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SOCIAL AGGRESSION MEDIATED VIA KIN SELECTION AND ECOLOGICAL COMPETITION IN THE AMAZON MOLLY, *POECILIA FORMOSA*

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SOCIAL AGGRESSION MEDIATED VIA KIN SELECTION AND ECOLOGICAL COMPETITION IN THE AMAZON MOLLY, *POECILIA FORMOSA*

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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ABSTRACT

The maintenance of sexual reproduction is still a largely unresolved question in evolutionary biology, and one of the most puzzling aspects of this is the co-existence of sexual and asexual species. This often leads to widespread niche overlap, posing an interesting challenge to the Competitive Exclusion Principle. Despite this, there seems to be something preventing either the sexuals or asexuals from outcompeting the other. I investigated this using the clonal fish, the Amazon molly (*Poecilia formosa*). Females produce daughters that are genetically identical to themselves and each other, and are sexual parasites of their parental species, the sailfin molly (*P. latipinna*) and the Atlantic molly (*P. mexicana*). They overlap and compete in many aspects of their ecological niche, behavioral, and life history parameters. The 'behavioral regulation hypothesis' predicts that the maintenance of this species complex is driven by adaptive male mate choice. However, little attention has been given to counter-adaptations that may allow Amazon females to thwart male mate choice. One of the most likely means of circumventing male choice is aggression towards the preferred sexual females. Here, I investigated two questions: 1) How costly is female-female aggressive behaviors in Amazon mollies in comparison to sailfin females, and 2) How does the social environment help regulate aggression in Amazon females (i.e., kin selection). First, I found that Amazon females were on average more aggressive over time when compared to their sexual hosts, sailfin molly females. Amazon females, however, incurred a higher cost of being aggressive. Aggressor females of this species had a lower body fat content than their conspecific

recipients. By contrast, sailfin females showed no differentiation between aggressors and recipients. Secondly, Amazon mollies have the ability to distinguish between different clonal lineages: clonal females prefer genetically identical, clonal sisters to slightly different clones. Intriguingly, they do this using multiple sensory modalities, including visual and/or chemical cues. In natural water, wild caught females preferred clonal sisters when chemical information was available, while the preference using visual information was non-existent. Most importantly, however, they scale their aggressive behaviors according to the relatedness to other females: they are more aggressive to non-related clones. In conclusion, female competition can be quantitatively measured and that these aggressive behaviors are costly to perform. I demonstrate that even in species with very small genetic differences between individuals, kin selection can be adaptive. Indeed, even minute differences in relatedness (extremely close kin are favored over very close kin) can provide enough substrate for the evolution of kin recognition. Their discriminatory abilities and regulation of behavior provides a powerful example of natural selection in species with limited genetic diversity.

INTRODUCTION

The Competitive-Exclusion Principle, also known as Gause's Law, states that two species cannot co-exist on the same limited resources; if similar species coexist within the same ecological niche there must be differences in the way they utilize those resources (Gause 1934; Hutchinson 1959). Otherwise, one species will outcompete and suppress- if not eliminate- the other (Mittelbach 2011). Within this principle, the competitive-relatedness hypothesis predicts that more closely related species will be less likely to coexist in a pair-wise comparison. Although this hypothesis has thus far yielded mixed support, there is enough evidence to suggest that this hypothesis should be accounted for when investigating two species in regards to the Competitive Exclusion Principle.

Sexual/unisexual species complexes produce interesting challenges when investigating this theory. First, unisexual gynogenetic vertebrate species (those that still require sperm to reproduce; see Chapter 1) are obligate sperm-parasites to their host species. Secondly, the majority of all known gynogenetic females are products of such hybridization and, therefore, closely related to each other. As such they overlap in their ecological niche, and it is likely that many of these species utilize the same resources. One such sexual/unisexual species complex includes the sailfin molly (*Poecilia latipinna*) and their sexual-parasite the Amazon molly (*P. formosa*). They show strong niche overlap and likely compete for several key resources. For instance, both sailfin and Amazon mollies consume the same diet at the same rate, and are not affected by either heterospecific or conspecific competition. Life histories are also very similar

between the two species; they have equivalent fecundity and similar size at birth. Both species are also very similar in the boldness of their feeding behaviors, and they even share comparable parasite species and parasite loads. In addition, they tend to form linear hierarchies between species, typically based on the size of the individuals within the shoal (D. Bierbach, personal comm.). Consequently, these two species overlap and compete in many aspects of their ecological niche, life history parameters, and behaviors. This poses a challenge in understanding the maintenance of these species complexes because it violates previous niche and competition theories.

One hypothesis that predicts the maintenance of these species is the 'behavioral regulation hypothesis.' This hypothesis predicts that this sexual/unisexual species complex is driven by adaptive male mate choice (Schlupp and Riesch, 2011). Male sailfin mollies show a clear preference for mating with sexual females when compared to unisexual females. One way males can achieve surplus mating opportunities with these sexual females is when the sexual females copy the mate choice of the unisexual females. However, this hypothesis does not take into account how Amazon mollies may thwart the males' preferences for sexual females. I predict that Amazon mollies are using aggressive behaviors towards the preferred sexual females to circumvent male mate choice. To date, there has been little research in female-female aggression in these fish; the only things present in the literature are short descriptions of the occurrence of these behaviors. Amazon females seem to be more aggressive than their sexual counterparts, and have been documented chasing sexual females away from males. Here, my research will address several questions in regards to this prediction: 1) How do the aggressive behaviors differ between

Amazon and sailfin mollies? 2) What are the costs of aggression on Amazon mollies in comparison to sailfin molly females (Chapter 1)? 3) Do Amazon females recognize and prefer clonal sisters to non-sisters? 4) What mechanism(s) do females use to identify clonal sisters from non-sisters? 5) Does clonal recognition occur in natural environments? 6) Finally, do females adjust their aggressive behaviors according to relatedness (Chapter 2)?

CHAPTER 1

Effects of Female-Female Aggression in a Sexual/Unisexual Species Complex

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ABSTRACT

The maintenance of sexual reproduction is still a largely unresolved question in evolutionary biology, and one of the most puzzling aspects of this is the co-existence of sexual and asexual females. This often leads to widespread niche overlap, posing an interesting challenge to competitive exclusion theory. In this study, we investigate how the aggressive behaviors between females in a sexual/unisexual mating complex of mollies (*Poecilia latipinna* and *P. formosa*) differ, and the effects these behaviors have on both the performer and receiver of aggressive acts. We exposed females to five treatments: 1) alone (control); 2) a large and small sexual female; 3) a large sexual female and a small unisexual female; 4) a small sexual female and a large unisexual female; and 5) a large and small unisexual female. We found that *P. formosa* females were on average more aggressive over time when compared to their sexual hosts, *P. latipinna*. The females of *P. formosa*, however, incurred a higher cost of being aggressive. Large, aggressor females of this species had a lower body fat content than their conspecific recipients. By contrast, *P. latipinna* females showed no differentiation between aggressors and recipients. Here, we combine life-history methodology and behavioral observations to show that female competition can in fact be quantitatively measured and that these aggressive behaviors are costly to perform. The adaptive value of this inter-species aggression is not yet completely understood, but sexual competition, access to resources, and position within the shoal are all possible functions.

Keywords: body condition, female-female aggression, female competition, *Poecilia formosa*, *Poecilia latipinna*, sexual/unisexual mating system

INTRODUCTION

Asexuality in vertebrates is very uncommon, and asexual amphibians and fishes are always sperm dependent (Avise 2008). Their eggs must be pseudo-fertilized by the sperm of heterospecific males to trigger embryogenesis. In evolutionary biology such species are used to investigate the maintenance of sexual reproduction (Schlupp & Riesch 2011). Given the classical costs of males and meiosis/recombination (e.g. the two fold cost of males and passing on only half of the genes), asexual reproduction should have a short-term advantage - especially in stable environments - and is predicted to outcompete sexual reproduction (Maynard-Smith 1978; West et al. 1999; Schlupp 2009).

In ecology, the co-existence of sexual and asexual species poses another interesting challenge. In some sexual/asexual species complexes, both species occur in vastly overlapping, if not identical environments, most likely because the asexual females are sperm dependent and have to follow males. Moreover, asexual vertebrates are typically natural hybrids, which already predicts a high degree of overlap with the parental species. These, in turn, are usually the species that provide sperm. This poses a challenge in understanding the maintenance of these species complexes because it violates niche and competition theories. The Competitive Exclusion Principle, or Gause's law, states that two species cannot co-exist on the same resources; and therefore, if similar species co-exist within the same ecological niche there must be differences in the way they utilize those resources (Gause 1934;

Hutchinson 1959). Otherwise, one species will outcompete and suppress, if not eliminate, the other (Mittelbach 2011).

In the sexual/unisexual mating system we study, the females that make up the complex are ecologically extremely similar: females of two species, *Poecilia latipinna* and *P. formosa,* compete for males (Hubbs and Hubbs 1932; Schlupp et al. 1991; Schlupp et al. 1994; Riesch et al. 2008; Schlupp 2009) and other resources. These females live in large, open shoals that vary in the ratio of each species throughout the year (Schlupp 2009). Therefore, the frequency at which females encounter heterospecific females varies throughout the breeding season - requiring them to adjust their behaviors accordingly.

Poecilia latipinna is a sexual species found in backwaters, streams, springs and pools ranging along the Atlantic coast from northern Mexico to southern North Carolina. *Poecilia formosa* is found along the Gulf of Mexico from Texas through northern Mexico (Costa and Schlupp 2010). *Poecilia formosa* is a natural hybrid of *P. latipinna* and *P. mexicana* (c.a. 120,000 generations ago; Schartl et al. 1995; Stöck et al. 2010). It is also a gynogenetic, unisexual species that reproduces using sperm of males of either the *P. latipinna* or *P. mexicana* to initiate embryogenesis (Hubbs and Hubbs 1932; Schartl et al. 1995). The male's DNA, however, is typically not incorporated into the offspring's genome, resulting in only indirect reproductive benefits for males who mate with these females (i.e., mate choice copying; Schlupp et al. 1994; Heubel et al. 2008). Nonetheless, males have been shown to discriminate against *P. formosa* females (Schlupp et al. 1991; Ryan et al. 1996; Gabor & Ryan 2001; Schlupp and Plath 2005; Riesch et al. 2012). Sexual females are significantly

more likely to have sperm in their genital tract and to be pregnant than the unisexual females (Riesch et al. 2012). Thus, the social environment in which mixed groups of *P. latipinna* and *P. formosa* co-occur sets the stage for complex social and competitive interactions both within and between species (Schlupp 2009).

Importantly, competition between females of the two species is not just for mates, but these species are also very similar in several other ecological traits. For instance, both *P. latipinna* and *P. formosa* consume the same diet, at the same rate, and are not effected by either heterospecific or conspecific competition (Heubel 2004; Fischer and Schlupp 2010; Scharnweber et al. 2011a; Scharnweber et al. 2011b). Life histories are also very similar between the two species: both reproduce about every 30 days, have equivalent fecundity (Schlupp et al. 2010), and similar sizes at birth (Tobler and Schlupp 2010), although *P. formosa* neonates have a lower life expectancy in low food environments (Tobler and Schlupp 2010). Size-classes of females overlap at maturity, both Amazons and sailfins range between 25 mm to a maximum of 65 mm, producing considerable heterogeneity in the social environment (Heubel 2004; Riesch et al. 2008). Furthermore, both species are very similar in their boldness (e.g., taking risks to gain resources; Scharnweber et al. 2011c), and they even share comparable parasite species and parasite loads (Tobler et al. 2005). In addition, they frequently form linear hierarchies between species in mixed shoals, typically based on the size of the individuals within the shoal (Bierbach, pers. comm.). Finally, females of both species prefer to mate with larger males (Marler and Ryan 1997) and consequently, these two species overlap and compete in multiple aspects of their ecological niche, life history, and behaviors.

Given the wide overlap in ecological niches, what are the mechanisms that regulate coexistence? The 'behavioral regulation hypothesis' predicts that the maintenance of this species complex is driven by adaptive male mate choice (Schlupp and Riesch 2011). However, little attention has been given to counter-adaptations that may allow *P. formosa* females to thwart male mate choice (Schlupp et al. 1991). One of the most likely means of circumventing male choice is aggression towards the preferred sexual females. For instance, *P. formosa* females were found to chase females, both *P. latipinna* and *P. mexicana*, away from the males (Schlupp et al. 1991). Mate choice has been the only context thus far, for which female-female aggression has been identified (Schlupp et al. 1991; Foran and Ryan 1995; Heubel and Plath 2008). As such, we predicted that in *P. formosa*, female-female aggression is directed towards the heterospecific species more often, and at more escalated and vigorous level, than to conspecifics due to the extremely high relatedness found within the unisexual species (Hamilton 1963). *Poecilia latipinna*, on the other hand, is predicted to show equivalent female-female aggression towards both heterospecifics and conspecifics due to competition over the same preferred males (i.e., possible sperm limitation in preferred males; Stockley and Bro-Jørgensen 2011).

In this context it is important to consider the costs of aggression both for the aggressor and the receiver. The costs of competition and aggression among females, when compared to the same level in males, are higher due to: 1) additional energy demands of gestation and parental care, and 2) lower reproductive rates (Rosvall 2011a; Stockley and Bro-Jørgensen 2011). Ultimately, the energetic costs of aggression for the aggressor can influence the frequency, duration, and the recipients

of such behaviors (Stockley and Bro-Jørgensen 2011). Previous research has shown that the cost of certain behaviors (e.g., sexual harassment; Makowicz and Schlupp 2013) influences the overall body fat content of individuals. It has also been shown that reproducing females endure a cost at the expense of body fat (i.e., fat accumulation decreases with reproduction; Monteith et al. 2013). In another livebearing fish, *Gambusia holbrooki*, late-stage pregnant females were more aggressive and had greater metabolic costs for their aggressive interactions, posing a risk to not only themselves but also their offspring (Seebacher et al. 2013). Indeed, the costs of female-female aggression have been shown to have a direct negative effect on the offspring; for instance, highly aggressive female tree swallows (*Tachycineta bicolor*) have small, lower-quality chicks (Rosvall 2011b). In dark-eyed juncos (*Junco hyemalis*), aggressive females spend less time brooding nestlings and produce smaller, lighter offspring when compared to their less aggressive counterparts (Cain and Ketterson 2013). This is, however, not always the case, as the offspring of aggressive female White's skinks (*Egernia whitii*) seemed to have a slight increase in survival rates (Sinn et al. 2008). Therefore, understanding how the costs of particular social behaviors (i.e., aggression) influence the body fat content, and thus potential reproduction and survival, are essential to understanding how species are able to co-exist in similar environmental conditions.

Therefore, in this study, we investigated four questions: 1) What are the effects of female-female aggression on the overall body fat content of each individual (i.e., which individual is most affected in terms of body fat content, the aggressor or the recipient, *P. formosa* or *P. latipinna*)? 2) How do the sexual and unisexual species

differ in their aggressive behaviors? 3) Does the size or species of the partner female influence how aggressive a female is? 4) Do the behaviors of the females change over exposure time? We predict that: 1) larger females will behave more aggressively towards smaller females irrespective of species (Almeida et al. 2014); 2) Amazon mollies will behave more aggressively than sailfin mollies, irrespective of body size (Schlupp et al. 1991; Foran and Ryan 1995; Heubel and Plath 2008); and 3) Aggressor females will have lower body condition than recipient females (Monteith et al. 2013).

METHODS

FISH MAINTENANCE

Specimens were collected in 2010 from Comal Springs (29˚42'46.86" N; 98˚08'8.57" W) in New Braunfels, Texas. They were transported to the University of Oklahoma campus using aerated 20 L coolers. Female *P. latipinna* and *P. formosa* were maintained separately in several 50 × 30 × 25 cm aquaria (length × width × height) at the University of Oklahoma in Norman. Two weeks before the tests, individuals were transferred to smaller (3.8 L) isolated tanks where visual contact with other fish was prevented. Females were maintained at a temperature of 27˚C, under a 12: 12-hour light: dark cycle, and fed daily *ad libitum* with commercial flake food (Tetramin flakes) to help alleviate aggression in a nutrient-rich environment. All fish were non-virgins, however, all females were in comparable reproductive states (not pregnant) at the start of this experiment.

EXPERIMENTAL DESIGN

After a two week period of isolation females were weighed (g), measured (standard length, mm), and randomly assigned to one of five treatments: 1) female alone (as a control; either a sexual, *P. latipinna*, or unisexual female, *P. formosa*); 2) a large and small sexual, *P. latipinna* female together; 3) a large sexual, *P. latipinna* female and a small unisexual, *P. formosa* female together; 4) a small sexual, *P. latipinna* female and a large unisexual, *P. formosa* female together; and finally 5) a large and small unisexual, *P. formosa* female together. Larger females ranged between 40-60 ±3mm, while smaller females ranged between 28-39 ± 3mm. There was at least a 6mm size difference between the large and small individuals. We choose not to include same-sized female treatments in this study because previous data indicated that when females are partnered with same-sized females, the time they spend behaving aggressively falls in between values found when they are partnered with a larger or smaller individual (see below, Makowicz et al. 2010, Makowicz, Moore, Schlupp unpublished data, Makowicz, Tiedemann, Schlupp unpublished data, Table S1). Therefore, we did not anticipate any large effects that would justify including these treatments.

Treatment tanks were the small aquaria (3.8 L) used during the isolation phase and contained two females in the treatment groups and a single female in the control group. A sponge filter was placed in the center of the tanks, large enough to also acted as a refuge to diffuse aggression towards the recipient females. Visual contact between each treatment tank was prevented by placing cardboard between tanks. Tanks received a water change once a week and at the same time the weight and

standard length was recorded for each fish. The behavioral experiment lasted for a total of 5 weeks.

Behavioral observations

Aggressive behaviors (bites and overall time spent interacting aggressively with partner female; Parzefall 1969, 1989) for each female were recorded. Behavioral recordings of both females were taken by the observer for 5-minutes every third day, starting from two hours after the initial set up. Females were fed in the morning (between 8 and 10 am) and behavioral recordings occurred in the afternoon (between 12 and 2 pm). In addition, the aggressiveness, whether each female was the aggressor or recipient of any given pair, was also recorded. We define the aggressor as the individuals that initiated and perform the majority of the aggressive behaviors (i.e., bites, chases, and tail beats), and the recipient as the individual that received the majority of the aggressive behaviors. Behavioral observations were recorded for 24 days for a total of 9 measurements for each tank. All observations were also conducted in front of each tank including the single female treatment to control for the presence of the observer. Control females did not display any aggressive behavior during the observations, however.

Life history data

To determine the effects of female-female aggression on body fat, individuals were reweighed and measured, then sacrificed in ice water after the last behavioral observations on the final day (AVMA Guidelines for the Euthanasia of Animals:

2013 Edition, section S6.2.2 (6); Wilson and Carty 2008) and preserved in 37% formaldehyde. Body fat was measured through determining the soluble fat content via ether extractions using the protocol of Reznick and Endler (1982) and Riesch et al. (2010). In short, the reproductive tissue of all females was removed via dissection and both the somatic and the reproductive tissues (and embryos if applicable) were dried for 10-12 days at 45 ˚C. Afterwards all somatic, reproductive and embryonic tissues were reweighed and placed into petroleum ether for a minimum of 6 hours, allowing ether to remove all non-structural fat from the tissues. More soluble fat indicates better body condition in the fish. The ether bath was repeated until the solution was clear, indicating the extraction of any non-structural fats. Once a clear bath was reached, the individual, reproductive and embryonic tissues were reweighed one final time. Reproductive allocation is the proportion of tissue dedicated to reproduction and was measured as the total weight of the reproductive tissue divided by the weight of the reproductive tissue plus the weight of the somatic tissue. This method allows us to measure the somatic weight, reproductive weight, the percent of fat (used to indicate body fat), and the reproductive allocation of females (Riesch et al. 2010).

Statistical Analysis

We used a repeated-measures ANOVA to analyze the behavioral data (SPSS v 17.0). The dependent variables were the overall time spent being aggressive, and the number of bites performed. The fixed factors were the focal female's species, the partner's species, and the size of the female relative to her partner (large or small). Together these factors make up the three parameters of the treatment design. We

included the relative size difference between focal and partner female and the aggressiveness (whether or not she was the aggressor or the recipient) of the female as covariates. Relative size difference was calculated by dividing the standard length of the partner female by the focal female, and then normalized via an arc sin square-root transformation. A linear regression analysis was also used to investigate the relationship between aggression and body size, along with aggression and the relative size difference between the focal female and partner female.

For the life history data, a multivariate GLM was used. The dependent variables were somatic lean weight, body fat, and reproductive allocation. The fixed factors were the focal female's species, the partner's species, with relative size difference and females aggressiveness as covariates. Furthermore, relative risks analysis (with Fisher's exact test; Motulsky 1995) was conducted on the unexpected mortality rates experienced.

Results

Behavior

We used a total of 177 females in this study, 88 of them were *P. latipinna* and 89 were *P. formosa*. Sample sizes for treatments for the behavioral data and the life history data (including 67 *P. latipinna* and 70 *P. formosa*) can be found in Table 1. The relative size difference between the focal and partner females did not have a significant effect on the time females spent being aggressive ($F_{(93)}= 0.206$, $p= 0.993$) or the number of bites performed ($F_{(93)}= 0.170$, $p= 0.997$) and was henceforth removed from the model. We found that females changed the time they spent

performing aggressive behaviors over the time span of the experiment ($F_{(94)}$= 8.371, p< 0.0001) and the number of bites given ($F_{(94)}$= 3.009, p= 0.003; Table 2). The time females spent performing aggressive behaviors and the number of bites administered was not influenced by the focal species, but rather by the partner species, the size, and the aggressiveness of the female (Table 3). Regression analyses showed that the aggressiveness of the females was significantly related to her body size ($F_{(1)}$= 1619.936, p< 0.0001, R= 0.961) and the relative size difference between the female and her partner ($F_{(1)}$= 61.052, p< 0.0001, R= 0.558).

Large *P. latipinna* females tended to vary in their social aggression when paired with either conspecific or heterospecific partners (Table 4). When paired with smaller conspecifics, larger females eventually increased aggression over time even if they initially started out as a recipient. Only a single small *P. latipinna* female remained an aggressor during the entire duration of the experiment. When paired with smaller heterospecifics, recipient females always remained recipients and were never aggressive. However, of the aggressor females, large *P. latipinna* females increased their aggressive behaviors over time; while small *P. formosa* aggressors decreased their aggressive behaviors over time, even though these smaller females were always the aggressor. Nonetheless, *P. latipinna* females were always more aggressive at the start of the experiment when compared to *P. formosa* females, and there was less variation in aggressive behaviors over time (Fig. 1). On the other hand, large *P. formosa* females were always aggressor females. They dramatically increased the time spent being aggressive and the number of bites performed by the end of the

experiment, more so when paired with a smaller *P. latipinna* female than a *P. formosa* female (Table 4; Fig. 1).

LIFE HISTORIES

Relative size differences did not influence the body fat ($F_{(1)}$= 0.259, p= 0.612), or reproductive allocation ($F_{(1)}$= 0.213, p= 0.645) of the females, but somatic lean weight was significantly influenced by the relative size difference between them ($F_{(1)}$= 4.241, p= 0.042). The focal female species significantly influenced the somatic lean weight ($F_{(1)}$= 44.152, p< 0.0001) and the body fat content ($F_{(1)}$= 12.491, p= 0.001) of the females. This however, did not influence the reproductive allocation ($F_{(1)}$= 0.740, p= 0.391). Partner species significantly influenced the somatic lean weight ($F_{(2)}$= 19.207, p< 0.0001), and the body fat ($F_{(2)}$= 5.540, p= 0.020), but not the reproductive allocation ($F_{(2)}$= 0.000, p= 0.993) of the females. Aggressiveness (whether females were the aggressor or the recipient) significantly influenced the somatic lean weight ($F_{(1)}$= 40.948, p< 0.0001) and the reproductive allocation ($F_{(1)}$= 9.704, p= 0.002), and tended to influence the body fat content ($F_{(1)}$= 1.692, p= 0.096), although this was not significant. There was an interaction between the focal species and partner species with somatic lean weight ($F_{(2)}$= 14.534, p< 0.0001), but not reproductive allocation ($F_{(2)}$= 1.012, p= 0.316) or body fat content ($F_{(2)}$= 2.479, p= 0.118). Finally, there was a significant interaction between the focal species, partner species, and the aggressiveness of the females in only somatic lean weight ($F_{(1)}$= 4.099, p= 0.045). *P. formosa* females were significantly larger than *P. latipinna* females, and aggressor females of both species were significantly larger than the

recipient females (Fig. 2). Aggressor females also tended to have a higher reproductive allocation than the recipient females, most prominently in *P. latipinna* females. However, this was not significant. On average, focal *P. formosa* females had less body fat content than *P. latipinna* focal females and both species seemed to have more body condition when partnered with a *P. latipinna* female (Fig. 2 and 3). There was no difference in body fat content between aggressor and recipient *P. latipinna* females, but there is a reduction in body fat of the aggressor *P. formosa* females when compared to the recipient conspecifics (Fig. 2).

Only 6 females had yolked eggs at the termination of the study, and 3 of these females became pregnant using stored sperm. All of these females were *P. formosa*, where five females were aggressors and one was a control. Of the five females in the treatments, four were in treatment 4 (large and small conspecifics) and one was in treatment 3 (large *P. formosa* and small *P. latipinna*).

MORTALITY

A total of 18/177 females died prematurely (roughly 10% of the total number used in this study): 61% of those died with *P. formosa*, as partner, and 39% with *P. latipinna*. Of the females that died, 78% were smaller than their partner, 67% were *P. latipinna* and 78% were the recipient female. A relative risk analysis showed that deceased females were 1.61 times more likely to have die when partnered with *P. formosa* than *P. latipinna*, although this was non-significant (Fisher's exact test: p= 0.325). Of the deceased females, *P. latipinna* were 1.95 times more likely than *P. formosa* to die (Fisher's exact test: p= 0.209), smaller females were significantly 3.23

times more likely to die than large females (Fisher's exact test: p= 0.022) and recipient females were 2.8 times more likely to die than aggressor females (Fisher's exact test: p= 0.070).

DISCUSSION

In the present study we quantified the cost female-female aggression has for both aggressor and recipient individuals under natural size variations. There were four major results: 1) *P. formosa* aggressor females had lower body condition than recipient conspecifics, although this was not the case in *P. latipinna*. 2) *P. formosa* tended to behave more aggressively towards their partner, and be significantly larger. 3) *P. formosa* females increase their aggressive behaviors over time more so than *P. latipinna* females. 4) Large females were more likely to be aggressive compared to small females, independent of species. However, in *P. formosa*, small females were more aggressive towards their larger partner than the small *P. latipinna* females.

Large, aggressor females seem to be using more energy when behaving aggressively towards other females. These behaviors seem to be physiologically more costly, although there may be an unknown evolutionary benefit not yet identified that outweighs the short-term costs, such as access to better foraging areas, or access to males. Aggressiveness in both species is positively correlated with both the aggressors' body size and the relative difference between the aggressor and her partner. Indeed, they tended to also have a higher reproductive allocation, although this was not significant. Of the aggressor females, *P. formosa* were more likely to be aggressive - to the point of causing unexpected mortality - than *P. latipinna*. When *P.*

formosa and *P. latipinna* were isolated there was no significant difference in their body fat; however, when competing with other individuals, *P. formosa* females tended to always have significantly less body fat than their sexual hosts (Fig. 2 and 3). Aggressor females were significantly more likely to be antagonistic towards smaller females than larger females; and of the deceased females, they tended to be either recipients or *P. latipinna*.

Interestingly, behavioral changes over time occurred in both *P. latipinna* and *P. formosa* females. *Poecilia latipinna* females were more aggressive at the start of the experiment when compared to *P. formosa*. However, this reversed at the end of the experiment with *P. formosa* behaving significantly more aggressive than the *P. latipinna* females. Also, *P. latipinna* females spent equal amounts of time being aggressive to both conspecific and heterospecific partners over time, although they tended to spend more of this time biting *P. formosa* partners than *P. latipinna* partners. *Poecilia formosa* females, initially, gave more bites to conspecifics, but over time switched to being more aggressive to heterospecific partners. These results suggest that *P. latipinna* females are more reserved with their aggressive behaviors when exposed to the same partner females over time. *Poecilia formosa*, however, become more aggressive and less tolerable towards their partners.

Interestingly, small *P. formosa* females were more aggressive towards their sexual hosts than large *P. formosa*. Small *P. formosa* females performed four times more aggressive bites and spent six times longer being aggressive than their large *P. latipinna* partners (Table 2 and 4), although this diminished over time. In comparison, small *P. latipinna* females rarely were aggressive towards larger *P. formosa* or *P.*

latipinna partners. This suggests that there are behavioral differences in aggressive behaviors between *P. latipinna* and *P. formosa* females, and even between different size classes within species (Supplemental Table 1).There may be an evolutionary advantage for aggression in general, irrelevant of size, in *P. formosa*; for instance, it may allow females to better compete with their sexual hosts (see below). However, we found that there is, indeed, a cost to female-female aggression in these fish, with an even greater cost to the asexual females. *Poecilia formosa* had significantly lower body condition in general, which suggests that they are physiologically more different from their sexual host than originally hypothesized. But aggressor females pay an even higher cost; they use roughly 35% more energy (a correlate of body fat content) on aggression when compared to aggressive sexual females and 29% more energy than their more passive conspecifics. This leads to new questions: What are possible benefits to balance the high cost of aggression in the Amazon molly? And how does this play a role in the maintenance of this species complex?

Surprisingly, *P. latipinna* females behaved more aggressively towards conspecifics than towards heterospecifics (Table 2, 3, 4; see also Scharnweber et al. 2011b; Heubel and Plath 2008). Thus, our prediction that *P. latipinna* females would be equally aggressive towards both conspecifics and heterospecifics due to sperm limitation was not supported in this study. Furthermore, the reverse was found for *P. formosa* females, which were less aggressive to conspecifics but more aggressive towards heterospecifics, which supports our prediction (Table 2, 3, 4; Heubel and Plath 2008). Several hypotheses that might explain this female-female aggression in general have been proposed (Rosvall 2011a): 1) females compete for males that

provide direct benefits (i.e., nutrients, nesting sites, parental care, foraging sites etc.); 2) competition and aggression during offspring-rearing (maternal-only care of offspring); and 3) the non-adaptive hypothesis (a behavior that is favored in one sex exists in the other by default via genetic correlation; Rosvall 2011a). None of these hypotheses can be applied to the sexual/unisexual species complex we study here because neither males nor females provide any parental care or direct benefits to each other or the offspring after birth, and the aggression cannot be maintained by selection for aggression in males for the unisexual species. Although the actual benefits that correlate with these particular behaviors are still unknown, we suggest four possible explanations for the female-female aggression found in this study: 1) female competition for preferred mates; 2) interference competition over food resources, 3) shelter, or 4) position in the water-column or shoal (Rosvall 2011a; Stockley and Bro-Jørgensen 2011).

First, it is possible that sexual females may be competing for access to the best mate (i.e., the male with the "good genes"). Female competition occurs less frequently than male competition, but can be as intense or more so (Stockley and Bro-Jørgensen 2011; Stockley and Campbell 2013; Clutton-Brock and Huchard 2013). Being the first female to mate with males with "good genes" would be beneficial; for instance, the female may receive more sperm, a longer copulation period, or higher quality sperm (Smith et al. 2009; Hettyey et al. 2009; Stockley and Bro-Jørgensen 2011). Therefore, females that are larger, more attractive, and/or show more vigor or female-female aggressive behaviors may be better able to mate first with their preferred male (Petrie et al. 1992). *Poecilia formosa*, may have evolved several

behaviors to increase male attention when compared to their sexual female counterparts that are generally more passive (Schlupp et al. 1991; Heubel and Plath 2008).

Another hypothesis for increased aggression within *P. latipinna* and the aggression between *P. formosa* and *P. latipinna* is competition for food resources (Rosvall 2011a; Stockley and Bro-Jørgensen 2011). Due to niche overlap, both species of females use essentially identical food resources and forage at similar rates (Fisher and Schlupp 2010). If a female can increase her available food resources, theoretically this would increase her potential body size, thus indirectly benefiting her chances to mate with her preferred male. This may account for the aggression we found in the small *P. formosa* towards the much larger *P. latipinna*. Previous research has shown that male *P. latipinna* prefer to mate with conspecifics rather than heterospecifics, and with larger, more attractive females than smaller females (Schlupp et al. 1991); however, body size over rides this preference for conspecific female (i.e., males are more willing to mate with larger heterospecifics rather than smaller conspecifics; Schlupp et al. 1991). Smaller *P. formosa* may display aggression to increase access to resources, to attain larger body size, to then overcome the male's preference for conspecific females. Indeed, *P. formosa* females do increase their aggressive behaviors more towards same-sized heterospecifics rather than conspecifics when competing over a food resource (Supplemental Table 1; Makowicz, Moore, Schlupp unpublished data). However, in the present study, females were fed *ad libitum* to help reduce this type of aggression.

Finally, females may behave aggressively to gain access to shelter or for

position within the water-column/shoal. Several species of fishes have been shown to increase aggressive behaviors towards both conspecifics and heterospecifics when defending a shelter (Shulman 1985). In the Eurasian perch (*Perca fluviatilis*), individuals are more aggressive towards conspecifics when competing for shelter than food resources (Mikheev et al. 2005). These shelters help reduce predation rates and are a limited resource within an environment. Indeed, both shelter and position within a shoal or water-column are pertinent when avoiding predation. Position within the water-column predicted the amount of aggressive behaviors Pumpkinseed sunfish (*Lepomis gibbosus*) received and was influenced by the different size classes (Almeida et al. 2014). Also, in mosquitofish (*Gambusia holbrooki*), position within the shoal was not influenced by dominant status (Burns et al. 2012). Nonetheless, it is possible that both Amazon (*P. formosa*) and sailfin mollies (*P. latipinna*) may behave aggressively when limited shelters are available or position in the water-column or shoal.

Due to the hybrid origin of *P. formosa*, it would be beneficial to investigate the effects of aggression in their maternal species, *P. mexicana*, also, which is relatively unknown. In addition, investigating how aggression fluctuates in high and low food resource or male availability between species addresses this aggression as if it were indeed a result of interference competition or mate competition. Here, we attempted to capture female-female aggression in *P. formosa* and *P. latipinna* using females of different sizes to simulate natural social environmental conditions; however, to truly understand the dynamics of aggression, similar sized females should also be assessed. Behavioral differences between *P. latipinna* and *P. formosa*

females may play a prominent role in allowing these species to overlap in the same ecological niche. Here, we show that aggression varies between these two species to play an important role in the interactions of this species complex. Nevertheless, more research is required on female competition within and between these species to fully understand the maintenance of this sexual/ unisexual mating systems.

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Table 1: Sample sizes for each treatment for both the behavioral data and the life history data.

TREATMENTS	BEHAVIOR	LIFE HISTORY
Control *P. latipinna*	N/A	13
Control *P. formosa*	N/A	16
Large and Small *P. latipinna*	38	29
Large and Small *P. formosa*	36	27
Large *P. latipinna* and Small *P. formosa*	36	13 and 12
Large *P. formosa* and Small *P. latipinna*	38	15 and 12

Table 2: The average (± SD) of female aggressive behaviors (bites and time) for both *P. latipinna* and *P. formosa* females across the different treatments. Treatments were 1) a large and small conspecific female; 2) a large female paired with a small heterospecific; and 3) a small female paired with a large heterospecific. The aggressor females were the individuals that were frequently performed the aggressive behaviors, and the recipient females were the individuals that received the aggressive behaviors and were rarely aggressive.

		P. latipinna			*P. formosa*		
Treatment		Large/Small	Large	Small	Large/Small	Large	Small
Bites	Aggressor	9.671 +/- 7.31	6.998 +/- 5.76	-	10.183 +/- 9.19	29.193 +/- 13.44	29.193 +/- 13.44
	Recipient	0.518 +/- 1.31	0	0.062 +/- 0.16	-	0.181 +/- 0.48	0.181 +/- 0.48
Time (s)	Aggressor	7.351 +/- 5.60	4.169 +/- 3.32	-	7.947 +/- 10.17	26.530 +/- 10.57	26.530 +/- 10.57
	Recipient	0.603 +/- 1.57	0.018 +/- 0.04	0.028 +/- 0.07	-	0.086 +/- 0.24	0.086 +/- 0.24

Table 3: Summary table of the repeated-measures ANOVA on both time and bites performed.

	Statistics	
	Time	**Bites**
Behavior	**F_{94}=8.371, *p*<0.0001**	**F_{94}=3.009, *p*=0.003**
Behavior*Relative Size Difference	F_{93}=0.206, *p*=0.993	F_{93}=0.170, *p*=0.997
Behavior*Focal Species	F_{94}=0.780, *p*=0.635	F_{94}=0.635, *p*=0.764
Behavior*Partner Species	**F_{94}=15.917, *p*<0.0001**	**F_{94}=2.924, *p*=0.004**
Behavior*Aggressiveness	**F_{94}=15.756, *p*<0.0001**	**F_{94}=2.459, *p*=0.015**
Behavior*Size	**F_{94}=17.741, *p*<0.0001**	**F_{94}=2.610, *p*=0.010**
Behavior*Focal Species*Partner Species	F_{94}=0.714, *p*=0.694	F_{94}=0.573, *p*=0.816
Behavior*Partner Species*Size	**F_{94}=14.307, *p*<0.0001**	**F_{94}=4.264, p<0.0001**
Behavior*Partner Species*Aggressiveness	**F_{94}=14.274, *p*<0.0001**	**F_{94}=4.396,p<0.0001**
Behavior*Size*Aggressiveness	**F_{94}=9.335, *p*<0.0001**	**F_{94}=2.374, *p*=0.018**
Behavior*Partner Species*Size*Aggressiveness	**F_{94}=16.349, *p*<0.0001**	**F_{94}=2.772, *p*=0.006**

Table 4: Average difference (± SD) between the first behavioral measurement and the last day for both *P. formosa* and *P. formosa* females. Aggressor females (both *P. latipinna* and *P. formosa*) increase the number of bites performed on the last day of behavioral measurements when compared to the first day. *P. formosa* females also increase substantially in the overall time performing aggressive behaviors towards *P. latipinna* partner when compared to *P. formosa* females. Note, the focal species was always the larger than their partner females.

Focal Species	Partner Species	Size	Aggressiveness	N	Bites		Time	
					First Day	Last Day	First Day	Last Day
P. latipinna	*P. latipinna*	Large	Aggressor	17	6.47±14.99	11.88±12.64	4.56±11.48	8.47±10.74
			Recipient	2	0±0	0±0	0±0	0±0
		Small	Aggressor	1	13.00±2.83	3.50±4.95	26.25±33.48	1.94±2.74
			Recipient	18	2.77±11.14	0.59±2.43	1.98±8.09	0.58±2.74
	P. formosa	Large	Aggressor	14	3.93±7.80	10.79±9.55	2.73±4.85	6.43±5.38
			Recipient	4	0±0	0±0	0±0	0±0
		Small	Aggressor	4	25.25±25.25	16.75±21.79	20.69±22.84	8.81±10.21
			Recipient	14	0±0	0±0	0±0	0±0
P. formosa	*P. latipinna*	Large	Aggressor	19	4.63±3.73	23.05±35.20	3.07±19.50	19.50±41.58
		Small	Recipient	19	0.05±0.23	0±0	0.13±0.48	0±0
	P. formosa	Large	Aggressor	18	5.94±7.58	16.22±17.91	4.09±4.21	12.65±18.68
		Small	Recipient	18	0±0	0±0	0±0	0±0

FIGURE LEGENDS

Fig. 1 The average number of bites performed and the time spent being aggressive towards their partner on the first day (blue dots) and last day (red dots) of the experiment for both *P. latipinna* and *P. formosa* females. The dashed lines represent a visual difference in responses between focal *P. latipinna* and *P. formosa* females for the same partner species on the first day (blue line) and last day (red line). Focal *P. latipinna* females were more aggressive on the first day when compare to *P. formosa* females; however, the sexual females did not vary much in aggressive behaviors over the course of the experiment. Although *P. formosa* females were not initially more aggressive, they were significantly more aggressive towards their partner at the conclusion of the experiment.

Fig. 2 The average somatic lean weight (black line), body fat content (blue line), and reproductive allocation (red line) of *P. latipinna* and *P. formosa* females. Focal *P. formosa* had a significantly lower body condition and were significantly larger than *P. latipinna* females. Females of both species did not vary in their body fat content and reproductive allocation when housed separately (control treatment). The control females that were kept alone (none; 3rd row) had similar body condition for both species.

Fig. 3 The average ± SE body condition (fat (g) / somatic tissue weight (g)) of *P. latipinna* and *P. formosa* females based on their aggressiveness (Aggressor- black;

Recipient- grey; and Alone- white). There was no difference between the body fat content of aggressor and recipient *P. latipinna* females. *P. formosa* females tended to incur a higher cost of aggression via a lower body fat content when compared to the recipient females. The control females that were kept alone (white) had similar body condition for both species.

Figure 1

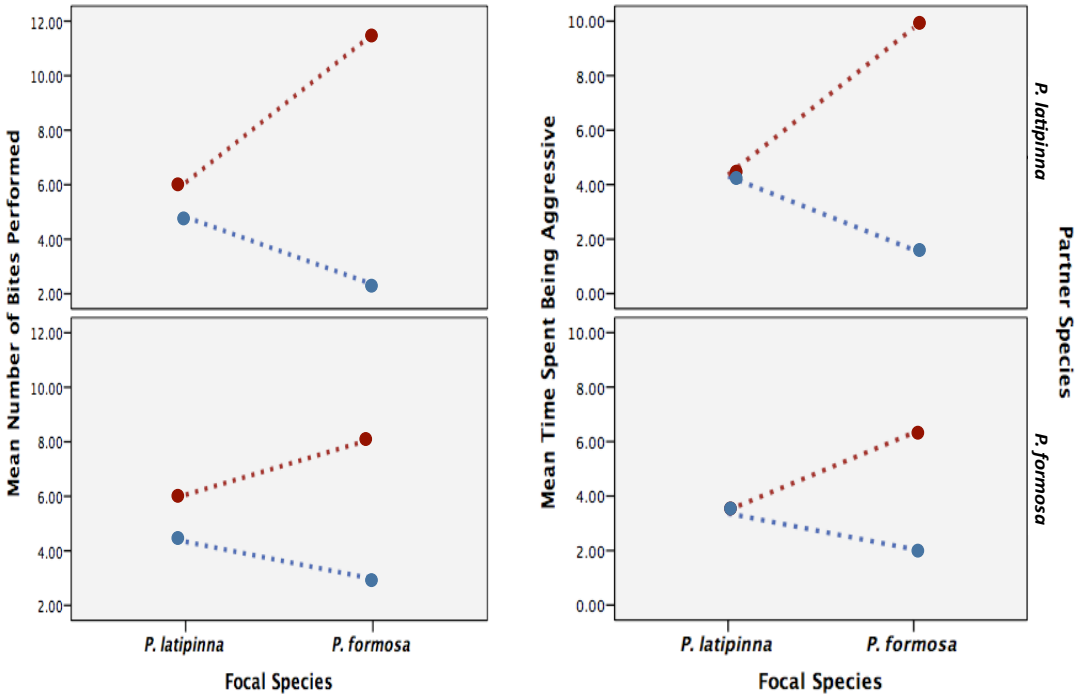

Mean Number of Bites Performed
12.00
10.00
8.00
6.00
4.00
2.00
P. latipinna
P. formosa
Focal Species
Mean Time Spent Being Aggressive
10.00
8.00
6.00
4.00
2.00
0.00
P. latipinna
P. formosa
Focal Species
Partner Species
P. latipinna
P. formosa

Figure 2

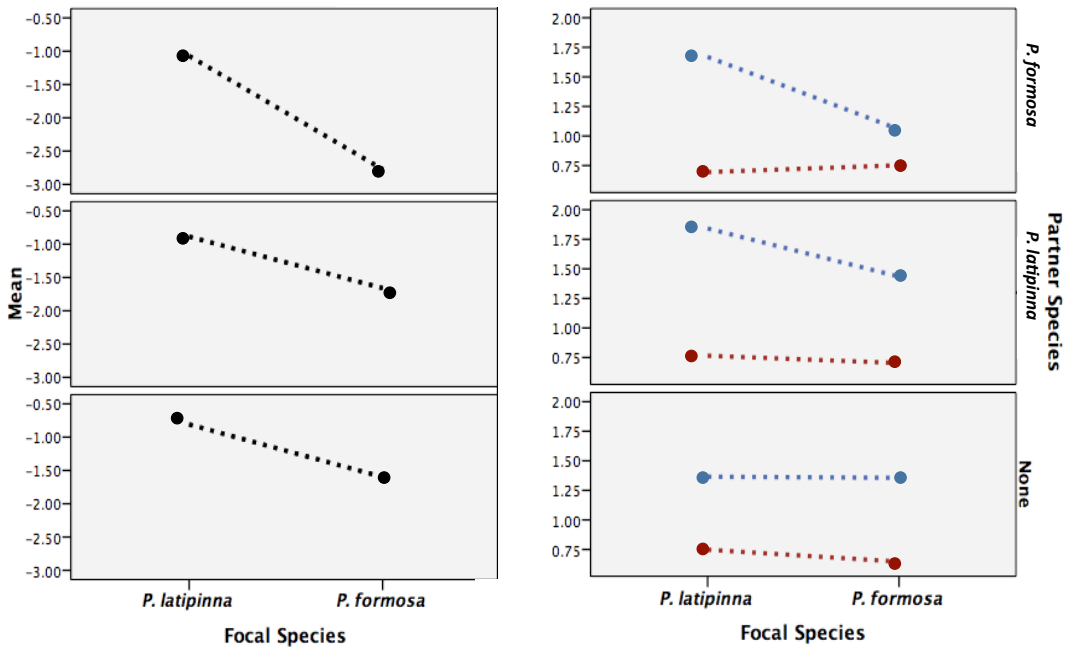
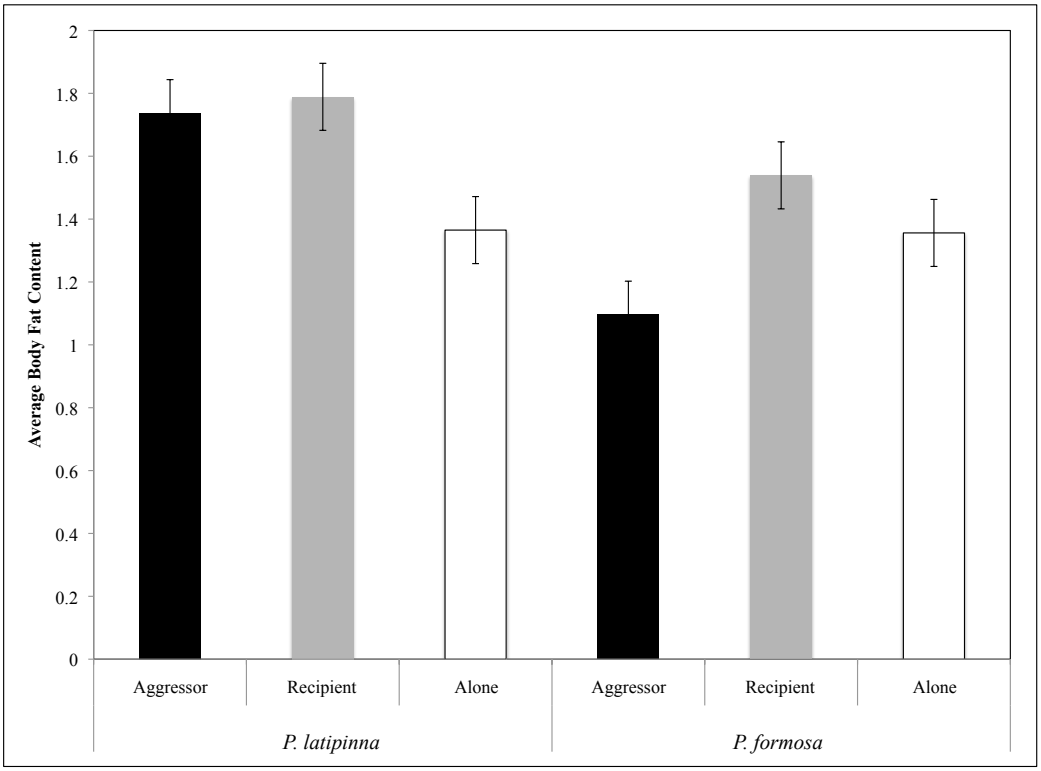

Mean
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
P. latipinna
P. formosa
Focal Species
2.00
1.75
1.50
1.25
1.00
0.75
P. formosa
P. latipinna
None
Partner Species
P. latipinna
P. formosa
Focal Species

Figure 3


Average Body Fat Content
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Aggressor
Recipient
Alone
Aggressor
Recipient
Alone
P. latipinna
P. formosa

CHAPTER 2

Extreme Kin Recognition in a Clonal Fish, *Poecilia formosa*

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ABSTRACT

Relatedness strongly influences social behaviours in a wide variety of species. For most species a full sibling with on average 50% of the genes shared is the highest typical degree of relatedness[1,2]. It is widely accepted that differences in relatedness massively drive evolution and influence numerous behaviours. So far, however, this is poorly understood in species with unusually high relatedness between individuals, such as clonal organisms. And although there has been some investigation into clonal invertebrates and yeast, nothing is known about kin selection in social, clonal vertebrates. Here we show that a clonal fish, the Amazon molly (*Poecilia formosa*), has the ability to distinguish between different clonal lineages. We find that clonal females prefer genetically identical, clonal sisters to slightly different clones. Intriguingly, they do this using multiple sensory modalities. Most importantly, however, they scale their aggressive behaviours according to the relatedness to other females: they are more aggressive to non-related clones. Our results demonstrate that even in species with very small genetic differences between individuals, kin selection can be adaptive. Indeed, even minute differences in relatedness (extremely close kin are favoured over very close kin) can provide enough substrate for the evolution of kin recognition. Their discriminatory abilities and regulation of behaviour provides a powerful example of natural selection in species with limited genetic diversity. In providing the first experimental demonstration of kin selection in a clonal vertebrate, we anticipate future research into a more mechanistic approach using this clonal study system, such as the specific mechanisms of recognition and the effects of epigenetics. Indeed, we believe

this study sets up a wonderful opportunity to study the genomic mechanisms underlying kin recognition.

Keywords: clonal selection, female-female aggression, female competition, kin recognition, kin selection, *Poecilia formosa*, sexual/unisexual mating system

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MAIN TEXT

Kin selection theory predicts that cooperative and altruistic behaviours scale with relatedness[1-4], strongly favouring close relatives. But how large must the difference in relatedness be for kin recognition to occur[3]? To address this, we need to understand just how relatedness shapes social behaviour in species with the highest possible relatedness between individuals: clonal organisms. Like monozygotic twins in humans, clonal organisms are genetically extremely similar, sometimes completely identical. Young human twins are almost impossible to tell apart by the naïve observer, but with some experience there are often subtle differences that allow us to distinguish between individuals[5]. While some clonal invertebrates are capable of detecting and favouring full clonal sisters, others lack the ability to discriminate between their own and other clonal lineages[6-11]. Indeed, it would seem that a major form of evolutionary selection, kin selection, is eliminated because there are no available recognition mechanisms. These findings raise three important questions: how much genetic distance is required for this extreme form of kin recognition to evolve, is selection eliminated because there are no available recognition mechanisms, and what is the adaptive benefit of clonal recognition? We investigated these questions using an ameiotic, clonal fish, the Amazon molly (*Poecilia formosa*), which naturally occurs in mixed groups of different clones[12,13].

The Amazon molly is a natural hybrid species that reproduces via sperm-dependent parthenogenesis, or gynogenesis, and originated in a single hybridization event between *P. latipinna* and *P. mexicana* approx. 100,000 generations ago[12]. The diploid eggs of *P. formosa* are pseudo-fertilized by either male *P. latipinna* in south

Texas or *P. mexicana* in Mexico, and typically the male genome is not incorporated into the offspring, leading to identical daughter clones[14]. Mollies are livebearing and have internal fertilization, and sexual and asexual females compete for the same males[15].

Gynogenesis results in populations of Amazon mollies that are genetically relatively uniform, yet clonal lineages may occasionally diversify by introgression, mutation, or gene conversion[13], and several different clonal lineages are known to coexist within the same population[12,13].

Amazon mollies show great similarities with their sexual hosts in their ecological niche, including feeding behaviour, mating preferences, parasite loads, life history traits, fecundity, and survivorship[16-20]. This competition should favour targeted aggressive behaviours, which in turn should favour species and potentially clonal recognition. In the present study, we test the ability of *P. formosa* to distinguish clonal sisters (i.e., females of the same clone funded by the same mother) from non-sisters, both in the laboratory and the field. We further established the sensory systems used in this recognition, tested if aggressive behaviours scale directly with relatedness, and provide an adaptive explanation for the evolution of clonal recognition.

Given the high genetic similarity with other clonal lineages, competition for resources, low dispersal rates of the clones, and the social environment in which they occur, we hypothesize that Amazon mollies show the ability to detect different clonal lineages and adjust their aggressive behaviours accordingly.

To test this hypothesis, we created six clonal lineages by mating virgin Amazon mollies from populations collected from the entire geographical range of the species to

sailfin molly males (Fig. 5, Table 1). Clonality of each lineage was confirmed using microsatellites (Tables 2, 3, 4). The results show that our clonal lineages exhibit: 1) high degrees of relatedness within each clonal lineage of or close to the value of 1; and 2) there is less relatedness between clonal lineages in comparison (Table 5, 6). We define clonal sisters as those individuals that are genetically identical, based on microsatellites, to the focal females and are descendants of the same founding mother. Non-sister individuals are defined as females that originate from a different, more distant clonal lineage and are not genetically identical to the focal females.

Using standard binary choice tests, we determined if individuals from the six clonal lineages preferred to associate with clonal sisters over non-sisters. We found that five of six clonal lineages exhibited a significant preference for their clonal sisters (Fig. 1), indicating that they distinguish between clonal lineages. To determine whether this result was due to familiarity, we kept sisters from one clone (Comal Spring, TX) in two groups, under the same conditions for over nine months (average life expectancy is 1-3 years, and sexual maturity is reached around 3 months of age) and tested the offspring also using standard choice tests for their ability to recognize clonal sisters. If the recognition mechanism was based on familiarity, females should be unable to recognize unfamiliar clonal sisters. We found that females preferred clonal sisters that were unfamiliar to non-sisters ($t_{(15)}$=3.362, p=0.005), and familiar clonal sisters to unfamiliar clonal sisters ($t_{(14)}$=2.966, p=0.011; Fig. 6). This result indicates that familiarity is not necessary for clonal recognition, but may strengthen the preference. Therefore, we hypothesized that a genetically based recognition mechanism for phenotype matching must be adaptive for Amazon mollies[21]. We were

able to confirm our findings in a field experiment, in which wild Amazon mollies from their site of origin (Weslaco), in natural water, were allowed to choose between wild caught individuals and non-sisters from a known, but distant laboratory lineage ($F_{(1,19)}$=4.926, p=0.039).

For human observers, it is nearly impossible to distinguish between different clonal lineages, yet Amazon mollies show clear preferences for clonal sisters. Given the paucity of genotypic and phenotypic differences that may provide information of which to base their preference, we asked: which sensory information is used to assess clonal identity? We concentrated on visual, chemical, and tactile information, all of which has been shown to be important in livebearing fishes. Using a repeated-measures design, we tested what signal or combination of signals might be used by Amazon mollies to distinguish clonal sisters from non-sisters. All sensory modalities in isolation and in combination were sufficient for clonal recognition, although there was no significant difference among sensory modalities (Mechanism: $F_{(3,15)}$=0.955, p=0.439; Fig. 2). Within each modality, post-hoc t-tests indicate that females showed the strongest preference for clonal sisters when only visual cues were present ($t_{(17)}$=3.608, p=0.002); nonetheless, they still showed a significant preference when chemical cues ($t_{(17)}$=2.694, p=0.015), a combination of chemical/mechanical ($t_{(17)}$=2.135, p=0.048), or visual/chemical cues were presented ($t_{(17)}$=2.722, p=0.014). Female activity, however, was higher when chemical cues were present, and they entered the preference zones that included the clonal sisters more often ($F_{(1,17)}$=8.285, p=0.010). In addition, there was no difference in the strength of clonal recognition in the presence of unimodal and bimodal cues ($F_{(1,70)}$=1.256, p=0.266), suggesting that

discrimination is not improved using more than one sensory channel. This lends support to the conclusion that signals are often redundant, conveying comparable cues[22]. Most importantly, we were able to find the same effect in our field experiment. As in the laboratory, wild caught females preferred clonal sisters when chemical information was available ($t_{(19)}$=3.805, p=0.001), while the preference using visual information was non-existent ($t_{(19)}$=0.310, p=0.760; Fig. 3). This was likely due to naturally high turbidity of the water[21] as Amazon mollies are found in both turbid and clear environments and it is possible that they may rely more on either visual or chemical cues depending on the environment they live in.

Nonetheless, there are various visual (i.e., body shape, pigment cell quantity and expression, etc.) and chemical cues (dietary, MHC genes, maternally inherited micro-biomes, etc.) in which clones may differ. We investigated body shape as a potential visual cue, and found females from clone CS7a were significantly different from the females from clone VI/17 in body shape (Right: $F_{(9,44)}$=9.592, p<0.0001; Left: $F_{(9,44)}$=6.235, p<0.0001). Overall, Amazon females from clone CS7a had deeper bodies, a more terminal mouth, a larger head, and a slightly longer and deeper caudal-peduncle (Fig. 7, 8). Body symmetry, however, did not differ between the clonal lineages CS7a and VI/17 ($F_{(1,52)}$= 2.264, p=0.138). For a potential chemical signal, we evaluated how diets may influence individual preference via chemical only cues. We found that females retain a preference for clonal sisters on a different diet over non-sisters on the same diet ($t_{(33)}$=3.643, p=0.001; Fig. 9) and prefer to spend more time interacting with clonal sister shoals, regardless of the diet they were on, as compared

to non-sister shoals ($t_{(47)}$=2.132, p=0.038; Fig. 10). This suggests that diet alone is not sufficient to alter kin recognition in these fish.

The presence of clonal recognition in an asexual vertebrate is interesting in itself, but a key question is what adaptive benefit Amazon mollies might derive from clonal recognition. Due to intraspecific competition and the extensive niche overlap between Amazons and their sexual hosts, we hypothesized that females may show more aggression towards non-sisters (and heterospecific sexual females) than clonal sisters to acquire access to limited resources, like food and potentially mates[15, 23-24]. We designed an open field experiment measuring the aggressive behaviours of females that were allowed to interact with either clonal sisters or non-sisters. Females behaved more aggressively towards non-sisters ($F_{(6,29)}$=2.490, p=0.046; Fig. 4), as would be predicted if clonal recognition is used in regulation of aggression. We also conducted a forced-choice experiment, which showed similar results ($F_{(1,17)}$=8.981, p=0.002) (Fig. 11).

In sexual species, kin recognition is between closely related and distantly related individuals that are more genetically distinct (i.e., individuals share either 50% or 25% of genes). It is likely to evolve when siblings overlap in time and space, and are able to recognize each other independently of context and familiarity[3]. The same parameters would hold true for asexual species; however, there is a much smaller genetic difference between individuals, which may suggest relaxed selection on other preferences. Alternatively, with female clones being genetically identical to one another, females may be using self-referential phenotype matching, which would perceptually be an easier task than doing so in a sexual species.

With their uncanny ability for clonal recognition, Amazon mollies are one of the most extreme examples corroborating the predictions of kin selection theory, where aggression is regulated in a way that extremely close (i.e., genetically identical) kin are favoured over very close kin. Given the substantial genetic similarity found throughout the whole species[12, 13], this indicates that it is likely beneficial for clones to be able to recognize each other and regulate competition in a way that favours extremely close kin; even minute differences in relatedness provide enough substrate for kin recognition. We believe that the discrimination ability found in Amazons could be a powerful example of natural selection in action.

METHODS

ESTABLISHING SINGLE-CLONE POPULATIONS

A single female each from six different populations across the geographic range of *Poecilia formosa* (Texas: San Marcos County 101, Comal Spring, Weslaco; Mexico: San Ignacio, VI/17, and III/9; Fig. 5) was isolated and kept with a male *P. latipinna* (all originating from Comal Spring, TX) to initiate the founding clonal lineages (Fig. 5, Table 1). Populations were maintained in outdoor tanks (1000L) during the summer and indoor tanks in the winter. Fish were fed tropical fish flakes *ad libitum* in addition to natural food (e.g., algae) growing in the tanks. After several generations (about 4 ± 2 generations), individuals were collected and tissue samples were taken to confirm that the population was a single clonal lineage. We used 12 microsatellites to analyze the genetic divergence between the 7 different populations

(Table 2)[25]. We isolated the DNA from the individual fin clips using DNeasy DNA extraction kits (QIAGEN), amplified the DNA using Polymerase chain reaction (PCR). An ABI 3100 automatic sequencer was used to determine fragment sizes. We then compared loci, H_0, H_E, of each of the different clonal lineages. We also assessed divergence among lineages, by calculating the F_{ST} values among lineages, both locus-wise and across all loci, and by performing exact tests of differentiation using Markov Chain Monte Carlo simulations (Table 2, 3)[26]. Genotypes of females indicate that each female within the clonal lineages is indeed identical to one another. We calculated the genetic identity and relatedness coefficient within and among the clonal lineages (Tables 5 and 6)[27, 28]. We found that although all Amazons are closely related, females clustered together based on clonal lineage. We also wanted to investigate clonal recognitions within a population, and to do this we isolated two different clones from Comal Spring, TX. These clones only differed at two microsatellite loci (GA-V18: 122-144 vs. 122-148; GT-II33: 182-182 (homozygous) vs. 178-182). Together this allows us to address the minimum genetic distance required for clonal recognition to occur within a population and between populations. Note that one clonal lineage, Comal Spring 7a, was genetically indistinguishable from that of San Ignacio with the 12 microsatellites that we tested for (Table 4**)**.

CLONAL RECOGNITION

A standard binary choice test (Fig. 12)[27-31] was used, allowing *P. formosa* to choose between clonal sisters and non-sisters (a clone from another population). Prior to the experiment, focal females were isolated from their clonal sisters for a minimum

of one week. Partner females were placed in clear, perforated Plexiglas cylinders (to allow chemical, visual, and mechanical signals) at each end of the experimental tank (61 cm length x 39 cm width x 30 cm height). The association time (s) females spent in the preference zone with a stimulus female was recorded. To prevent any side bias, focal females were tested twice, with the second trial having the partner females switching sides[28-29,31]. These two trials were added together and the strength of preference (SOP)[32] scores were calculated as:

$$\frac{\text{Total time spent with Stimulus 1}}{\text{(Total time spent Stimulus 1 + Total time spent Stimulus 2)}}$$

These SOP scores were calculated for both the sister and non-sister clone, then √arc(sin) transformed to normalize the data. Paired *t*-tests were used to then compare the SOP scores for sister and non-sister clones in SPSS (ver. 17) (Fig. 1).

MECHANISM OF RECOGNITION

Populations:

The focal females and clonal sisters came from a stock population originally collected from Río Purificacíon in Nuevo Padilla (VI/17), Mexico. The non-sister stimulus females came from a population originating from Comal Springs, Texas. Both stock populations were founded using a single female, and have been genetically confirmed to be identical to one another (see above; Table 3). These populations were also used in the above study and were shown to be able to recognize and prefer identical clonal sisters. Fish were maintained in a 12 light: 12 dark photoperiod, and fed fish flakes (Tetramin) *ad libitum* daily. Focal females were

randomly selected from the stock populations and then isolated from clonal sisters one-week prior to conducting the experiments in a separate 75.7 L tank. Both stimulus female populations were maintained under similar conditions in separate 37.9 L tanks.

Experimental Setup:

To prevent residual chemical signals, prior to each individual experiment, the experimental tanks, Plexiglas cylinders and Plexiglas sideboards were washed with soapy water, and then rinsed thoroughly. A second round of cleaning followed using 3% hydrogen peroxide to thoroughly clean the tanks and Plexiglas from any chemical that may influence the behavior and preference of the focal fish[33]. After tanks were clean, the experimental tank was filled with ionized de-ionized water (700-1000 ppm). White Plexiglas was placed on the bottom and both long sides of the tank to prevent any influence by the presence of the experimenter. Treatment Plexiglas cylinders were randomly assigned and placed in each end on the tank (Fig. 12).

Experimental Procedures:

Experimental treatments consisted of: 1) allowing visual only signals of the stimulus females to be passed to the focal fish using solid, clear Plexiglas cylinders; 2) allowing visual and chemical signals to the focal fish using clear cylinders perforated with small 3mm holes; 3) allowing chemical only signals to the focal female using solid black cylinders perforated with small 3mm holes (corresponding to an area of about 7mm^2 per hole); 4) allowing only chemical and mechanical (lateral

line) signals to be received by the focal female using black cylinders with large 5mm holes (equivalent to about 20mm^2 per hole); 5) a side bias control using clear empty cylinders; and 6) a color control to compare the response effects of one empty black and a clear cylinder. The area perforated was the same for cylinders with small or large holes. These treatments were chosen to explore the three main sensory channels used in these fishes[34] (visual, chemical and mechanical; Note: auditory communication has yet to be detected in this species of fish[35] and therefore was not incorporated in this study) as a possible mechanism of an individual to identify between different clonal lineages[36-37].

Once the tanks were set up, the stimulus fish (size matched females, +/- 3mm) were placed in each cylinder at the end of the experimental tank (Fig. 12). A clear, large-hole (5 mm) Plexiglas cylinder (allowing visual, chemical, and mechanical signals through during acclimation) was placed in the center of the tank, and then the focal female was added and allowed to acclimate for 10 minutes. After acclimation, the experimental trial was run for 10 minutes using the Viewer system (a video tracking system; Bioserve GmbH, Germany, 2005)[38] to track and record the time spent near each stimulus and the number of times the focal fish entered each preference zone. At the conclusion of the trial, each female was placed into an individual tank overnight, which allowed us to identify each focal and stimulus female. Measurements of the water chemistry (Horiba water quality monitor: pH, salinity, temperature, and dissolved oxygen) were taken to confirm that the water quality remained constant (data not shown). The next day followed the same procedures for the following treatment until all 6 treatments were completed. The

order in which the treatments were presented and the side each stimulus female was placed was randomized. During the entire duration of the experiments females were maintained in similar lighting conditions as above, and fed frozen mosquito larvae.

Statistical Analysis:

Strength of preference scores (see above) were calculated using the time spent within the preference zone, and then √arc(sin) transformed to normalize the data. A repeated-measures GLM was run using "treatments" as the within-subject variable. We found the "Preference*Treatment" interaction was non-significant ($F_{(3,68)}$= 0.522, p= 0.669). However, "Preference" alone was highly significant ($F_{(1,68)}$= 31.122, p< 0.0001). Post hoc tests (Tukey HSD) showed no significant differences between the different mechanism treatments. Therefore, we used paired *t*-tests to compare the clonal sister and non-sister SOP scores for post-hoc differences.

To analyze differences between unimodal (e.g. visual only) and bimodal (e.g. visual plus chemical) sensory mechanisms a repeated-measures GLM was used using "mode" as the within subject factor. Again, there was a highly significant difference for the "preference" ($F_{(1,70)}$= 31.879, p< 0.0001), however, no significant difference between unimodal and bimodal signals ($F_{(1,70)}$= 1.256, p= 0.266). Hence, we reported the post-hoc paired *t*-tests to demonstrate the minute differences between the treatments (Fig. 2).

Confirmation of Treatment Efficiency:

Prior to experimentation, we validated the construction of the Plexiglas cylinders and that they were only allowed the specific signals for each type of cylinder to be released. To demonstrate the diffusion of the chemical cues from the inside of the Plexiglas cylinders we ran several trials using food coloring. Using an identical set up as previously mentioned, we placed a fish inside the cylinder and then added 10 drops of red food coloring (Ingredients: water, propylene glycol, FD&C red, and propylparaben; Note: this was not harmful to the fish, IACUC R13-006) and video recorded the amount of time it took to diffuse to the preference zone. We ran this trial for all four types of Plexiglas cylinders to confirm their construction (i.e., to confirm that the solid cylinders was indeed only allowing the visual only signals and did not leak chemical signals; Fig. 13).

CLONAL RECOGNITION IN NATURAL WATER CONDITIONS

We wanted to determine if clonal recognition was an artefact of the laboratory or if it indeed occurs in natural habitats[36]. To do this, we tested a population of Amazon mollies (Weslaco, Texas), which have already displayed clonal recognition in the lab (see above), to determine if they can recognize sister clones under natural water conditions. In addition, we wanted to test if Amazon mollies can use visual only signals to recognize clonal sisters from non-sisters, or if turbidity, which is high in many of the Amazon mollies natural habitats, would make chemical signals more dominant when visual accuracy is limited.

Experimental Procedures:

Non-sister clone population was a population that originated from Mexico (see above). In practice, we collected fishes from a site in Weslaco, Texas, separated them into two aerated containers (one for focal females, the other for clonal sisters; 45.4L), and transported them to a local hotel[41]. They were allowed to settle in over night and were then used in the experiment described below. Water (76L) from the field site that included native chemical and visual "noise" (i.e., pheromones and turbidity), was also brought into the hotel to allow us to test if the recognition signals are still detectable in their natural environment[36]. We used this water in the subsequent tests. We had to dilute the water due to naturally high turbidity (day of collection: 246 NTU; average: 242.3±120.1; maximum: 482.5; minimum: 82.9), therefore, we added 19L of spring water to 38L of native water. Although diluting the water would likely have influenced both chemical and visual signals of the natural water source, our results suggest that even with this diluted water the visual signals were still impacted by the turbidity. Due to naturally high turbidity levels of this environment and the inability of identifying different clones visually, a field study within the natural water source was impossible; therefore, we were required to use this experimental design in the field. Indeed, it would be unethical to introduce the non-sisters from Mexico into an open field design, thus we designed the above protocol to best address this question. Tanks and other equipment used, was identical to those used in the laboratory experiments described above. All fish were kept in aerated 45.4 L containers.

We performed 2 different experiments: 1) testing chemical only signals using black Plexiglas cylinders, perforated with 3 mm holes, and 2) testing visual only

signals with solid clear Plexiglas cylinders. The sister individual was a wild caught female from the fieldsite in Weslaco, Texas, while the non-sister individual was from a population in Mexico (see above). A standard binary choice test (Fig. 12)[29-33] was used. At the conclusion of the first experiment (i.e., test for chemical signals) females were then tested in the second experiment (i.e., test for visual signals), using the same procedure. The SOP scores were calculated and a repeated measures GLM was used to compare the two different experiments, and *t*-tests were used as post-hoc analyses to compare with-in the experiment (Fig. 3).

MECHANISM FOR CLONAL RECOGNITION: DIFFERENCES IN BODY SHAPE

To determine the degree of visually detectable differences between clones, we assessed the degree of morphological divergence between females used in the experiments. The females from the mechanism and aggression experiments (above) were sedated with MS222 after the completion of the final behavioural experiment to take lateral photographs of both their left and right sides using a dissecting microscope (SZXZ-ILLT) and SPOT software. Fourteen standard landmarks (tip of pre-maxillary, most posterior point of skull, anterior and posterior insertion points of dorsal fin, dorsal and ventral insertion points of caudal fin, anterior and posterior insertion points of anal fin, anterior insertion of pelvic fin, isthmus, dorsal and ventral insertion points of pectoral fin, dorsal most part of the operculum, and the centre of eye; Fig. 14) were analysed using geometric morphometrics (tpsDIG2)[42-45]. Digital photo files were converted into an nts file to control for the size variation among the fish (tpsUtil)[46]. A weighted matrix of the shape variables and centroid size was

created using the aligned, size-corrected landmarks (tpsRelw)[47]. The shape variables and centroid size matrixes were then used in the statistical analyses[45].

Centroid size did not significantly influence the shape variables, so it was removed from the model. Also, neither focal females nor their sister clones showed significant differences for either their left (p=0.152) or right (p=0.497) side, hence we combined these and used "clonal lineage" as the independent variable. We used a Principle Component Analysis to reduce the number of shape variables (N=28 for each specimen). All factors with an Eigen value of one or greater were kept[48], resulting in nine factors for both right and left side, which explained 82.315% of the variation in the right side and 79.132% in the left side. A Multivariate GLM was then used to analyse the two different clonal lineages (fixed factors) with the PCA factors for the shape variables as the dependent factors. Clonal lineages were significantly different from each other (Right: $F_{(9,44)}$=9.592, p<0.0001; Left: $F_{(9,44)}$=6.235, p<0.0001; Fig. 7, 8), showing that there are detectable morphological differences between the two populations.

DIET INFLUENCE ON CHEMICAL RECOGNITION

Fish Maintenance:

Fish originated from two of the single clonal lineages above (San Ignacio and Weslaco). Pregnant females were collected from stock tanks and isolated in individual tanks (3.8L) until they had offspring. Adult females were then returned to the stock tanks. Broods were raised together for a total of five weeks in 12/12hour light/dark cycle and fed *ad libitum* brine shrimp and flake food. At 5 weeks of age,

juveniles were raised individually in 3.8Ltanks on either: 1) a high protein (crude protein 52% min.) diet, 2) a low protein (crude protein 37% min.) diet, or 3) a 50/50 mix of the high protein and low protein (crude protein 44.5% min.) diet. Visual communication between the tanks was prevented to avoid visual imprinting from the neighbouring tanks as the juveniles grew. Each tank had weekly 2/3 water changes. The room temperature was maintained at 30°C and the tank temperature averaged 27.8°C during the duration of the experiment. Individuals were raised until 22-34 weeks old prior to the start of the behavioural experiments.

Preference Choice Test:

A standard binary choice test (Fig. 12)[29-33] was used. However, stimulus females were placed into black perforated Plexiglas rectangular cylinders on either end of the experimental tank (18.9L). These black cylinders allowed focal females to make their choice solely based on chemical cues. After the experiment was finished the focal and stimulus females were returned to their appropriate individual tanks. We used five, randomized treatments to assess whether females would retain clonal recognition when females were placed on different diets: 1) a clonal sister on different v. non-sister on same; 2) a clonal sister on different v. non-sister on mix; 3) a clonal sister on mix v. non-sister on same; 4) a clonal non-sister on same v. non-sister on different; and 5) a clonal non-sister on same v. non-sister on mix. Focal females were retested every 24-hours until they complete all five treatments. If a female did not respond within the first 5 minutes of the trial, the trial was terminated, and the female was replaced into her appropriate tank and retested the next day.

Females that were used as stimulus females were not tested as focal females until after one week has past. Females that were focal females were used as stimulus females only after all 5 treatments were complete.

Shoal Preference Function Test:

Shoaling preference was analysed with using a preference function test (Fig. 15). Using a block design, we randomly tested half of the females as focal females and used the other half to compose the stimulus shoals; after one week, the females were switched and the second half of the females were tested as focal female with the first half as was used as stimulus females. We used five randomized treatments, and one treatment was tested every 24 hours: 1) a clonal sister shoal on the same diet, 2) a clonal sister shoal on a different diet, 3) a non-sister clonal shoal on the same diet, 4) a non-sister clonal shoal on a different diet, and 5) a control where the shoal Plexiglas cylinder was present in the test tank but empty. Focal females were placed in a perforated Plexiglas cylinder on the side of the tank opposite the shoal Plexiglas and allowed to acclimate for 5 minutes. Once the focal female's cylinder was removed she was allowed to swim freely for 10 minutes. We recorded the time (s) females spent in both the preference zone (17.8cm) and the interaction zone (included the stimulus Plexiglas cylinder plus one body length from the stimulus females, 10.5cm).

Statistical Analysis:

For the preference test, we calculated the SOP scores for time spent with the stimulus females. These scores were then √arc (sin) transformed to normalize the data. We used a repeated-measures GLM to compare preference scores across the

different treatments, with "treatment" and "stimulus type" being the within-subject factors. We used the age of the fish at the time of testing, the population the females originated from, and whether they had the same mother as covariates. These factors were non-significant and were therefore removed from the model (Age, $F_{(1,20)}$=0.415, p=0.923; Population, $F_{(1,18)}$=0.088, p=0.916; Mother, $F_{(1,20)}$=1.336, p= 0.291).

For the shoal preference function test, we used a repeated-measures GLM with "treatment" and "zone" as within-subject factors, and with "clone type" and "diet" as between-subject factors. We used "block" as a covariate, however, this did not have a significant effect on either the type of stimulus ($F_{(4,16)}$=1.109, p=0.387) or the zone ($F_{(1,19)}$=0.215, p=0.648) and we removed it from the model.

Results

Preference Choice Test:

Although there was no significant effect between treatments ($F_{(2,19)}$=0.184, p=0.833) and no significant interaction between treatment*stimulus ($F_{(4,17)}$=2.255, p=0.106), we did find a significant difference between the types of stimuli ($F_{(1,20)}$=5.573, p=0.029). Post-hoc t-tests indicate that females significantly preferred clonal sisters on a different diet to non-sister clones on the same diet ($t_{(33)}$=3.643, p=0.001; Fig. 9). However, we did not find any significant preference for: 1) clonal sister on a mixed diet to clonal sisters on the same diet ($t_{(34)}$=-0.473, p=0.639); 2) clonal sisters on a different diet to non-sisters on a mixed diet ($t_{(30)}$=-0.259, p=0.797); 3) non-sister clones on the same diet to non-sister clones on a mixed diet ($t_{(33)}$=1.145, p=0.260); and 4) non-sister clones on the same diet to non-sisters on a different diet ($t_{(34)}$=0.765, p=0.450).

Shoal Preference Function Test:

We found that stimulus female had a significant effect ($F_{(4,17)}$=15.567, p<0.0001, Fig. 10), and the time they spent in each zone was significantly different ($F_{(1,20)}$=427.045, p<0.0001). However, there was no interaction between clone type or the diet type a focal female was on with either the stimulus female or the zone (Treatment*Clone, $F_{(4,17)}$=0.521, p=0.721; Treatment*Diet, $F_{(4,17)}$=0.337, p=0.849; Treatment*Clone*Diet, $F_{(4,17)}$=2.395, p=0.091; Zone*Clone, $\underline{F}_{(1,20)}$=0.769, p=0.391; Zone*Diet, $\underline{F}_{(1,20)}$=0.122, p=0.730; Zone*Clone*Diet, $\underline{F}_{(1,20)}$=0.185, p=0.672). Post-hoc t-tests indicate that females preferred to spent more time with clonal sisters on a different diet to non-sister clones on a different diet in both the preference zone ($t_{(23)}$=2.792, p=0.010) and the interaction zone ($t_{(23)}$=2.909, p=0.008; Extended Data Fig. 7). They also tended to spend more time with clonal sisters on a different diet to non-sisters on the same diet in the preference zone ($t_{23)}$=2.027, p=0.054), although there was no significant difference between clonal sisters on different diets to clonal sisters on the same diet for either zone (preference zone, $t_{(23)}$=-0.847, p=0.406; interaction zone, $t_{(23)}$=-0.725, p=0.476). Finally, females preferred to spend more time in the interaction zone when the stimulus female was a clonal sister, irrespectively of diet type, when compared to non-sisters ($t_{(47)}$=2.132, p=0.038; preference zone, $t_{(47)}$=1.761, p=0.085) and this effect was not seen when evaluating based on diet alone for either zone (preference zone, $t_{(47)}$=-0.258, p=0.797; interaction zone, $t_{(47)}$=0.073, p=0.942).

KIN SELECTION AS A MEANS TO REGULATE AGGRESSION

Forced-Choice Experiment:

Using the same females as the above mentioned experiment investigating mechanisms, focal females were tested for their aggressive behaviors towards clonal sisters and non-sisters[40] using two experimental designs: 1) a forced-choice (i.e., one stimulus female at a time)[41], and 2) free-swimming with choice (i.e., both stimulus females at the same time)[42]. Fish were given one week of rest. Since these fish appear identical to the human eye, focal females had half of the dorsal fin clipped for identification. Both clonal sister and non-sister females underwent the same handling procedures as the focal female, although their fin was not clipped. All females were allowed to rest from handling for three days, prior to any trials.

Aggression was measured in a direct-contact (stimulus and focal female able the directly interact with one another) experimental tank (19L) with either a clonal sister or non-sister (Fig. 16**)**. At the start of the experiments, both focal female and stimulus female were placed in separate, clear Plexiglas cylinders. After a 5-minute acclimation period females were released from the cylinders and behavioral measurements (bites, tail beats, and overall time spent being aggressive) were started at the first sign of aggression and ran for 10 minutes. We measured all three behaviors, both given to the stimulus females and received from the stimulus females. If there was no aggressive interaction after 10 minutes, the trial was terminated and the female was retested after 24 hours. At the end of the trial both females were placed back into their individual tanks. Focal females were retested 24 hours later with the other partner, either the clonal sister or non-sister that was not tested the day before, following the same procedure.

Statistical Analysis:

A repeated-measures GLM was employed using "Clone" and "Behavior" as the within subject factors. Behaviors were significantly different from one another ($F_{(2,16)}$= 5.134, p= 0.019), suggesting that females receive particular behaviors more frequently from the stimulus females than others (i.e., more bites than tail beats). Clone type also significantly influenced the amount of aggression received from other females ($F_{(1,17)}$= 8.981, p= 0.008). Moreover, there was a positive interaction between behavior and clone type ($F_{(1,16)}$= 5.337, p= 0.017), which demonstrates that females received more aggression via bites but not tail beats from non-sister individuals when compared to clonal sisters. Although the amount of aggression focal females performed towards the other females was positively influenced by clone type ($F_{(1,17)}$= 7.412, p= 0.014; Fig. 11), the actual behaviors did not significantly differ from one another, nor was there and interaction between behavior and clone type (Behavior: $F_{(2,16)}$= 2.373, p= 0.125; Behavior x Clone interaction: $F_{(2,16)}$= 2.373, p= 0.125).

Free-Swimming Experiment:

After one week of rest following the forced-choice aggression experiment, either the clonal sister and non-sister female had the caudal fin clipped for identification (focal females were still identifiable via the dorsal fin clip), the third female and focal females underwent the same handling procedures, although their fin was not clipped. All females were allowed to rest from handling for three days, prior to the open field, free-swimming aggression trials. Aggression was measured in a

direct-contact experimental tank (19L) with all three females together to give the focal female a choice between the two different stimuli (Fig. 17). At the start of the experiments, both focal female and stimulus females were placed in separate clear, Plexiglas cylinders. After a 5-minute acclimation period females were released from the cylinders and behavioral measurements (bites, tail beats, and overall time spent behaving aggressively; we again measured all three behaviors, both given to the stimulus females and received from the stimulus females) were started at the first sign of aggression and ran for 10 minutes. If there were no aggressive interactions among the three females after 10 minutes, the trial was terminated and the focal female was retested in 24 hours. At the end of the trial all females were placed back into their individual tanks. After the completion of the experiment, females were allowed to recover and regenerate their fins.

Statistical Analysis

A multivariate GLM was run using "clone" as the fixed factor and the behaviors (bites given, tail beats given, time given, bites received, tail beats received, and time received) as the dependent variables. The results show that the clone type did have a significant effect on how aggressive females would behave ($F_{(6,29)}$= 2.490, p= 0.046). Indeed, clone type significantly influenced the total time females spent performing aggressive behaviors ($F_{(1)}$= 5.866, p= 0.021), the number of aggressive bites a female received from a stimulus female ($F_{(1)}$= 6.668, p= 0.014), and the total time females received aggressive behaviors from the stimulus females was marginally non-significant ($F_{(1)}$= 4.001, p= 0.054; Fig. 4). However, clone type did not influence

the number of tail beats given or received and the number of bites given ($F_{(1)}$= 2.125, p= 0.154; $F_{(1)}$= 2.125, p= 0.154; $F_{(1)}$= 2.305, p= 0.138, respectively).

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Table 1.

Tested Population	Location	Drainage Basin	Coordinates	
			North	West
San Marcos (C101)	Texas, USA	Guadalupe River	29°51'25.83	97°53'47.96
Comal Spring	Texas, USA	Guadalupe River	29°42'46.82	98°8'8.25
Weslaco	Texas, USA	Río Grande	26°7'14.52	97°57'41.44
Río Purificacíon, Barretal (III/9)	Mexico	Río Pánuco	24°4'42.85	99°7'21.76
Río Purificacíon, Nuevo Padilla (VI/17)	Mexico	Río Pánuco	24°02'35.59	98°54'15.98
San Ignacio	Mexico	Río San Fernando	24°51'53.2	99°20'02.7

Table 1. Population origins. This table shows the test population origins across the range of *P. formosa*, indicating the location, drainage basin and the coordinates of the original population collection site). There were 2 populations from the northern range (San Marcos (C101) and Comal Spring), 2 populations from the midpoint (Weslaco and San Ignacio), and 2 populations from the southern range (Río Purificacíon, Barretal (III/9) and Río Purificacíon, Nuevo Padilla (VI/17).

Table 2.

Locus	Characteristics in *Poecilia formosa*							
	Population	***n***	**Allele No.**	**Allele size**	H_O	H_E	***P***	F_{ST}
GA-II41 **Genbank#** AJ810469	Co101	5	2	118 - 126	0.800	0.533	0.429	0.083
	3VI/17	11	2	118 - 126	1.000	0.524	0.003	
	4III/9	12	2	118 - 126	1.000	0.522	0.002	
	W5-Weslaco	7	2	118 - 126	1.000	0.538	0.037	
	6SI-SanIgnacio	5	2	118 - 126	1.000	0.556	0.126	
	7aCS-7aComalSpring	11	2	118 - 126	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	118 - 126	1.000	0.524	0.003	
GA-I47A **Genbank#** AJ810468	Co101	4	2	133 - 155	1.000	0.571	0.314	0.240
	3VI/17	11	2	133 - 155	1.000	0.524	0.003	
	4III/9	12	2	133 - 155	1.000	0.522	0.002	
	W5-Weslaco	7	2	133 - 159	1.000	0.538	0.037	
	6SI-SanIgnacio	5	2	133 - 155	1.000	0.556	0.128	
	7aCS-7aComalSpring	11	2	133 - 155	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	133 - 155	1.000	0.524	0.003	
GT-II33 **Genbank#** AJ810474	Co101	5	2	178 - 182	0.800	0.533	0.429	0.397
	3VI/17	11	1	182	0.000	0.000	-	
	4III/9	12	1	182	0.000	0.000	-	
	W5-Weslaco	7	1	182	0.000	0.000	-	
	6SI-SanIgnacio	5	1	182	0.000	0.000	-	
	7aCS-7aComalSpring	11	1	182	0.000	0.000	-	
	8bCS-8bComalSpring	11	2	178 - 182	1.000	0.524	0.003	
GA-V18 **Genbank#** AJ810470	Co101	5	3	122 - 148	1.000	0.644	0.176	0.221
	3VI/17	11	2	122 - 148	1.000	0.524	0.003	
	4III/9	12	2	122 - 148	1.000	0.522	0.002	
	W5-Weslaco	7	2	122 - 148	1.000	0.538	0.037	
	6SI-SanIgnacio	5	2	122 - 144	1.000	0.556	0.127	
	7aCS-7aComalSpring	11	2	122 - 144	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	3	122 - 148	1.000	0.602	0.006	
GA-I26 **Genbank#** AJ810456	Co101	4	2	160 - 194	1.000	0.571	0.314	0.201
	3VI/17	11	2	160 - 194	1.000	0.524	0.003	
	4III/9	12	2	160 - 194	1.000	0.522	0.002	

	W5-Weslaco	7	2	160 - 194	1.000	0.538	0.038	
	6SI-SanIgnacio	5	2	160 - 194	1.000	0.556	0.126	
	7aCS-7aComalSpring	11	2	160 - 194	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	160 - 194	1.000	0.524	0.003	
GA-III28 **Genbank#** AJ810459	Co101	4	2	215 - 241	1.000	0.571	0.315	0.303
	3VI/17	11	2	215 - 241	1.000	0.524	0.003	
	4III/9	12	2	215 - 241	1.000	0.522	0.002	
	W5-Weslaco	7	2	237 - 241	1.000	0.538	0.038	
	6SI-SanIgnacio	5	2	215 - 241	1.000	0.556	0.126	
	7aCS-7aComalSpring	11	2	215 - 241	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	215 - 241	1.000	0.524	0.003	
GA-III29B **Genbank#** AJ810460	Co101	5	2	255 - 257	0.000	0.356	0.111	0.889
	3VI/17	11	2	255 - 265	0.000	0.173	0.047	
	4III/9	12	1	255	0.083	0.083	1.000	
	W5-Weslaco	7	1	261	0.000	0.000	-	
	6SI-SanIgnacio	5	1	257	0.000	0.000	-	
	7aCS-7aComalSpring	11	1	257	0.000	0.000	-	
	8bCS-8bComalSpring	11	1	257	0.000	0.000	-	
GT-I41 **Genbank#** AJ810472	Co101	5	1	148	0.000	0.000	-	-
	3VI/17	11	1	148	0.000	0.000	-	
	4III/9	12	1	148	0.000	0.000	-	
	W5-Weslaco	7	1	148	0.000	0.000	-	
	6SI-SanIgnacio	5	1	148	0.000	0.000	-	
	7aCS-7aComalSpring	11	1	148	0.000	0.000	-	
	8bCS-8bComalSpring	11	1	148	0.000	0.000	-	
GA-IV42 **Genbank#** AJ810462	Co101	5	3	198 - 204	1.000	0.644	0.175	0.316
	3VI/17	11	2	198 - 202	1.000	0.524	0.003	
	4III/9	12	2	198 - 202	1.000	0.522	0.002	
	W5-Weslaco	7	2	198 - 202	1.000	0.538	0.038	
	6SI-SanIgnacio	5	2	198 - 204	1.000	0.556	0.127	
	7aCS-7aComalSpring	11	2	198 - 204	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	198 - 204	1.000	0.524	0.003	
GA-I29B **Genbank#** AJ810458	Co101	4	2	229 - 255	1.000	0.571	0.314	0.228
	3VI/17	11	2	229 - 255	1.000	0.524	0.003	
	4III/9	12	2	229 - 255	1.000	0.522	0.002	

	W5-Weslaco	7	2	229 - 257	1.000	0.538	0.037	
	6SI-SanIgnacio	5	2	229 - 255	1.000	0.556	0.127	
	7aCS-7aComalSpring	11	2	229 - 255	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	229 - 255	1.000	0.524	0.003	
GT-II49 **Genbank#** AJ810466	Co101	5	2	342 - 382	1.000	0.556	0.128	0.219
	3VI/17	11	2	342 - 382	1.000	0.524	0.003	
	4III/9	12	2	342 - 382	1.000	0.522	0.002	
	W5-Weslaco	7	2	342 - 382	1.000	0.538	0.037	
	6SI-SanIgnacio	5	2	342 - 382	1.000	0.556	0.126	
	7aCS-7aComalSpring	11	2	342 - 382	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	342 - 382	1.000	0.524	0.003	
GA-III49A NEU **Genbank#** AJ810461	Co101	5	3	394 - 412	1.000	0.644	0.174	0.181
	3VI/17	11	2	394 - 408	1.000	0.524	0.003	
	4III/9	12	2	394 - 408	0.917	0.518	0.014	
	W5-Weslaco	7	2	394 - 414	1.000	0.538	0.037	
	6SI-SanIgnacio	5	2	394 - 408	1.000	0.556	0.127	
	7aCS-7aComalSpring	11	2	394 - 408	1.000	0.524	0.003	
	8bCS-8bComalSpring	11	2	394 - 408	1.000	0.524	0.003	

Table 2. Characteristics of the 12 microsatellites. Here, summary statistics of the 12 microsatellites (plus Genbank acquisition numbers) that were used in differentiating the 7 different clonal lineages of *P. formosa* are shown, i.e., population, sample size, the number of alleles, the observed heterozygosity of the current generation (H_0), the expected heterozygosity (H_E), the probability of Hardy-Weinberg-Equilibrium (HWE),i.e., $H_0 = H_E$ (P), and the F_{ST} value of all populations at that particular locus. Note that loci are generally expected not to be in HWE in Amazon mollies, due to the lack of sexual recombination.

Table 3.

	Co101	3VI/17	4III/9	W5-Weslaco	6SI-SanIgnacio	7aCS-7aComalSpring	8bCS-8bComalSpring
Co101	-	0.148**	0.157**	0.248**	0.000	0.000	0.000
3VI/17	0.00040**	-	0.000	0.251***	0.210**	0.232***	0.244***
4III/9	0.00060**	1.00000	-	0.258***	0.221**	0.241***	0.253***
W5-Weslaco	0.00085**	<0.00001***	<0.00001***	-	0.298**	0.318***	0.320***
6SI-SanIgnacio	0.00970*	<0.00001***	<0.00001***	<0.00001***	-	0.000	0.000
7aCS-7aComalSpring	0.00045**	<0.00001***	<0.00001***	<0.00001***	1.00000	-	0.006***
8bCS-8bComalSpring	0.39495	<0.00001***	<0.00001***	<0.00001***	0.00035**	<0.00001***	-

Table 3. Genetic divergence. Genetic divergence among the 7 clonal lineages of *P. formosa*. Above the diagonal are the F_{ST} values and below the diagonal are the *P*-values from the Markov Chain Monte Carlo exact test. Statistical significance after sequential Bonferroni correction for multiple pairwise comparisons is indicated at an experiment-wise error rate α (*α=0.05; **α=0.01; *** α=0.001).

Table 4.

	C101	VI/17	III/9	Weslaco	San Ignacio	Comal Spring 7a	Comal Spring 8b
C101	-	654.1	653.9	415.5	573	28.1	28.1
VI/17	6	-	22.51	249.8	100.4	635	635
III/9	6	1	-	255.4	90	634.1	634.1
Weslaco	8	6	6	-	196.2	399.8	399.8
San Ignacio	4	4	4	6	-	552.8	552.8
Comal Spring 7a	4	4	4	6	0	-	0
Comal Spring 8b	3	5	5	7	2	2	-

Table 4. Loci difference and geographic range. This table shows the geographical distance (km) between the 7 different clonal lineages above the diagonal. Below the diagonal are the number of loci that are different in their allelic pattern between the different clonal lineages. Interestingly, the San Ignacio clonal lineages is located 552.8 km south of Comal Spring, yet the Comal Spring 7a lineage is identical to the San Ignacio lineage (for the 12 microsatellites that we tested them for) and not to a sympatric clonal lineage Comal Spring 8b.

Table 5.

	C101	VI/17	III/9	Weslaco	San Ignacio	Comal Spring 7a	Comal Spring 8b
C101	0.825	0.772	0.772	0.650	0.892	0.892	0.906
VI/17		0.992	0.989	0.750	0.791	0.791	0.750
III/9			0.997	0.708	0.788	0.788	0.747
Weslaco				1.000	0.667	0.667	0.625
San Ignacio					1.000	1.000	0.951
Comal Spring 7a						1.000	0.951
Comal Spring 8b							0.986

Table 5. Genetic Identity. The genetic identity[28] within each clonal lineage is higher (i.e., more closely related among each other; 1.000= 100% genetically identical within clonal lineage) than between clonal lineages, with exception of C101. The lack of higher genetic identity with the C101 clonal lineage may be reflected in the lack of behavioural evidence for kin recognition in this clonal lineage.

Table 6.

	C101	VI/17	III/9	Weslaco	San Ignacio	Comal Spring 7a	Comal Spring 8b
C101	0.115	-0.153	-0.153	-0.77	0.454	0.454	0.525
VI/17		0.960	0.944	-0.264	-0.057	-0.057	-0.264
III/9			0.985	-0.477	-0.072	-0.072	-0.279
Weslaco				1.000	-0.684	-0.684	-0.896
San Ignacio					1.000	1.000	0.752
Comal Spring 7a						1.000	0.752
Comal Spring 8b							0.929

Table 6. Relatedness Coefficient. The coefficient of relatedness[27] within each clonal lineage is higher than between clonal lineages, with exception of C101. R=1: identical twins/clones; R=0.5: clonal populations as related to each other as full siblings would be in an outcrossing, sexual species; and R<0: less identity than at random (i.e., individuals are as dissimilar to each other as unrelated individuals would be in outcrossing, sexual species with the lower numbers indicating the more unlikely related the lineages are). Note: the underlying logic of R is assuming sexual reproduction of diploid organisms, and therefore, these values are only considered an approximation in clonal organisms.

Figure 1.

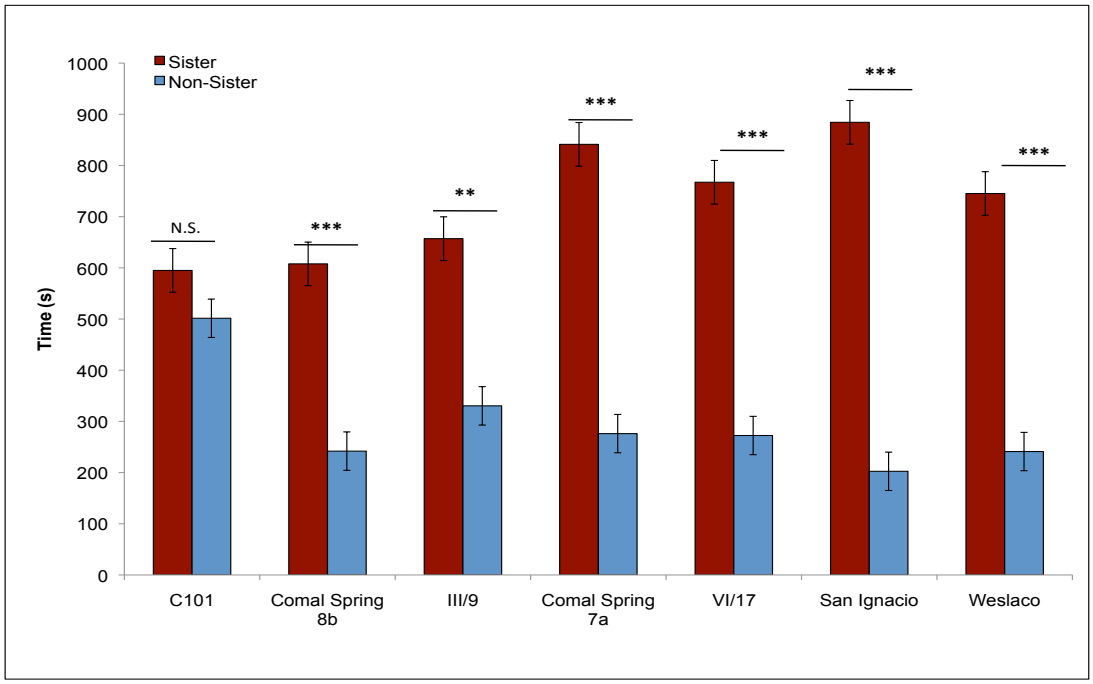

Figure 1. Female Preferences. The average time ± SE female preferences for clonal sisters (red) and non-sisters (blue) in six different populations across the range of *P. formosa.* County 101, San Marcos, TX, $t_{(25)}$=1.567, ***p*=0.13**; Comal Spring, TX (8b), $t_{(18)}$=5.989, ***p*<0.0001**; III/9 Río Purificación, Barretal, MX, $t_{(16)}$=3.484, ***p*=0.003**; Comal Spring, TX (7a), $t_{(15)}$=5.21, ***p*<0.0001**; VI/17 Río Purificación, Nuevo Padilla, MX, $t_{(15)}$=7.258, ***p*<0.0001**; Baños de San Ignacio, MX, $t_{(5)}$=13.279, ***p*<0.0001** and Weslaco, TX, $t_{(23)}$=6.291, ***p*<0.0001**. Females from 5 of the 6 populations showed a significant preference for clonal sisters over non-sisters when visual, chemical and mechanical information was present. For unknown reasons, C101 clonal lineage had relatively low relatedness, likely leading to a lack of kin recognition in this population.

Figure 2.

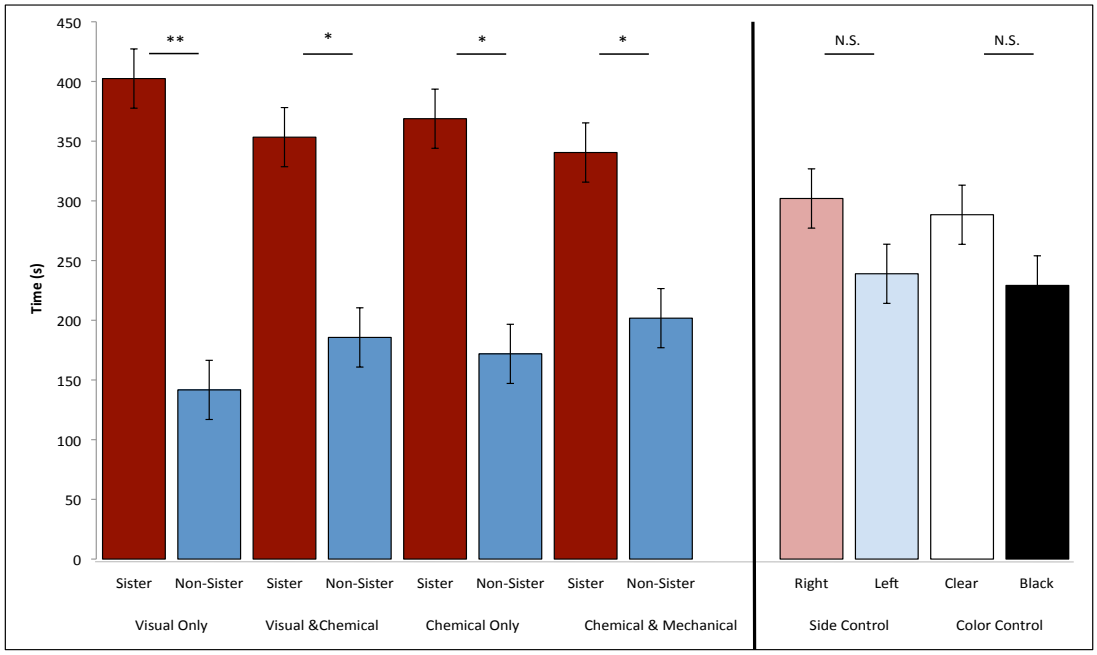

Figure 2. Mechanism Experiment. The average time ± SE females spent with a clonal sister (red) and a non-sister (blue) in the four different treatments (visual signals only, $t_{(17)}$=3.608**, *p*=0.002**; visual-chemical signals, $t_{(17)}$=2.722, ***p*=0.014**; chemical signals only, $t_{(17)}$=2.694, ***p*=0.015**; chemical-mechanical signals, $t_{(17)}$=2.135, ***p*=0.048**). Females showed a stronger preference to only visual signals. The two control treatments demonstrate that there was no bias for the right (light red) or left (light blue) sides of the experimental tank ($t_{(17)}$=0.551, p=0.588) and there was no bias for the clear (white) cylinder or the black (black) cylinder ($t_{(17)}$=0.699, ***p*=0.494**).

Figure 3.

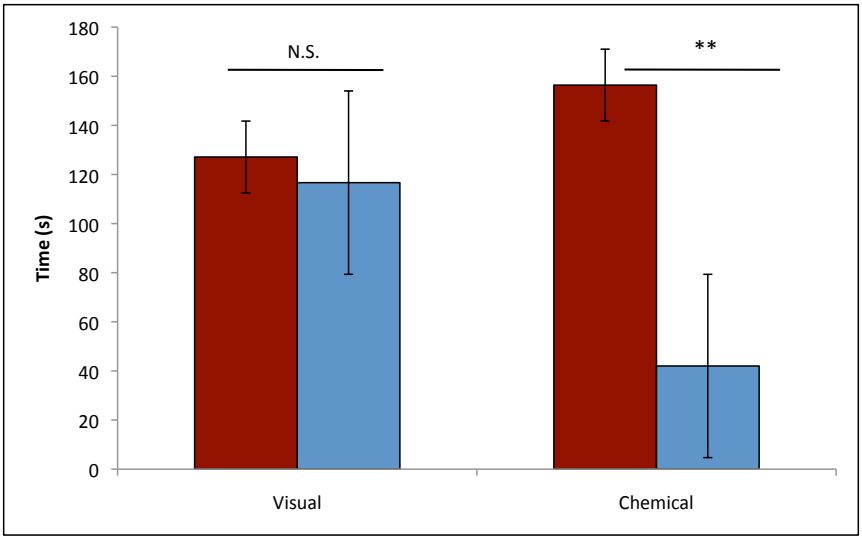

Figure 3. Field study of clonal recognition: Field study of clonal recognition. We tested clonal recognition for both visual signals only and chemical signals only in a naturally turbid stream located in Weslaco, TX. We found that females loose the ability to discriminate between clonal sisters (red) and non-sisters (blue) when only visual signals are available, most likely due to the naturally high turbidity ($t_{(19)}$= 0.310, p= 0.760). On the other hand, females were able to recognize clonal sisters compared to non-sisters when only chemical signals were present ($t_{(19)}$= 3.805, ***p*= 0.001**).

Figure 4.

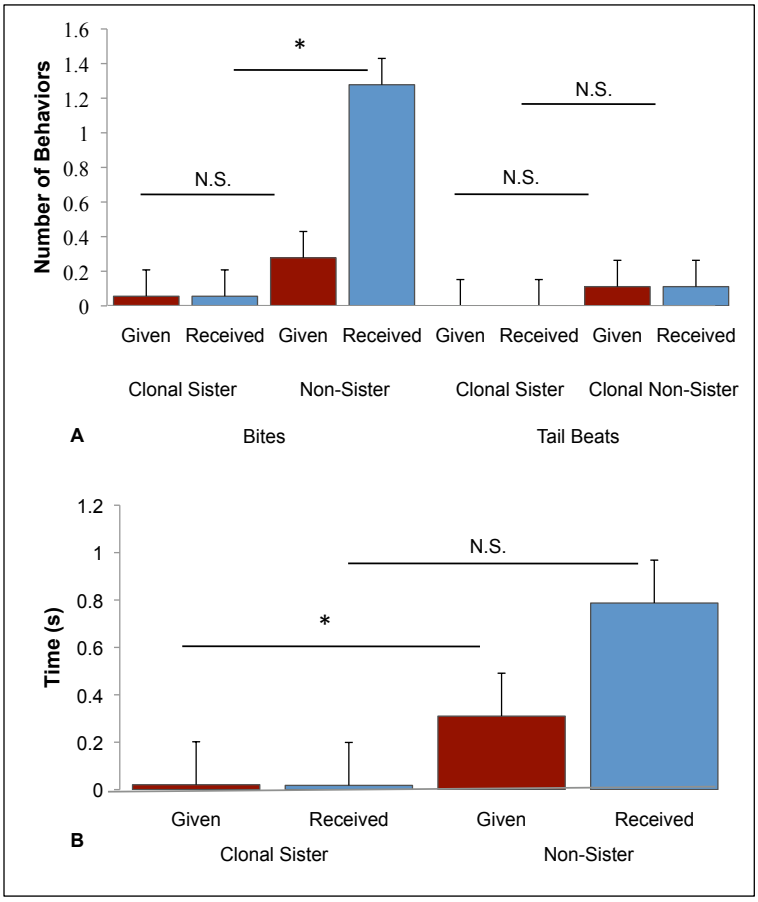

Figure 4. Aggression Experiment. This experiment tested the aggression levels of females when given a choice between a clonal sister and non-sister (average ± SE). Females received (blue) significantly more bites (A. given: $F_{(1)}$=2.305, p=0.138; received: $F_{(1)}$=6.668, ***p*=0.014**) and spent significantly more time performing (given= red) aggressive behaviors (B. given: $F_{(1)}$=5.866, ***p*=0.021**; received: $F_{(1)}$=4.001, p=0.054) towards non-sisters when compared to clonal sisters. There was no significant difference in performing tail beats (A. given: $F_{(1)}$=2.125, p=0.154; received: $F_{(1)}$=2.125, ***p*=0.154).**

Figure 5.

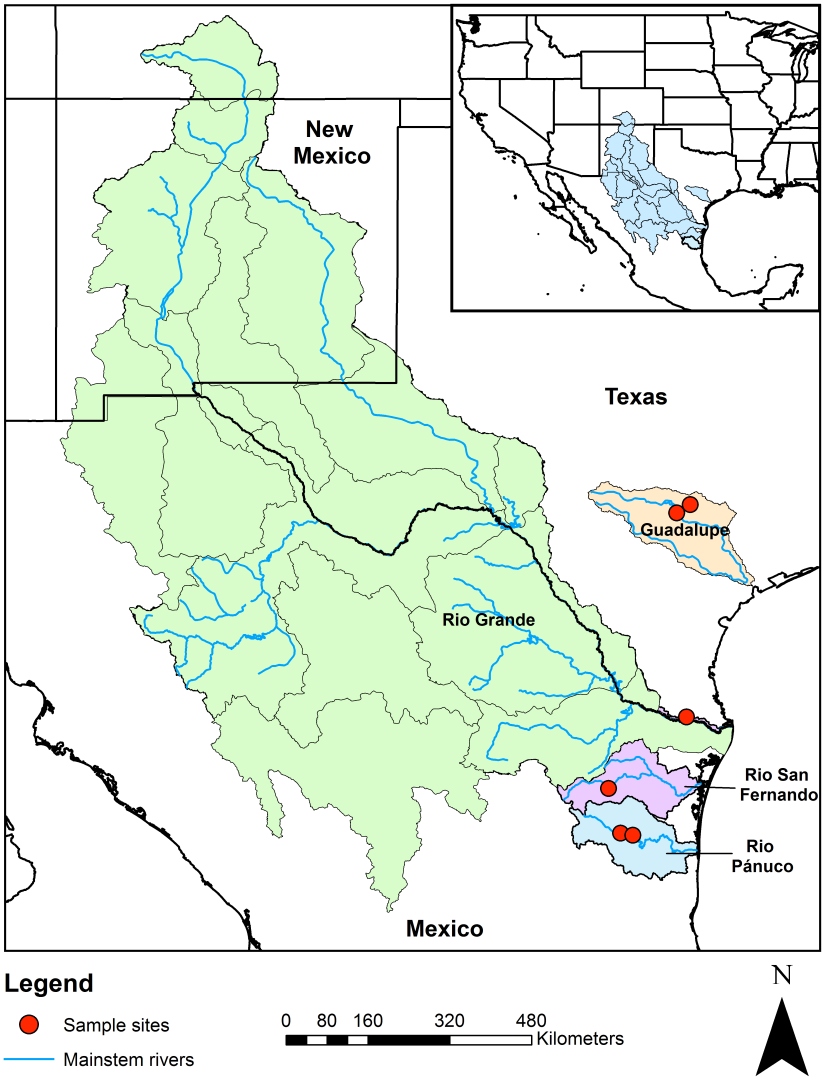

Figure 5. Map of Population Localities. Six populations (red dots) of *P. formosa* were used in the female preference study: San Marcos (County 101), Comal Spring, Weslaco, San Ignacio, VI/17, and III/9. These populations are part of four different river drainage basins across Texas and Mexico: Guadalupe (orange; San Marcos (County 101) and Comal Spring), Río Grande (green; Weslaco), Río San Fernando (purple; San Ignacio), and Río Pánuco (blue; VI/17 and III/9).

Figure 6.

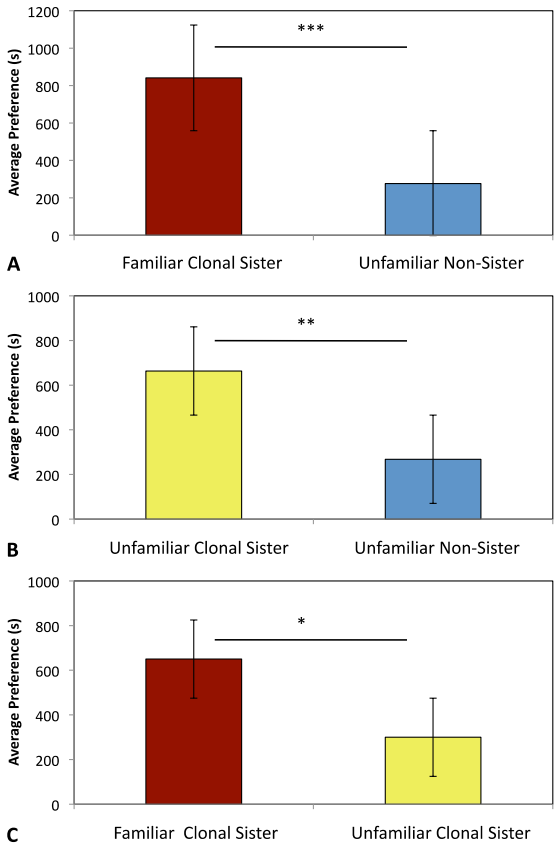

Figure 6. Preferences for Familiarity. The average ($\bar{x}$ ± SE) for female preferences of clonal sisters and non-sisters was not due to familiarity. In each experiment, 15 females were given a choice between a familiar clonal sister (red), an unfamiliar clonal sister (yellow), or an unfamiliar non-sister (blue) after a 10-month isolation period from clonal sisters. **A.** Females from Comal Spring, TX, familiar clonal sisters and unfamiliar non-sisters, $t_{(15)}$=5.213, p<**0.0001**; **B.** Unfamiliar clonal sisters and unfamiliar non-sisters, $t_{(15)}$=3.362, p=**0.005**; **C.** Familiar clonal sisters and unfamiliar clonal sisters, $t_{(14)}$=2.966, p=**0.011**. Females maintain the preference for clonal sisters (regardless of familiarity) and prefer familiar clonal sisters to unfamiliar clonal sisters.

Figure 7.

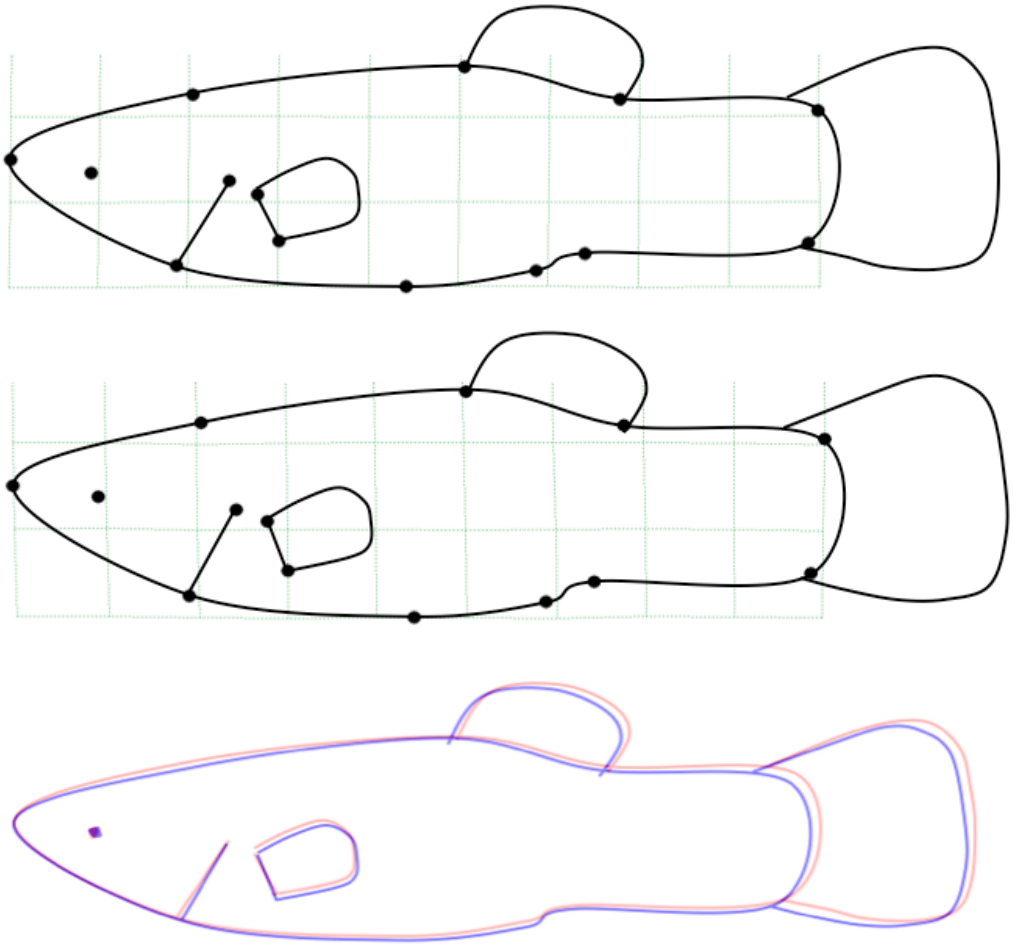

Figure 7. Morphology Between Clones (1X). Geometric morphometric results at normal resolution (1X) of both the Amazon mollies from the Mexico population (top, focal and sister clones, red), and the Texas population (middle, non-sister clones, blue). The bottom demonstrates the differences in morphology between the two populations by overlaying of both body shapes to show the minute differences in morphology. Overall, morphology was significantly different between Amazons from the Texas and Mexico population (Right: $F_{44}= 9.592$, $p< 0.0001$; Left: $F_{44}= 6.235$, $p< 0.0001$). Females from Texas had deeper bodies, a more terminal mouth, a larger head, and a slightly longer and deeper caudal-peduncle.

Figure 8.

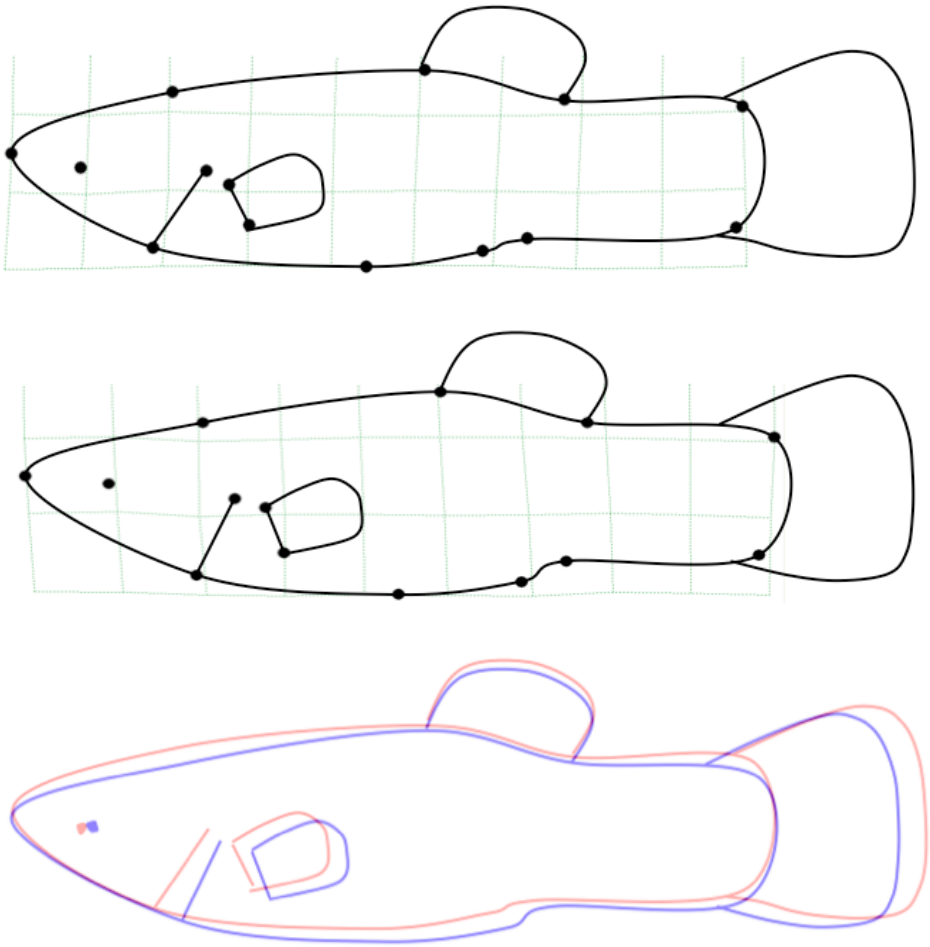

Figure 8. Morphology Between Clones (3X). Geometric morphometric results at three-times the resolution (3X) of both the Amazon mollies from the Mexico population (top, focal and sister clones, red), and the Texas population (middle, non-sister clones, blue). The bottom demonstrates the exaggerated differences in morphology between the two populations by overlaying of both body shapes to show the minute differences in morphology.

Figure 9.

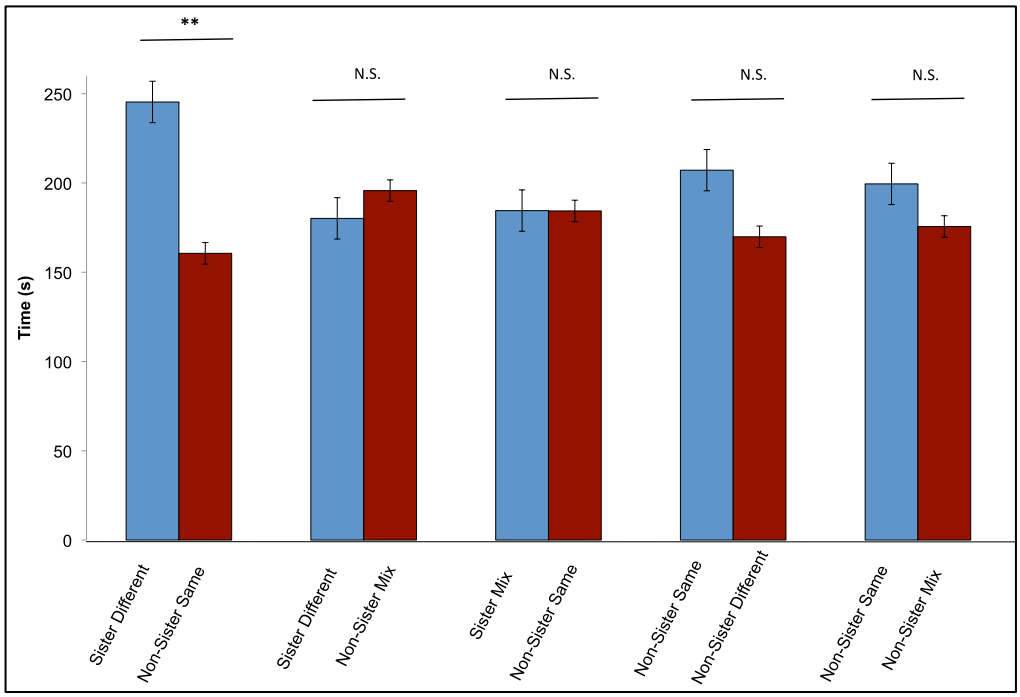

Figure 9. Diet Influence on Chemical Recognition of Clonal Sisters. Juvenile kin preference for clonal sisters and non-sisters when raised on the same diet, a mixed diets or a different diet. Female juvenile Amazon mollies spent significantly more time with clonal sisters on a different diet to non-sisters on the same diet ($t_{(33)}$=3.643, ***p*=0.001**). There was no significant differences between clonal sisters on a different diet and non-sisters on a mixed diet ($t_{(34)}$=-0.473, p=0.639), clonal sisters on a mixed diet and non-sisters on the same diet ($t_{(30)}$=-0.259, p=0.797), non-sisters on the same diet and non-sisters on a different diet ($t_{(33)}$=1.145, p=0.260), or non-sisters on the same diet and non-sisters on a mixed diet ($t_{(34)}$=0.765, p=0.450).

Figure 10.

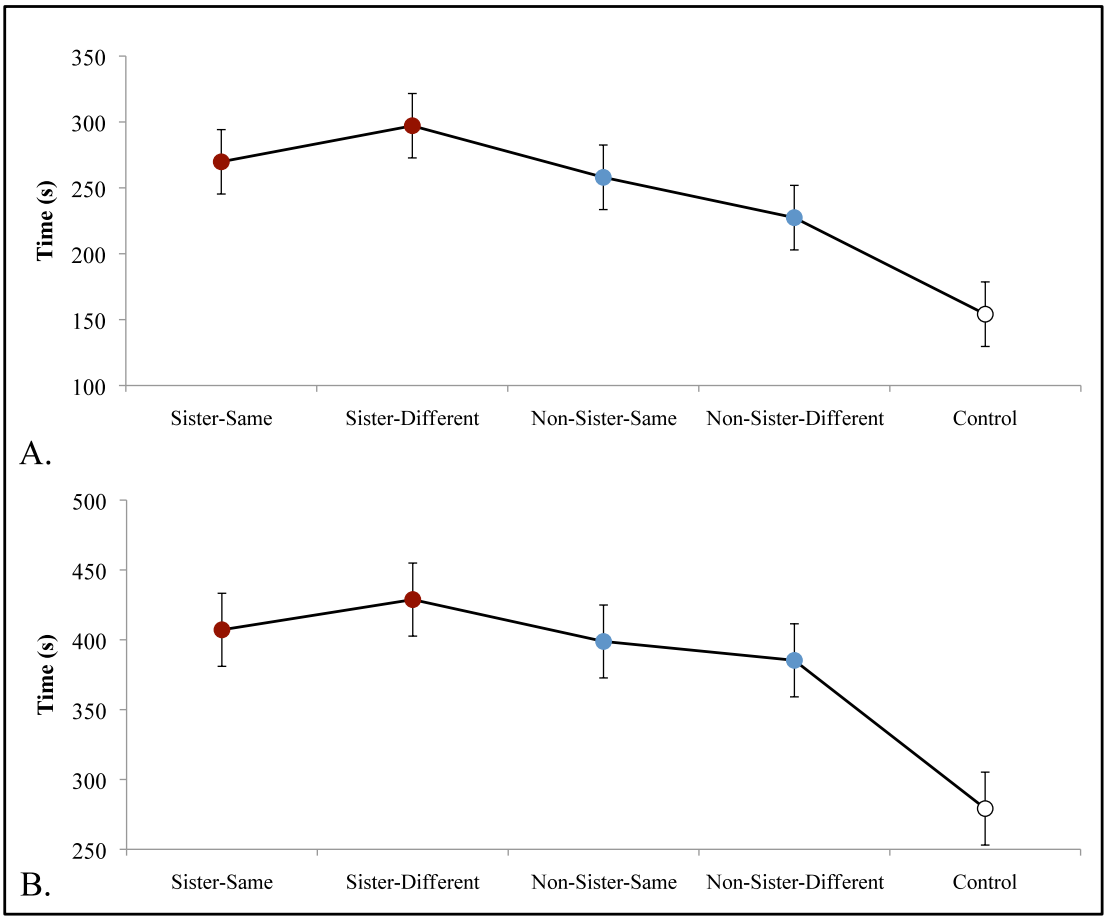

Figure 10. Shoaling Preference Experiment. Average time (s) ± SE females spent near the stimulus females in (A.) the interacting zone and (B.) the preference zone. Females spent significantly more time with the clonal sisters (red) on a different diet in both zones (preference zone: $t_{(23)}$=2.792, ***p*=0.010**; interaction zone: $t_{(23)}$=2.909, ***p*=0.008**) when compared to non-sisters (blue) on a different diet. They also tended to spend more time with clonal sisters on a different diet to non-sisters on the same diet (preference zone: $t_{(23)}$=2.027, p=0.054), and with clonal sisters in general, regardless of diet, that with non-sisters (interaction zone: $t_{(47)}$=2.132, ***p*=0.038**).

Figure 11.

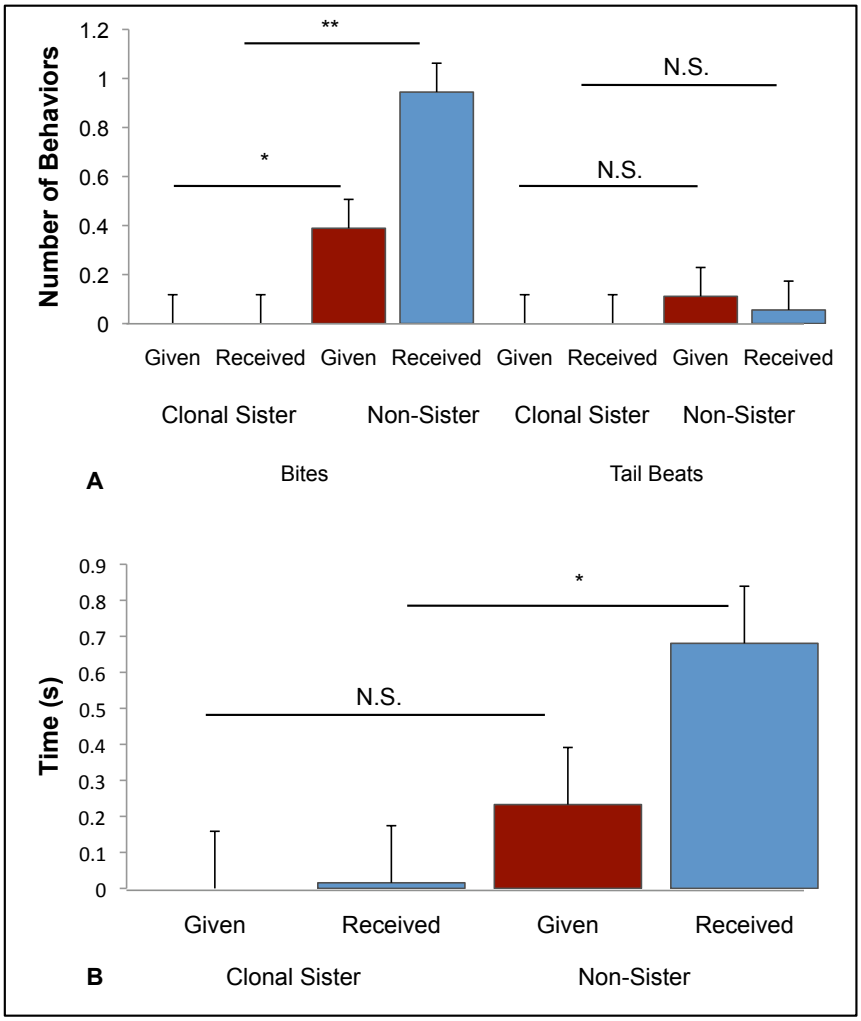

Figure 11. Aggression Experiment #1: Forced Choice. Female aggression was tested in a forced choice design to measure the baseline aggression levels toward clonal sisters and non-sisters for each female ($\bar{x}$ ± SE). Females gave (red) and received (blue) significantly more bites (**A.** given: $t_{(17)}$=-2.715, p=**0.015**; received: $t_{(17)}$=-3.308, p=**0.004**), and spent more time being aggressive (**B.** given: $t_{(17)}$=-2.078, p=0.053; received: $t_{(17)}$=-2.330, p=**0.032**) towards non-sisters when compared to clonal sisters. There was no significant difference in performing tail beats (given: $t_{(17)}$=-1.000, p=0.331; received: $t_{(17)}$=-1.000, p=0.331).

Figure 12.

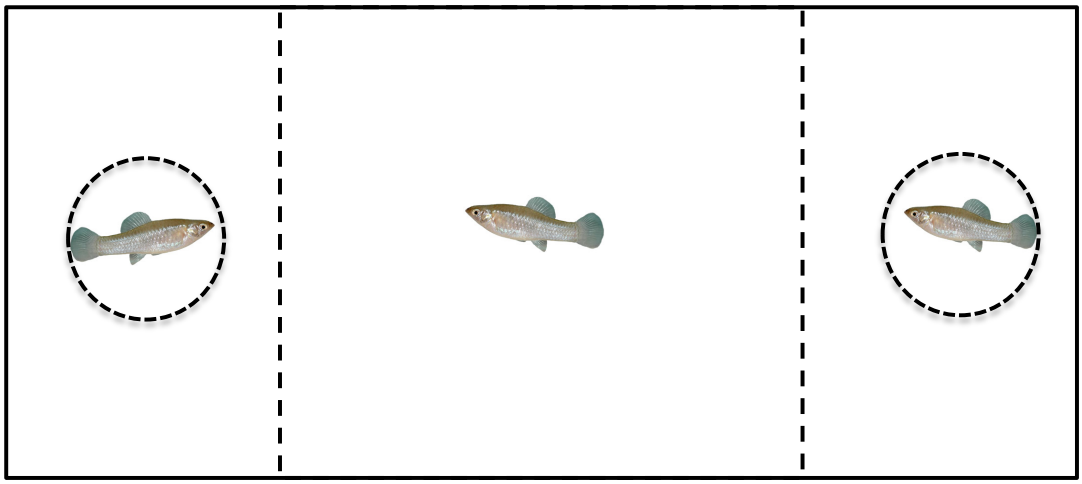

Figure 12. Female Preference Experimental Set-up. Standard choice test allowing visual, chemical and mechanical cues, where the focal female swims freely between both stimuli. The dashed circles represent the clear, perforated cylinders and the dashed lines represent the preference zones. The amount of time she spends in each zone reflects a preference for the stimulus in that zone. This set-up was used in the initial female preference experiment and familiarity experiment. It was then adjusted for the mechanism experiment, where the dashed circles selectively excluded selected mechanism pre treatment requirements.

Figure 13.

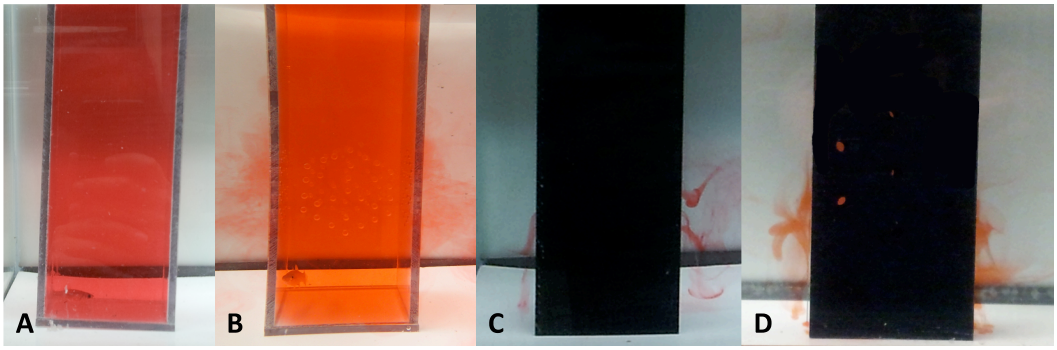

Figure 13. Plexiglas Cylinder Construction and Functions. We tested the construction of each Plexiglas cylinder to validate its proper construction and function. Each picture illustrates the diffusion of water from inside each of the 4 Plexiglas cylinders to the preference zone (**A**. visual only signals; **B**. visual and chemical signals; **C**. chemical only signals (each perforated hole had a small area, about 7 mm^2, to reduce hair cell stimulation); and **D**. chemical and mechanical signals (each perforated hole had a large area, about 20 mm^2, to allow hair cell stimulation)). A female was placed inside each cylinder to provide normal water disturbance, food coloring was added to the cylinders, then set-up was recorded for the full 10-minute acclimation period. Diffusion usually occurred within 2-4 minutes after food coloring was added, demonstrating that female chemical signals would be present in the preference zones after the 10-minute acclimation period.

Figure 14.

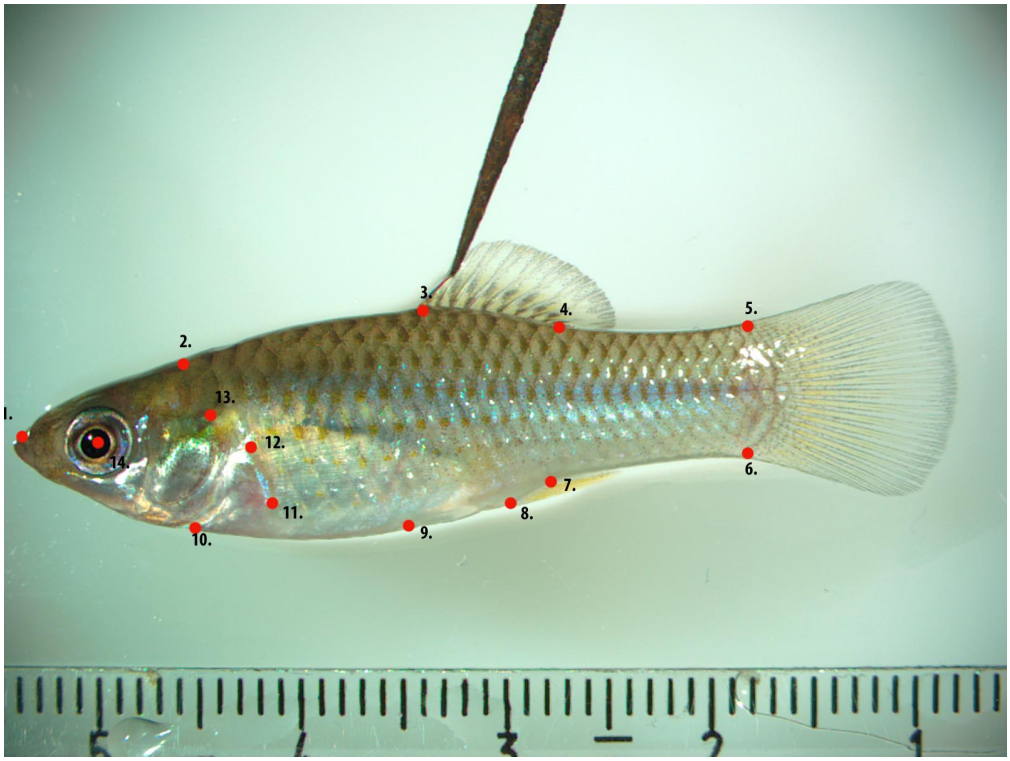

Figure 14. Morphological landmarks. The 14 landmarks used in the geometric morphometrics depicted on a photo of a *Poecilia formosa* female. 1) tip of pre-maxillary, 2) most posterior point of skull, 3) anterior and 4) posterior insertion points of dorsal fin, 5) dorsal and 6) ventral insertion points of caudal fin, 7) posterior and 8) anterior insertion points of anal fin, 9) anterior insertion of pelvic fin, 10) isthmus, 11) ventral and 12) dorsal insertion points of pectoral fin, 13) dorsal most part of the opercle, and 14) the center of eye.

Figure 15.

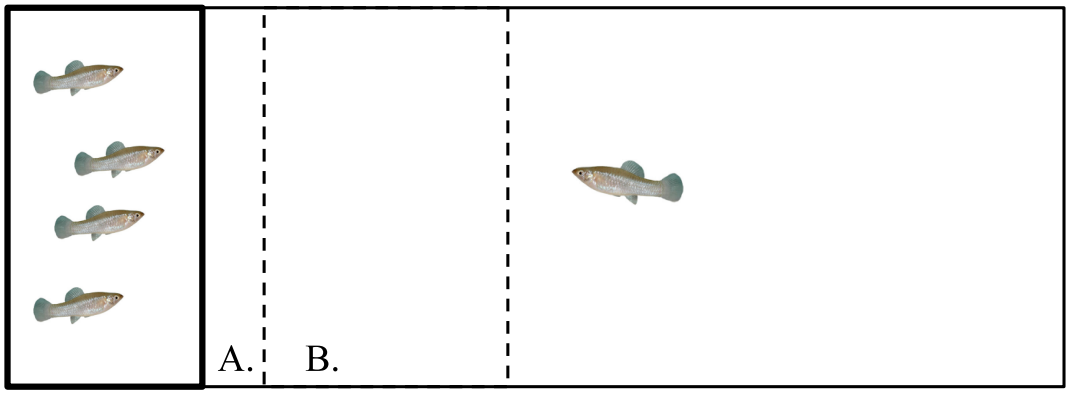

Figure 15. Shoaling Preference Experimental Tank. This represents a diagram of the experimental preference function test used to evaluate the overall time spent with the four different shoal types. The thick solid black line represents the clear, perforated Plexiglas cylinder that the stimulus shoal was kept in. The first dashed line (A.) represents the interaction zone (10.5 cm) and the second dashed line (B.) represents the preference zone (17.8 cm).

Figure 16.

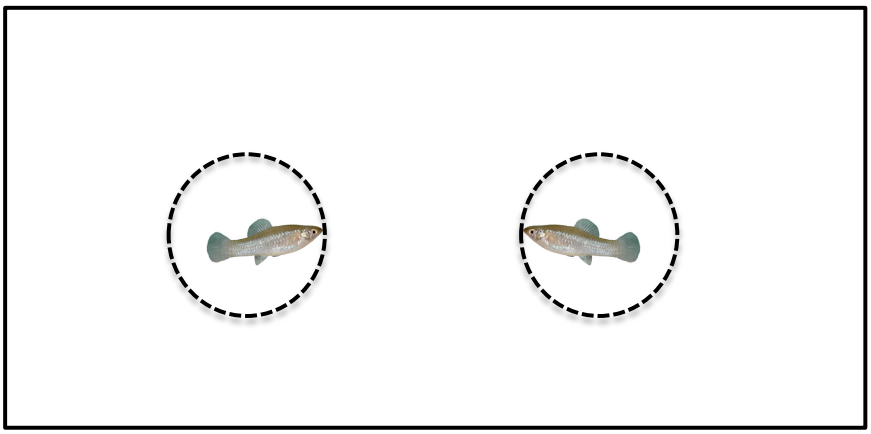

Figure 16. Female Aggression Experimental set-up #1: Forced Choice. This figure is a representation of the experimental tank for the female aggression experiment to test without a choice. The experiment was used to measure the baseline aggression levels toward clonal sister and non-sister for each female. Females were placed into clear, perforated Plexiglas cylinders (dashed circle) in the center of the tank along with a stimulus female, either a clonal sister or a non-sister. Females were released after a 10-minute acclimation period, allowed to swim freely and interact with each other. Aggressive behaviors and overall time spent being aggressive was recorded for the duration of 10 minutes. Females were the tested again with the other stimulus female after 24 hours.

Figure 17.

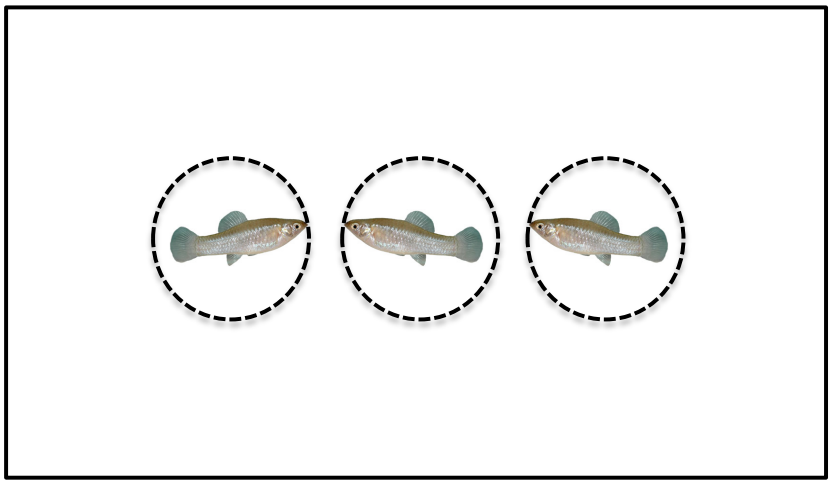

Figure 17. Female Aggression Experimental set-up #2: Free-swimming with Choice. This figure is a representation of the experimental tank used to test the aggression levels when females are given a choice between either clonal sisters or non-sisters. Females were placed into clear, perforated Plexiglas cylinders (dashed circle) in the center of the tank along with stimulus females, a clonal sister and non-sister. Females were released after a 10-minute acclimation period, allowed to swim freely and interact with each other. Aggressive behaviors and overall time spent being aggressive was recorded for the duration of 10 minutes.

Conclusion

For the past three decades, research has focused primarily on understanding the maintenance of sexual-asexual mating complexes. As a result, with respect to the sailfin-Amazon system mentioned here, we now understand how male mate choice may actually be maintaining the stability of these species. Surprisingly, however, we still lack sufficient understanding on if and how Amazon mollies are circumventing male mate choice. My hypothesis argues that female Amazons are able to regulate their aggressive behaviors to counter-act male preference for the sexual females. In my first chapter, I demonstrated that aggression is costly for both species, but more so for the Amazons. Indeed, Amazon females were inclined to perform aggressive behaviors more often towards their sexual host when compared to conspecifics. The next step to understand female-female aggression in this system was investigating how Amazon mollies behave towards different clonal lineages within the species, and if they can regulate their aggression on a more precise level. In my second chapter, I and was able to identify several important aspects in this system.

I have demonstrated that Amazon mollies can distinguish between different clonal lineages and prefer to spend more time associating with clonal sisters to non-sisters. I have also ruled out the familiarity hypothesis, showing that although familiarity helps strengthen the preference, it is not necessary for the recognition and preference to occur. Amazon mollies can use either or both visual and chemical cues to recognize and prefer clonal sisters. Indeed, when turbid water from a natural population was used, females were still able to recognize clonal sisters but only when

chemical cues were present. Fourthly, I was able to show that body shape, not symmetry, may be used as possible visual cues and was able to rule out any dietary effects on chemical cues. Finally, Amazon females are able to regulate aggressive behaviors according to their degree of relatedness: being more aggressive to heterospecific sexual females, less aggressive to non-sister clones, and even less aggressive to clonal sisters. Together, this research suggests that there are more complex social interactions occurring in this system than originally perceived. Indeed, there are five particular avenues that are promising for future research: 1) investigating other possible chemical cues that can be used for recognition; 2) determining other possible visual cues used for recognition; 3) ruling out the lateral line as a mechanism for recognition; 4) exploring if female-female aggression is used to drive away preferred, sexual females to obtain matings with good quality males; and 5) examining other potential roles for female-female aggression.