ZEBRAFISH
Volume 5, Number 1, 2008

DOI: 10.1089/zeb.2007.0513

Are Designer Guppies Inbred? Microsatellite Variation in Five Strains of Ornamental Guppies, *Poecilia reticulata*, Used for Behavioral Research

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Abstract

Inbred lines are an important tool of genetic studies of all traits, including behavior. Independently derived strains of ornamental "designer" guppies are readily available and predicted to be inbred; however, little is known about actual levels of inbreeding in any of these strains or whether these lines differ in genetic traits that have not been under strong directional artificial selection. We genotyped five designer strains of guppies known to vary in their responses to predator cues and a wild reference population to determine whether designer strains show evidence of inbreeding and whether the strains differed from each other at five microsatellite loci. The designer strains exhibited lower allelic diversity and observed heterozygosity than the wild population. Observed heterozygosity departed significantly from expected heterozygosity for most markers in all five strains of designer guppies. Inbreeding coefficient (f) comparisons between the wild reference population and the designer strains show considerable inbreeding in the designer strains. F_{is} values for the designer strains also provide evidence of inbreeding. Finally, F_{st} values indicate that the designer strains differ significantly from each other and the wild population. We therefore concluded that designer guppies are inbred compared to wild populations and differ among strains, making them useful tools for genetic studies of behavioral or life history traits.

Introduction

Inbred lines have long been used in genetic studies of phenotypic diversity at all levels of organization and have become an invaluable tool in neuroethological, physiological, and behavioral research. Inbred lines are commonly used to map quantitative trait loci (QTL) and candidate genes underlying behavior,[1–4] investigate the heritability of all types of traits,[5–7] and provide tester strains against which other strains, inbred or outcrossed, may be compared during behavioral research.[8,9] In addition to these more traditional investigations, inbred strains are used in behavioral research to investigate the influence of social environment on behavior and development,[10–12] for example, by controlling the potential for interactions at the genetic level between social partners.[13] The vast majority of such studies make use of a handful of traditional model species: rats, mice, *Drosophila*, and zebrafish.[8,14–17] Developing inbred lines of nonmodel organisms, therefore, offers the potential to more deeply explore how traits, particularly behavioral traits, may evolve in nature.

An excellent nonmodel species in which to develop inbred lines is the Trinidadian guppy

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(*Poecilia reticulata*). Guppy behavior has been extensively studied in the wild and in the lab using wild-type populations.[18–20] Guppies perform a suite of social and antipredator behavior in the wild,[21–23] including predator inspections where an individual or small group approaches potential predators to obtain information about the threat or deter predation (reviewed by Kelley and Magurran[24]). Guppy life history evolution resulting from differences in predation regime has become a textbook example of evolution in the wild.[25,26] Complementing the rich history of evolutionary and behavioral research on guppies, many independently derived and potentially inbred lines of "designer" guppies have been produced by aquarists who have artificially selected guppies for a variety of traits. Of the hundreds of strains of designer guppies commercially available, several strains have been behaviorally phenotyped and found to respond as wild-type fish do to predatory stimuli.[27] Individuals of one such strain (½Green) react in a similar fashion to wild-type fish in response to sometimes subtle differences in their specific social group,[13] suggesting that these lab strains retain wild-type behaviors appropriate for quantitative genetic studies of behavior.

Despite the ubiquity of such strains in aquarium settings, designer guppies have been only rarely utilized for research of any kind (but see the work by Sheridan and Pomiankowski[28]). Although such strains are generally predicted to be inbred, specific breeding histories are typically unavailable and little is known about their genetic variability.[29] Non-wild-type male body color and fin morphology are typically hemizygous or homozygous recessive traits (reviewed by Houde[30] and Oosterhout *et al.*[31]). As such, strains that breed true for male body color or fin morphology are likely homozygous for a number of traits; however, these traits have been under strong directional artificial selection. Other regions of the genome, such as neutral microsatellites, may not exhibit reduced diversity if selective breeding for traits or drift resulting from small breeding populations has also not resulted in inbreeding in general. Designer strains of guppies, if inbred, have the potential to greatly enrich the available genetic tools that can be applied to understanding behavioral and life history traits known to impact guppy fitness and evolution in the wild, as well as studies of behavioral and life history evolution in general. For example, inbred lines provide greater power to explore the genetic architecture and underlying sources of variation for phenotypic traits by controlling sources of genetic variation.[32] We therefore sought to quantify the degree of inbreeding in several strains of designer guppies known to respond to social and predator cues to explore their utility in quantitative genetic studies of behavior and behavioral evolution.

Materials and Methods

Strain selection and husbandry

Four strains of ornamental guppies (½Green, ½Yellow, Blue, and Snakeskin; described in Bleakley *et al.*[27]) were obtained in May 2004 and a fifth strain (Red-Cobra) in May 2005 from a breeder, S. Rybicki (Angels Plus, Olean, NY). The strains were chosen to maximize morphological diversity, and thus differ in a number of characteristics including color, body size, and fin morphology at sexual maturity. The initial four strains are also known to vary in their behavior, including their responses to predator stimuli.[27] The strains were maintained in multiple strain-specific tanks with larval fish moved randomly from breeding tanks into rearing tanks to maintain admixture within each strain. The fish were kept on a 14:10 light:dark cycle with constant water temperatures $\sim 24^{\circ}$C. They were fed twice daily 6 days per week with Hikari Fancy Guppy Food™.

Most guppy body colors other than the wild type are hemizygous or homozygous recessive traits (reviewed by Houde[30] and Oosterhout *et al.*[31]). F1 and F2 progenies were assessed for coloration upon adulthood and found to breed true in all cases,[27] including the Red-Cobra, which was assessed after the other four strains. Strains that breed true for such body coloration are likely to exhibit reduced genetic diversity for at least body color and fin morphology, as those traits have been under strong artificial directional selection. However, the breeder maintained three independent lines for each strain, crossing between lines only when necessary to combat inbreeding depression for viability and

reproductive traits[27] (Rybicki personal communication to B.H.B., 2005). No information is available, however, about the number of lines from any particular strain represented in the initial lab populations. Each of the designer strains was therefore of unknown inbreeding upon arrival in the lab, whereupon each strain was initiated with approximately eight females and eight males. All strains except the Snakeskin line experienced bottlenecks early in their lab history associated with probable inbreeding depression or husbandry problems. These bottlenecks reduced the populations to a single breeding male or female in four of the five strains. Each designer strain was subsequently maintained in the lab for 6 to 10 generations without any outcrossing between strains. For comparison to a natural population, a sixth group of fish was collected in March of 2006 from a high-predation population on the Aripo River in Trinidad near those used in previous studies of life history and behavioral evolution (e.g., Magurran *et al.*[20]). For consistency, we will hereafter refer to the wild population as the wild-type strain.

Tissue collection and genotyping

Tissue was obtained from 18 to 23 animals from each strain (Table 1). Individuals were picked haphazardly from the designer strains. Approximately one-third of the individuals sampled died of natural causes prior to the experiment and were stored in 1.5 mL Eppendorf tubes at −20°C. Sampling within each strain represented several generations. Measures of diversity are therefore assessed assuming an average of eight generations in the lab. The remainder of the individuals were removed randomly from their strain-specific community tanks. Sixteen wild individuals and seven lab-reared wild-conceived F1 progeny known to belong to different maternal families were utilized from the wild-type strain. Live fish were removed from their tanks and placed in an 80 mg/L solution of MS-222. Upon becoming nonresponsive, usually within 60 s, an individual was gently moved using a plastic spoon onto sterile cotton moistened with the anesthetic solution. The fin was clamped using forceps and clipped using surgical scissors to obtain approximately 3 mm^2 of tissue from the distal edge of the caudal fin. Tissue samples were placed immediately into individual 1.5 mL Eppendorf tubes and kept on ice until all fin clips were completed. The individual was then placed into a recovery tank containing antifungal medication to prevent infection and allowed to return to responsiveness. Altogether, each individual was anesthetized for less than 3 min.

DNA was extracted from the fresh or previously frozen tissue using standard phenol-chloroform extraction.[33] Extracted DNA was amplified at five microsatellite regions using primers from pret45, pret46, pret49,[34] pr80, and pr172.[35] Amplification of pret45, pret46, and pret49 occurred in 10 μL reactions, which included 0.2 mM of each dNTP and 1× buffer, 1 mM magnesium chloride ($MgCl_2$), 0.2 μM of each primer, 0.5 U *Taq* polymerase (Promega, Madison, MI), and approximately 10–20 ng/μL genomic DNA. Amplification of pr80 and pr172 occurred in 15 μL reactions and included 0.2 mM of each dNTP and 1× buffer, 1 mM $MgCl_2$, 3 pmol of each primer, 1 U *Taq* polymerase (Promega), and approximately 10–20 ng/μL genomic DNA. All reactions were completed in Eppendorf™ Thermocyclers utilizing the reaction conditions reported in Watanabe *et al.*[34] and Becher *et al.*[35] with two exceptions: annealing temperatures were optimized and adjusted to 58°C for pret49 and 56°C for pr172. Following amplification, the allele sizes and genotypes at each microsatellite locus were characterized using an ABI 3730 capillary sequencer and Genemapper software.

Statistical analysis

For each locus, the total number of alleles and observed heterozygosity were calculated. We calculated F_{st} between all pairs of strains and F_{is} for each locus within each strain and averages of all loci for each population using Fstat for Windows 2.9.3.2.[36] The remaining statistical analyses were carried out in JMP 5.0.1a (SAS Institute, Cary, NC, 1989–2002). We compared total alleles present in all strains using an ANOVA to look for differences among strains, and secondly, a Wilcoxon signed-rank test where the wild-type strain and the global maxima for the experiment provided expected values against

Table 1. Summary Statistics for All Strains (per Locus): Total Number of Individuals Sampled; Total Number of Alleles Identified; Frequency of Most Common Allele; Observed Heterozygosity (H_0); Expected Heterozygosity (H_E); Significance for Deviation from Hardy-Weinberg Equilibrium Obtained from χ^2 Comparison of Observed and Expected Heterozygosity; F_{IS} Values; and the Inbreeding Coefficient (F) for Each Designer Strain Relative to the Wild Population

Locus		*Snakeskin*	*Red-Cobra*	*½Green*	*½Yellow*	*Blue*	*Wild*
Pret45	n (123)	22	18	20	21	19	23
	Alleles (8)	1	2	5	3	3	5
	H_0	0[a]	0.056	0.2[b]	0.143[b]	0.053[b]	0.522
	H_e	0	0.056	0.523	0.222	0.104	0.475
	χ^2–HW	1	1	0.0002	0.0001	<0.0001	0.9949
	F_{is}	NA	0	0.529	0.362	0.5	−0.102
Pret46	n (122)	22	18	20	21	19	22
	Alleles (22)	8	4	5	7	7	11
	H_0	0.545[b]	0.556[b]	0.5[b]	0.571[b]	0.263[b]	1
	H_e	0.77	0.528	0.635	0.656	0.454	0.768
	χ^2–HW	0.0004	0.7418	0.2019	<0.0001	0.0075	0.0199
	F_{is}	0.298	−0.053	0.216	0.13	0.425	−0.01
Pret49	n (108)	20	17	19	18	20	14
	Alleles (11)	4	3	2	3	4	8
	H_0	0.650[b]	0.353[b]	0.053[b]	0.722[b]	0.4[b]	0.214
	H_e	0.654	0.456	0.053	0.532	0.433	0.845
	χ^2–HW	0.8627	<0.0001	1	0.3865	<0.0001	<0.0001
	F_{is}	0.006	0.232	0	−0.373	0.079	0.753
PR80	n (124)	22	18	20	21	20	23
	Alleles (15)	4	4	3	3	3	11
	H_0	0.25[b]	0.529[b]	0.105[b]	0.167[b]	0.6[b]	0.773
	H_e	0.32	0.577	0.153	0.427	0.522	0.802
	χ^2–HW	<0.0001	0.3027	0.0006	0.0002	0.7151	<0.0001
	F_{is}	0.222	0.084	0.315	0.615	−0.154	0.046
PR172	n (108)	19	16	19	19	15	20
	Alleles (10)	5	2	5	2	3	6
	H_0	0.579[b]	0[b]	0.895[b]	0.053[b]	0.333[b]	0.2
	H_e	0.56	0.226	0.624	0.053	0.543	0.321
	χ^2–HW	0.0004	<0.0001	0.5783	1	0.5798	<0.0001
	F_{is}	−0.034	1	−0.45	0	0.394	0.382
All loci	n (117)	21	17.4	19.6	20	18.6	20.4
	Alleles	22	15	20	18	20	41
	H_0	0.265	0.278	0.359	0.31	0.33	0.483
	H_e	0.457	0.366	0.397	0.366	0.413	0.641
	F_{is}	0.123	0.196	0.094	0.109	0.202	0.236
	F	0.45	0.42	0.26	0.36	0.32	

All loci category reports mean values for all statistics.
[a] $p < 0.05$ and
[b] $p < 0.0001$ for difference between observed heterozygosity in the designer strain compared to observed heterozygosity in the wild reference population.
NA, not applicable; χ^2−HW, p value for deviation from Hardy-Weinberg equilibrium.

which each designer strain was compared. We then calculated expected and observed heterozygosity for each locus within each strain and compared them using a χ^2 test to detect deviations from Hardy-Weinberg equilibrium. To compare observed levels of heterozygosity to predicted levels of inbreeding for a known history of inbreeding, we calculated expected heterozygosity given half-sib ($\frac{1}{2}\mathrm{Het}_{n-1} + \frac{1}{4}\mathrm{Het}_{n-2}$) or cousin ($\frac{1}{2}\mathrm{Het}_{n-1} + \frac{1}{4}\mathrm{Het}_{n-2} + 1/16\mathrm{Het}_{n-3}$) mating for 0 to 30 generations (n)[37] assuming complete heterozygosity in generation 0 ($\mathrm{Het}_0 = 1$).

Results

Levels of inbreeding

We estimated levels of inbreeding in four ways: overall allelic diversity, observed heterozygosity, coefficient of inbreeding (f), and the

population parameter F_{is}. Wild-type fish had somewhat fewer alleles than the global maximum (reflecting all alleles identified in this experiment; $\chi^2 = 2.2$, df = 1, $p = 0.138$). All designer strains were characterized by significantly reduced allelic variation compared to both the wild fish and the global maximum (Table 1; $F_{6,34} = 8.74$, $p < 0.0001$; $\chi^2 = 20.23$, df = 6, $p = 0.0025$). Designer strains did not differ from each other in overall levels of allelic diversity (Table 1; $F_{4,24} = 0.43$, $p = 0.78$). A single allele was the most common for the pret45 locus in all designer strains and the wild population, reaching fixation in the Snakeskin strain and near fixation in the Red-Cobra, ½Yellow, and Blue strains (Table 1). The most common allele in all other sampled loci varied among strains, but was often near fixation.

In the majority of cases, levels of observed heterozygosity were significantly lower than expected levels of heterozygosity for each marker within a designer strain and in some of the markers in the wild-type strain (Table 1). In four cases (pret45 in Snakeskin and Red-Cobra, pret49 in ½Green, and pr172 in ½Yellow), observed levels of heterozygosity did not differ from expected. However, these loci were fixed or extremely close to fixation with one or two total alleles, limiting the potential magnitude of divergence from Hardy-Weinberg equilibrium. More heterozygotes than expected were observed in three cases: the ½Yellow strain at the pret49 locus, the Blue strain at the pr80 locus, and the ½Green strain at the pr172 locus. Levels of observed heterozygosity in the designer strains were almost always significantly less than in the wild fish (Table 1). If we assume for the purposes of comparison that the designer strains are descended, at least in part, from Trinidadian guppies and that Hardy-Weinberg equilibrium is not being violated, then the inbreeding coefficient (f) may be calculated for each designer strain using the wild Aripo River fish as a reference population and the equation $f = 1 - H_f/H_r$, where H_f is the observed heterozygosity of the designer strain and H_r is the observed heterozygosity of the reference population.[38,39] Although many of the loci were not in Hardy-Weinberg equilibrium, this nonetheless provides a means of directly comparing levels of observed heterozygosity between designer and wild strains and allows comparisons to wild guppy populations known to have undergone bottlenecks. The calculated inbreeding coefficients of 0.26 to 0.45 indicate substantial levels of inbreeding in the designer strains compared to the wild fish (Table 1).[39] Lastly, observed levels of heterozygosity best matched those predicted by five to eight generations of second degree (half-sib) or fourth degree mating (cousin–cousin and aunt–nephew), respectively (Fig. 1), corroborating the timing of known bottlenecks in the lab.

F_{is} values ranged from −0.45 to 1, but in general were positive (Table 1). Negative F_{is} values for each locus can reflect significantly more heterozygotes than expected or other complexities of population structure, while large, positive F_{is} values indicate high levels of inbreeding.[40] Despite sometimes wide variance in F_{is} for each locus within a strain, mean F_{is} values were positive for all strains, including the wild population, which had the highest mean F_{is} value.

Differences among strains

Differences among strains were assessed using F_{st}, a measure of population subdivision in nature. Measures of F_{st} range from 0 to 1, with values above 0.2 indicating strong population subdivision and genetic differentiation.[41] Measures of F_{st} ranged from 0.25 to 0.49 for all pairs of strains (Table 2). As such, all strains were determined to be genetically distinct from each other at these five loci.

Discussion

Levels of inbreeding

Although no strain of designer guppies was completely homozygous for all the loci sampled, they were more inbred than a wild strain of fish. We found broad concurrence between several measures indicative of inbreeding: allelic diversity, levels of observed versus expected heterozygosity, and inbreeding coefficients. Four of the designer strains experienced severe bottlenecks within the lab and overall reduced effective population sizes; however, the strains retained some genetic diversity across all five of the sampled loci. Although

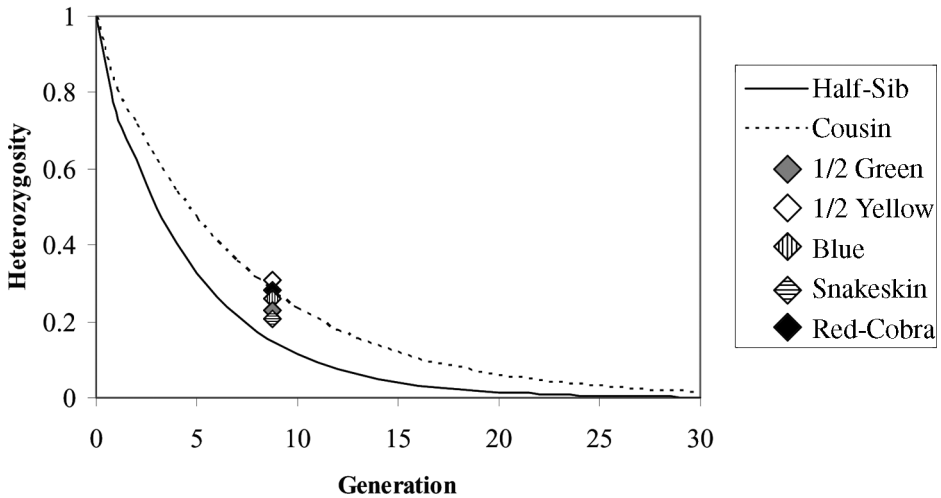

FIG. 1. Expected loss of heterozygosity given entirely half-sib (second degree) or cousin (fourth degree) mating system, assuming initial stock is fully heterozygous. Average levels of observed heterozygosity for each designer strain are plotted at generation eight, the average generation during which the strains were sampled.

single females became founders in three of the designer strains, they were almost certainly multiply mated (e.g., Pitcher *et al.*[42]) prior to the bottleneck and are likely to have stored sperm,[43] allowing more variation to pass into the next generation than in traditional isofemale lines. Individuals that died of natural causes were included in the sampling, potentially increasing measures of observed heterozygosity by including purged deleterious alleles that may have contributed to their deaths. However, the alleles sampled are expected to be neutral and thus should not be impacted by selection operating through inbreeding depression (reviewed by Keller and Waller[44]). The observed heterozygosity of the strains fell within theoretical predictions for the rate of loss of genetic diversity over several generations of half-sib mating, assuming complete heterozygosity upon entry to the lab (Fig. 1). Sampling within each strain represented several generations, thus providing a measure of average homozygosity within each strain and potentially an inflated measure of heterozygosity. The small number of founders combined with the bottlenecks experienced within the lab may have reduced genetic diversity compared to the original strains maintained by the breeder; however, the Snakeskin and Red-Cobra strains did not experience bottlenecks while in the lab but exhibited similar levels of inbreeding to the other strains. Several more generations of inbreeding within the lab would be predicted to further reduce existing levels of variation and eventually lead to fixation of alleles at more loci.

The inbreeding coefficient (f) provided an additional measure of inbreeding. Shared ancestry based on geographical location, and Hardy-Weinberg equilibrium are both assumed in the f measure. Violations of these assumptions can lead to underestimates of ac-

Table 2. F_{ST} Comparison Between All Pairs of Strains

	Snakeskin	*Red-Cobra*	*½Green*	*½Yellow*	*Blue*	*Wild*
Snakeskin	0					
Red-Cobra	0.393	0				
½Green	0.275	0.477	0			
½Yellow	0.233	0.410	0.258	0		
Blue	0.343	0.356	0.494	0.473	0	
Wild	0.254	0.368	0.353	0.272	0.321	0

tual inbreeding for both f and F_{is} by overestimating identity by descent among populations.[44] No explicit information is available about the geographical origins of the designer guppy strains. Guppies used to found designer strains potentially originate from anywhere within the full range of guppies, including Trinidad, Venezuela, and Guyana. The designer guppies carried alleles not found in the wild fish, with all identified alleles falling within reported size variation for alleles for all markers,[34,35] suggesting that the designer strains do reflect ancestors from multiple geographical locations, violating the assumption of shared geographical origin. However, this violation potentially results in underestimating inbreeding in the designer strains compared to the wild-type reference population. The measured magnitude of f in the designer strains compared to the wild-type reference population combined with measures of observed heterozygosity (H_0) and expected heterozygosity (H_e) and significantly greater allelic diversity in the wild-type fish compared to designer strains therefore provide strong evidence that the designer strains are inbred.

Levels of allelic diversity, mean levels of observed versus expected heterozygosity, and inbreeding coefficients all provided evidence that despite some genetic variation, the designer strains were inbred relative to a wild reference population. The F_{is} results, however, are not fully consistent with the other measures of inbreeding. Although mean F_{is} values indicate moderate inbreeding within each of the designer strains, the greatest mean F_{is} value was obtained for the wild population. F_{is} is strongly impacted by population substructure and sex ratio, ultimately measuring the degree of assortative mating within the population.[45] Female guppies have strong mating preferences in the wild,[46] yielding assortative mating, potentially inflating F_{is} for the wild population. Further, ornamental strains used in this study typically exhibited female-biased sex ratios, as designer males appear to be more susceptible than females to disease and minor environmental perturbations (B.H.B. personal observation). Females in these strains are likely to mate with all or nearly all males in a group tank both as a result of male scarcity and because males are better able to coerce females in tanks where they cannot escape a persistent male. As such, the assumptions underlying the F_{is} calculation may be violated, impacting the estimates of F_{is} and potentially underestimating inbreeding in the designer strains.

Strain differentiation

Our F_{st} data indicate significant differentiation between the designer strains in the five loci sampled. These data are consistent with measures of population subdivision in wild guppy populations, which are moderately to greatly subdivided.[47,48] The designer strains are known to vary in body coloration, fin morphology, and size at sexual maturity[27]; however, reduced genetic variation within a strain for these traits and differentiation among the strains are predicted because strong directional selection is expected to reduce additive genetic variation for those traits. Directional selection should not impact levels of variation in neutral genes. One microsatellite marker, PRET45, maps to a linkage group associated with yellow body coloration and therefore may have experienced selection in all of the lines.[49] The other markers are yet to be associated with traits under selection and are presumed to be neutral. Inbreeding and drift are, however, potent forces for differentiation in neutral genetic variation between small reproductively isolated groups.[41] In general, designer strains were more different from each other than any designer strain was from the wild population. Different strains may originate from stock obtained from different geographical locations, reflecting the base phenotypic variation for developing such morphologically distinct lines. Allelic diversity within a locus for many designer strains was near zero, while the wild fish were more variable at all loci. Drift is likely to exaggerate differences among strains with little variation increasing the disparity between the designer strains.[41]

Utility of designer guppies for genetic studies

The designer strains can be characterized as inbred and differ significantly from each other for the loci sampled. Reduced genetic variation combined with known differences in coloration, morphology, and behavior[27] make designer

guppies a potentially useful tool for quantitative genetic studies of all types, including behavioral evolution. Quantitative genetic models that explore the expression and/or evolution of traits typically partition the influences of genetics and environment on an individual's phenotype.[50] Inbred lines increase the power of such studies.[32,51] For example, inbred lines can be used to finely partition the effects of minute genetic changes, such as single nucleotide polymorphisms on complex phenotypes in *Drosophila*.[4] While the designer guppy strains are not clonal, as the *Drosophila* strains above are, they nevertheless offer the opportunity to better control genetic influences on phenotype to enhance our understanding of the impact of genetics and all types of environmental influences on the expression and evolution of traits that are ecologically important. For example, guppies have been used as a model system for exploring sexual selection.[52–55] Male guppy coloration is thought to provide an honest signal of his genetic quality by which females may choose among males,[31] but females may simply be more likely to mate with males exhibiting novel coloration, rather than the brightest males available,[56] and female preference is labile in the presence of predatory threats[57] and food availability.[58] Inbred lines could be used to more precisely explore the underlying causes of female preference[59] by controlling both the direct effects of genes on female preference and on male coloration or allowing direct explorations of interactions between female genotype and environmental conditions such as the presence of a predator or food availability.

Acknowledgments

Steve Rybicki of Angels Plus, NY, generously provided the designer guppies used to create the inbred lines. N. Combs, S. Pearish, D. Parker, R.M. Peace, and W. Smith all assisted significantly with aspects of fish husbandry. Comments from two anonymous reviewers improved the manuscript. Funding for this research was provided by IU Biology, the CISAB Shared Lab, and an NSF DDIG (IOB: 0508791); an ABS Grant-in-Aid of Student Research; and a CISAB Graduate Scholar Fellowship to B.H.B. and an NSF grant (IBN: 0130880) to E.D.B. III. The CISAB Shared Lab and IU CGB provided laboratory space and/or equipment. This research was carried out under the approval of the IUB-IACUC protocol #06-143.

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