Original Article

Does sensory expansion benefit asexual species? An olfactory discrimination test in Amazon mollies

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INTRODUCTION

“Doomed to extinction,” “dead-ends,” and “evolutionarily hopeless”: These are the terms used to describe asexual vertebrates (Maynard Smith 1978; White 1978; Vrijenhoek 1989), and with good reason. Asexual vertebrates appear as stubby twigs on the vertebrate tree of life, where only 0.1% of species are asexual (Bell 1982; Judson and Normark 1996). The scarcity of asexual vertebrates is surprising, both because asexuality is widespread in less complex life forms and because asexuality is thought to have many advantages over sexuality (Otto and Lenormand 2002). The gynogenetic Amazon mollies (*Poecilia formosa*; Hubbs CL and Hubbs LC 1932), a poeciliid fish formed by hybridization over 100,000 years ago. We test whether Amazon mollies can use only olfactory cues to detect conspecifics, detect heterospecific males, and discriminate between males infected with a common parasite. We further explore whether a female’s size, a proxy for age, explains any variation in her behavior. We find strong evidence that Amazon mollies use olfactory cues to detect conspecifics but surprisingly may avoid heterospecific males based on olfactory cues alone. We find no evidence that females use chemical cues to discriminate between infected and noninfected males. We also find that smaller Amazon mollies are more likely to use chemical cues. This study highlights the potential importance of sensory systems in asexual vertebrates.

Key words: asexual, chemical communication, expansion hypothesis, gynogenetic, hybrid sensory, Poeciliidae.

Why are asexual vertebrates so rare? One seldom explored avenue to understanding the evolutionary persistence of extant asexual species is their sensory ecology—how they perceive and respond to the environment. Asexual species formed by hybridization have been hypothesized to have an expanded sensory repertoire because they carry 1 allele from each of their parental species, including alleles that impact sensory function. The ability to detect odors in the environment is a likely candidate for this expansion but has never been explored in this context. Here, we explore the olfactory abilities of the asexual Amazon molly, a gynogenetic fish formed by hybridization 100,000 years ago. We test whether Amazon mollies can use only olfactory cues to detect conspecifics, detect heterospecific males, and discriminate between males infected with a common parasite. We further explore whether a female’s size, a proxy for age, explains any variation in her behavior. We find strong evidence that Amazon mollies use olfactory cues to detect conspecifics but surprisingly may avoid heterospecific males based on olfactory cues alone. We find no evidence that females use chemical cues to discriminate between infected and noninfected males. We also find that smaller Amazon mollies are more likely to use chemical cues. This study highlights the potential importance of sensory systems in asexual vertebrates.

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has a broader spectral sensitivity than those in either parental species, consistent with a mixture of opsin genes from the 2 parental species (Korner et al. 2006; Sandkam et al. 2013).

Olfaction—the ability to detect odorants in the environment—is a more likely candidate for sensory expansion, however. Odors are bound by olfactory receptor proteins in the olfactory epithelium (Hino et al. 2009). These olfactory receptor proteins are encoded by single genes (Ache and Young 2005), are co-dominant (Chess et al. 1994), and can have high specificity to bind specific ligands (Katada et al. 2005; Kaupp 2010; Leary et al. 2012). Odorant receptor genes are much more plentiful than opsin genes that mediate color vision. In teleosts, there are only a handful of opsin (9 in zebrafish and guppies; Chinen et al. 2003; Hoffmann et al. 2007) but 100–140 olfactory receptor genes (Alioto and Ngai 2005; Nimura and Nei 2005). Because Amazon mollies are hybrids, they contain one copy of the odorant receptor proteins—the proteins that bind odorants in the environment—from each parental species. Hence, hybrids may be able to bind a greater diversity of odorants and experience more diverse olfactory capabilities than either of their parental species, which may contribute to the continued existence of this asexual species.

Though olfaction may be an important modality for Amazon mollies and is a prime candidate for sensory expansion, its use by this species is completely uncharacterized. Olfaction mediates ecologically important behaviors in related species of fish, such as selecting mates (Shoet and Watt 2004; Fisher and Rosenthal 2006), identifying conspecífics (Crapson de Caprona and Ryan 1990), and avoiding predators (Brown and Godin 1999; Coleman and Rosenthal 2006), suggesting it might be an important modality for Amazon mollies as well. Understanding how these asexual fish use olfaction will shed light on the sensory system of this species and may offer some insight into the continued existence of the Amazon molly.

Here, we explore the olfactory abilities of the asexual Amazon molly. Amazon mollies prefer to shoal with conspecific females (Schlupp and Ryan 1996), but they also must mate with a male of one of their parental species to induce embryogenesis (Hubbs CL and Hubbs LC 1932; Schlupp 2005). Therefore, we ask whether Amazon mollies can use chemical cues to detect conspecific females and heterospecific sailfin molly males. Because adaptation is generally impeded in asexual species due to lack of genetic recombination, asexuals are often an easy target for parasites (Hamilton 1980; Bell 1982). Amazon mollies tend to avoid parasitized heterospecific males based on visual cues (Tobler et al. 2006), perhaps to mitigate risk of parasite exposure. We therefore also tested whether Amazon mollies can discriminate between heterospecific males parasitized by a common parasite. We further explore whether olfactory detection and discrimination vary with a female’s age. To test these questions, we built a custom flow tank and measured the amount of time Amazon mollies spend in the chemical plumes of different individuals. We predicted that Amazon mollies would use chemical cues to detect both conspecifics and heterospecifics, as well as to avoid infected heterospecific males.

**METHODS**

**Animals**

We caught Amazon and male sailfin mollies in Fort Brown Resaca, Brownsville, TX (25.897192, −97.492935) and acclimated the fish to the laboratory for at least a month before testing. All fish were housed on a 13/11 light/dark light cycle and fed TetraMin flake food daily and brine shrimp weekly. All females were isolated from males for more than 30 days, the average time between broods (Farr and Travis 1986; Snelson et al. 1986), to control for any differences in receptivity among females that may affect their behavior. Amazon mollies ranged in standard length from 24.7 to 48.4 mm and the male sailfins ranged from 35.5 to 41.5 mm. We initially planned to perform the same battery of tests described below with one of the Amazon molly’s parental species, the sailfin molly. Unlike the Amazon mollies, the sailfin mollies that we tested (n = 9) did not display normal behaviors in the experimental tank. As the sailfin mollies failed to explore the experimental tank in our assay (sailfin mollies moved on average 12% of the distance that Amazon mollies moved in the tank; Wilcoxon rank sum test, P < 0.001), we did not feel confident in our ability to reliably, meaningfully test olfaction in sailfin molly females and excluded them from the experiment.

Experiments were performed in accordance with IACUC protocol AUP-2013-00156.

**Experimental setup**

We designed and built a custom flow-through tank of Plexiglas to test olfactory detection and discrimination. The aquarium measured $45 \times 45 \times 15 \text{cm}^3$ (width × length × height) and consisted of 3 compartments: 2 stimulus compartments separated from a main arena by opaque dividers that served as light baffles. Built like a privacy fence, the baffles allowed water and any water-borne chemical stimuli to flow from the stimulus compartments to the focal compartment, but did not allow the fish on either side of the divider to see each other (Figure 1).

We used a submersible pump (Superior Pump model 91250) to expel aerated water at 78–80 °F from a reservoir through 2 flow meters (Key Instruments) and into the aquarium at 3 gal/h through each of 2 inflows. The water flowed continuously through each stimulus compartment, across the main arena of the aquarium, and out the back of the aquarium throughout all trials. Extensive dye testing showed that chemical plumes from the 2 inlets remain distinct for at least 10 min (Figure 1). We therefore limited all trials to 10 min. We covered the sides of the aquarium in white felt to minimize reflections and enable tracking of the focal individual (see below). The tank was fit by 2 full spectrum Marineland Natural Daylight fluorescent lights positioned at the edge of the left and right sides of the tank. After each trial, we drained and washed the tank with hydrogen peroxide (McLennan and Ryan 1999) to remove any traces of chemical cues.

**Data collection**

We recorded each trial with a Microsoft LifeCam Studio webcam mounted above the aquarium. We developed computer code using version 2.3 of the OpenCV library of functions for the Python programming language to automate data collection (Bradski 2000). This program allowed us to make data collection repeatable, unbiased, and exact. The program extracts each frame of the video from an image of the tank without fish, allowing rapid identification of the fish in the tank. The program outputs the coordinates of the center of the fish. Whenever a frame was dropped or the fish was obscured by a reflection, we interpolated its position assuming it moved in a straight line. We validated this program’s accuracy by comparing the results of program with those of a blind observer for 15 trials. We found a strong correlation between time spent in each area of the tank from the program and from the blind observer (r = 0.97, P < 0.001). An example of a fish’s path output by the program is shown in Figure 1.
perceive the chemical signatures of other Amazon mollies. This experiment was similar to the control experiment with 2 exceptions. First, we socially isolated the focal female for at least 7 days prior to testing to ensure social motivation. Second, we placed a conspecific female in one of the stimulus compartments and allowed her to acclimate to the tank for 5 min before placing the focal female in the tank (Figure 2b). The other stimulus compartment was left empty. Though the results from the control experiment did not indicate that focal females tended toward one side of the tank, we nonetheless switched the side of the tank the stimulus female occupied between each focal female trial to control for any side bias. Stimulus females were only used once a day. We predicted that the focal female would spend more time on the side of the tank containing the stimulus female.

**Male sailfin detection and discrimination experiment**

We performed a third experiment using male sailfin mollies that were either infected with black spot disease or not. Infected males are easy to identify because of the black-spotted phenotype induced by the trematode parasite. For these trials, we placed 2 size-matched (<2 mm) males, one infected and one noninfected, into separate stimulus compartments for 5 min of acclimation (Figure 2c). We then placed a focal female into a clear jar in the neutral zone for an additional 5-min acclimation period. We then removed the jar, turned on the pump, and began the trial. As in Gabor and Aspbury (2008), each stimulus male was isolated from females at least 24 h prior to the experiment to ensure sexual motivation and was only tested once a day.

This experiment, coupled with the control experiment, allowed us to ask whether Amazon mollies use chemical cues to detect sailfin molly males and to discriminate between males. First, if Amazon mollies can detect the odor of males, we predicted they should spend more time in the association zones in this experiment compared with the control experiment. Second, if Amazon mollies use chemical cues to discriminate infected from noninfected individuals, we predicted that females would spend a greater amount of time in the association zone adjacent to the noninfected male.

**Data analyses**

We divided the focal arena of the aquarium into 3 parts of equal width: a left association zone, a neutral zone, and a right association zone (Figure 1). We assume that a fish in an association zone is experiencing the odor plume of the fish in the associated stimulus compartment, though the odor is likely more concentrated upstream (Figure 1). We wrote code in the R statistical programming language (R Core Team 2015) to extract the zone of the tank the focal female occupied in each frame. We then used the frame rate of each video to calculate the number of seconds the focal fish spent in each of the 3 zones.

We used paired t-tests to determine whether the differences between the time spent in the 2 association zones within a given experiment differed from 0. We used Shapiro-Wilk tests in R to ensure that these differences were normally distributed, validating our use of parametric statistics. Because female age, as measured by standard length, is often associated with discrimination between stimuli in poeciliid fish (e.g., Morris et al. 2006; Rios-Cardenas et al. 2007; Wong et al. 2011), we also used simple linear models to test for associations between the size of the focal female and the time spent in the association zone of a conspecific and an uninfected sailfin male. We predicted that females should be better able to use...
chemical cues as they age and grow larger. Standardized residuals from these models were checked for normality using Shapiro–Wilk tests in R. We plotted the fitted model values against the standardized residuals to ensure the assumption of constant variance was satisfied.

To determine whether females were able to use odor to detect males, we compared the amount of time females spent in the neutral and association zones in the male sailfin experiment relative to the control experiment. If females could detect heterospecific male odors, we predicted females would spend more time investigating the association zones where male chemical stimuli were present compared with control trials where no olfactory stimuli were present. We found the opposite pattern and conducted additional analyses to dissect whether the female’s movement patterns were likely explained by avoidance of male odor. First, we conducted a finer-scale spatial analysis of each female’s behavior by defining a subset of each association zone we call the stimulus zone extending 7 cm (~2 fish standard lengths) immediately downstream of each baffle. We calculated the proportion of time each fish spent in these stimulus zones in the male sailfin experiment compared with the other 2 experiments. Second, fish may spend more time in the neutral
zone if they frequently move between the 2 association zones, perhaps to assess the difference in chemical stimuli between the 2 sides of the tank. We therefore compared the number of times a fish crossed from one zone into any other zone in the 3 experiments. We call each of these events a transition.

If females were avoiding heterospecific male odor, we predict that the responses for each of these metrics would differ between the male sailfin experiment and the first 2 experiments that lacked heterospecific odor. We used an analysis of variance (Anova) with orthogonal contrasts to test whether the means of the response in the first 2 experiments differed from the mean response in the male sailfin experiment. The second, fixed contrast was between the control and conspecific experiments and never approached statistical significance (all $P > 0.5$). We log transformed the number of seconds in the neutral zone and square-root transformed the number of transitions to meet the assumptions of Anova. All tests were 2 tailed.

**RESULTS**

Our study consisted of 3 experiments. The first experiment was a negative control to ensure the fish were not inherently attracted to one side of our experimental tank in the absence of any chemical cues. We found no evidence that Amazon mollies spent more time on one side of the tank than the other (Figure 2a; paired $t$-test, $n = 19$, $t = 1.07$, $P = 0.297$).

The second experiment was designed to test whether Amazon mollies use olfactory cues to detect conspecifics. We found that
Amazon mollies spent almost twice as much time in the association zone adjacent to the conspecific than in the association zone that contained no chemical stimulus, a difference that is highly statistically significant (Figure 2b; paired t-test, n = 22, t = 3.39, P = 0.003). Furthermore, contrary to our expectation that older, larger females would be better able to use olfactory cues, we found modest (linear model, n = 22, r² = 0.19) but statistically significant (P = 0.040) evidence that smaller Amazon mollies spend more time with the chemical cues of conspecific females compared with larger Amazon mollies (Figure 3a). On average, each millimeter increase in standard length resulted in 10 fewer seconds spent in the chemical plume of the conspecific.

The third experiment was designed to test whether olfaction mediates detection of sailfin molly males and discrimination between infected and noninfected males. We found no consistent difference in the amount of time an Amazon molly spent associating with the chemical cues of an infected male compared with those of a noninfected male (Figure 2c; n = 27, t = −0.48, P = 0.639). There was also no statistically significant association between female size and time spent near the chemical plumes of infected males (Figure 3b; n = 27, r² = 0.01, P = 0.645).

Intriguingly, we found evidence that Amazon mollies may be avoiding the scent of male sailfin mollies. Amazon mollies spent about 70% more time in the neutral zone of the tank during the male sailfin molly experiment than in the other 2 experiments that lacked male scent, a statistically significant difference (Figure 4a; Anova orthogonal contrast, t = 3.669, P < 0.001). This increased time spent in the neutral zone might simply be an outcome of increased movement between the association zones because a fish must pass the neutral zone as she moves from one association zone to the other. There were significantly more transitions between zones in the male sailfin molly experiment than in the 2 other experiments (Figure 4b; Anova orthogonal contrast, t = 2.26, P = 0.027). Despite this, females spent significantly less time in the stimulus zone near the baffles where the odor is concentrated when they were in the association zones than females in the control or conspecific experiment (Figure 4c; Anova orthogonal contrast, t = −2.13, P = 0.037), providing some support for avoidance of male odor. We also found evidence for a size-dependent basis for this putative avoidance, as there was a marginally statistically significant negative association between female size and time in stimulus zones (Figure 3c; n = 27, r² = 0.14, P = 0.056; with outlier removed: n = 26, r² = 0.28, P = 0.005).

**DISCUSSION**

We tested the ability of asexual Amazon mollies to use olfaction to detect conspecifics and heterospecific males, and to discriminate between parasitized heterospecific males. We predicted that mollies would use olfaction to mediate these behaviors if olfaction has undergone a sensory expansion. We found evidence that Amazon mollies can use chemical cues to detect both conspecifics and heterospecifics. However, we found no evidence that Amazon mollies use chemical cues to discriminate among heterospecific males that vary in parasitization status.

Female Amazon mollies exhibited a strong preference to associate near conspecific odor plumes compared with a no-odor blank (Figure 2b). This willingness to associate with the chemical cues of a conspecific varied with female size: Smaller Amazon mollies spent more time in the association zone near the conspecific than larger mollies (Figure 3a). Because poeciliid fish grow as they age, these small females are young (Snelson 1982). This result parallels a study of 2 nascent stickleback species (Gasterosteus spp.), which found that juvenile fish show a stronger tendency to shoal than adults (Kozak and Boughman 2008). A study of Xiphophorus birchmanni swordtails that found that olfaction was especially important to newborn fish for identifying conspecifics (Coleman and Rosenthal 2006). It seems plausible that detection of conspecifics may be critical for earlier-life stages that are more dependent on shoaling for evading predators and finding food.

Our experiments make clear that Amazon mollies can use olfaction to detect conspecifics. This ability has been demonstrated in a number of related fish, including guppies and swordtails (Crapon de Caprona and Ryan 1990; McLennan and Ryan 1997; McLennan and Ryan 1999; Shohet and Watt 2004), but rarely in mollies. Chemical cues are insufficient for species recognition in male sailfin mollies (Asbury et al. 2010) and are not used by female Atlantic mollies for discriminating between large and small males (Plath, Körner, et al. 2005) or between well-fed and starved males (Plath, Heubel, et al. 2005). Female Atlantic mollies adapted to living in caves, however, use nonvisual cues to discriminate between males based on size (Plath, Körner, et al. 2005) and nutritional state (Plath, Heubel, et al. 2005), though they do not use chemical cues to detect males infected with a common bacterium (Plath 2004). Our experiments therefore lend limited support to the idea that hybrid Amazon mollies may undergo a sensory expansion in the olfactory modality, in that we observe evidence for olfactory detection of both conspecifics and heterospecifics, a rarity in other mollie species.

Although many poeciliids may use olfactory cues to detect or discriminate among conspecifics, olfactory detection of heterospecifics is less commonly tested. Many fish species tend to prefer to associate with a conspecific chemical cue compared with a heterospecific cue (Crapon de Caprona and Ryan 1990; Hankison and Morris 2003; Rafferty and Boughman 2006). The 2-stimuli dichotomous choice paradigm used in these studies, however, limits our ability to disentangle a preference of conspecifics from avoidance of heterospecifics. Studies with Xiphophorus swordtails that have tested olfactory heterospecific recognition directly by pairing odorless water with a heterospecific olfactory cue have generally found that species spend more time with a heterospecific olfactory cue than a water control (McLennan and Ryan 1997; Wong et al. 2005). Interestingly, in this study, we found evidence that Amazon mollies may use olfactory cues to avoid heterospecific sailfin males—females spent significantly less time in the association zones when male sailfin odor was present in the tank than when it was absent. This is especially intriguing because without mating with these males, Amazon mollies cannot trigger embryogenesis and reproduce. One possibility is that sailfin mollies of both sexes share a common odor component that can be detected by Amazon mollies. Amazon mollies and sailfin molly females compete with each other for males and behave aggressively toward each other both in the presence and absence of sailfin molly males (Foran and Ryan 1994). When given a choice between shoaling with a conspecific or heterospecific, females of both species prefer to not shoal with the heterospecific (Schlupp and Ryan 1996). If there is a sailfin odor shared by males and females, it might benefit the Amazon molly to avoid this scent in the absence of any visual cues: Moving toward the chemical stimulus could risk an aggressive encounter with a female sailfin molly and greater competition for a mate.

Our experiment also sheds some light on a common explanation for the rarity of asexuality: the Red Queen hypothesis. The
rapid evolution of parasites—with large populations and short generation times—to their host generates a changing environment that provides a particular challenge for slowly adapting asexual species, which become easy targets for parasites (Hamilton 1980; Bell 1982). Because asexual species lack genetic recombination, evolving resistance to these rapidly changing parasites is particularly difficult. The Red Queen hypothesis predicts that asexual species should have more parasites than their sexual relatives, a prediction that is realized in snails, fish, geckos, and worms (Lively et al. 1990; Moritz et al. 1991; Johnson 2000; Hakoyama et al. 2001; Michaels et al. 2001; Mee and Rowe 2006), but not Amazon mollies (Tobler and Schlupp 2005). This lack of parasite load between Amazon mollies and related sexual species may be due to a possible behavioral adaptation: In visual discrimination assays, Amazon mollies avoid associating with heterospecific males that bear signs of black spot disease, a condition caused by the parasite trematode *Urolophus* spp. (Tobler et al. 2006). Both sexual species whose hybridization resulted in the Amazon molly, the sailfin and Atlantic Molly (*Poecilia latipinna* and *Poecilia mexicana*; Stock et al. 2010), fail to show this avoidance behavior (Tobler et al. 2006), suggesting avoidance of parasitized fish is a derived trait in asexual Amazon mollies. An increased aversion to parasitized individuals in the asexual species may partly explain why sexual sailfin mollies and asexual Amazon mollies have similar rates of parasitism.

Our experiments did not support the idea that hybrid Amazon mollies underwent a sensory expansion in the olfactory modality leading to increased olfactory discrimination between males infected with this same parasite. This is particularly unexpected given that asexual species are likely more susceptible to parasitic infection. Why might this be? One possibility is that vision, which Amazon mollies use to identify parasitized conspecifics (Tobler et al. 2006), has some inherent advantages over olfaction for identifying infected males—detection of parasitized individuals at a greater distance, for example. Perhaps the conspicuous black spots of infected males are a sufficiently reliable and salient cue that evolving olfactory discrimination is not advantageous. Indeed, olfactory avoidance of parasitized individuals is probably more likely when the parasite does not induce a conspicuous visual phenotype as it does here. Female rats and mice, for example, can use olfactory cues to discriminate healthy male rats from those infected with an internal parasite that does not induce a conspicuous phenotype as it does here (Kavaliers and Colwell 1995; Willis and Poulin 2000).

The history of recombination in hybrid species is important for understanding whether it might undergo a “sensory expansion” (Sandkan et al. 2013). Mitochondrial and nuclear microsatellite evidence suggest that Amazon mollies are frozen *F₁* hybrids that only originated once (Stock et al. 2010). If Amazon mollies are truly frozen *F₁* hybrids, then they must contain 1 haploid genome from each of their parent species. However, a recent study found evidence of a possible history of recombination in the Amazon molly, most likely mitotic gene conversion, resulting in higher levels of homozygosity than expected for an asexual hybrid species across some loci (Da Barbiano et al. 2013). If recombination has played a prominent role in the evolutionary history of this species, a sensory expansion may be less likely.

In conclusion, we found no evidence that asexual Amazon mollies use olfaction to discriminate between males infected with a common parasite and those that are not, though they do use olfaction to mediate detection of conspecifics and may avoid heterospecifics based on olfactory cues. We also find that younger Amazon mollies place a greater reliance on olfactory cues when detecting conspecifics. These experiments provide partial support for a sensory expansion in the olfactory modality, as olfactory detection of conspecifics and heterospecifics has never been clearly demonstrated in mollies. Sensory expansion in the olfactory modality may be a more universal feature of asexual vertebrates, nearly all of which are of hybrid origin (Vrijenhoek 1989). Ultimately, studying the sensory systems of asexual vertebrates is likely to yield insight into one of biology’s most persistent questions, the evolution and maintenance of sex.


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