Cascading indirect genetic effects in a clonal vertebrate

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Author contributions

This study was conceived by AMM and KAH, data collected by AMM and CR, analyzed the data by KAH, AMM and DB, and the manuscript was written by AMM and KAH.

Data archiving

All data will be archived in Figshare upon acceptance.

Abstract

Understanding how individual differences among organisms arise and how their effects propagate through social groups are fundamental questions in behavioral biology. Genetic variation among social partners can influence individual phenotypes, creating individual differences that might then have cascading effects in social groups. Using a clonal species, the

Amazon molly (*Poecilia formosa*), we test the hypothesis that such indirect genetic effects (IGE) propagate beyond individuals that experience them firsthand. We tested this hypothesis by exposing genetically identical Amazon mollies to social partners of different genotypes, and then moving these individuals to new social groups in which they were the only member to have experienced the IGE. We found that the differences in aggression experienced in genetically different social environments carried over into new social groups to influence the exploratory behaviors of individuals that did not directly experience the previous social environments. Our data reveal that IGE can propagate beyond the individuals that directly experience them in Amazon mollies and possibly in many group-living species. Theoretical and empirical expansion of the quantitative genetic framework developed for IGE to include cascading and other types of carry-over effects will facilitate understanding of among-individual variation, social behavior and its evolution.

1. Introduction

Interactions among individuals define the social environment and individual differences have long been known to influence these interactions [1, 2]. Understanding how individual differences arise and how their effects propagate through social groups are fundamental questions in behavioral biology. One cause of both individual variation and propagation of effects in social groups are indirect genetic effects (IGE) [3-5]. IGE arise when an individual's phenotype is influenced by the genotype of its social partners, and they have been documented to affect behavioral, life history, and morphological traits in a wide variety of taxa [e.g., 6-16]. Most of the IGE literature focuses on how stimulus genotypes influence the phenotype of focal individuals. While understanding these dyadic interactions is important, much less is known about IGE on group-level characteristics or the degree to which IGE can propagate to affect phenotypes of individuals that do not experience them firsthand. Because IGE can profoundly affect phenotypes, fitness, and the rate and direction of evolutionary change [17-19],

54 understanding possible cascading or carry-over effects within social groups is necessary to
55 understand behavioral variation and evolution.

56 There have been two studies, to our knowledge, that have investigated IGE beyond
57 those caused by dyadic interactions. The first used fruit flies (*Drosophila melanogaster*), to
58 measure first-order IGE on male aggressive behavior (i.e., how the genotype of stimulus
59 individuals influences the phenotypes of individuals with which they interact) and second-
60 order IGE (i.e., effects of the stimulus genotypes on the interaction between two other members
61 of the group) [20]. This experiment showed that the stimulus genotypes differed in their first-
62 order effects on individuals, and on second-order effects on interactions between other group
63 members. The second study, also using *D. melanogaster*, reported that the genotype of stimulus
64 individuals influenced emergent, group-level behavior of focal individuals [21]. Specifically,
65 these investigators reported that, if individuals of the stimulus genotype were more cohesive
66 (i.e., closer to one another, on average), then the focal individuals were also more cohesive, and
67 interactions between stimulus and focal individuals were less frequent.

68 Together, these two experiments indicate that IGE can extend beyond the direct effects
69 of one individual on another. However, it remains unknown whether IGE previously
70 experienced by one or a few group members can influence the behavior of individuals that were
71 never exposed to the IGE. That is, can IGE propagate beyond individuals that experience them
72 firsthand? Previous work indicates that individual group members can influence group
73 behavior [1, 22-26]. However, this literature has generally not focused on prior social experience
74 as a factor that generates differences between influential group members [but see 27, 28], and
75 we know of no studies that implicate IGE as a cause of such differences. Because many
76 organisms exhibit either dispersal or fission-fusion social structure, understanding IGE caused
77 by prior social environments is critical to understanding the evolution and ecology of collective
78 behaviors. Furthermore, it is challenging to measure prior influence of IGE because it is difficult
79 to replicate group genotypic composition and genetically-based differences in social experience
80 in sexually-reproducing species.

Naturally clonally-reproducing organisms provide an opportunity to measure these effects outside of model species and without inbreeding or complex breeding designs. The Amazon molly (*Poecilia formosa*) is a gynogenetic, all-female species [29] that arose from a single hybridization event between a male sailfin molly (*Poecilia latipinna*) and a female Atlantic molly (*Poecilia mexicana*) about 100,000 generations ago [30, 31]. Although reproduction is clonal, females require sperm from a male of one of the ancestral species (sailfin or Atlantic molly) to initiate embryogenesis of unreduced ova [32]. Many distinct clonal lineages arose from the original diploid lineage through mutation or complete and/or partial incorporation of paternal genetic material (i.e., through triploidy or acquisition of michrochromosomal-sub fragments of paternal chromosomes), which can be stable and transmitted to subsequent generations [32, 33]. Furthermore, new evidence shows high inter-clonal transcriptional variation which suggests that different clonal lineages can adapt to different environments through long-term selection on transcriptional fitness [34]. Together, this genetic diversity within a gynogenetic species produces opportunities in which social interactions occur on multiple levels: within-clone interactions, among-clone interactions, and interspecies interactions between Amazons and their sexual hosts. While the interactions between Amazon mollies and their hosts has been the focus of many investigations over the past forty years [e.g., 27, 335-38], little attention has focused on the social interactions within and among the different clonal lineages. Nonetheless, previous research suggests that clonal lineages vary in the social behaviors [39] and this variation may be via functional differences in transcription landscapes [34].

In natural populations, the number of clonal lineages that co-occur can vary dramatically from a single lineage to more than a dozen [40-42]. Therefore, the degree of competition and the frequency with which females encounter conspecifics of different lineages can vary greatly across time and space. One of the first studies to investigate social behaviors among different clones reported that females could distinguish between lineages, associate preferentially with fish of their own lineage, and were more aggressive toward unrelated clones [39]. Other studies have reported that different features of the social environment can influence

social behavior, especially aggression, within and among clonal lineages, including early dominance interactions [43] and the degree of familiarity among individuals [44, 45]. These data suggest that individual behavior depends in part on the clonal composition of the social environment; that is, IGE are likely to be important regulators of phenotypic variation and social dynamics in natural populations.

We leveraged clonal variation in Amazon mollies to test the hypothesis that IGE propagate beyond individuals that experience them firsthand. This hypothesis predicts that variation in behavior generated by IGE in a previous social environment will influence the behavior of naïve individuals when an animal with this prior experience joins their group. To distinguish this effect from first- and second-order IGE, we use the term 'cascading IGE'. Based on extensive literature indicating that individual differences in behavior affects group-emergent phenotypes [reviewed by 1, 2], we also predicted that cascading IGE would influence group-emergent behavior in these fish. We tested these predictions by exposing genetically identical Amazon mollies to social partners of different genotypes, and then moving these individuals to new social groups in which they were the only member to have experienced IGE.

2. Material and methods

(a) Study Specimens

Three distinct clonal lineages were used in this study, but all lineages were descended from individuals collected from the Río Purificacíon in Nuevo Padilla, Mexico (24°4′42.85″N, 99°7′21.76″W) originated from single-lineage stock populations kept in a greenhouse at the Mission Road Research Facility of Florida State University. Both Clone 1 (VI/17 Schartl) and Clone 2 (VI/17 AMM#11) are diploid with microchromosomes, although the microchromosomes are distinctly different between the two lineages [39, 46]. The focal clone (VI/17 3N) is a triploid without any microchromosomes.

Two weeks prior to initiating the experiment, we marked all fish with elastomer tags (two 3mm subcutaneous marks anterior and/or posterior of the dorsal fin) to allow us to

identify focal individuals within each long-term social environment tank. To recover, fish were placed in 113.6L aquaria treated with Stress Coat+® (API®) and sea salt (Instant Ocean®), with each aquarium containing an average of ten sister clone individuals. Females were all virgins and, thus, were all receptive but not pregnant at the time of the trials. They were fed daily ad libitum with commercial fish food (Tetramin® tropical flakes). Experiments occurred from August to November 2019, and fish were exposed to natural light cycles during the course of the experiment.

(b) Long-term social environments

Focal females were placed into 18.9L aquaria in one of three different long-term social environments: (1) 1 focal female + 2 sister clones; (2) 1 focal female + 2 females from a Clone 1; and (3) 1 focal female + 2 females from Clone 2. That is, each aquarium contained 1 focal fish and 2 "social partner" fish. The partner fish genotypes, but not the genotype of focal fish, differed among treatments. All aquaria contained sand and two small PVC pipe fittings (2 cm) for shelter, with one long side and two short sides covered with blue tarp to prevent visual communication with neighboring tanks. Each social-environment treatment was replicated 12 times for a total of 36 experimental tanks. Experimental tanks were set up using a randomized complete block design (one replicate of each treatment per block) over the course of two weeks until all 12 blocks were complete. All females ranged between 27 and 38 mm in standard body length with a maximum size difference among females within each social environment of 4 mm.

To characterize differences in the social environment induced by the three different social treatments, we measured social interactions in the experimental tanks at 9 different times over the course of the experiment: 10 min after placing the focal fish in the social environment (week 0), weekly for the first four weeks thereafter (weeks 1-4), and then biweekly until a total of 12 weeks of exposure (weeks 6, 8, 10, and 12). Behavior measured at week 0 represents a baseline because females had no prior exposure to social treatments at this time point. Social behavior in the experimental tanks consisted mainly of aggressive interactions (bites, tail beats,

and chasing); few affiliative or neutral behaviors (e.g., swimming in the same direction or foraging simultaneously within 2 body lengths) were observed outside an aggressive context (e.g., proceeding or following biting, chasing or tail beating). We counted the number of bites and tail beats performed, and the total time (s) spent performing these behaviors and chasing other females. Tail beats were rarer than bites, and the distribution was zero-inflated. We, therefore, summed the total number bites and tail beats observed, and separately summed the total time spent in aggressive interactions to produce two overall measures of aggression: total number of aggressive acts and total time spent in aggression. Both measures were log-transformed before analysis, after adding 1 to account for zero values. We excluded one datum (block 7, clone 1 treatment, week 3) due to it being an extreme outlier: 174 aggressive acts (2.3x higher than the maximum number of acts in any other tank), and 190s of total aggression (2.2x larger than the maximum time spent being aggressive in any other tank). Individual identification was not possible during the trial while fish were in motion and visible only from one side. Therefore, we used the total number and duration of these behaviors across all fish in the trial to characterize the social environment within the tank. These assays were recorded by a live observer blind to the treatments for a duration of 10 minutes.

(c) Naïve-group tests

Each focal female was introduced to a pair of novel ('naïve') social partners three times over the course of the experiment (at 0, 4, and 12 weeks). A different pair of naïve social partners was used at each of these trials, and those partner fish were not used with any other focal female. We measured the average behavior of these naïve-groups before exposing focal fish to genetically different long-term social environments (week 0) and after 4- and 12-weeks of exposure (see figure 1). To do so, individual focal females were removed from their rearing tank (at week 0) or their long-term social environment tank (at weeks 4 and 12) and placed in a "naïve-group" test chamber with two unfamiliar females from the same clonal lineage as the focal fish, size matched to the focal fish (± 4mm), and in the same reproductive state. These

novel fish were drawn from monoclonal, non-breeding rearing tanks similar to those from which focal and stimulus females originated and were, therefore, not exposed to the experimental social environments experienced by the focal females. After we introduced the focal fish into the naïve-group test chamber, we video recorded all three fish for 10 minutes, after which the focal female was removed and placed back into her experimental social environment (figure 1).

The naïve-group test chamber was an open field, circular tank (55.9 cm diameter), with half the bottom and corresponding sides painted white and the other half grey. This test chamber was placed inside a frame covered with blue tarp to minimize external disturbance. All water in the chamber was replaced with clean freshwater prior to every test. In the center of the frame, a camera (JVC Everio 1920x1080 HD video camcorder) was suspended 1.1 m above the tank. All videos were edited to remove the first and last 2 minutes of recording (VideoPad Video Editor by NCH sofware©, v. 8.40) to allow for acclimation to the experimental tank and to remove any influence of camera or experimenter movement at the beginning and end of trial. All cropped videos were 6 minutes long and were analyzed by a blind observer using EthoVision XT (Noldus, v14). Within the EthoVision program, we distinguished the three individuals throughout the analyses and acquired movement and position data (Cartesian coordinates) for all three individuals. Although fish could be individually tracked, the focal individual could not be distinguished from the novel partner fish on the videos; therefore, we did not calculate separate metrics for focal and novel partner fish. We extracted the following measures from EthoVision: distance traveled (cm), velocity (cm/s^2), frequency entering white zone, duration in white zone (s), latency to enter white zone (s), frequency entering grey zone, duration in grey zone (s), time spent immobile (s; freezing behavior), and distance between individuals (cm; shoaling distance).

We interpret these behaviors to reflect stress-related behavior and tendency to be exploratory. More stressed individuals are less active, travel shorter distances at lower velocity, spend more time frozen and in the grey zone (negative phototaxis), and are closer together; less

stressed individuals tend to be more exploratory and cover more distance, move at higher velocity, enter zones more frequently, spend more time in the white zone and less time frozen, and haver more distance between individuals [47, 48]. We also gathered baseline data on these behaviors by following the same procedure at the start of the experiment, before the focal fish had experienced the experimental social treatments (time 0; figure 1: Pre-exposure).

(d) Ethics

All fish tanks included substrate and enrichment and were maintained with weekly water changes throughout the duration of this study. Fish never suffered from food deprivation or injuries during this study. This research was approved by the Institutional Animal Care and Use Committee of Florida State University (1704 and 201900038).

(e) Analyses

There were no significant differences in size (SL) among focal females in different social treatment groups, nor treatment-associated differences in size among the social partners fish used in the long-term and naïve-group trials (electronic supplemental material, table S1). Nevertheless, we included SL of focal and partner fish as covariates in subsequent analyses because there was a non-significant trend for Clone 1 and Clone 2 social partner fish to differ in SL (electronic supplemental material, table S2)

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

We assessed the correlation structure of the two measurements of aggression to determine if they could be adequately represented by principal components (PC), and then used the first PC from this analysis as our measure of aggression (see Results). To determine if aggression was influenced by social treatment group, we used this PC score as the dependent variable in general linear mixed models that accounted for the repeated measures on each group. In addition to the social treatment group, initial models included fixed effects of

exposure time (weeks), treatment-by-time interaction, the baseline (week 0) measure of aggression PC1 in each group, focal female standard length (log-transformed), and the average standard length of the social-partner females (log-transformed). A random within-subjects effect with group ID as the subject was used to account for repeated measures on groups; initial models also included a random effect due to experimental block. The fixed effects of baseline aggression and size of social partners never approached significance in initial models (electronic supplemental material, table S3A), and the random block effect was consistently near zero and never significant. These terms were, therefore, not included in the final models. This and other analyses of general linear mixed models were conducted using SAS *Proc Glimmix* in SAS v. 9.4 (SAS Institute Inc. 2013. SAS/STAT 9.4. SAS Institute Inc., Cary, NC.). Because repeated observations were not equally spaced in time, we used a covariance structure that allows for unequally-spaced observations (a 1-dimensional spatial structure, implemented with the *sp(pow)* option). Within-subject variance estimates were allowed to vary by treatment group, by using the *group* option. Post hoc comparisons of treatment group means were conducted using the simulation method of [49], as implemented by using the *adjust=simulate* option.

(e.2) Naïve-group tests.

To determine the extent to which the presence of the focal individual influenced behavior in the naïve-groups, and thus to measure cascading IGE, we calculated two kinds of metrics: those that described average behavior of the 3 members of the group, and those that described individual behavior of fish within the group. For both analyses, we included seven of the movement variables (distance traveled (cm), velocity (cm/s^2), frequency entering white zone, duration in white zone (s), latency to enter white zone (s), time spent immobile (s; freezing behavior), and distance between individuals (cm; shoaling distance)); the frequency entering and duration in the gray zone was redundant with information for entering and duration in the white zone, so we used only the data for the white zone in the analyses.

Average behavior of naïve-groups. We assessed the correlation structure of the 7 behaviors to determine if they could be adequately represented by principal components (PC). The six behaviors that described movement or physical position in the enclosure were all moderately to highly correlated with one another ($0.4 < |r| < 1.0$), but they were not correlated with the average shoaling distance between fish (all $|r| < 0.2$) (electronic supplementary material, figure S1A), indicating that a PCA should include the 6 movement/position variables, but that shoaling distance should be analyzed separately. We used the first PC from this analysis as our measure of the movement and position of fish (see Results), and we used the log-transformed average shoaling distance as a measure of a group cohesion, since it arises from the relative positions of all three members of the group.

To determine if these two measures of naïve-group behavior were affected by the social environment experienced by a single member of the group, we used them as dependent variables in general linear mixed models that accounted for repeated measures on a group, implemented in SAS *Proc Glimmix*. Initial models included fixed effects of the long-term social environment of the focal fish, time in the long-term social environment, size of the focal female, the mean size of the novel partner females, mean size of the long-term social partners, and baseline behavior (i.e., before exposure of the focal female to long-term social environments). To assess whether the effect of the long-term social environment was mediated by aggression experienced by the focal female, initial models also included a measure of aggression averaged over the 4 weeks prior to the naïve-group test (PC1 of aggressive behaviors averaged over those 4 weeks). A within-subjects random effect with focal female ID as the subject was used to account for the three different naïve-group trials in which each focal female was used; initial models also included a random effect due to experimental block. Neither the size-related fixed effects nor the summary measure of aggression ever approached significance in the initial models (electronic supplemental material, table S3B and C), so only treatment and exposure time (and their interaction) were retained in the final models. The block random effect was always near zero and never approached significance, so it was dropped from the final models.

We used the compound symmetry covariance structure because it fit the data better than alternative structures (by AICC and BIC metrics). Post hoc comparisons of group means were conducted as described above.

Behavior of individuals in naïve-groups. The main purpose of this analysis was to determine if differences in the average behavior among groups was attributable to all members of a group behaving similarly or to specific individuals within the group. For example, if the behavior of the three females within a group was very similar, then average differences among groups reflect the behavior of all group members. Alternately, if individuals within groups behaved differently from one another, then between-group differences could have been driven by the divergent behavior of a single group member. The former, but not the latter would support cascading IGE because it would indicate that non-focal behavior was influenced by the prior social experience of the focal fish. Our primary measure of similarity of the behavior of individuals within naïve-groups was the intraclass correlation coefficient (ICC). ICC values near 1 indicate that individuals within a group behaved very similarly to one another, whereas lower values of ICC indicate substantial differences in behavior among members of the group.

We first investigated the correlation structure of the same 8 behaviors described above but measured on individuals rather than the mean of the 3 fish in a group. Similar to the group-average data, the position/movement variables were moderately to highly correlated with each other, but not with shoaling distance (electronic supplementary material, figure S1B). We, therefore, summarized the movement/position behavior of individual fish using the first PC of the 6 movement/position metrics (table 1). As in the group-averaged data, behaviors associated with exploration loaded positively on PC1 (distance, velocity, and duration and frequency in the white zone), while behaviors associated with reluctance to explore loaded negatively on PC1 (freezing, latency to enter white zone, table 1). Again, we considered positive values of the first PC to indicate a tendency to be exploratory and negative values a tendency to be stressed. We used the log-transformed individual shoaling distance (the mean distance of a single individual from her group mates during a trial) as a measure of individual tendency to shoal.

We then calculated the ICC of the individual exploratory behavior scores and the individual shoaling distances as the ratio of the between-group variance to the total variance. These variance components were calculated using SAS *Proc Mixed* with default settings and a single random effect corresponding to the group ID.

3. Results

(a) High correlations found within the aggression behaviors and among the movement/position variables

We found that the two measures of aggression (number of acts and time spent) were highly correlated (R^2=0.803, p<0.0001), with the first PC explaining 96.9% of the total variation (electronic supplementary material, figure S2A). For the average behaviors of the naïve-groups, the first PC summarizing the 6 movement/position variables explained 72.4% of the total variation, and it was the only PC with an eigenvalue >1 (table 1, electronic supplementary material, figure S2B). In this PCA, behaviors associated with exploration loaded positively on PC1 (distance, velocity, and duration in the white zone, and frequency entering white zones), while behaviors associated with stress loaded negatively on PC1 (freezing, latency to enter white zone, table 1). We therefore considered positive values of PC1 to indicate a tendency to explore, and negative values to indicate lack of exploration or stress-like behaviors.

(b) Long-term social environments differ in social behavior

Long-term social groups in which the focal fish was housed with two females of its own clonal lineage exhibited more aggression than groups where the social partners were Clone 1 or Clone 2 fish (figure 2A, table 2A, effect estimates provided in electronic supplementary material, table S4). On average, fish in the Monoclonal environment performed 60% more aggressive acts than fish in the Clone 1 environment (14.45 ± 1.49 vs. 9.04 ± 1.27 acts per 10-minute observation bout, respectively; fish in the Clone 2 environment performed 11.04 ± 1.18 aggressive acts per bout, on average). Post hoc tests indicated that the Monoclonal social environment elicited significantly more aggressive behavior than the Clone 1 environment

($p<0.001$), but no other contrasts were significant after adjustment for multiple tests (Monoclonal vs Clone 2: $p=0.087$; Clonal 1 vs Clone 2: $p=0.114$; table 2A; electronic supplementary material, figure S3).

(c) Genetic differences in prior social experience for one group member affected behavior of all members of the naïve-groups.

The long-term social environment experienced by the single focal fish in a naïve-group affected the exploratory behavior of the entire group (table 2B, figure 2B, and electronic supplementary material, figure S4, effect estimates for fixed effects provided in electronic supplementary material, table S5). Indeed, the social environment explained 43.1% of the total variation in the exploratory/stress PC1 scores [50]. This result is particularly striking because all focal and stimulus fish were members of a single clonal linage and, therefore, genetically identical [39]. Specifically, groups in which the focal individual experienced the Monoclonal long-term social environment exhibited more stress-related behavior (negative values on exploratory PC1) than groups in which the focal individual experienced Clone 1 or Clone 2 social environments (post-hoc tests: Monoclonal vs Clone 1, $p=0.021$; Monoclonal vs Clone 2, $p=0.004$). Naïve-groups in which the focal fish had experienced social environments containing Clone 1 and Clone 2 did not differ from each other after correction for multiple tests ($p=0.259$).

The mean shoaling distance in the naïve-groups was unaffected by the social environment experienced by the focal fish, duration of exposure, or their interaction (table 2C, effect estimates for fixed effects provided in electronic supplementary material, table S5, figure S5).

(d) Individuals within naïve-groups behave very similarly.

Focal and stimulus fish within the naïve-groups were unfamiliar with one another and had different social experiences prior to the trials. Focal fish were drawn from the long-term social environments, whereas stimulus fish were all genetically identical, all of similar age and

size, and all had similar prior social experience that differed substantially from that of the focal fish. Moreover, there was substantial variation in behavior across different trials, as indicated by the significant effects of long-term social environment described above. Nevertheless, the three individuals in a given trial behaved in a remarkably similar manner (ICC for individual exploratory behavior: 0.913; ICC for individual shoaling distance: 0.953). Figure 3 shows representative tracking data for 3 different trios from the naïve-group tests (see electronic supplementary material, figure S6 for 12 additional representations). The striking visual similarity of tracking patterns within a given trial is reflected in very high ICC estimates across each treatment for both individual exploratory behavior (Monoclonal: 0.917; Clone 1: 0.924; Clone 2: 0.897) and for individual shoaling distance (Monoclonal: 0.941; Clone 1: 0.970; Clone 2: 0.930); variance components and significance tests are reported in electronic supplementary material, table S7. That is, less than 10% of the total variation in behavior occurred among the three females within a given trial, despite the substantial differences in behavior among trials that is evident in figure 3 and electronic supplementary material, figure S6. These high ICC values indicate that all three individuals within a given trial exhibited highly similar behavior, despite their different prior experience.

Discussion

Elucidating the heritable causes of individual and group-level behavior is necessary to understand the evolution of social traits. Here, we demonstrate that phenotypic effects of genetically different social environments (IGE) carry over to a novel social environment to influence the behavior of individuals that did not experience IGE. This cascading effect is distinct from 'second-order' IGE [20, 21], in which the presence of genetically different individuals influences interactions between other group members. Our results, therefore, expand the scope of IGE by demonstrating that they can influence phenotypes even when there is no genetically-based variation present within groups. Given the prevalence of dispersal and fission-fusion social structure, there is substantial opportunity for cascading IGE in nature.

Recognizing cascading IGE in natural populations and quantifying their influence on phenotypes and evolvability will be challenging, however. Field research coupled with genetic analysis, or transplant experiments, could reveal effects that related individuals have on new social partners, but this would require tracking individuals that disperse into new social groups. Such investigations can be facilitated by using species with clonal reproduction (e.g., many microbes and plants, and some vertebrates and invertebrates). In these systems, high relatedness within clones will make cascading IGE easier to detect. Sexual species in which relatedness is known and social interactions can be recorded after dispersal from natal groups could provide additional opportunities to measure cascading IGE.

The cascading IGE we observed was associated with different levels of aggression that focal fish experienced in the long-term social environments. Somewhat surprisingly, it was the social environment containing fish of the same clone as the focal animals that exhibited the most aggression (and the naïve-groups containing these focal fish exhibited the most stress behaviors). Previous studies found that Amazon mollies exhibited less aggression towards sister clones when compared to non-sister clones [39, 51]. However, a different focal clonal lineage was used in those studies, suggesting that responses to sister and non-sister clones (and, therefore, first-order and cascading IGE) vary across genotypes. Consequently, we predict that higher levels of aggression within the social environment result in more stressed individuals, regardless of whether that social environment is monoclonal or composed of non-sister clones; thus, fish should exhibit less exploratory behaviors when in new social group. Furthermore, this kind of interaction between the direct effect of an individual's genotype and IGE can produce frequency-dependent and other forms of balancing selection that can maintain, or rapidly erode genetic variation [52, 53]. The possibility that similar effects could arise from the interaction of direct genetic variance and cascading IGE warrants future empirical and theoretical investigation.

In this experiment, it is possible that the cascade of IGE that we observed occurred because focal females in Clone 1 and Clone 2 treatments experienced a genetic change in the

social environment when they moved into the naïve-groups, but focal fish from the Monoclonal treatment did not. This would predict a significant difference between the Monoclonal treatment and both Clone 1 and Clone 2 (which we do find), but not between Clone 1 and Clone 2 (for which we found only a non-significant trend). Nevertheless, our data support the conclusion that genetically identical fish (the naïve partners) behave differently depending on genetic variation in the prior social environment experienced by another member of the group (the focal female). Whether cascading IGE depend on the degree of genetic similarity between past and current social partners should be a focus of future research.

We detected no effects of exposure time within the long-term social environments on aggression in those environments or on cascading IGE in the naïve-groups. Time-course effects on first-order IGE have been found in mosquitofish [53, 54], and increased exposure time led to higher aggression in previous studies of Amazon mollies [44, 45]. However, the time course effects of IGE reported in mosquitofish occurred during maturation, whereas the fish in our experiment were fully mature at the start of the study. The two studies that reported exposure-time effects on aggression in Amazon mollies maintained the animals at considerably higher density than that used in our experiment (44: 1.9 L / fish; 45: 4 L / fish; the present study: 6.3 L / fish), suggesting that exposure-time effects could be density-dependent.

The relatively low density in our long-term social environments might also account for lack of treatment or cascading effects on shoaling distance, despite strong effects on exploratory behavior. Anderson et al. [21] found that second-order IGE influenced social cohesion in *D. melanogaster*, and the extensive literature on leadership in social organisms indicates that differences among individual group members can substantially influence group-emergent behaviors such as shoaling [55, reviewed in 1]. We, therefore, predict that cascading IGE could be an important source of individual variation that generates group-emergent phenotypes [26, 56]. In our experiment, groups consisted of only 3 individuals, which might limit the tendency of these fish to shoal. Experiments that use larger groups and enclosures that allow more

457 flexibility in fission-fusion dynamics could determine the extent to which cascading IGE
458 influence group-emergent phenotypes.

459 In summary, IGE propagate beyond individuals that directly experience them in
460 Amazon mollies and possibly in many group-living species. These cascading IGE are a
461 potentially important cause of individual differences that can lead to the emergence of leaders
462 and followers, shoaling, swarming, and other group-emergent phenotypes. Theoretical and
463 empirical expansion of the robust quantitative genetic framework developed for IGE to include
464 cascading or other types of carry-over effects will facilitate understanding of social behavior
465 and its evolution.

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605

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

616 behaviors.

617

Level	Model	Measurement	PC1 loading
Group-averaged	PC1 exploratory/stress	Total distance traveled	0.4571
		Velocity	0.4565
		Frequency entering white zone	0.4516
		Duration in white zone	0.3237
		Latency to enter white zone	-0.3053
		Time spent frozen in place	-0.4251
Individual-level	PC1 exploratory/stress	Total distance traveled	0.4716
		Velocity	0.4711
		Frequency entering white zone	0.4484
		Duration in white zone	0.3080
		Latency to enter white zone	-0.2605
		Time spent frozen in place	-0.4380

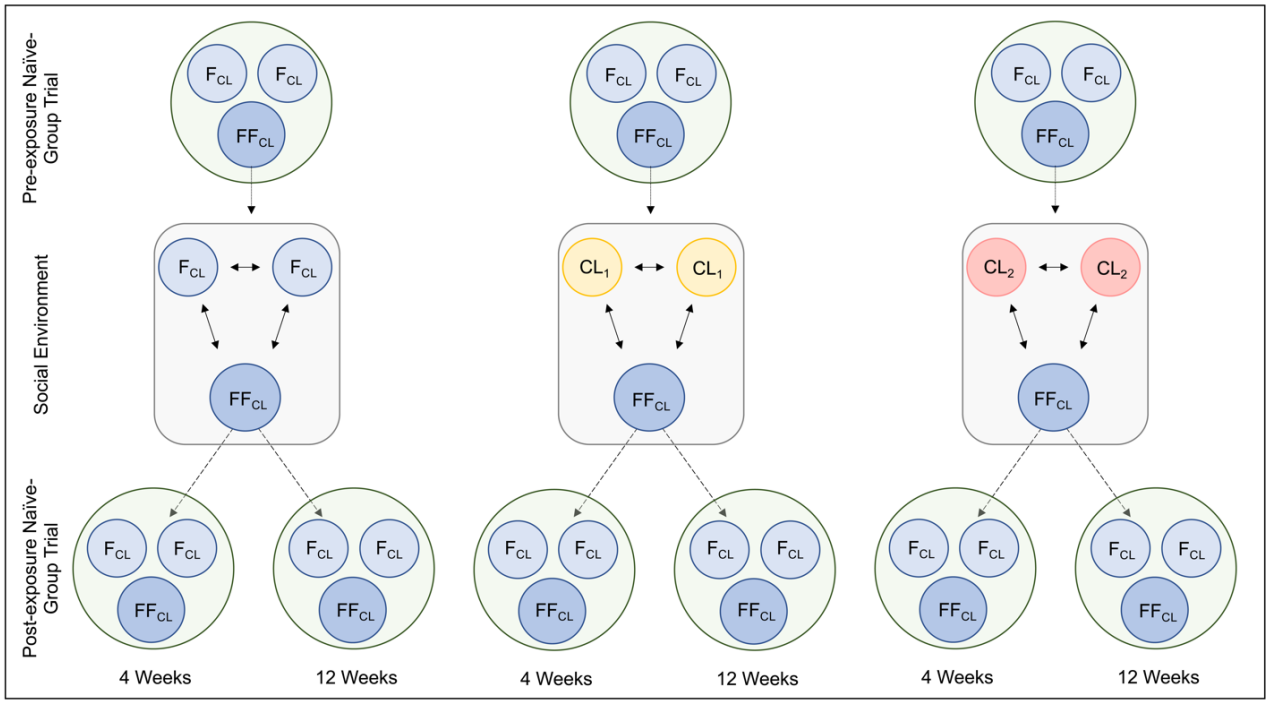
618

619 **Table 2:** Test statistic and p-value for statistical model of aggression PC1, exploratory/stress
620 behaviors (PC1), and shoaling behaviors as dependent variables.

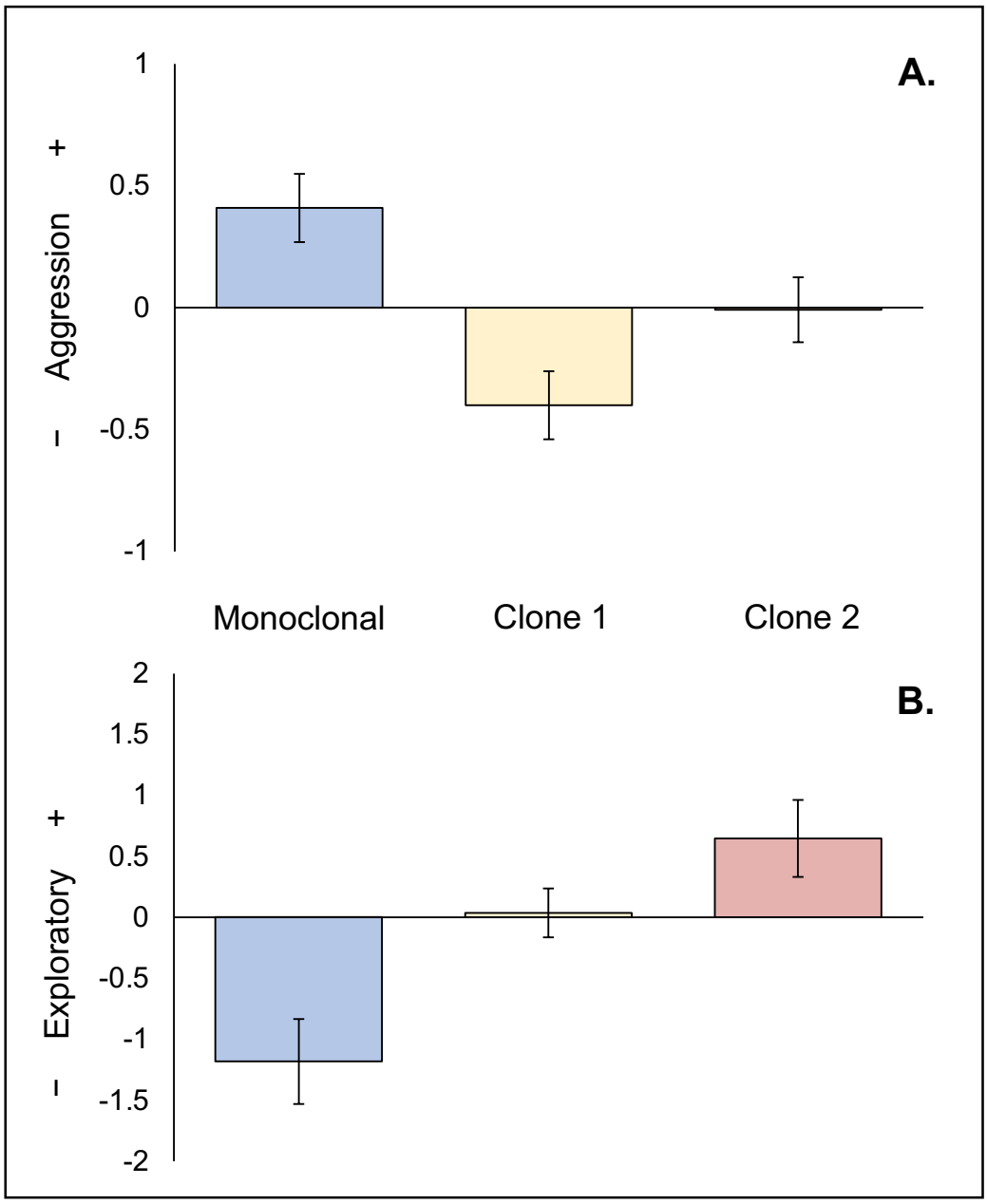
Model	Effect	Statistic	P-value
A. Aggressive behavior in long-term social treatments (PC1)			
	Focal female standard length	$F_{1,208.1} = =3.26$	0.072
	Social environment	**$F_{2,250.3} = 7.70$**	**<0.001**
	Exposure time	$F_{7,221.2} = 1.30$	0.253
	Social environment*Time	$F_{14,232.6} = 0.69$	0.788
B. Exploratory/stress behavior in naïve-group trials (PC1)			
	Social environment	**$F_{2,19.91} = 7.55$**	**0.004**
	Exposure time	$F_{1,23.29} = 0.55$	0.466
	Social environment*Time	$F_{2,16.03} = 0.78$	0.476
C. Shoaling distance in naïve-group trials			
	Social environment	$F_{2,20.04} = 0.60$	0.560
	Exposure time	$F_{1,26.93} = 0.07$	0.793
	Social environment*Time	$F_{2,20.1} = 3.20$	0.062

621

622 **Figure 1:** Schematic of experimental design illustrating the focal females (FF_{CL}) tested for pre-
623 exposure exploratory behaviors with two novel sister clones (F_{CL}) at week 0. Focal females were
624 then transferred into one of the three different social environments: Monoclonal (FF_{CL} + 2 F_{CL});
625 Clone 1 (FF_{CL} + 2 CL_1); or Clone 2 (FF_{CL} + 2 CL_2). After 4 and 12 weeks of exposure to these
626 social environments, the exploratory behaviors of the focal females were tested again with novel
627 F_{CL} individuals. Note that the F_{CL} partners of the FF_{CL} were different individuals at each time
628 period. That is, each individual F_{CL} was included in only one trial.



629 **Figure 2:** Least square means +/- standard error from PC1 for (**A.**) aggression focal females
630 were exposed to in each social environment (Monoclonal (blue); Clone 1 (yellow); Clone 2
631 (pink)). Positive values indicate more aggression. (**B.**) Exploratory/stress behaviors in the naïve-
632 group trials. Group-averaged exploratory behaviors with positive values indicating more
633 exploratory behaviors and negative values indicate less exploratory and more stress behaviors.



634 **Figure 3:** Visual representation of high intraclass correlation among individuals in the same
635 naïve-group trial. Within each treatment category (Monoclonal (blue), Clone 1 (yellow), Clone 2
636 (pink)) each row represents tracks of the three individuals in a single naïve-group trial. At each
637 time point (Pre-exposure (Pre), 4 wk, and 12 wk) the focal fish and two naïve partners were
638 tracked. For a given treatment, the same focal female was present at each time point, but her
639 two social partners were different individuals across time point.