

Variation in Anti-Predator Behavior Among Five Strains of Inbred Guppies, *Poecilia reticulata*

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Quantitative genetic studies frequently utilize inbred strains of animals as tools for partitioning the direct and indirect effects of genes from environmental effects in generating an observed phenotype, however, this approach is rarely applied to behavioral studies. Guppies, *Poecilia reticulata*, perform a set of anti-predator behaviors that may provide an ideal system to study how complex behavioral traits are generated. To assess the utility of ornamental guppies in quantitative genetics studies of behavior, we assayed five morphologically distinct strains of ornamental guppies for response to predator cues and for variation in response among strains. Despite individual variation, all five strains responded to predator cues and differences among strains were found for all assayed behaviors, including measures of boldness and predator avoidance.

KEY WORDS: Anti-predator behavior; inbred strain; *Poecilia reticulata*; quantitative genetics.

INTRODUCTION

Inbred strains of animals have played an increasingly important role in behavioral, physiological, and neuroethological research in recent years. Inbred strains may serve as “standard strains” against which outcrossed strains may be compared during behavioral phenotyping (Blizard *et al.*, 2005). Phenotyping inbred strains can provide information about the heritability of all types of biological traits, including behavior (e.g. Biesiadecki *et al.*, 1999; Isles *et al.*, 2004; Mackay *et al.*, 1996), and is frequently used as a tool for identifying and mapping genes (e.g. Anholt and Mackay, 2001; Baum *et al.*, 2005; Park *et al.*, 2003). In addition to these more common uses, inbred

strains of animals may also be an extremely important tool for exploring the impacts of selection on suites of morphological and behavioral traits and for generating empirical tests of quantitative genetics theories such as indirect genetic effects theory. However, inbred strains are rarely utilized outside of a few model systems such as rats, mice, *Drosophila* and zebrafish (e.g. Blizard *et al.*, 2005; Bothe *et al.*, 2004; Flint, 2003; Guo, 2004; Robison and William, 2005) or employed in studies of social interactions or the evolution of social behavior.

Genetic correlations between morphology and behavior can result in the evolution of both traits when selection is applied to only one. While behavioral evolution may frequently precede the evolution of morphology (West-Eberhard, 1983), selection on morphology or physiology can lead to correlated changes in behavior. For example, flight distance in red foxes is negatively correlated with mutant coat-color, docility in Norway rats is correlated with black coat color (reviewed in Price, 1984) and morphology and escape behavior are genetically correlated in

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garter snakes (Brodie, 1989). With the exception of hatchery-raised salmonids (e.g. Yamamoto and Reinhardt, 2003), the impacts of correlated selection for morphological traits on behavior in fishes have been poorly explored. Ornamental guppy strains have been under strong artificial selection for many morphological traits and may therefore provide a genetic tool for better understanding how correlated selection on morphology or physiology may be expected to impact the evolution of behavior.

Quantitative genetic studies of behavior typically involve exploring the contributions of genes and the environment to a behavioral phenotype. Studies of heritability typically partition out general environmental effects, but social animals experience both general and social environments, with the social environment containing its own genes. Because social behaviors are frequently defined in the context of interactions, considering the impacts of social partners and their genes may be very important for understanding how social behavior is expressed and evolves. One approach to understanding how genes carried in social partners may impact an individual's phenotype is provided by indirect genetics theory (IGEs) (Moore *et al.*, 1997; Wolf *et al.*, 1999). For example, the specific IGE case of maternal effects has been well studied, with genes that control begging behavior in offspring impacting the provisioning behavior of parents (e.g. Agrawal *et al.*, 2001; Hager and Johnstone, 2003; Kolliker *et al.*, 2000). IGEs imposed by unrelated social partners remain less well-studied because isolating the environmental effects provided by the social group from the direct genetic effects of genes carried in the focal individual is difficult. Inbred strains are extremely useful in teasing apart the direct effects of genes and the indirect effects of social environment in two ways. Inbred strains first supply a way to control the direct genetic contribution to behavior by providing focal individuals that are nearly homozygous. Second, inbred strains provide the means to control the genetic component of the social environment by holding the genes in social partners constant. Although many inbred strains of model organisms, including rats (Blizard *et al.*, 2005), fruit flies (Anholt and Mackay, 2001), and zebrafish (Robison and William, 2005), display behavioral differences (e.g. open field sensitivity, alcohol sensitivity, and startle response, respectively), relatively few assayed behaviors are expressed in a social context and no studies to date have utilized inbred strains to explore IGEs on behavior in unrelated individuals.

Understanding how quantitative traits in general, and IGEs specifically, may evolve in nature additionally requires inbred strains that retain and display ecologically relevant traits that can be compared to wild type relatives. Common guppies, *Poecilia reticulata*, have a long and rich record of life history and behavioral evolution studies (e.g. Dugatkin and Alfieri, 1992; Ghilambor *et al.*, 2002; Reznick *et al.*, 1990; Rodd and Sokolowski, 1995). As part of a suite of anti-predator responses, guppies exhibit different levels of boldness and perform "inspections" when presented with potential predatory threats in the wild (Dugatkin, 1988). Inspection behavior responds to the differing selective pressures associated with different predation regimes, with individuals from high predation populations inspecting more frequently than guppies from low predation populations (Dugatkin and Alfieri, 1992; Reznick *et al.*, 2001).

Utilizing guppies as a model for exploring the genetics of socially expressed behavior requires a number of inbred lines displaying phenotypic differences. Guppies are bred, and inbred, by hobbyists seeking to produce extreme morphological variation, producing literally thousands of independently maintained inbred lines of ornamental guppies that are commercially available. Several studies have utilized inbred lines of ornamental guppies to explore the genetics of coloration (cited in Lindholm *et al.*, 2005; Watanabe *et al.*, 2005), however, no studies have employed inbred guppies in studies of social behavior or behavioral evolution. As such, the goals of this study were 2-fold. First, we sought to determine if strains of guppies inbred in captivity for many generations retain the capacity to respond appropriately to predatory stimuli. Second, we wished to ascertain if strains of guppies inbred for morphological differences, but without regard for behavior, display differences in their responses to predatory stimuli. We therefore tested five commercially obtained inbred lines of "designer" guppies for responsiveness to predator stimuli and variation among strains in their responses.

METHODS

Five strains of common guppies maximizing phenotypic variation were obtained from Steve Rybicki of Angels Plus in Olean, NY. The strains: Red, Blue, $\frac{1}{2}$ Yellow, $\frac{1}{2}$ Green, and Snakeskin varieties varied in body coloration, fin morphology and male size at the onset of sexual maturity (Fig. 1).

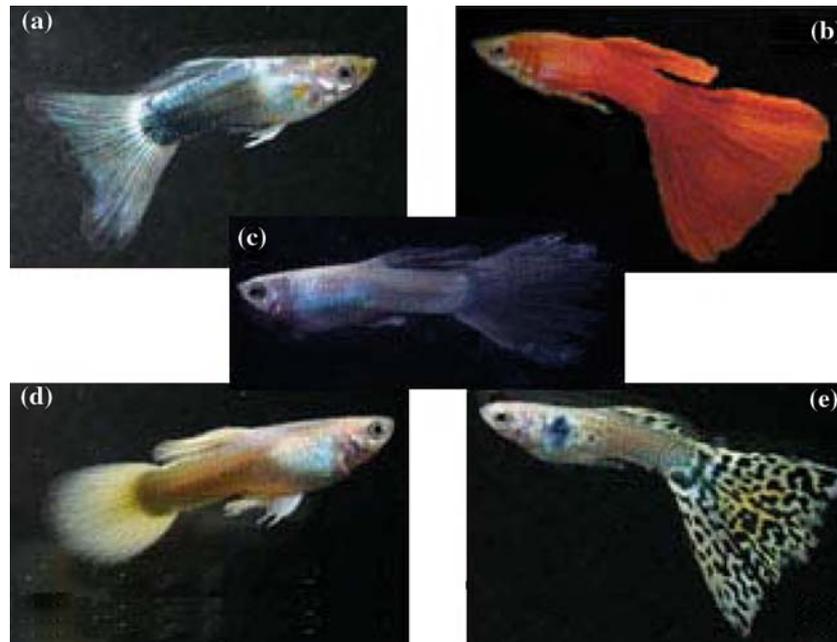


Fig. 1. Males from all five tested ornamental strains: (a) $\frac{1}{2}$ Green, (b) Red, (c) Blue, (d) $\frac{1}{2}$ Yellow, (e) Snakeskin.
Photo credit: Patrick Alexander.

The $\frac{1}{2}$ Yellow and $\frac{1}{2}$ Green strains correspond to breeder strains termed “half-black yellow” and “half-black green”, respectively, with black coloration on the anterior half of the fish. Individual breeders maintain separate lineages and utilize different names for morphologically similar strains. However, of the strains used in this study, the Reds, Snakeskins, and both “ $\frac{1}{2}$ ” strains phenotypically resemble other previously described inbred varieties: Red Tail, Tuxedo and Green Snakeskins, respectively (cited in Lindholm *et al.*, 2005). All individuals were sexually mature, with all females arriving and being tested while pregnant.

F1 and F2 progeny were collected throughout the experiment and assessed at maturity for patterns of coloration to estimate levels of homozygosity. Inbreeding full siblings for as few as 11 or 12 generations raises levels of homozygosity to nearly 90% (Crow, 1983). Information from the breeder indicates each strain derives from three closely related lineages maintained in parallel. Each lineage is inbred for variable lengths of time with infrequent outcrosses between lineages only when inbreeding depression becomes evident (Rybicki, 2005). Most body colors other than the wild type are homozygous recessive traits (reviewed in Houde, 1997). In the absence of direct knowledge of the exact number of generations

for which a lineage has been inbred, an approximation of homozygosity may be obtained by observing whether the selected color pattern breeds true in the F1 generation. All five inbred strains bred true for male body coloration in the F1 and F2 generations, indicating a high level of homozygosity in the parental generation for body color and fin shape.

The fish were maintained in strain-specific tanks kept at approximately 25°C with a 14:10 light:dark cycle and were fed Hikari Fancy Guppy Food™ twice per day (at approximately 9:00 am and 4:00 pm) on non-test days, and once after testing on test days. All individuals were naïve to interactions with any fish beyond their own strain, including all predatory fish. Five males and five females of each strain were tested, for a total of 10 individuals per strain. Individuals can be identified by small differences in coloration, fin morphology and size. Deaths associated with a disease outbreak in the middle of the experiment precluded testing all of the $\frac{1}{2}$ Yellow individuals and six Blue individuals in part three of the experiment.

Individuals were selected at random from each strain until all 10 individuals of each strain had been tested, and all individuals were tested between 9:00 am and 2:30 pm, the period during which these fish are most active, to minimize variation in behavior

associated with time of day. Individuals were exposed to three predator stimuli in sequential order: part one, visual presence of a predator model; part two, presence of predator chemical cue; and part three, the presence of both the chemical and visual cues. A generalized cichlid model was used, providing an “attack cone” visual stimulus, which is a potent threat cue for many small fish (reviewed in Kelley and Magurran, 2003). Chemical cue was obtained by placing a 5” standard length Midas Cichlid (*Amphilophus citrinellus*) in a 20 gallon aquarium for 12 hours, during which time it was not fed. One liter of water was removed and stored at 40°F for the duration of the study, approximately 12 days, to minimize degradation. Chemical cue samples were removed from the refrigerator in the morning, stored on ice for the day and the remainder discarded at the end of the day. Chemical cues were used on consecutive days within the trial periods and no differences in behavior across days were identified to suggest that the cue degraded over time.

The testing tank comprised a 10 gallon aquarium, lit from overhead with a SunStick™ Natural Wavelength fluorescent light, and divided into three sections: predator stimulus zone, close proximity zone (the area closest to the predator stimulus), and a neutral zone. The predator stimulus zone was isolated

from the rest of the tank by a permanent clear, water-permeable barrier and a removable opaque barrier. The tank was backed with a black and white grid in 1” increments for scoring position within the tank (Fig. 2). The tank was filled with 7 gallons of filtered water treated with AmQuel and Nova Aqua Water treatment. The testing tank was rinsed and the water changed completely between trials to prevent residual chemical cues, particularly alarm cues, from transferring between test subjects.

Each focal individual was caught from the strain-specific community tank and isolated in a small opaque container for 5 minutes prior to testing. The fish was then introduced to the testing tank in the neutral zone and allowed to acclimate for 30 seconds. Ornamental guppies have likely been strongly selected to rapidly disregard human disturbance. In all cases, the guppies quickly resumed normal activity (typically less than 15 seconds) after being introduced into the testing tank, therefore requiring only a short acclimation period. After acclimation, the focal individual was filmed for 3 minutes, during which time it was not exposed to any predator stimulus (the “pre-stimulus period”). At the end of 3 minutes, the opaque barrier was removed allowing the guppy to see the visual stimulus or empty predator zone. In parts two and three, 10 ml of chemical cue were

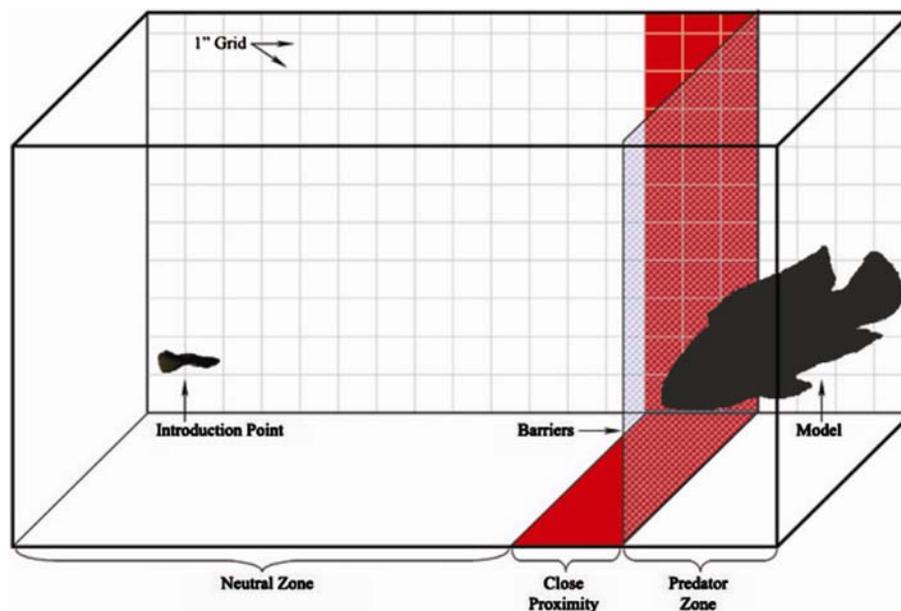


Fig. 2. Test-tank set-up showing predator model, barrier, focal introduction point, scoring grid on the tank back and area of close proximity (shaded area).

added simultaneously to the water in the predator zone and allowed to diffuse through the permeable barrier. Focal individuals were allowed to acclimate for 30 seconds and then filmed for an additional 6 minutes (the “post-stimulus period”). At least 2 days separated parts one, two and three for all fish. Video was captured from a Cannon, ZR-10 digital camcorder directly to a PC laptop, using FireWire™ and Windows Movie Maker, and saved as Windows Media Files (.wmv) at 30 fps and 189 kbps.

The videos were subsequently scored for several anti-predator behaviors using an event recorder (Ha, 1990). Time spent in close proximity to the predator stimulus along with number of inspections provide measures of boldness in the presence of a predator, while time spent in agitated swimming, the number of drops to the bottom and time spent frozen on the bottom of the testing tank provide measures of threat response. The data were converted to proportions of time spent out of pre-stimulus and post-stimulus periods of the trials and number of events per minute. The data were analyzed in JMP (1989–2005) using a mixed model (Standard Least Squares/EMS) with the strain and sex of the individual as random variables and the cue and period as repeated measures. Cue type was treated as a repeated measure rather than an experimental variable because the experiment was not intended to detect differences in response between cue types, but rather to maximize the chance of obtaining a response by providing a variety of cue types. No interaction effects were detected ($F_{4,233}=0.19-0.95$, $p>0.43$ for strain \times cue interactions and $F_{2,233}=0.0074-2.1224$, $p>0.13$ for strain \times sex interactions in all behaviors) during the initial analysis and were therefore removed from the final model. *Post-hoc* comparisons between strains were completed using Tukey’s HSD, which produces a conservative estimate of significant differences between all pairs, given multiple comparisons and unequal sample sizes (JMP, 1989–2005).

RESULTS

Guppies altered their behavior significantly in the post-stimulus period. Across all strains, the guppies spent a significantly greater proportion of time in agitated swimming ($F_{1,233}=29.28$, $p<0.0001$; Fig. 3) and spent a significantly greater proportion of time in close proximity to the stimulus area ($F_{1,233}=7.36$, $p=0.0072$; Fig. 3) in the post-stimulus period. The guppies also greatly increased the number of drops to the bottom in the presence of the predator stimulus

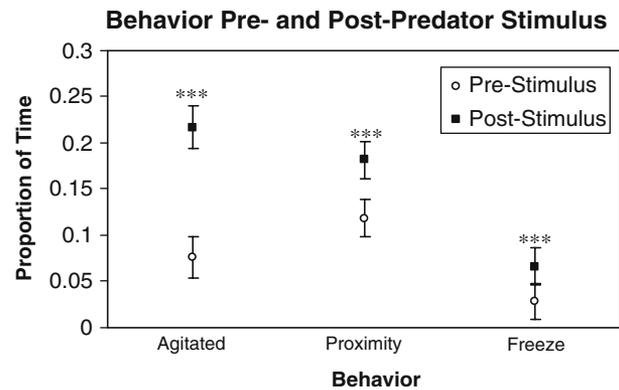


Fig. 3. Least squares means (\pm s.e.) for proportion of time engaged in behavior before and after the presentation of predator cues across all strains. *** $p<0.001$.

($F_{1,233}=10.46$, $p=0.0014$; Fig. 4). The guppies displayed a trend toward increases in the time spent frozen ($F_{1,233}=2.75$, $p=0.099$; Fig. 3) and the number of inspections displayed ($F_{1,233}=3.11$, $p=0.079$; Fig. 4), however, the increases were not significant. While small sample size may have decreased our ability to detect differences among strains for both of these behaviors, power for these analyses are 0.98 and 0.96, respectively, with both requiring sample size greater than 100 individuals to obtain statistical significance at $\alpha=0.05$.

The five strains varied with respect to all measured behaviors. The Snakeskins spent a significantly greater proportion of time frozen than did the Reds or $\frac{1}{2}$ Greens ($F_{4,233}=5.6401$, $p=0.0002$; Fig. 5a). $\frac{1}{2}$ Greens spent a significantly greater proportion of time in agitated swimming than did all other strains

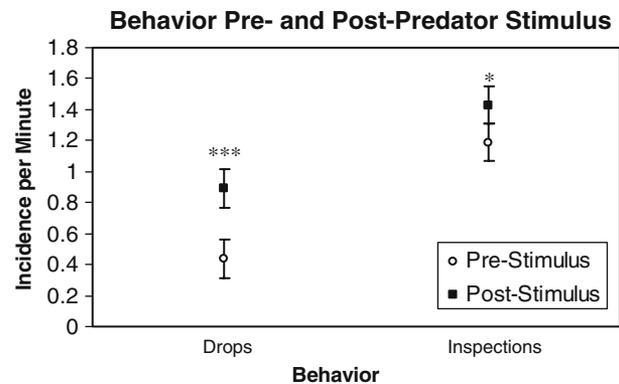


Fig. 4. Least squares means (\pm s.e.) for number of inspections or drops per minute before and after the presentation of predator cues across all strains. *** $p<0.001$, * $p<0.10$.

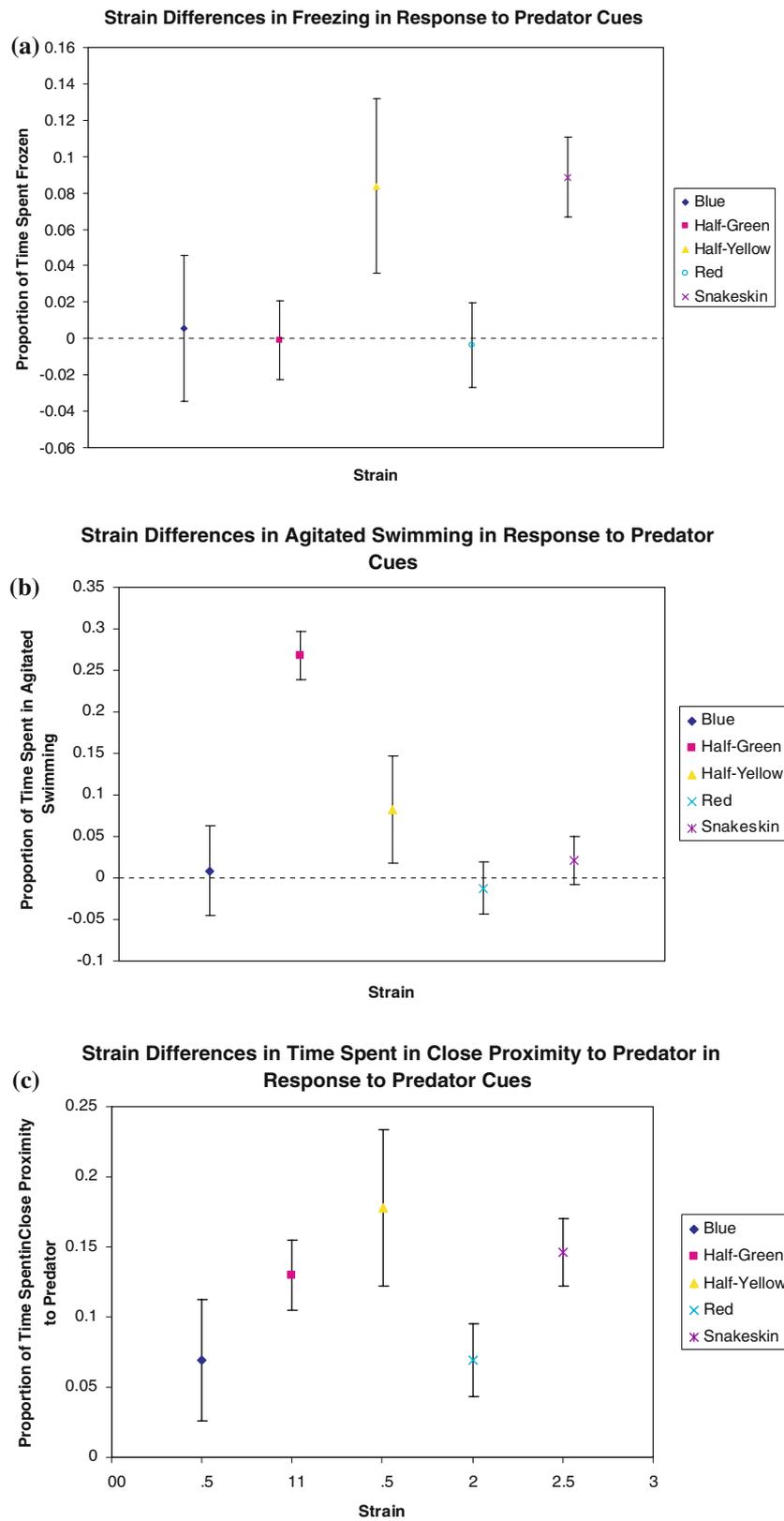


Fig. 5. Least squares means (\pm s.e.) for proportion of time spent (a) frozen, (b) agitated, and (c) in close proximity to the predator zone by strain.

($F_{4,233} = 19.3861$, $p < 0.0001$; Fig. 5b). Snakeskins displayed a trend toward spending a greater proportion of time in close proximity to the predator stimulus than did Blues or Reds ($F_{4,233} = 2.3361$, $p = 0.0564$; Fig. 5c). The $\frac{1}{2}$ Greens performed significantly more inspections per minute than did Snakeskins or Blues ($F_{4,233} = 4.9653$, $p = 0.0007$; Fig. 6a) and $\frac{1}{2}$ Greens performed significantly more drops per minute than did any other strain ($F_{4,233} = 11.6469$, $p < 0.0001$; Fig. 6b).

DISCUSSION

Despite being isolated from predatory influences for many generations, inbred guppies retain the ability to recognize and respond to predatory cues, increasing the frequency of anti-predator behavior in response to predator stimuli. Moreover, strains vary in their response to predatory cues in several ecologically relevant behaviors. $\frac{1}{2}$ Greens appear to be

generally more active than other strains, producing more drops to the bottom, more inspections and spending a greater proportion of time in agitated swimming. However, absence of interactions between cue type and strain for all behaviors indicate that differences among strains do not simply result from differences between $\frac{1}{2}$ Green guppies and the other four strains. Additionally, Snakeskins differ from Reds in the proportion of time spent frozen in response to predator stimuli. Small sample size, especially in the $\frac{1}{2}$ Yellow strain resulting from several deaths due to infection during the course of the experiment, increased the within strain variance exhibited in several behaviors. Larger sample size would likely reduce the observed within strain variance and allow for finer distinction between the responses among strains.

Behavioral differences in response to predator stimuli were found in all behaviors surveyed, perhaps surprisingly as the strains were initially chosen to maximize morphological diversity rather than behavioral differences. Anti-predator behavior has not specifically been under artificial selection in these ornamental strains. However, body coloration and fin morphology have been strongly selected for by breeders. Body coloration and boldness are correlated in male guppies in the wild (Godin and Dugatkin, 1996) suggesting that body patterns and behavior are in linkage disequilibrium in the wild, either as the result of selection or physical linkage between suites of genes. Linkage between morphology and behavior is an expected result of selection (e.g. Brodie, 1992; Brooks and Endler, 2001; Sinervo *et al.*, 2001). Background body coloration is a complex quantitative trait. For example, yellow body coloration can be mapped to 35 linkage groups as well as a number of unlinked loci (Watanabe *et al.*, 2005). As such, artificial selection on body color is likely to exert indirect selection on behavior, driving divergence in behavior as well as morphology between strains, if genes for the behavior are linked with those for body coloration.

Although inbred strains have rarely been used to address how social behavior evolves, they provide useful genetic tools for isolating the direct genetic effects of genes carried within individuals and indirect genetic effects of genes carried within social partners. Ornamental guppies retain the ability to respond appropriately to predator cues, both visual and chemical, despite many generations of selective breeding for morphological variation in the absence

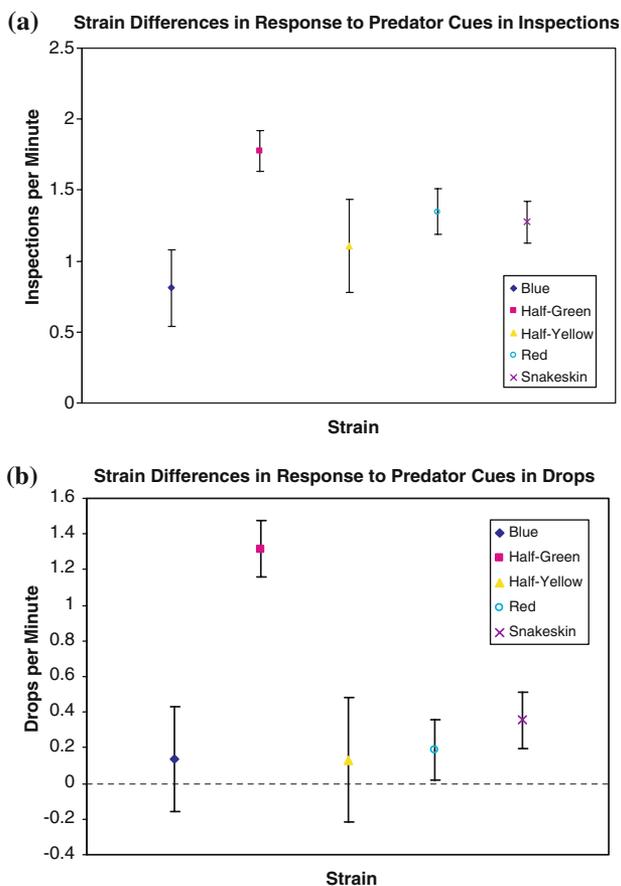


Fig. 6. Least squares means (\pm s.e.) for (a) inspections and (b) drops per minute by strain.

of predators. Ornamental guppy strains therefore provide both genetically and behaviorally distinct strains displaying ecologically relevant behavior that may be capitalized on for quantitative genetic studies of social behavior, specifically within an indirect genetic effects framework. Indirect genetic effects studies utilizing combinations of inbred and wild guppies, controlling various aspects of the genetics underlying anti-predator behavior, will allow novel exploration of selection pressures on and the evolution of complex behavioral traits.

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