Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2018), 29(2), 289–300. doi:10.1093/beheco/arx180

Original Article

Pervasive indirect genetic effects on behavioral development in polymorphic eastern mosquitofish

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Received 12 May 2017; revised 30 October 2017; editorial decision 23 November 2017; accepted 29 November 2017; Advance Access publication 25 December 2017.

The social environment can dramatically influence the development and expression of individual behavior. Indirect genetic effects (IGE) arise when variation in the social environment depends on genotypic differences among social partners. Their role in generating variation and influencing evolutionary dynamics has become increasingly recognized in recent years, but less attention has been paid to how IGE arise during development. We measured the development of IGE using a discrete natural polymorphism in male coloration and associated behaviors in eastern mosquitofish (*Gambusia holbrooki*). We observed substantial IGE and direct genetic effects on behavior. For some behaviors, IGE changed and even reversed direction over the 16 weeks of the experiment, indicating important developmental dynamics. Interaction between IGE and direct genetic effects for some behaviors suggests that melanistic males were less responsive to a genetic change in their social environment than nonmelanistic males were. Alternately, social partners might vary less in the behavior they direct toward melanistic males. Color morphs differed in mating behavior and in aggressive behavior they received from social partners, but not in direct measures of aggression. Therefore, even apparently innate differences in behavior between morphs could arise as indirect effects of differences in behavior directed toward them by social partners. These results indicate that some differences attributed to melanism in this and other species might result from color morphs experiencing different social environments. Deducing the developmental and social origins of these indirect effects is therefore critical for understanding melanism-associated behavioral variation in the many species in which it occurs.

Key words: **associate effects, behavioral syndrome, genotype-environment correlation, GxG epistasis, Poeciliidae, social niche construction.**

INTRODUCTION

Behavior is influenced by genetic and environmental variation, and the social environment, including during development, is an important source of environmental variation (Crook 1972; West-Eberhard 1979; Krebs and Davies 1991; Krause et al. 2010; Sokolowski 2010; Stamps and Groothius 2010; Sachser et al. 2013). This variation in the social environment can be created by factors such as density (Chapman et al. 2008), relatedness (Magellan and Magurran 2009), presence of adults, parents or siblings (Arnold and Taborsky 2010; Miller et al. 2012; van Leeuwen et al. 2014; Hesse et al. 2015), and sex ratio (Gracceva et al. 2011). An important source of social environmental variation is that due to the genetic identity of social partners (Griffing 1967; Moore et al. 1997; McGlothlin and Brodie III 2009; Bailey and Moore 2012), which can create indirect genetic effects (IGE) whenever phenotypes are influenced by genetic differences among social partners (Moore et al. 1997; Wolf and Brodie III 1998; Wolf et al. 1999; Bijma 2014). The role of IGE (also known as associate effects Griffing 1967, 1976; McGlothlin et al. 2010) in generating phenotypic variation and in influencing evolutionary dynamics has become increasingly recognized during the past 20 years, both in the development of a robust theoretical literature and with empirical demonstrations of IGE in many different organisms (Wolf et al. 1998; Wolf 2000; Shuster et al. 2006; Bijma and Wade 2008; McGlothlin et al. 2010; Saltz and Nuzhdin 2014).

Far less attention has been paid to how IGE might be influenced by an individual's early experience and whether IGE change during an individual's lifetime (Trubenová et al. 2015). The expression of behavioral traits can be highly plastic (e.g., Agrawal 2001), so the type and magnitude of IGE might respond to an individual's early experience or change substantially through an individual's lifetime. Several studies have shown that IGE experienced by juveniles subsequently affect traits expressed in adults (Buttery et al. 2010; Saltz et

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al. 2012; Teseo et al. 2014). However, whether and how IGE emerge and change over a developmental time course has rarely been investigated, except in the context of parental effects on offspring morphological development (Cheverud et al. 1983; Riska et al. 1985; Skrypzeck et al. 2000; Hunt and Simmons 2002, see also Saltz 2017 for an example of time-course effects on adult behavior).

Here, we use a discrete natural polymorphism in male coloration and associated behaviors in eastern mosquitofish (*Gambusia holbrooki*) to measure IGE across developmental timescales. The simplified genetics of this system permits the creation of replicated, genetically distinct social environments without the need for using inbred lines or clonally reproducing species. Using a polymorphism that segregates within populations also allows IGE to be attributed to genotypes on the range of genetic backgrounds in which they occur in nature. In addition, because the polymorphism we used results in visible differences between genotypes (body color), we are able to integrate results of laboratory experiments with those of field studies (Kraft et al. 2016) to understand how IGE operate in nature.

Eastern mosquitofish are small (~15–30 mm) livebearing fish in the family Poeciliidae, native to the southeastern United States (Kushlan 1980; Wooten et al. 1988). In natural populations, a majority of males display silver (S) body coloration, whereas a minority (0–15%) are mottled black-and-white or "melanistic" (M) (Horth 2004; Figure 1). This discrete natural polymorphism is regulated by a Y-linked factor that is constitutively expressed in some populations and temperature sensitive in others (Angus 1989; Regan 1961; Horth 2006). M males have been reported to be more aggressive than S males (Martin 1977), more likely to chase and attempt to mate with females, and to elicit more evasive behaviors from S conspecifics (Horth 2003). These results indicate that adult M and S males differentially affect their social partners, meaning that they can produce IGE.

We recently reported that M males have more social partners and especially more female social partners than S males, both in natural populations and in the lab (Kraft et al. 2016). M and S males therefore differ in the social environments they experience; i.e., they exhibit genetic variation in social niche construction (Saltz and Foley 2011; Saltz and Nuzhdin 2014). Genotype-environment correlation has also been reported in dark and light morphs of Eleonora's falcon (*Falco eleonorae*), in which nesting pairs clustered by color within a single breeding area (Gangoso et al. 2015). Variation among genotypes in social niche construction produces genotype-environment correlations that can inflate (or diminish) phenotypic differences among individuals and can alter the evolutionary potential of phenotypes and of the environments that those phenotypes construct (Donohue et al. 2005; Saltz and Nuzhdin 2014).

This evidence indicates that adult mosquitofish can experience direct genetic effects (DGE), IGE, and variation in social niche construction due to the M/S genetic polymorphism. However, we do not know if genetic variation in the social environment influences behavior or development of young mosquitofish, if IGE interact with DGE to influence individual behavior, or how these effects change over time. Because polymorphisms involving melanism have been associated with behavioral differences in many species (reviewed by Ducrest et al. 2008), the importance of understanding how social environment influences color-associated behavior extends well beyond mosquitofish. Melanistic body color is associated with variation in aggression, mating and other behaviors in fruit flies (Takahashi 2013), salmonid fish (Kittilsen et al. 2009), reptiles (Mafli et al. 2011), birds (Roulin et al. 2000; Scriba et al. 2014), and mammals (Bubenik and Bubenik 1985; West and Packer 2002; Graipel et al. 2014). Indeed, this association is so well known in vertebrates that it has been characterized as a behavioral syndrome and hypothesized to be driven by extensive pleiotropy in the melanocortin system (Ducrest et al. 2008). Understanding if and how the social environment influences behavior in a system exhibiting melanism polymorphism can therefore shed light on this hypothesis. For example, evidence for IGE and social niche construction in eastern mosquitofish suggests that behavioral differences associated with melanism can arise, at least in part, from a propensity for different color morphs to experience different social environments.

To address these questions, we reared young M and S males in 2 social environments in which the only difference was the genotype of social partners with which the focal animals could interact. Specifically, young males were reared with adult females and either M or S adult males, and their social behavior was assessed repeatedly over 16 weeks of development. Because the M/S polymorphism is the basis for genetic variation both in the social environment and in the focal animals, this experimental design can reveal IGE (if behavior varies in response to 2 different social contexts), DGE (if color morphs differ), and IGE-by-DGE interaction (if behavior is influenced by the interaction between social environment and focal male color). Because focal male behavior was measured repeatedly over 16 weeks, we could also ascertain if and how these effects depended on the time period over which different social environments were experienced.

METHODS

General husbandry, breeding, and experimental design

All focal juvenile males and adult social partners in the following experiments were offspring of wild fish collected from a population at Newport Spring, FL. Male color in this population is not temperature sensitive (Horth 2006), and M males comprise ~10% of the male population (personal observation). Fish were reared and housed in 38-L aquaria at Florida State University. Tanks were equipped with full spectrum lighting, a gravel substrate, a filter, and artificial plants for structure. The sides of each tank were covered to prevent individuals from receiving visual cues from adjacent tanks.

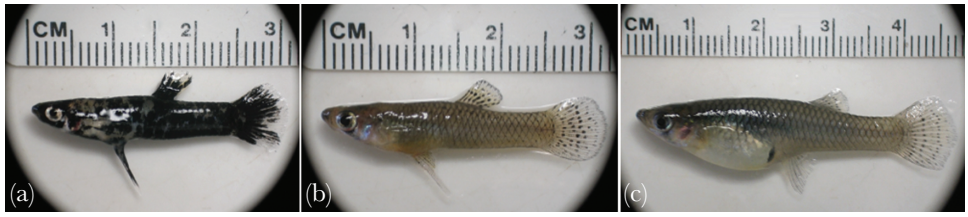

Figure 1
Silver male (left), melanistic male (center), and female (right) eastern mosquitofish.

All fish were fed twice on weekdays; fish received flake food in the morning (either Tetramin or Spirulina) and brine shrimp nauplii or flake food in the afternoon. On weekends, fish were fed flake food once during the day. Overhead lights and full spectrum tank lights were on a 12:12 h schedule (8:00 AM to 8:00 PM) during the first part of replicate 1 (2013), but were later switched to a 14:10 h schedule (7:00 AM to 9:00 PM) to promote breeding for the second replicate (2014). Temperatures were maintained between 22 °C and 26 °C. This study took place over the course of 2 years; different wild fish used as the progenitors in the 2 years, resulting in 2 replicates or blocks in the experimental design.

The gestation period for female mosquitofish is 2–3 weeks and females may be fertilized within 1 week following a birth, indicating that these females can reproduce as frequently as every 3–4 weeks during the breeding season (Pyke 2005). We therefore placed groups of 3–6 wild-caught females in tanks without males for a minimum of 30 days to allow for any broods conceived in the wild to be born. After this period, 1 or 2 females were placed with 1 or 2 males of the same color morph (either M or S) to form a breeding group. This procedure allowed us to predict the color morph of male offspring from each breeding group and to ensure that focal juvenile fish reared from the same breeding group (see below) would develop the same color morph.

The day juveniles were born, their broods were transferred to a single 10-gallon tank. Subsequently, we inspected fry at least once per week for development of M male color pattern or of the gonopodium, characterized by fusion of the rays on the anal fin to produce an intromittent organ (Pyke 2005). Either of these events indicated that the developing fry was male, rather than female. Note that while these events mark the beginning of maturation, male poeciliids are not sexually mature, and they continue to grow, until full development of the gonopodium, which takes up to 8 weeks in related species (Schreibman and Kallman 1977; Kallman and Borkoski 1978; Farr and Travis 1989). We therefore refer to these fish as "maturing" or "juvenile" males, rather than as adults. Once two developing males of the same brood could be identified, both males were removed from the brood tank and one of each pair was placed in each of two social contexts (described below). We split pairs of brothers in this way to control for maternal and common-environment effects that can influence juvenile behavior and development. Maternal effects have been studied extensively in the context of IGE (Wolf and Brodie 1998; Wolf et al. 1999; Moore and Moore 2003; Head et al. 2012); however, here we explored the role of postnatal interactions with unrelated social partners rather than maternal effects. Brothers were always placed in their respective social context on the same day (i.e., at the same age). Practically, this is the earliest stage at which males and females can be reliably distinguished. Moreover, this is a biologically realistic procedure. Young eastern mosquitofish interact mainly with other young fish in nature; young juveniles occupy shallower, more heavily vegetated littoral areas than adults (personal observation). Once juveniles reach sizes at which maturation begins, they move to deeper water where they shoal with adults. This experimental strategy unavoidably results in variation in the age of males when they entered the experiment (range = 23–109 days), which was taken into account in the data analysis (see below). The experimental design resulted in a hierarchical data structure: individual focal juveniles were nested within pair (a single pair was selected from each brood), and broods were nested within breeding group (i.e., breeding groups produced more than one brood).

For a given pair of male siblings, one focal juvenile was placed in a social environment consisting of 2 adult females and 2 adult M males ("MM") and the other was placed in a social environment consisting of 2 adult females and 2 adult S males ("SS"). Thus, each experimental tank contained 1 juvenile and 4 adults. Therefore, the density and sex ratio were consistent across treatments, and the only difference between treatments was the color morph of the adult male social partners. All female and male social partners used in the experiment were at least 1 year older than the focal juveniles and all were lab-reared. Fish that comprised a single focal juvenile's social environment were taken from different tanks in the laboratory to prevent any history of interactions with social partners from affecting behavior. Any social partner deaths during the course of the experiment were noted and these individuals were replaced with another individual of the same color and sex.

Focal juveniles were reared and observed in these social environments for 16 weeks from the day they were added to the study. Only one estimate of the lifespan of wild mosquitofish in nature is available. In a population in the Everglades, the maximum lifespan of a small sample of males was estimated as 108 days (Haake and Dean 1983). This is likely an underestimate of maximum longevity in the wild, and mosquitofish typically live much longer in the laboratory. Nevertheless, we reasoned that observing males past 16 weeks would extend the study beyond the lifespan of males we would expect to find in nature. We excluded pairs from the final analysis if one member of the pair died before the end of the experiment. This procedure resulted in removal of 4 pairs in replicate 1 and 1 pair in replicate 2. As a result, we used $N = 4$ S pairs and $N = 7$ M pairs from replicate 1 and $N = 4$ S pairs and $N = 5$ M pairs from replicate 2 in the final analysis, resulting in a total of $N = 8$ replicates of S juveniles in the MM context, $N = 8$ replicates of S juveniles in the SS context, $N = 12$ replicates of M juveniles in the MM context, and $N = 12$ replicates of M juveniles in the SS context.

Behavioral development in social contexts

Immediately following the addition of a focal juvenile to their social environment, the individual's behavior and interactions with its adult social partners were recorded for 10 min using a Kodak Playsport Camera. Following this initial observation (observation 0), behavior and interactions of each focal juvenile within his social environment were recorded for 10 min once per week for 16 weeks (observations 1 through 16), producing 17 observations on each focal individual. The behavior of pairs of focal juveniles in their respective tanks was always recorded on the same day. We recorded the following focal juvenile behaviors using JWatcher software (Blumstein et al. 2006): follow, flee, chase, swim, freeze, gonopodial swing, display, nip at social partner, nip by social partner, and not visible. Operational definitions of these behaviors are described in Supplementary Table S1. Focal males were not visible when they remained motionless behind filters or plastic plants; hereafter, we refer to this behavior as "hiding." Gonopodial swings and nips were recorded only as count variables because durations were too brief to be reliably recorded. For other behaviors, we recorded both durations and occurrences. The social partner (either male or female) toward which the focal juvenile directed interactive behaviors (e.g., follow or flee) was also recorded. We note that some scored behaviors, e.g., "nips by partner," depended on the behavior of the social partner, but we did not quantify social partner behavior independently.

An observer with knowledge of the hypotheses (B.K.) scored these videos. Therefore, to assess the possibility that unconscious bias influenced the results, a blind observer trained in identifying mosquitofish behavior also scored a subset of the videos. We compared the duration that these observers scored behaviors using Spearman correlations, and we used general linear models of log-transformed duration and count data to determine if observers differed significantly in scoring patterns. In these analyses, we did not distinguish between behaviors directed toward male or female social partners, instead analyzing total durations and counts of behavior directed at males and females combined. Correlations between observers were high and positive (Supplementary Table S2). While there were a few behaviors for which differences in counts or durations between observers were statistically significant (4 of 15 analyzed behaviors, Supplementary Table S3), for no behavior was there a significant interaction between observer and male color, or between observer and social context. These analyses suggest there was little unconscious bias on the part of observer B.K. that would have biased scoring with respect to focal male color or social context.

Statistical analysis

The same behaviors directed toward male and female social partners (e.g., "follow") were treated as separate behaviors in the analysis (e.g., "follow male" and "follow female"). We removed behaviors that occurred in fewer than 25% of all observations to eliminate excessive zero inflation. For the remaining behaviors (follow female duration, follow male duration, flee female duration, flee male duration, freeze duration, exploratory swim duration, follow female count, follow male count, flee female count, flee male count, nip female count, nipped by male count, and freeze count), we performed a principal component analysis (PCA) on data scaled to have unit variance using the "prcomp" function of the R "stats" package (R Core Team 2015). To account for variation in the amount of time available for scoring behavior while the focal fish was visible, time spent hiding was not used in the PCA, but instead used as a continuous covariate in the statistical models described below. Because remaining motionless out of sight of the observer could be a salient behavior, we analyzed hiding behavior separately from the principal components (PCs).

Scores of PCs that accounted for >10% of the variance were retained and analyzed separately using linear mixed models with repeated measures in SAS "Proc Mixed" (SAS Institute Inc. 2013). In these models, repeated weekly assays of focal male behavior were accounted for by using trial date as the repeated measure and focal male ID as the subject effect in the "repeated" statement. We chose the covariance structure of the repeated measures on individuals by comparing the AIC_C scores for different structures. We also allowed for separate covariance estimates across the 2 experimental blocks (years) by using the "group=year" option in the "repeated" statement. Potential nonindependence of the male pairs and of groups of male pairs derived from the same breeder tanks was accounted for by using breeder tank and male pair nested within breeder tank as random effects, using the "random" statement. We also included a random effect of time in experiment, as a categorical effect, to account for time effects that were not captured by the linear and quadratic fixed effects. In all mixed linear models, we used restricted maximum likelihood estimation and estimated denominator degrees of freedom using the Kenward–Roger method, which accounts for random effects, adjusts for small-sample bias in parameter estimates and standard errors, and is appropriate for correlated error models (Littell et al. 2006; Bell et al. 2013; Bell et al. 2014). Unless otherwise stated, data met the assumptions of linear mixed models (data and residuals were approximately normally distributed and homoscedastic). For effects with more than 2 levels, post hoc comparisons between least-square means were adjusted using the "simulate(cvadjust)" option in "Proc Mixed" to control for multiple testing.

We evaluated the following fixed-effect predictors for PCs and for time spent hiding: male color morph, social context, male age when he entered the social context treatments ("initial age" hereafter), and time (weeks) since beginning of experiment. Initial inspection of trait means across age and time indicated little evidence of nonlinear trends in dependent variables with respect to initial age, but substantial curvature for some trends with respect to time in experiment. Initial age was, therefore, used as a continuous linear predictor, whereas both linear and quadratic effects of time in experiment were evaluated. All interactions between these effects, up to the three-way interactions, were evaluated in initial models, and nonsignificant (NS) interactions between fixed effects ($P > 0.20$) were removed in a stepwise fashion. We also included experimental block (year) and the Julian date of the trial in initial models, and removed if NS; the year effect was always NS and was only retained in the statistical model for PC2 (where $P = 0.06$). We used time spent hiding as a continuous covariate in analyses for the PCs to account for differences among individuals and trials in the time available for scoring other behaviors. This variable was retained in all models even if NS. Finally, diurnal time of the observation (minutes past midnight) was used as a continuous covariate in initial models, but was removed from all final models because it was NS. Continuous covariates (time in experiment, initial age, and time spent hiding) were standardized to have mean zero and unit variance. We report only the final models here.

To compare the relative contributions of IGE, DGE, and other explanatory variables in the statistical models, we calculated Cohen's f^2 estimates (Cohen 1988) for each explanatory variable (i.e., male color, social context, time in experiment, and initial age). This measure compares different explanatory variables within the same model, is appropriate for hierarchical and repeated-measures data, and accommodates both categorical and continuous predictors (Selya et al. 2012). The local effect size version of Cohen's f^2 is the proportion of variance uniquely accounted for by main effects and interactions involving a given explanatory variable (e.g., social context), over and above that of all other explanatory variables in the model. We used the approach and calculations described in Selya et al. (2012) in these analyses. Because all dependent variables were standardized to have the same mean and variance, model effect estimates can also be used to compare the relative influence of different explanatory variables. We, therefore, report model effect estimates along with statistical tests from the final models.

RESULTS

Principal components analysis of behavior

The first 5 PCs (of 13 total) explained 76% of the variance in the multivariate behavioral data (Supplementary Table S4). Each of these PCs explained at least 10% of the variance, and each had an eigenvalue (and standard deviation) > 1. Loadings of behaviors on the first 5 PCs are shown graphically in Supplementary Figure S1. In the mixed-model analyses of these 5 major PCs, the covariance structure that minimized AIC_C was either a first-order autoregressive moving average ("type=arma(1,1)," PC1, PC2, PC4, and

hiding) or a heterogeneous first-order autoregressive ("type=arh(1)," PC3 and PC5), which allows for different variances across observation periods (Littell 2006). We report variance and covariance estimates from the final models for each trait in Supplementary Table S5.

The PCs were readily interpreted in terms of the individual behaviors that contributed most strongly to each one (Supplementary Table S4, Supplementary Figure S1). PC1 captured variation in following females, fleeing from males, and freezing behavior, with high scores on this axis corresponding to less following of female social partners and more freezing and fleeing from male partners. All social behaviors loaded negatively on PC2, so high scores on this axis corresponded to low levels of all social behaviors. Fleeing from and being nipped by male social partners and following females had the most negative loadings, so high scores on this axis corresponded to low fleeing and being nipped by male partners and low following of females. For PC3, high values represented more fleeing from females, while for PC4, high values represented less freezing behavior. PC5 captured variation in following males and nipping at females, with high values representing high following and low nipping.

IGE, DGE, and interactions between IGE and DGE were pervasive in these data. For hiding behavior and 4 of 5 major PCs, at least one factor involving color morph, or social context was significant (Table 1). In total, 13 tests involving color morph and/or context were significant at $P < 0.05$, whereas only 4 would be expected by chance (in the full statistical model for each trait, 12 terms involved color and/or context, and 6 full models were fit, for a total of 72 tests). In these tests, a significant effect of color morph represents a DGE. A significant effect of context represents an IGE because social contexts were defined by the genetic composition of animals that made up those contexts. Consequently, a significant color-by-context interaction represents IGE × DGE interaction, i.e., that IGEs differ between the 2 genetically determined color morphs.

Indirect genetic effects of social context

Hiding, PC1, PC3, and PC5 all exhibited significant main effects or interactions involving social context (Table 1, Figure 2). For PC3, a significant main effect of social context indicated that males in social contexts containing M males fled more from females than males in S social contexts (Figure 2e). For hiding, PC1 and PC5, social context interacted significantly with time in experiment. Despite significant non-linear time effects for PC1 and PC5, the general pattern was for larger effects of social context later in the experiment (Figures 2a, c, and f), suggesting that the effects of social context developed gradually over several weeks. These patterns indicate that, relative to the social context containing S males, the M context induced more hiding and freezing, more fleeing and less following of social partners, and more nipping at females. In other words, the M social context induced more behavior that can be categorized as defensive or subordinate, and the S context induced more offensive or dominant behavior. The only exception to this general pattern is that males in the M social context nipped more at females. In poeciliids, males nipping females is generally interpreted as a reproductive behavior, not aggression (Sumner et al. 1994; Houde 1997; Plath et al. 2007; Schlupp et al. 2010).

For PC1 (24% of variation in the PCA) and for hiding behavior, social context accounted for more individual variation than did any other explanatory variable in the model (Table 2). For PC3 (11% of variation in PCA), time in experiment and initial age accounted for the most individual variation, but social context accounted for much more variation than did male color morph. These effect-size estimates (along with the model effect estimates shown in Table 1) indicate that for hiding behavior and for a substantial proportion of the variation in social behavior, IGE due to the social environment were more influential than DGE due to genetically determined color morph.

For PC1, the color-by-context interaction was significant, reflecting that the effect of social context was greater for S than M males (Figure 2b, post hoc tests for differences between social contexts for S focal males $t = 2.26$, df = 23.9, unadjusted $P = 0.033$, adjusted $P = 0.131$; post hoc tests for M males, $t = 0.64$, df = 18, unadjusted $P = 0.53$, adjusted $P = 0.795$). The same pattern was evident for PC3 (Figure 2f), although the color-by-social context interaction did not reach significance for this PC (Table 1, PC3 post hoc test for differences between contexts for S males $t = 3.27$, df = 116, unadjusted $P = 0.001$, adjusted $P = 0.007$; for M males, $t = 1.33$, df = 139, unadjusted $P = 0.185$, adjusted $P = 0.542$). These results suggest that the social environment containing M males, which in general produced more defensive or subordinate behavior, had a larger effect on S focal males than on M focal males.

In addition to the time in experiment effects described above, social context interacted with initial age for PC1 and PC3 (Table 1C and D, Figure 2d and g). For PC1, this interaction reflected that males in the M social context tended to freeze and flee from males less and follow females more if they were older when entering the experiment (slope = −0.33, $t = -1.85$, df = 52, $P = 0.07$). In contrast, there was little or no trend for males in the S social context (slope = 0.15, $t = 0.75$, df = 32.1, $P = 0.46$). For PC3, the interaction reflects that males in the M social context were more likely to flee from females if they were older when they entered the experiment (slope = 0.23, $t = 2.81$, df = 37.8, $P = 0.008$), whereas males in the S social context were unaffected by initial age (slope = 0.03, $t = -0.35$, df = 36.4, $P = 0.73$). Remember that age at entry into the experiment was determined by initiation of maturation, so this effect is not necessarily driven by age per se.

Direct genetic effects of color morph

All PCs except for PC4 (together accounting for 66% of PCA variation) exhibited significant main effects or interactions involving color (Table 1). The color-by-context interaction for PC1 is described above. PC2 exhibited a significant main effect of color. On average, S focal males fled and were nipped more by male social partners and followed females more than did M males (Table 1B, Figure 3a). The significant color-by-time in experiment interaction for PC2 indicates that this difference between S and M males appeared after the first week of the experiment and persisted until the 15th week. Moreover, S males changed their behavior more over time than did M males (Figure 3b). PC3 also exhibited a significant color-by-time in experiment interaction in which S males fled more from females during the first half of the experiment, but this pattern was reversed in the second half (Figure 3c). Again, S males changed more over time than did M males. Finally, the main effect of male color for PC5 indicates that M males followed their male social partners more and nipped females less than did S males (Figure 3c).

Table 1

Final results of mixed-model analysis of behavior in different social contexts

A. Hiding	Estimate	DF_{num}	DF_{den}	*F* value	*P* value
Color	0.016	1	42.9	0.01	0.940
Context	−0.375	1	44.1	3.4	0.072
Time in experiment	0.412	1	34.6	1.87	0.180
Time in experiment*color	−0.493	1	149	2.01	0.158
Time in experiment*context	**−0.750**	**1**	**147**	**5.18**	**0.024**
(Time in experiment)^2	−0.319	1	33.1	1.81	0.187
(Time in experiment)^2*color	−0.485	1	150	2.04	0.155
(Time in experiment)^2*context	0.576	1	149	3.22	0.075
Age0	−0.022	1	72.6	0.06	0.806
B. PC1					
Color	0.420	1	17.9	0.2	0.664
Context	0.183	1	21.2	2.47	0.131
Color*context	**−1.176**	**1**	**23.1**	**4.89**	**0.037**
Time in experiment	0.063	1	37.1	3.24	0.080
Time in experiment*context	0.968	1	170	2.62	0.107
(Time in experiment)^2	**−0.013**	**1**	**36**	**5.74**	**0.022**
(Time in experiment)^2*context	**−1.433**	**1**	**169**	**5.72**	**0.018**
Age0	−0.331	1	24	0.32	0.575
Age0*context	**0.489**	**1**	**20.3**	**5.04**	**0.036**
Hiding	**0.225**	**1**	**572**	**9.06**	**0.003**
C. PC2					
Color	**−0.704**	**1**	**5.49**	**7.82**	**0.035**
Context	−0.087	1	33.5	2.59	0.117
Color*context	−0.552	1	32.8	1.47	0.234
Time in experiment	**−0.975**	**1**	**59.4**	**16.37**	**0.0002**
Time in experiment*color	**−0.661**	**1**	**200**	**6.44**	**0.012**
Time in experiment*context	1.042	1	200	0.22	0.636
Time in experiment*color*context	−1.547	1	199	1.87	0.173
(Time in experiment)^2	**0.408**	**1**	**57.2**	**6.13**	**0.016**
(Time in experiment)^2*color	**0.695**	**1**	**201**	**7.53**	**0.007**
(Time in experiment)^2*context	−0.941	1	200	0.04	0.851
(Time in experiment)^2*color*context	1.672	1	200	2.25	0.135
Age0	0.094	1	27.1	4	0.056
Age0*context	0.297	1	44.2	2	0.165
Hiding	0.459	1	606	58.65	<.0001
Date	0.375	1	6.14	5.53	0.056
D. PC3					
Color	0.173	1	14.8	0.04	0.849
Context	**−0.167**	**1**	**132**	**11.99**	**0.0007**
Color*context	−0.399	1	124	3.4	0.068
Time in experiment	−0.384	1	13.7	2.2	0.161
(Time in experiment)^2	0.364	1	14.1	0.61	0.447
(Time in experiment)^2*color	**−0.332**	**1**	**111**	**9.2**	**0.003**
Age0	0.230	1	15.4	2.45	0.138
Age0*context	**−0.258**	**1**	**134**	**6.34**	**0.013**
Hiding	**−0.169**	**1**	**262**	**14.59**	**0.0002**
E. PC4					
Color	−0.017	1	15.9	0.01	0.941
Context	0.200	1	31.7	1.79	0.190
Time in experiment	0.486	1	32.6	3.48	0.071
(Time in experiment)^2	−0.536	1	32.6	2.86	0.100
(Time in experiment)^2*color	0.194	1	144	2.19	0.141
Age0	0.159	1	21.4	2.68	0.117
Hiding	0.074	1	528	2	0.158
F. PC5					
Color	**−0.208**	**1**	**184**	**6.29**	**0.013**
Time in experiment	**−0.146**	**1**	**10.8**	**10.23**	**0.009**
Time in experiment*color	−0.111	1	203	1.73	0.190
Context	0.037	1	213	0.21	0.644
Time in experiment*context	**−0.762**	**1**	**188**	**5.42**	**0.021**
(Time in experiment)^2	**0.089**	**1**	**12.7**	**6.6**	**0.024**
(Time in experiment)^2*context	**0.807**	**1**	**193**	**5.42**	**0.021**
Age0	−0.070	1	209	3.07	0.081
Hiding	−0.244	1	334	38.53	<.0001

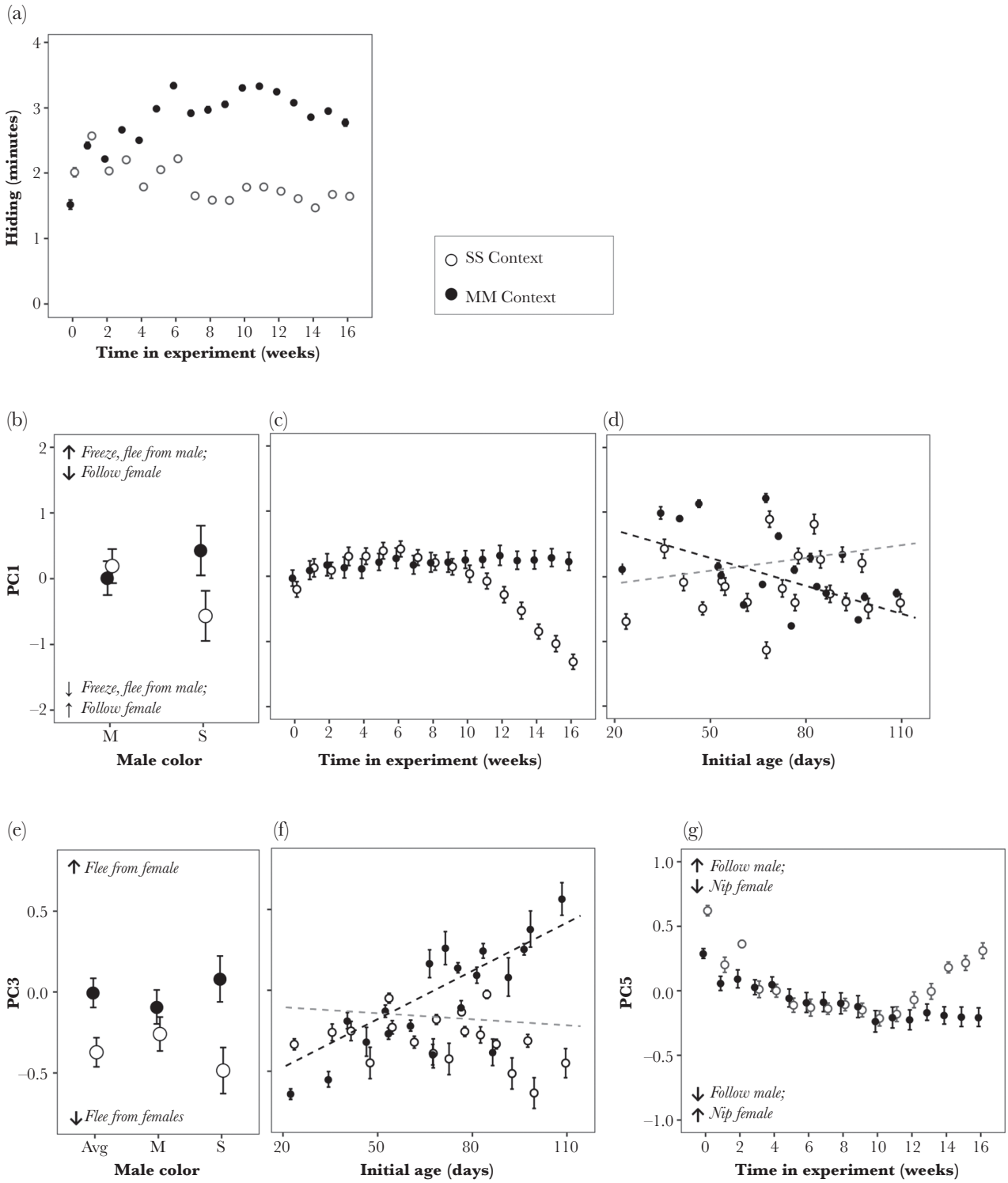

Figure 2

Indirect genetic effects on focal male behavior. Main effects and interactions due to social context. Statistical results and *P* values given in Table 1. Symbols show least-square means (BLUPs for interactions with a continuous predictor) ± standard errors corrected for other terms in the model, and are jittered to improve legibility. Open circles indicate values for a social context containing adult S males (SS context); closed circles indicate values for a social context containing adult M males (MM context). Arrows in one panel for each PC indicate behaviors that loaded most strongly on that PC and the direction of loadings. When there are multiple graphs for the same trait, they are plotted on the same y-axis scale. A, C, and G: interaction between social context and time in experiment for hiding, PC1 and PC5, respectively. B: interaction between social context and focal male color for PC1. D and F: Interaction between social context and initial age for PC1 and PC3, respectively; dashed lines show the linear trends with initial age for S (gray line) and M males (black line). E: Main effect of social context and (nonsignificant, $P = 0.07$) interaction of social context and focal male color for PC3; "Avg": least-square means for the main effect; "M": least-square means for M males; "S" least-square means for S males.

Table 2
Cohen's f^2 estimates of proportion of variance explained by main effects and interactions of each explanatory variable in the final model

A. Hiding	Cohen's f^2
Male color	0.004
Social context	0.052
Time in experiment	0.022
Initial age	0.002
B. PC1	
Male color	0.008
Social context	0.075
Time in experiment	0.070
Initial age	0.023
C. PC2	
Male color	0.162
Social context	0.050
Time in experiment	0.171
Initial age	0.054
D. PC3	
Male color	0.001
Social context	0.011
Time in experiment	0.023
Initial age	0.019
E. PC4	
Male color	0.007
Social context	0.008
Time in experiment	0.017
Initial age	0.035
F. PC5	
Male color	0.015
Social context	0.006
Time in experiment	0.027
Initial age	0.015

DISCUSSION

Pervasive indirect genetic effects associated with color polymorphism

If genetic variation in the social environment affects ecologically relevant phenotypes, IGE can profoundly alter selection and evolution. Although IGE have been demonstrated in many taxa, little is known about how they arise during development, or whether they contribute to behavioral differences such as those attributed to color polymorphisms. By rearing eastern mosquitofish that differed genetically in color in groups that varied in the genotypes of social partners, we found pervasive IGE on behavior; IGE were substantial and statistically significant in 4 of 6 behavioral traits (hiding, PC1, PC3 and PC5). We also observed developmental change in IGE influencing young males. In 3 of 6 traits (hiding, PC1, and PC5), the effects of genetic variation in the social environment became more pronounced over the course of the experiment. To our knowledge, this is one of the first reports of a developmental time course for behavioral IGE (Saltz 2017).

These IGE, and the size of their effects, indicate that the genetic composition of the social environment can influence behavior to at least an extent as great as genetically determined melanism. In addition to the IGE described above, IGE × DGE interactions indicated that some differences between morphs were apparent only in some social contexts, a form of genotype-by-environment interaction. In addition, DGE for some behaviors that were not significantly influenced by the social environment nonetheless depended on the time that males spent in the social treatments. PC2 and PC3, which together accounted for 31% of the variation in the PCA, were both influenced by an interaction between color morph and time in experiment. For PC2 (reflecting variation in fleeing from and being nipped males and following females) the interaction was due to differences between morphs increasing during most of experiment. For PC3, (reflecting variation in fleeing from females) differences between morphs reversed during the course of the experiment. These patterns suggest that some behavioral differences between morphs are not displayed immediately, but develop only after prolonged exposure to social partners, and that the differences can reverse over time. Overall, our data support the hypothesis that differences in M and S social behavior arise from both inherent differences and from the social environments they experience.

Our previous work indicated that M and S males experience different social environments even when they live in the same groups and are measured in the same natural populations (Kraft et al. 2016). We found that adult M males had more nearby social partners, and especially more female social partners, than did S males. Therefore, differences between M and S males might arise at least in part from the morphs experiencing differing social environments, even when they live in the same social groups. In other words, some differences between genotypes could result from genotype-environment correlation (Saltz and Nuzhdin 2014; Saltz 2017). This possibility has received little attention in the literature on color-associated behavioral polymorphism (but see Gangoso et al. 2015).

To illustrate this point, note that data from the current experiment do not strongly support the hypothesis that melanistic individuals are inherently more aggressive than nonmelanistic ones. The only "aggressive" behavior that was frequent enough in focal males to include in the analysis was nipping of female social partners. In many poeciliids, this behavior is a normal part of mating, not aggression (Sumner et al. 1994; Houde 1997; Plath et al. 2007; Schlupp et al. 2010). Moreover, M males nipped females less than did S males (Figure 3d). PC2 and PC5 provide the strongest evidence of inherent differences in behavior between young M and S males because they were both influenced by a significant main effect of color morph. These were also the only traits for which color morph explained more behavioral variation than did social context. These patterns reflected that, compared to S focal males, M males followed and nipped females less, fled and were nipped less by males, and followed males more.

These results parallel those of Horth (2003) in which mating behavior differed between M and S morphs, but other differences were due to aggressive behavior *received by*, rather than "performed by," the 2 types of males. Even the apparently innate difference in following and nipping of females we observed could be an indirect effect of females exhibiting more avoidance behavior toward M than toward S males. Because we did not directly assay avoidance behavior of females, we cannot assess this possibility. Collection of detailed behavioral data on social partners and on all dyadic interactions could test this hypothesis in future studies.

More generally, the influence of social partner behavior toward melanistic and nonmelanistic individuals should be addressed in other systems characterized by color-associated behavioral polymorphisms. In most of these systems, it is an open question whether some apparently inherent differences between morphs are due to differences between morphs in behavior that social partners direct toward them. Such differences could arise if rarer melanistic individuals are less recognizable as conspecifics, or if small inherent differences are magnified by social partner response to those differences.

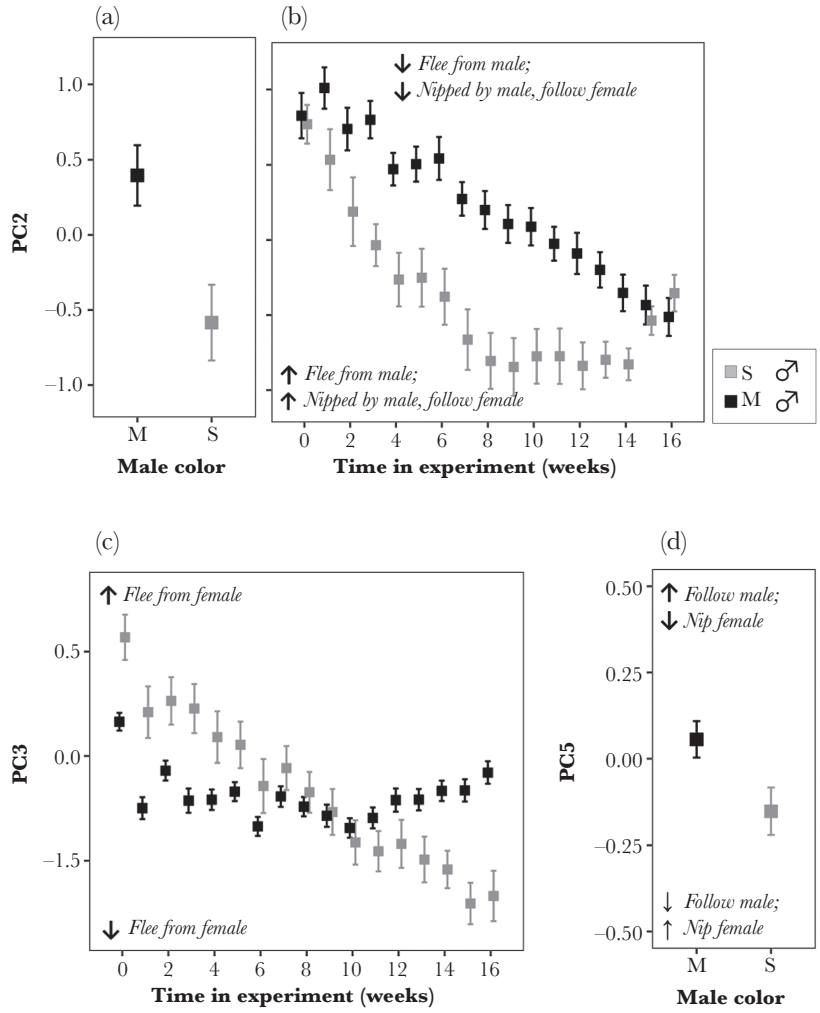

Figure 3

Direct genetic effects on focal male behavior. Significant main effects and interactions due to focal male color (M or S). Statistical results and *P* values given in Table 1. Symbols show least-square means ± standard errors corrected for other terms in the model and are jittered to improve legibility. Gray squares indicate values for S focal males; black squares indicate focal M males. Arrows in one panel for each PC indicate behaviors that loaded most strongly on that PC and the direction of loadings. A and D: main effect of focal male color for PC2 and PC5, respectively; B and C: interaction between focal male color and time in experiment for PC2 and PC3, respectively.

Indirect genetic effects and the maintenance of color-associated behavioral polymorphism

For a substantial fraction of behavioral variation, IGE interacted with DGE (PC1, which accounted for 24% of variation in the PCA). That is, the effects of genetic differences in the social environment depended on the genotype of the animal experiencing the social environment. This kind of interaction has been described as "GxG epistasis" (Wade 1998; Wolf & Brodie 1998; Wolf 2000, Buttery et al. 2010), reflecting that nonadditive interactions between genomes of different individuals are analogous to traditional epistasis (nonadditive interactions between loci in the same individual). Like traditional epistasis, GxG epistasis can produce complex forms of selection and rugged fitness landscapes (Wolf 2000; Wolf et al. 2004) and thereby contribute to the maintenance of polymorphism.

One form of selection that can emerge when IGE interact with DGE is frequency-dependent selection, where the fitness of a genotype depends on the frequency of that genotype in the population (Wolf 2000). For example, if individuals experience more aggression or competition from social partners that have similar genotypes than from partners with dissimilar genotypes, negative-frequency dependent fitness can arise. Negative-frequency dependence is an especially pertinent form of selection because it can promote and maintain genetic polymorphism (Ayala and Campbell 1974; Trotter and Spencer 2013), including behavioral and color polymorphism (Fitzpatrick et al. 2007; Hughes et al. 2013). More generally, IGE x DGE interactions can generate nontransitive fitness effects, where the fitness consequences of an interaction depend nonadditively on the genotypes that are interacting. Nontransitive fitness interactions, like frequency-dependence, can maintain polymorphism (Prout and Bundgaard 1977; Sinervo and Lively 1996; Trotter and Spencer 2007).

While we did not measure the fitness consequences of male behavior in this experiment, some results suggest that frequency-dependence resulting from IGE x DGE interaction could contribute to the maintenance of the color polymorphism. A substantial fraction of behavioral variation was characterized by a pattern in which S males responded more to IGE imposed by the social environment than did M males. PC1 and, arguably, PC3 showed this pattern (Figure 2b and e). For the behaviors captured by these

PCs, maturing S males were more responsive to genetic change in the social environment. Specifically, young S males displayed more defensive/subordinate behavior in the social environment containing M adult males. Greater flexibility of nonmelanic morphs has also been reported for reproductive strategies in owls (Emaresi et al. 2014), and this kind of variation in flexibility or plasticity has been suggested to be a mechanism that could promote melanin-based polymorphism (Roulin and Ducrest 2013).

In the case of eastern mosquitofish, greater flexibility might represent an advantage for S males in some contexts. A previous experiment showed that M males and adult females experienced lower survival in mesocosms with high M male frequency, but that S male survival was unaffected (Horth and Travis 2002). The authors suggested that increased M male aggression toward each other and toward females accounted for higher mortality rates at higher M frequency. If greater social flexibility of S males allows them to avoid deleterious consequences of high M frequency, then our results would be consistent with this suggestion.

Another explanation of more change in S males across social contexts is that young M males were more resistant to a stressful environment induced by adult M males. This interpretation is consistent with the hypothesis that melanistic individuals are more stress resistant in general than are nonmelanistic conspecifics (e.g., Ducrest et al. 2008). However, if resistance to social stress is beneficial, and environments with M males are more stressful, then the pattern we observed would generate positive frequency-dependent fitness, tending to destabilize the color polymorphism. Consequently, measuring the fitness consequences of the IGE and DGE associated with melanism will be critical for understanding if and how these effects contribute to the maintenance of genetic polymorphism in mosquitofish and other species.

Finally, a third possible interpretation for the apparently more flexible behavior of S males is that their social partners altered behavior across contexts, while the social partners of M males did not. That is, social partners might have behaved differently toward young S males in the S social context than in M social context, whereas they did not alter their behavior toward young M males across contexts. In this scenario, an interaction between social context and genotype of the focal animals would arise because social partners respond differently to young males of different color morphs. This possibility can be assessed in future experiments by quantifying all behavior of social partners across genetically different social environments.

CONCLUSIONS

Indirect genetic effects on male behavior were pervasive in this study and indicated that, for some behaviors, melanistic males were less responsive to a change in the social environment than were nonmelanistic males. We do not currently know the fitness consequences of this difference, but that knowledge is critical to understanding if and how IGE contribute to the maintenance of the melanism polymorphism in *G. holbrooki* and in the many other species that exhibit such polymorphism. Direct genetic effects were also apparent for some behaviors; melanistic and nonmelanistic morphs differed in mating behavior and in aggressive behavior received from social partners. Future studies color-polymorphic species should investigate to what extent even apparently innate differences between melanistic and nonmelanistic individuals arise from a tendency for the morphs to experience different social environments, or to receive different behaviors from their social partners, even when they are members of the same social groups.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

This work was supported by the National Science Foundation (IOS-1257735 to K.A.H. and J.T.)

We thank A. Buncy and E. Hoffner for help with experiments and Z. Culumber and K. Dixon for comments on drafts of the manuscript.

Ethical statement: All experiments were performed under protocol #1114 and protocol #1402 approved by the FSU Animal Care and Use Committee.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Kraft et al. (2018).

Handling editor: John Fitzpatrick

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