,19.46} = 5.13}$	**0.016**
	Exposure time	$R^2 = 0.023$	$F_{1,19.4} = 1.11$	0.306
	Social environment*Time	$R^2 = 0.089$	$F_{2,18.13} = 0.58$	0.568
C. Shoaling distance in naïve-group trials				
	Social environment	$R^2 = 0.056$	$F_{2,20.5} = 0.25$	0.779
	Exposure time	$R^2 = 0.003$	$F_{1,28.63} = 0.06$	0.816
	Social environment*Time	$R^2 = 0.242$	$F_{2,20.77} = 3.08$	0.067
D. Cumulative aggression on exploratory/stress behaviors in naïve-group trials (PC1)			$\mathbf{F_{1,19.66} = 5.42}$	**0.031**
E. Cumulative aggression on shoaling distance in naïve-group trials			$F_{1,26.3} = 1.19$	0.285

620

27

621   **Figure 1:** Schematic of experimental design illustrating the focal females (FF_{CL}) tested for pre-

622   exposure exploratory behaviors with two novel sister clones (F_{CL}) at week 0. Focal females were

623   then transferred into one of the three different social environments: Monoclonal (FF_{CL} + 2 F_{CL});

624   Clone 1 (FF_{CL} + 2 CL_1); or Clone 2 (FF_{CL} + 2 CL_2). After 4 weeks and then again at 12 weeks of

625   exposure to these social environments, the exploratory behaviors of the focal females were

626   tested again with novel F_{CL} individuals. Note that the F_{CL} partners of the FF_{CL} were different
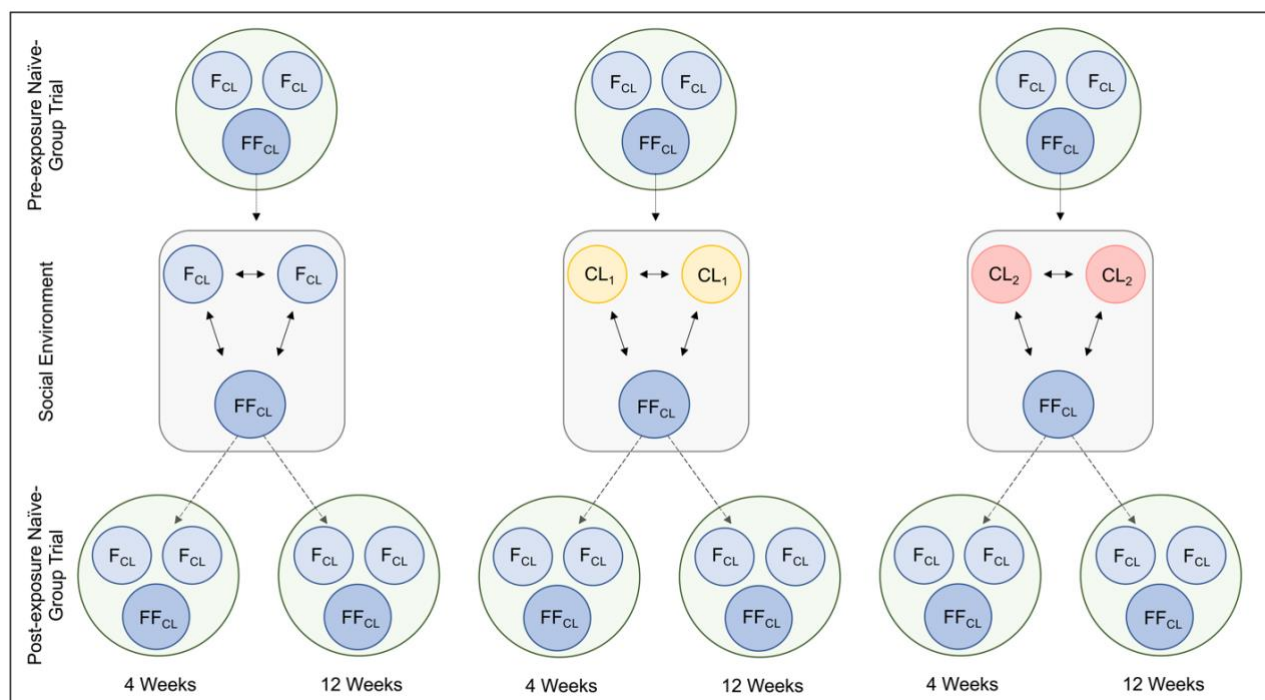

627   individuals at each time period. That is, each individual F_{CL} was included in only one trial.

28

628 **Figure 2:** Least square means +/- standard error for (**A.**) aggression PC1 for each long-term

629 social environment (Monoclonal (blue); Clone 1 (yellow); Clone 2 (pink)). Positive values indicate

630 more aggression. (**B.**) PC1 for exploratory/stress behaviors in the naïve-group trials. Group-

631 averaged exploratory behaviors with positive values indicating more exploratory behaviors and
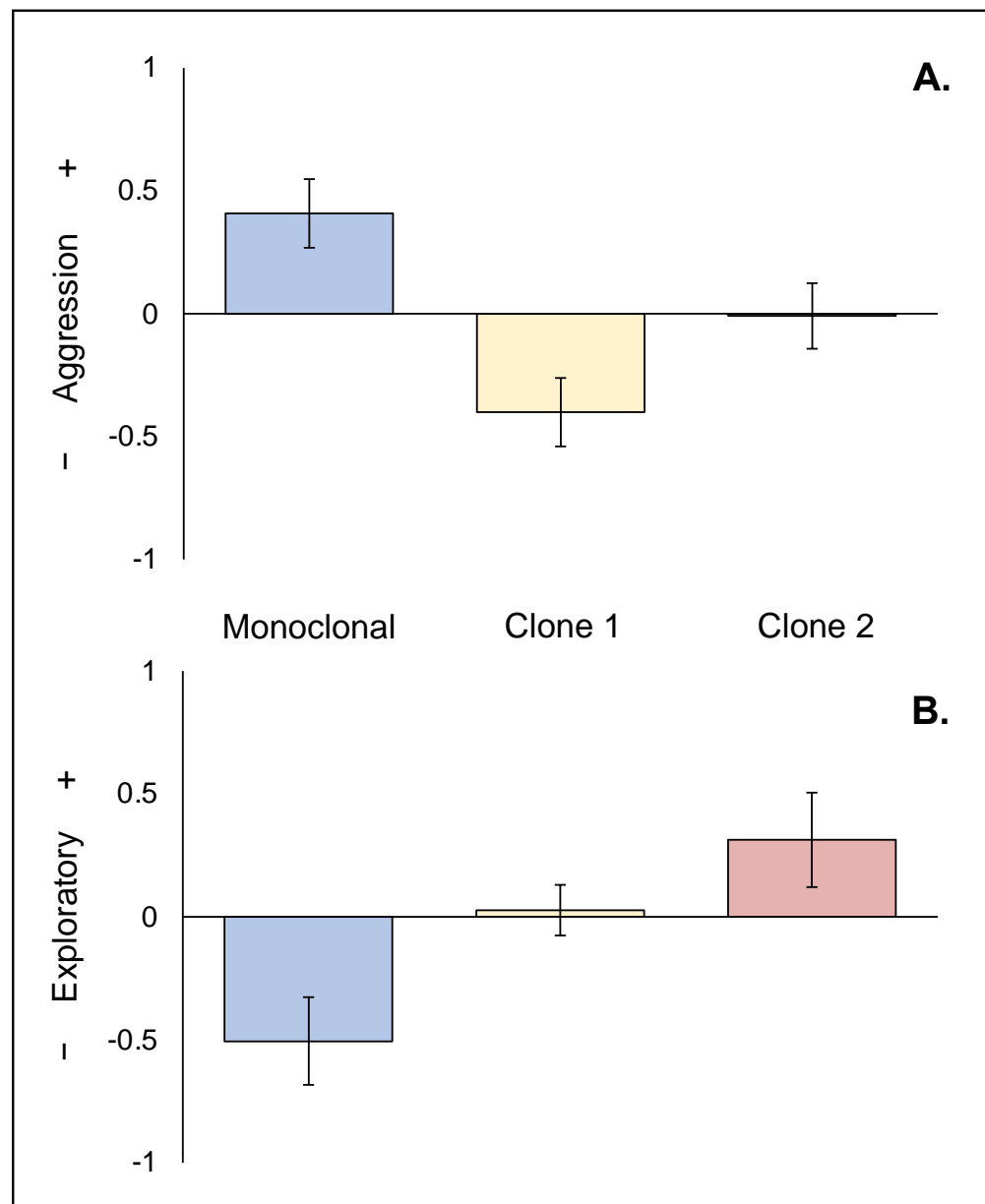
632 negative values indicate less exploratory and more stress behaviors.

633   **Figure 3:** In each panel, the different colors represent Monoclonal (blue), Clone 1 (yellow), Clone

634   2 (pink). (**A.**) Consistency of behavior of naïve-groups containing same focal female at 4 and 12

635   weeks exposure to long-term social environments (r^2 ± 95% confidence intervals). (**B.**)

636   Consistency of behavior of the 3 individuals within a single naïve-group trial (ICC ± 95%

637   confidence intervals). (**C.**) Visual representation of high intraclass correlation among individuals

638   in the same naïve-group trial. Within each treatment category (Monoclonal, Clone 1, Clone 2)

639   each row represents tracks of the three individuals in a single naïve-group trial. At each time

640   point (Pre-exposure (Pre), 4 wk, and 12 wk) the focal fish and two naïve partners were tracked.
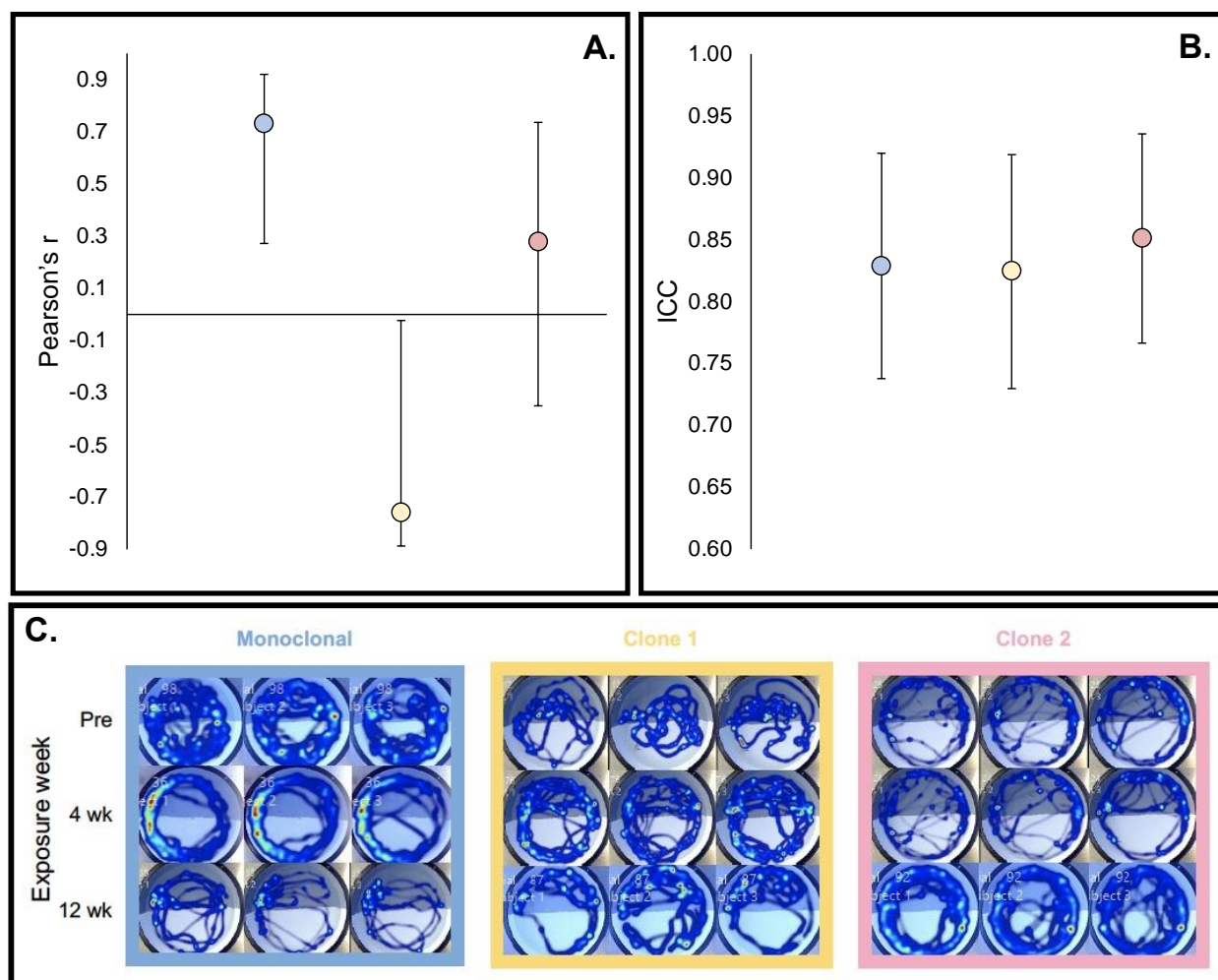
641   For a given treatment, the same focal female was present at each time point, but her two social

642   partners were different individuals across time points.