

## Lack of species discrimination based on chemical cues by male sailfin mollies, *Poecilia latipinna*

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**Abstract** When making mating decisions, individuals may rely on multiple cues from either the same or multiple sensory modalities. Although the use of visual cues in sexual selection is well studied, fewer studies have examined the role of chemical cues in mate choice. In addition, few studies have examined how visual and/or chemical cues affect male mating decisions. Male mate choice is important in systems where males must avoid mating with heterospecific females, as is found in a mating complex of *Poecilia*. Male sailfin mollies, *Poecilia latipinna*, are sexually parasitized by gynogenetic Amazon mollies, *P. formosa*. Little is known about the mechanism by which male sailfin mollies base their mating decisions. Here we tested the hypothesis that male sailfin mollies from an allopatric and a sympatric population with Amazon mollies use multiple cues to distinguish between conspecific and heterospecific females. We found that male sailfin mollies recognized the chemical cues of conspecific females, but we found no support for the hypothesis that chemical cues are by themselves sufficient for species discrimination. Lack of discrimination based on chemical cues alone may be due to the close evolutionary history between *P. latipinna* and *P. formosa*. Males from populations sympatric with Amazon mollies did not differentially associate with females of either of the two species when given access to both visual and chemical cues of the females, yet males from the allopatric population did associate more with conspecific females than with heterospecific females in the presence of both chemical and visual cues. The lack of discrimination by males from the sympatric population between conspecific and heterospecific females based on both chemical and visual cues suggests that these males require more complex combinations of cues to distinguish species, possibly due to the close relatedness of these species.

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## Introduction

Mate choice behaviors function along a continuum from reproductive isolation between species to assessment of conspecific individuals. Visual signals play an important role in both species recognition and mate assessment in many taxa (review in Andersson 1994). In addition, many taxa can use olfactory cues to both detect conspecifics and to discriminate between conspecifics and heterospecifics (review in Johansson and Jones 2007). Some of the cues that individuals use in mate assessment consist of multiple components (multi-component signals) or multiple sensory modalities (multi-modal signals; Candolin 2003). Increasing evidence suggests that mate choice may play a role in the evolution and maintenance of multiple signals in multiple modalities (Marchetti 1998; Møller et al. 1998; Hebets and Uetz 1999; Candolin 2003; Hankison and Morris 2003; McLennan 2003; Papke et al. 2007).

While females are generally considered the “choosy” sex (Andersson 1994), there is increasing evidence of males from many taxa also benefiting from mate choice discrimination (e.g., birds: Jones and Hunter 1993; Monaghan et al. 1996; Griggio et al. 2005; Pryke and Griffith 2007; insects: Engqvist and Sauer 2001; Bonduriansky 2001; Chenoweth et al. 2007; spiders: Hoefler 2007; fish: Gabor and Ryan 2001; Werner and Lotem 2003; Aspbury and Gabor 2004a, b; McLennan 2004; Schlupp and Plath 2005; Wong et al. 2005; Plath et al. 2006; Robinson et al. 2008; Gabor and Aspbury 2008; primates: Parga 2006). One reason why selection may favor male choice is because sperm production is energetically costly and can limit male reproductive success (Dewsbury 1982; Nakatsuru and Kramer 1982). Males may conserve energy by differentially producing or allocating sperm to certain females over others and thus exercise a form of male mate choice (Aspbury and Gabor 2004a, b; Schlupp and Plath 2005; Robinson et al. 2008).

The costs of mating that influence male choice may be elevated in systems where sexual species co-occur with sexually parasitic species and can influence the evolution of mate choice discrimination. Gynogenetic species consist of only females but require sperm from males of closely related bisexual species to initiate embryogenesis (first described in Hubbs and Hubbs 1932; review in Dawley 1989; Schlupp 2005). Males of the sexual species that mate with parasitic gynogenetic females gain no direct fitness benefits (but see Schlupp et al. 1994). While male mate choice for conspecific females may cause further divergence in female traits between the unisexual and bisexual species, females of parasitic species are genetically related to conspecific females, of their parental species, given their hybrid origin and thus might be falsely recognized as conspecific mates (Lima et al. 1996; Dries 2003). Therefore the use of multiple cues by males in mate assessment may aid in avoiding heterospecific matings.

One well studied example of a sexually reproducing species and a gynogenetic species is the sailfin—Amazon molly species complex. Male sailfin mollies (*Poecilia latipinna*) are sexually parasitized by the gynogenetic Amazon molly (*P. formosa*). Amazon mollies are of hybrid origin, and they must co-exist and mate with males of the parental species (*P. latipinna* and *P. mexicana*) to induce embryogenesis, but inheritance is strictly maternal (Hubbs and Hubbs 1932, 1946). When allowed direct access to conspecific and

heterospecific females, male sailfin mollies show a greater mating preference for conspecifics than for Amazon mollies, and males from sympatric populations have a greater preference for conspecifics over Amazon mollies than males from allopatric populations (Hubbs 1964; Ryan et al. 1996; Gabor and Ryan 2001). In addition, Gumm et al. (2006) showed that male sailfin mollies preferred to associate more with models of conspecific females than Amazon mollies. Aspbury and Gabor (2004b) found that male sailfin mollies also produced more sperm when in the presence of conspecific females than when in the presence of Amazon mollies, while Robinson et al. (2008) showed that male sailfin mollies undergo rapid spermiation when mating with conspecifics, but not when mating with heterospecific females.

The goal of this research was to determine if male sailfin mollies use chemical, visual, or a combination of cues from multiple sensory modalities to recognize females, and what cues they use to discriminate between species. We examined male mating preference by measuring both male sperm production, as well as male association time, using males from populations sympatric with and allopatric to Amazon mollies.

## Materials and methods

### Fish collection and maintenance

For all experiments, we isolated females from males for at least 30 days in 38-l glass aquaria. Ovarian cycles for mollies are about 30 days (Farr and Travis 1986; Snelson et al. 1986) and females used in the experiments were not visibly gravid. We tested only mature males, identified by the fusion of the anal fin into the gonopodium. Fish were maintained on a 14-h light: 10-h dark cycle using UV lighting, and were fed Ocean Start International Inc. Spirulina Flake mixed with Ocean Start International Inc. Freshwater Flake food (Ocean Start International Marine Laboratory Inc. Hayward, California, USA) twice daily and supplemented daily with live brine shrimp.

### *Experiment 1: Male sperm production—effect of chemical and visual cues of conspecific females*

The goal of this experiment was to examine if male sailfin mollies differentially produce sperm in the presence of conspecific females when given access to either chemical or visual cues. Sailfin mollies used in this experiment were collected from a population that is allopatric from Amazon mollies, and is found in the headwaters of the San Marcos River, Texas, USA (29.89°N, 97.82°W) in May 2004 and May–June 2005. Fish were tested from June to September, 2005. On day 0 of the experiment, we removed males ( $n = 40$ ) from their aquaria, and measured standard length (SL: snout to caudal peduncle). We then extracted sperm from all males, following established protocols (Aspbury and Gabor 2004a, b). We placed the spermatozeugmata from each male into individual micro-centrifuge tubes with 0.9% saline solution (0.9 g of NaCl/100 ml of water) and mixed each sample by pipetting. We counted sperm cells in five cells of an improved Hy-Lite Neubauer chamber hemocytometer (Reichert, Buffalo, New York, USA) under 400× magnification. The total number of sperm cells was determined by multiplying the mean cell count by the initial volume (100  $\mu$ l) of the sample and dividing by the volume of the hemocytometer (0.1  $\mu$ l).

**Table 1** Mean male and female standard length (mm)  $\pm$  SE for experiments 1–5

Expt.	Pop	Species	Cues	Male SL	Female SL
1	Allopatry	Conspecific	Chemical	30.69 $\pm$ 2.80	39.81 $\pm$ 0.501
		Conspecific	Visual	29.37 $\pm$ 1.70	40.00 $\pm$ 0.425
2	Sympatry	Conspecific	Chemical	30.13 $\pm$ 1.67	45.29 $\pm$ 0.934
		Heterospecific	Chemical	29.64 $\pm$ 1.13	44.00 $\pm$ 1.24
3	Allopatry	Conspecific	Chemical	34.48 $\pm$ 0.967	36.59 $\pm$ 1.01
		Heterospecific	Chemical	34.48 $\pm$ 0.967	38.45 $\pm$ 1.51
	Sympatry	Conspecific	Chemical	34.52 $\pm$ 0.940	39.42 $\pm$ 1.26
		Heterospecific	Chemical	34.52 $\pm$ 0.940	39.87 $\pm$ 1.43
4	Allopatry	Conspecific	Chemical	36.15 $\pm$ 0.900	38.81 $\pm$ 0.927
		Heterospecific	Chemical	36.15 $\pm$ 0.900	39.74 $\pm$ 1.06
	Sympatry	Conspecific	Chemical	32.99 $\pm$ 1.09	44.03 $\pm$ 1.39
		Heterospecific	Chemical	32.99 $\pm$ 1.09	44.47 $\pm$ 1.50
5	Allopatry	Conspecific	Visual, Visual + Chemical	31.39 $\pm$ 1.15	39.26 $\pm$ 1.52
		Heterospecific	Visual, Visual + Chemical	31.39 $\pm$ 1.15	39.50 $\pm$ 1.43
	Sympatry	Conspecific	Visual, Visual + Chemical	36.97 $\pm$ 0.839	41.87 $\pm$ 1.39
		Heterospecific	Visual, Visual + Chemical	36.97 $\pm$ 0.839	42.47 $\pm$ 1.38

Following sperm extraction, we placed individual males in 21 l aquaria with 14 l of water and gravel covering the bottom of the aquarium. Males were randomly assigned to one of two stimulus treatments ( $n = 20$  males per treatment) consisting of males presented with (1) visual cues or (2) chemical cues of a mature conspecific female. In the chemical cue treatment, we placed the female in an opaque plastic container (3 l, 12 cm diameter  $\times$  19 cm high) that had 1 mm holes poked throughout that permitted the penetration of chemical cues only. For the visual cue treatments, females were placed in a clear plastic container (3 l, 12 cm diameter  $\times$  19 cm high) with no perforations. On day 3 of the experiment, we measured female SL and added individual females to containers in the aquaria housing the individual males. On day 10 of the experiment, all fish were removed from the aquaria and sperm was extracted from all males. Males were excluded from analyses if they had no strippable sperm on days 0 and 10 (final  $n = 17$  in visual cue treatment and final  $n = 15$  in chemical cue treatment) (see Table 1 for fish SL).

#### *Experiment 2: Male sperm production—effect of chemical cues of conspecific and heterospecific females*

The goal of this experiment was to determine if male sailfin mollies differentially produced sperm when given access to the chemical cues only of either conspecific or heterospecific females. This experiment consisted of a similar design to experiment 1 but we used male sailfin and Amazon mollies collected from a sympatric population in Tamaulipas, Mexico (25.07°N, 98.02°W) in March 2003. Female sailfin mollies were collected from the allopatric population used in experiment 1 due to insufficient females from this sympatric population. Fish were tested from June to August, 2006. On day 0, we measured male SL using digital calipers and sperm was extracted. The males ( $n = 20$  males per treatment) were then placed in separate 21 l aquaria with 14 l of water and a thin layer of gravel substrate. Males were randomly assigned to a treatment of either the: (1) chemical cue of a

conspecific female or (2) chemical cue of a heterospecific female. On day 3, a mature female was placed in a chemical cue container (described in experiment one) in the male tank. On day 10 of the experiment, all fish were removed from the aquaria and sperm was extracted from all males. Males were excluded from analyses if they had no strippable sperm on both days 0 and 10 (final  $n = 18$  in conspecific treatment and final  $n = 18$  in heterospecific treatment) (see Table 1 for fish SL).

### *Experiment 3: Male association preference—conspecific and heterospecific chemical cue detection*

The goal of this experiment was to determine if male sailfin mollies from both an allopatric and a sympatric population with Amazon mollies detect chemical cues of heterospecific and conspecific females in an association time experimental design. For this experiment all fish in the sympatry trials were collected in March 2003 from another population in Tamaulipas, Mexico (25.30°N, 97.86°W). The male and female sailfin mollies in the allopatry trials were collected in 2003 and 2005 from the same population used in experiment 1, and the Amazon mollies were from a laboratory reared population from a population collected in 1989 and 1998 in Tamaulipas, Mexico (22.92°N, 98.07°W). We tested males from allopatry ( $n = 15$ ) and sympatry ( $n = 15$ ) in two different trials. Males from allopatry were tested from April to September 2003, and males from sympatry were tested from June to September 2003. One trial tested male detection of conspecific cues, and the other trial tested male detection of heterospecific cues. Each male was tested in both trials presented in random order in a 57 l (60 cm × 32 cm × 32 cm) aquarium split into three equal sections by lines drawn on the outside of the aquarium. The aquarium contained water to 17 cm depth. Sides of the aquarium were covered with black plastic to prevent disturbance from outside of the aquarium, but the front and back of the long side of the aquarium was covered with one-way film to allow the tester to see in without disturbing the fish. A 15°W Sun-Glo full spectrum fluorescent light (General Electric®) was suspended above the aquarium and the rest of the testing room was dark. For the conspecific treatment, on one side of the aquarium a conspecific female was placed into a chemical cue container (described in experiment 1; see Fig. 1 for image of testing chamber). The other side of the aquarium held the same type of container, but no stimulus fish was placed in the container. Similarly, for the heterospecific treatment, a heterospecific female was placed into a container on one side of the aquarium, and the other end of the aquarium held a container without a stimulus fish (see Table 1 for fish SL).

We acclimatized the focal male in the center of the aquarium for 10 min under a clear plastic cylinder (12 cm diameter) with 3 mm holes throughout the container. After we released the male, he could freely move in the aquarium and interact with the stimulus fish (in its container) and the empty container without physical or visual contact. We recorded the amount of time the male spent in either of the two preference zones (around the stimulus containers) of the aquarium for 10 min. After the first trial, to control for side bias, the aquarium was rotated 180° and testing was repeated after another 10 min acclimatization period before testing the male for an additional 10 min. We did not switch female side in the aquarium to prevent mixing of the chemical cue with the blank cue. Based on pilot studies using dyed water, we found that the water flow from within the isolating containers into the rest of the test aquarium was constant and symmetrical. The overlap of flow during the 20 min of testing was minimal. After each test in the aquarium, we emptied the water from the aquarium and cleaned both the aquarium and the cylinders



**Fig. 1** Example of aquarium set up for experiments 3–5. Shown are the containers used in the visual + chemical cue experiment. Similar containers are used in experiments 1–2

with 3% hydrogen peroxide solution to remove potential chemical cues and rinsed the containers with clean water (McLennan and Ryan 1997).

*Experiment 4: Male association preference—species discrimination based on chemical cues*

The goal of this experiment was to determine if males from the same sympatric and allopatric populations used in experiment 3 discriminate between the chemical cues of conspecific and heterospecific females. In this experiment,  $n = 25$  males from sympatry and  $n = 25$  males from allopatry were tested in a design that was identical to that in experiment 3 except that both stimulus containers contained size-matched (within 2 mm SL) conspecific and heterospecific females (Table 1). The stimulus containers were the same as those used in experiment 2 and allowed for passage of chemical cues only.

*Experiment 5: Male association preference—species discrimination based on visual cues and visual + chemical cues*

The goal of this experiment was to determine if males from the same sympatric and allopatric populations used in experiments 3 and 4 discriminate between conspecific and heterospecific females when given access to visual cues only of the females, or when given access to both visual and chemical cues. In this experiment,  $n = 25$  males from sympatry and  $n = 25$  males from allopatry were tested in a design similar to experiment 3. We tested males from allopatry from September–November 2003, and in June 2005 when more males were available to test. We tested males from sympatry from August to September 2003. Each male was a subject in two different trials. In the first trial, males were presented simultaneously with the visual cues of size-matched (within 2 mm SL) conspecific and heterospecific females (see Table 1 for fish SL). The females were placed in separate plastic cylindrical containers (12 cm diameter  $\times$  19 cm), that were clear and had no holes, at either end of the aquarium. In the second trial (20 min after the end of the first trial), each male was presented simultaneously with both the visual and chemical cues of the same size-matched conspecific and heterospecific females (Fig. 1). The females were

placed in plastic cylindrical containers (12 cm diameter  $\times$  19 cm) that were clear and had 1 mm holes poked throughout that permitted the penetration of potential chemical cues. The order of the trials was not randomized to eliminate the effect of chemical cues being present during visual cue only trials.

### Statistical analyses

In experiment 1, the number of sperm cells produced during the trials (day 10–day 0) was compared between the treatments using an unpaired *t*-test ( $\alpha = 0.05$ ). Because larger males produce more sperm than smaller males, and males produce more sperm when in the presence of larger females than when in the presence of smaller females (Aspbury and Gabor 2004a), we used simple linear regression to determine the effect of male and female SL on sperm production in each treatment.

In experiment 2, because of unequal variances between the treatments, the number of sperm cells produced during the trials (day 10–day 0 = priming response, sensu Olsén and Liley 1993) was compared between the treatments using Mann–Whitney *U* test ( $\alpha = 0.05$ ). We also used simple linear regression to determine the effect of male and female SL on sperm production within each treatment.

For each of the association preference experiments, our data sets showed unequal variances that could not be corrected with transformations of the data. We therefore used nonparametric statistics. We compared the amount of time males spent in the right preference section between trials within each treatment using a Wilcoxon signed ranks test ( $\alpha = 0.05$ ) for each of the experiments for discussion see Gabor (1999). All tests were two-tailed.

## Results

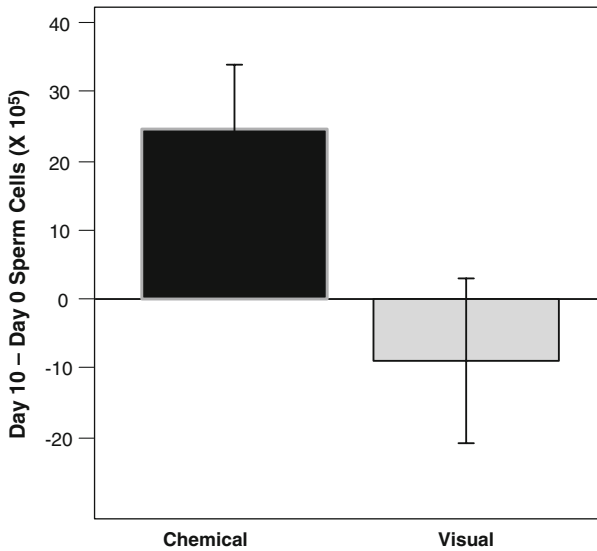
### Experiment 1: Male sperm production—effect of chemical and visual cues of conspecific females

Male sailfin mollies from an allopatric population produced significantly more sperm when presented with the chemical cues of a conspecific female than when presented with the visual cues of a conspecific female (unpaired *t*-test:  $t = -2.254$ ;  $P = 0.032$ ; Fig. 2).

There was no relationship between male SL and the amount of sperm produced in either treatment (chemical cue treatment linear regression:  $r^2 = 0.219$ ,  $P = 0.079$ ; visual cue linear regression:  $r^2 = 0.004$ ,  $P = 0.814$ ). There was also no relationship between female SL and the amount of sperm males produced in each of the two treatments (chemical cue treatment linear regression:  $r^2 = 0.005$ ,  $P = 0.803$ ; visual cue linear regression:  $r^2 = 0.038$ ,  $P = 0.454$ ). Furthermore, male SL and female SL did not differ between the chemical cue and visual cue treatments (Table 1; unpaired *t*-tests: male SL:  $t = -0.402$ ;  $P = 0.691$ ; female SL:  $t = 0.293$ ;  $P = 0.772$ ).

### Experiment 2: Male sperm production—effect of chemical cues of conspecific and heterospecific females

There was no significant difference in the amount of sperm produced (priming = day 10–day 0 cells) by male sailfin mollies from a sympatric population when presented with the



**Fig. 2** Mean ( $\pm 1$  SE) male sperm production (day 10–day 0 total sperm cells) given either chemical cues or visual cues of a mature conspecific female. Larger values indicate greater sperm production (relative to the day 0 baseline)

chemical cues of conspecific (mean  $\pm$  SE priming =  $492688 \pm 755861$ ) or heterospecific (mean  $\pm$  SE priming =  $392312.5 \pm 385695$ ) females (Mann–Whitney  $U$  test:  $U = 140.50$ ,  $P = 0.496$ ).

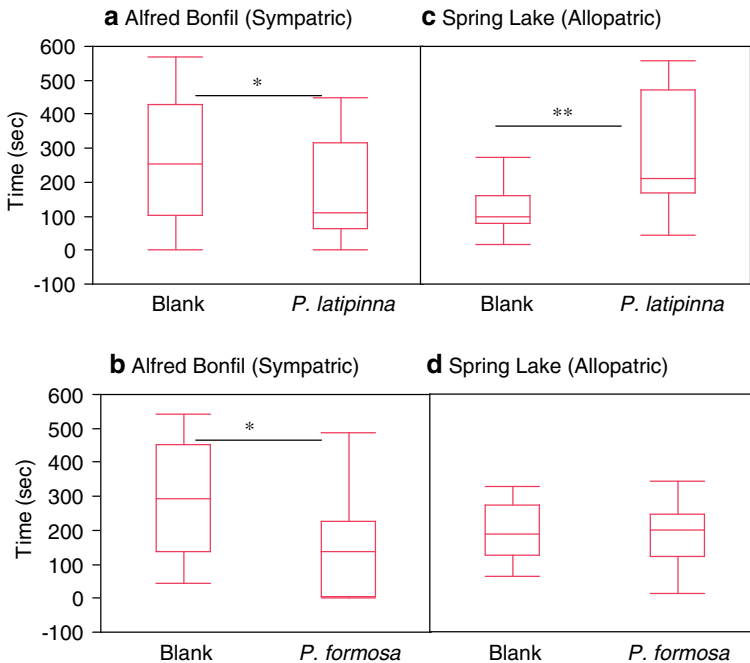
There was no relationship between male SL and the amount of sperm produced in either treatment (conspecific treatment linear regression:  $r^2 = 0.029$ ,  $P = 0.500$ ; heterospecific treatment linear regression:  $r^2 = 0.048$ ,  $P = 0.385$ ). There was also no relationship between female SL and the amount of sperm that males produced in each of the two treatments (conspecific treatment linear regression:  $r^2 = 0.000$ ,  $P = 0.989$ ; heterospecific treatment linear regression:  $r^2 = 0.000$ ,  $P = 0.902$ ). Furthermore, neither male SL nor female SL differed between the conspecific and heterospecific chemical cue treatments (Table 1; unpaired  $t$ -tests: male SL:  $t = 0.243$ ;  $P = 0.809$ ; female SL:  $t = 0.830$ ;  $P = 0.413$ ).

### Experiment 3: Male association preference—conspecific and heterospecific chemical cue recognition

Males from the sympatric population spent significantly more time in association with the blank stimulus than with the conspecific chemical cue stimulus (Wilcoxon signed-rank test:  $n = 15$ ,  $Z = 38$ ,  $P = 0.030$ ; Fig. 3a), and they also significantly preferred to associate with the blank stimulus over the chemical cue of a heterospecific female (Wilcoxon signed-rank test:  $n = 15$ ,  $Z = 37$ ,  $P = 0.035$ ; Fig. 3b).

Males from the allopatric population recognized conspecific female chemical cues, but showed no evidence of detection of the heterospecific female chemical cues. These males significantly preferred to associate with the chemical cue of a conspecific female over a blank stimulus (Wilcoxon signed-rank test:  $n = 15$ ,  $Z = -47.5$ ,  $P = 0.005$ ; Fig. 3c), but did not show a preference when given the choice between the chemical cue of a





**Fig. 3** Box plots for male sailfin mollies from a sympatric population time in association with: **a** blank stimulus versus the chemical cues of a conspecific female; **b** blank stimulus versus the chemical cues of a heterospecific female, and for male sailfin mollies from an allopatric population time in association with: **c** blank stimulus versus the chemical cues of a conspecific female; **d** blank stimulus versus the chemical cues of a heterospecific female. \*  $P < 0.05$  and \*\*  $P < 0.005$  Wilcoxon signed ranks test. Box plots show medians, first and third quartiles, and ranges

heterospecific female and the blank stimulus (Wilcoxon signed-rank test:  $n = 15$ ,  $Z = -7$ ,  $P = 0.709$ ; Fig. 3d).

#### Experiment 4: Male association preference—species discrimination based on chemical cues

Males from both the allopatric and sympatric population did not discriminate between conspecific and heterospecific female chemical cues. There was no difference in the time spent associating with the conspecific or heterospecific female (Table 2a).

#### Experiment 5: Male association preference—species discrimination based on visual cues and visual + chemical cues

When given access to only visual cues of conspecific and heterospecific females, males from both the allopatric and sympatric population did not discriminate between females. There was no difference in the time spent associating with the conspecific or heterospecific female (Table 2b).

When given access to both the visual and chemical cues of conspecific and heterospecific females, males from the allopatric population did discriminate between the females. Males spent more time associating with the conspecific female than with the

**Table 2** Mean  $\pm$  SE time (s) spent associating with the conspecific and heterospecific females when given access to: (a) chemical cues only (experiment 4), (b) visual cues only (experiment 5), and (c) both visual and chemical cues (experiment 5)

	Population	Conspecific cue	Heterospecific cue	Z	P
(a) Chemical	Sympatry	205.60 $\pm$ 30.10	145.16 $\pm$ 33.32	-43.00	0.255
	Allopatry	224.12 $\pm$ 31.37	180.40 $\pm$ 31.85	-31.50	0.408
(b) Visual	Sympatry	219.52 $\pm$ 25.45	225.16 $\pm$ 24.75	-5.00	0.890
	Allopatry	250.48 $\pm$ 43.56	177.80 $\pm$ 35.61	-63.00	0.071
(c) Visual + chemical	Sympatry	240.96 $\pm$ 34.99	248.68 $\pm$ 28.72	6.50	0.857
	Allopatry	315.96 $\pm$ 40.61	140.08 $\pm$ 26.30	-108.50	0.001

Z = Wilcoxon Sign-Rank test statistic

heterospecific female (Table 2c). Whereas, when given access to both the visual and chemical cues of conspecific and heterospecific females, males from the sympatric population did not discriminate between the females (Table 2c).

## Discussion

Male sailfin mollies recognized the chemical cues of conspecific females, as males from an allopatric population produced more sperm in the presence of conspecific female chemical cues than they did in the presence of conspecific female visual cues. At the behavioral level, males from the allopatric population also preferentially associated with the chemical cues of conspecific females over a blank stimulus, but showed no discrimination between a blank stimulus and the chemical cues of heterospecific females. This result could suggest that they do not detect the cue, or alternatively, it could suggest that they do detect it, but avoid the cue. One way to distinguish these possibilities would be to examine if males could be conditioned to respond to the cue (see Witte and Klink 2005). The males from allopatry also showed no discrimination between conspecific and heterospecific females when given access to chemical cues only.

Male sailfin mollies from a population sympatric with Amazon mollies also showed evidence of recognition of both conspecific and heterospecific females. These males avoided the chemical cues of heterospecific females, as evidenced by a significantly greater association time with the blank stimulus over the chemical cue of the Amazon molly. The males from this population also showed avoidance of the conspecific female chemical cue. One explanation for this result could be that the females used in the experiment were not females that the males found attractive based on the chemical cues alone (Farr and Travis 1986). The male sailfin mollies from the sympatric population also showed no evidence of discrimination between the chemical cues of conspecific females and those of heterospecific females.

We found no support for the hypothesis that male sailfin mollies use chemical cues alone for species discrimination, as manifest by a male's physiological response. Males given access to the chemical cues of conspecific females did not produce more sperm than males given access to the chemical cues of heterospecific females. One experimental design constraint may have obscured our ability to detect significant differences in sperm production between conspecific and heterospecific females; the conspecific females used in this experiment did not originate from the same population as the males. Males may have shown no increase in sperm production for these females if there are significant population

level differences in female chemical cues. This is an intriguing possibility that has yet to be tested. In a previous experiment, when male sailfin mollies had access to both visual and chemical cues, male sailfin mollies produced more sperm when in the presence of the conspecific female than when in the presence of an Amazon molly (Aspbury and Gabor 2004b). The combination of the current experiment with that of Aspbury and Gabor (2004b) could suggest that male sailfin mollies need a combination of visual and chemical cues to differentially produce sperm for conspecific and heterospecific females.

Although chemical cues of conspecific females may contain more information about their size, reproductive, or mating status than visual cues (Farr and Travis 1986; Roberts and Uetz 2005; Poschadel et al. 2006), these cues alone do not appear to play a role in species discrimination by male sailfin mollies. This is in contrast to previous research which showed that females from various *Xiphophorus* species can discriminate between conspecific and heterospecific males based on chemical cues alone (Capron de Caprona and Ryan 1990; McLennan and Ryan 1997, 1999, 2008; Fisher et al. 2006). Furthermore, McLennan and Ryan (2008) have shown that males can detect and discriminate between the chemical cues of conspecific and heterospecific males (e.g., males of *X. continens* prefer the odor of the males of *X. montezumae*). Perhaps because the costs of mate choice are higher for females than males there has been greater selection on females to attend to the chemical cues of potential mates than for males to do so. However, Wong et al. (2005) found that male swordtails (*Xiphophorus birchmanni*) do discriminate between conspecific and heterospecific females based on chemical cues. The lack of differential sperm production and association behavior by male sailfin mollies for conspecific over heterospecific females based on chemical cues alone may be due to the close evolutionary history of this unisexual–bisexual complex. For cues to lead to both recognition and discrimination in the receiver, the cues of both the conspecific species and the heterospecific species must contain both ancestral and derived information (cue complexes, sensu McLennan and Ryan 1999). Sailfin mollies likely represent one of the parental lineages of the Amazon molly (Avisé et al. 1991; Schartl et al. 1995), and therefore the chemical cues of Amazon mollies may not have diverged enough from the cues of female sailfin mollies. The occurrence of hybridization event(s) between *P. mexicana* and a sailfin species, suggests that chemical cues of both parental species are similar enough to each other to confound species discrimination.

One result that we did not predict was that male sailfin mollies from both allopatry and sympatry did not differentially associate with conspecific or heterospecific females when given access to only the visual cues of the females. This result is contrary to prior experiments that have evaluated the effect of visual cues on both male and female sailfin molly association preference for female conspecifics versus Amazon mollies (Schlupp et al. 1991, Schlupp and Ryan 1996, 1997; Gumm et al. 2006). One hypothesis that warrants further consideration is that there is geographic variation in the ways that male sailfin mollies discriminate between species or in the ability to discriminate based on visual cues. There is evidence of geographic variation in male sailfin molly mating preference (Gabor and Ryan 2001; Gumm and Gabor 2005). Therefore it is possible that the populations of males that we used in our studies differ behaviorally from the males from other populations used in the studies of Schlupp et al. (1991), and Schlupp and Ryan (1996, 1997) and Gumm et al. (2006).

We also found that males from sympatry did not differentially associate with females of either of the two species when given access to both visual and chemical cues of the females. However, males from the allopatric population did associate more with conspecific females than with heterospecific females in the presence of both chemical and visual

cues. These males preferred conspecific females over heterospecific females when given access to the multi-modal signals, but not when given access to the cues in isolation, suggesting that multi-modal signaling may be more effective for species discrimination by male sailfin mollies, as has been found by Aspbury and Gabor (2004b) for the same population and in other studies of female mate choice (review in Candolin 2003). The lack of discrimination by males from the sympatric population between conspecific and heterospecific females based on both chemical and visual cues suggests that these males may rely on additional cues to distinguish species. Several studies have demonstrated that male sailfin mollies do prefer to mate with conspecific over heterospecific females in the presence of chemical, visual, and tactile cues (Woodhead and Armstrong 1985; Ryan et al. 1996; Gabor and Ryan 2001; but see Schlupp et al. 1991). Male sailfin mollies from sympatry may require close range tactile access via gonoporial nibbling to assess more specific chemical cues of females (Farr and Travis 1986).

In conclusion, two main issues may be responsible for the variation in the results across experiments. First, there is geographic variation in male mate choice. Many factors that vary across populations (e.g., female densities, female and male sizes, ecological conditions) could influence the outcome of mate choice experiments. Therefore, it may be difficult to compare the results of multiple studies without knowledge of the source of the study animals. Second, while chemical cues are expected to provide important species recognition information, in systems where the species are closely related and share half their genes (such as with the mollies), this form of discrimination may be insufficient. In this system, it appears that the close relationship between these species results in males potentially needing multiple cues and sensory modalities.

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