

ORIGIN, PHYLOGEOGRAPHY AND EVOLUTIONARY ECOLOGY OF A
VERTEBRATE GYNOGENETIC SPECIES (*POECILIA FORMOSA*)
AND ITS SEXUAL HOSTS (*P. LATIPINNA*
AND *P. MEXICANA*)

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DEDICATION

This work is dedicated to my parents, who, from a very early age, have endorsed and nourished my curiosity, have supported my every choice, and have been an example of honesty, work ethics, and thirst for knowledge. Thank you for being who you are and helping me become the person I am today.

“... Considerate la vostra semenza
fatti non foste a viver come bruti
ma per seguir virtute e canoscenza”

(Dante Alighieri, Divina Commedia, Inferno canto XXVI, 118-120)

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ABSTRACT

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Unisexual (all female) vertebrates are extremely rare hybrids that comprise about 1% of all extant vertebrate species. Many selective forces act against unisexual individuals, and the persistence of these species, which reproduce via complete or modified parthenogenesis, has been of interest to evolutionary biologists for many decades. Theoretically, given their mode of reproduction, and, therefore, the lack of

genetic recombination, unisexual vertebrate lineages should be unable to respond quickly to selective pressures, as well as, accumulate deleterious mutations over time.

Additionally, when the unisexual species is sperm-dependent (relies on males of a closely related species to reproduce but does not recombine the genomes), additional pressures are added. For example, these females need to compete with both conspecific and heterospecific females to access mates, and, at the same time, selection should act on heterospecific males to avoid interspecific matings. Additionally, in the case of sperm-dependent unisexual females, the usually considered benefit of having a two-fold reproductive advantage of asexuals over sexually reproducing individuals becomes a cost. If resource differentiation has not occurred between the asexual hybrid and the sexual species (usually a parent species), then the fast population growth of the asexual population could cause asexual individuals to competitively exclude sexual individuals from resources. If this occurs, the asexual species will soon go extinct because males will be no longer available.

Unisexual-bisexual mating complexes, which are composed by a sperm-dependent unisexual species and its sexual host, are present in several taxa and have persisted longer than it would be predicted by theory. Several non-mutually exclusive hypotheses have been proposed to provide mechanisms that aid the maintenance of these rare mating complexes. The work herein relates the results of experiments that were performed to test these hypotheses using the unisexual-bisexual mating system of *Poecilia formosa* – *P. mexicana* and *P. latipinna* as a study system. No empirical support was found for the hypotheses, however, interesting and surprising aspects of the genomic

architecture of *P. formosa* were discovered. The persistence of *P. formosa* might be due to its high genetic variation and might not be as paradoxical as theory would predict.

CHAPTER I

INTRODUCTION TO UNISEXUAL-BISEXUAL MATING COMPLEXES AND HYPOTHESES PROPOSED FOR THEIR MAINTENANCE

Unisexual-bisexual mating complexes

The maintenance of sex presents a conundrum for evolutionary biology because the costs of sexual reproduction (such as the cost of producing males, the energy expenditure to find a mate, the exposure to diseases and the segregation of alleles) appear to be immediate and substantial whereas its benefits such as the facilitation of adaptations and the elimination of deleterious mutations, are postponed (reviewed in Avise 2008). However, the long-term maintenance of unisexual organisms is paradoxical as well because the advantages of asexual reproduction are all immediate (no cost of producing males and therefore exponential growth) but the long-term costs are substantial (e.g., accumulation of deleterious mutations and lack of genetic recombination to respond to environmental changes). Unisexual species are therefore predicted to be short-lived (Muller 1964; Maynard-Smith 1968; Kondrashov 1988; Beukeboom & Vrijenhoek 1998).

Sexual reproduction is the predominant reproductive mode in most vertebrates. Although unisexual reproduction is rare, several divergent vertebrate taxa exhibit some form of unisexuality (Dawley 1989; Avise 2008). Unisexual vertebrate species are

all-female lineages that arose via hybridization between two or more parental species and are extremely rare: they constitute only 0.1% of all extant vertebrate species (Avisé 2008). Even more rare are unisexual-bisexual mating systems, which are found when a unisexual species requires sperm from a closely related bisexual species for reproduction (Dawley 1989). Some unisexual sperm-dependent species reproduce by gynogenesis: the formation of an unreduced, unrecombined egg which needs sperm to start embryogenesis, but no genetic information from the sperm cell is inherited by the embryo (Dawley 1989). Gynogenetic individuals are all clones of one another within a lineage. Other unisexual sperm-dependent species reproduce by hybridogenesis: the production of a haploid egg that will be fertilized by sperm, but no syngamy of the genomes will occur and the offspring will, therefore, be half clones of the mother. The long-term maintenance of a gynogenetic or hybridogenetic species is paradoxical. Like every other unisexual species the lack of genetic recombination due to their mode of reproduction, causes a lack of genetic variation, which increases their susceptibility to extinction in changing environments (Maynard-Smith 1968), and results in the accumulation of deleterious mutations and eventual extinction (Muller 1964; Kondrashov 1988). Additionally, given their mode of reproduction, hybridogenetic and gynogenetic species are dependent upon their sexual host and must live in sympatry with their host or hosts. It has been shown that unisexual lineages have a two-fold reproductive advantage over sexual females due to the fact that they avoid the cost of producing males (Maynard-Smith 1971; Williams 1975). For gynogenetic (sperm dependent) species, this latter phenomenon presents a significant problem: their two-fold reproductive advantage will cause their intrinsic population growth to raise rapidly, and if no niche differentiation has

occurred between the sperm-dependent lineage and its host, then competition for resources will rise rapidly and the sexual species might be competitively excluded from resources. As a result, the extinction of the unisexual sperm-dependent species will soon follow (Beukeboom & Vrijenhoek 1998). Additionally, because male sperm donors do not gain any fitness advantage from heterospecific matings, the gynogenetic unisexuals are considered sexual parasites, and, as a result, selection should favor males that avoid mating with them.

Despite the many selective forces acting against unisexual species, there are several distributed across most vertebrate orders. Parthenogenetic species are known to exist in the Gekoonidae (Geckos), Luceritidae (wall or rock lizards), Gymnophthalmidae (spectacled lizards), Xantusiidae (Night lizards), Scincidae (Skinks), Typhlopidae (blind snakes), Pythonidae (pythons) and Sphyrnidae (hammerhead sharks). Unisexual sperm-dependent species, however, seem to be limited to amphibians and fishes and are found in several families of both orders such as Poeciliidae (livebearers), Cyprinodontidae (killifish), Cobitidae (loach fish), Cyprinidae (minnows), Atherinidae (silversides), Ambystomatidae (mole salamanders) and Ranidae (true frogs) (Avisé 2008 and references within).

The first vertebrate to be recognized as unisexual was the gynogenetic *Poecilia formosa* (Amazon molly) (Hubbs & Hubbs 1932), yet, surprisingly, its evolutionary history still remains relatively unexplored, as several important questions concerning the evolutionary history of *Poecilia formosa* and its sexual hosts (*P. latipinna* and *P. mexicana*) have not yet been resolved. The present work will try to shed some light on the evolutionary history of *P. formosa* and will use the *P. formosa*-*P. latipinna*-*P.*

mexicana unisexual-bisexual mating system to test various hypotheses that have been proposed to explain the maintenance of these rare but resilient mating systems.

Maintenance of unisexual-bisexual mating complexes: Proposed hypotheses. Ecological hypotheses

Frozen niche variation

Various hypotheses have been proposed to explain the maintenance of unisexual-bisexual mating complexes, and they are not necessarily mutually exclusive in that some focus on ecological factors and others on behavioral ones. The frozen niche variation (FNV) hypothesis proposed by Vrijenhoek (1984, 1989) suggests that unisexual lineages are frozen into a new niche by inheriting characteristics from both parental species and lacking recombination. Referring to Ghiselin (1974), Vrijenhoek (1998) additionally suggests that a unisexual species comprised of multiple clonal lineages will be more efficient at extracting resources than single clone lineage. The assumption of this hypothesis is that the clones are able to freeze the variation that was present in the parental gene pools and therefore each clone can exploit a different sub-niche. Therefore the unisexual hybrids can live in sympatry with the sexual hosts and sister hybrid lineages because they exploit different sub-niches and do not compete for resources. Schenck & Vrijenhoek (1986, 1989) found that there are strong dietary differences between different hemiclones of the hybridogen *Poeciliopsis monacha-lucida*. Additionally, field surveys in this system have revealed that unisexual females outnumber sexual females in populations where the hybridogens are multi-clonal, whereas the opposite happens in

populations where the unisexuals are monoclonal (Vrijenhoek 1979); suggesting that the presence of multiple clonal lineages in a population is advantageous to the hybridogenetic species. The FNV hypothesis has never been tested on a gynogenetic species (where individuals are all clones) and this hypothesis could only be plausible if the gynogenetic species arose from multiple hybridization events yielding multiple clonal lineages.

Asymmetric competition

The FNV hypothesis specifies that in order for coexistence between a sexual parasite and its host to occur, then different lineages of the parasite need to be present in the population to lower both intra and interspecific competition. However, theoretical models have shown that coexistence can occur even when niche differentiation has not taken place and only one lineage of the unisexual species is present in a population.

Doncaster et al. (2000) proposed that coexistence between a parthenogenetic and a sexually reproducing species with overlapping niches is possible if the effect that the asexual species has on the exploitative abilities of the sexual species (ability to consume the resource of interest) is smaller than the effect that the sexually reproducing individuals have among themselves. This is because if interspecific competition (of asexual over sexual) is higher than intraspecific competition in the sexual species, then the growth capacity of the sexual population would be too low to counteract the competitive pressure exerted by the asexual population (Doncaster et al. 2000). Schley et al. (2004) extended these results with an additional model focused on sperm-dependent species and found that as long as the unisexual parasite is a poorer competitor than its host, then coexistence is maintained. No empirical support to these two models has been

provided yet. Chapter two herein, will present a study performed to test the predictions of the models using the *P. formosa*-*P. latipinna* mating complex.

Metapopulation dynamics

Another model to explain the coexistence between a unisexual parasite and its host species when niche differentiation has not occurred was proposed by Kokko et al. (2008). Kokko et al. (2008) proposed that the maintenance of the unisexual species might be a by-product of the natural temporal dynamics of extinction and re-colonization of the sexual population. The model is very basic because it only takes into consideration the probabilities of colonization, probability of extinction and the colonization rate for the sexual host, but does not consider time lags or the spatial dimension of the metapopulation. As a result, the model assumes that no patches can exist that are only inhabited by the unisexual species, and the distance between demes is not considered. Kokko et al. (2008) suggest that extinction/colonization dynamics, coupled with a not fully developed mate discrimination mechanism in parasitized males, can be considered an alternative hypothesis to how unisexual-bisexual mating complexes are maintained over time. No empirical evidence to support this metapopulation dynamics hypothesis has been provided yet. In chapter three, I present a model that is similar to the Kokko et al. (2008) model, but doesn't assume that the extinction of the sexual host in a patch causes the immediate extinction of the parasite. As a result, parasites are allowed to inhabit a patch alone for one generation.

Behavioral hypotheses

Frequency-dependent mating

The first behavioral hypothesis proposed to explain the maintenance of mixed mating systems, is the “best-of-a-bad-job” hypothesis proposed in a model by Kawecki (1988). The payoff of mating for a male is a function of the total time the subject spends evaluating possible mates. As time dedicated to evaluation increases, payoff increases. The function reaches an asymptote when the maximum payoff is reached. The model also takes into consideration the relative social rank of the male. Subordinate males have shorter mate evaluation time because of the risk of being chased away or having to physically interact with a dominant male, and therefore increasing the costs of mating to the point that the mating costs overcome the benefits. The hypothesis that shorter assessment time causes mismating, was later supported by a model proposed by Schmeller et al. (2005). The Kaweki (1988) model was constructed for a mixed mating system situation and it suggests that subordinate males are prone to mate with unisexual heterospecific females because their mate evaluation time is minimized by the presence of dominant males in the population. The frequency of the mating mistakes on the part of subordinate males depends upon the frequency of dominant males and available mates. In a situation where mates are not a limiting resource and therefore male-male competition is low, subordinate males should be able to assess mates correctly and avoid mating with heterospecific females. Whenever the availability of conspecific females is low and intrasexual competition between males is high, then subordinate males are more likely to mate with heterospecific females. Although the existence of hierarchies has been

recorded in some mixed mating systems (McKay 1971; Balsano et al. 1983; Woodhead & Armstrong 1985), and some support for it was found by (Moore & MacKay 1971) no study has been performed to specifically test how the relative frequency of conspecific and heterospecific females (and therefore the intensity of intrasexual competition between males) can affect the mating behavior of subordinate males. Interestingly, a recent model proposed by Heubel et al. (2009) suggests that sometimes it may be advantageous for males to mate indiscriminately with both conspecific and heterospecific females depending on the cost of maintaining a strong mate preference, suggesting that mismating of subordinate males might not be as harmful to the sexual species as it was initially thought. In chapter three, I test the hypothesis that the frequency of heterospecific females affects the mate choice of parasitized males.

Study system

The Amazon molly, *Poecilia formosa*, was the first vertebrate to be recognized as unisexual (Fig. 1a; Hubbs & Hubbs 1932). *Poecilia formosa* is a gynogenetic lineage that belongs to the live-bearing fish family Poeciliidae. Its common name refers to the mythological Greek tribe of all female warriors. As with all other vertebrate unisexual species, *P. formosa* is of hybrid origins (Dawley 1989; Avise et al. 1991; Scharl et al. 1995). The maternal species is the shortfin Atlantic molly, *P. mexicana limantouri* (Fig. 1b), while the paternal species is still unknown, but it is proposed to be the sailfin molly, *P. latipinna* (Fig. 1a) or an extinct ancestor (Avise et al. 1991; Scharl et al. 1995). It is still unknown if *P. formosa* is the result of a single or multiple hybridization events, although genetic evidence strongly points to a single origin scenario (Stock et al. 2010).

Additionally, we still do not know if *P. formosa* is a simple F1 hybrid or a backcross hybrid. The fact that nobody has been able to re-synthesize *P. formosa* under laboratory conditions (Turner et al. 1980) suggests that it could be a complex backcross and not a simple F1 hybrid.

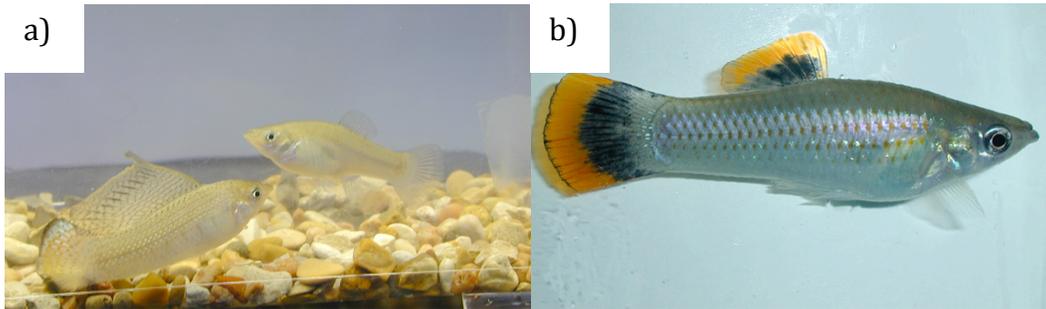


Figure 1. Pictures of *Poecilia formosa* (on the right in 1a), its maternal species *P. mexicana limantouri* (1b) and its paternal species *P. latipinna* (1a).

The gynogenetic mode of reproduction makes *P. formosa* a sperm dependent species. It therefore must live in sympatry with at least one of the parental species and depend on them for its maintenance over time. Theory predicts that unisexual gynogenetic lineages should not survive over long periods of time and will reach extinction relatively quickly (Muller 1964; Maynard-Smith 1968; Williams 1975; Bell 1982; Vrijenhoek 1984; Kondrashov 1988). However, dating of this complex using mtDNA, suggests it has persisted for 100,000 years (Avisé et al. 1991), although Dries (2000, 2003) suggests that the error associated with this estimation is broad.

The range of *P. formosa* overlaps the ranges of its parental species in northern Mexico at the mouth of the Rio Tuxpan through southern Texas at the Nueces river (Darnell & Abramoff 1968), and it is sympatric with *P. latipinna* in areas of coastal Northeastern Mexico and coastal South-East Texas, while it is sympatric with *P.*

mexicana throughout the south of its range (Fig. 2). In the Tamesí river basin in central-coastal Mexico, *P. formosa* parasitizes a third host species: *P. latipunctata* (Niemeitz 2002). Within areas of sympatry with *P. latipinna*, *P. formosa* appears to utilize a similar ecological niche as its parental species (Balsano et al. 1981). However, Balsano et al. (1985) found that *P. formosa* is found mostly downstream whereas *P. mexicana* is more abundant in headwaters localities, suggesting that these two species show niche differentiation. A recent study has showed that there are no differences in the gut contents of *P. formosa*, *P. latipinna* and *P. mexicana*, suggesting that no resource differentiation is present between the sexual parasite and its hosts (Scharnweber et al. 2011).

Poecilia formosa needs to inhabit localities already inhabited by either *P. latipinna* or *P. mexicana* because it highly depends on its sexual hosts for reproduction. Although *P. formosa* should not be expected to show a preference for any particular type of male since sperm is used only to trigger embryogenesis and no genetic information of the male is passed on to the offspring, behavioral studies have shown that *P. formosa* does indeed show mating preferences (Marler & Ryan 1997; Landmann et al. 1999; Körner et al. 1999). Differences in species recognition and discrimination have, however, been recorded among males of the parental species (Ryan et al. 1996; Alberici da Barbiano et al. submitted and refs within).

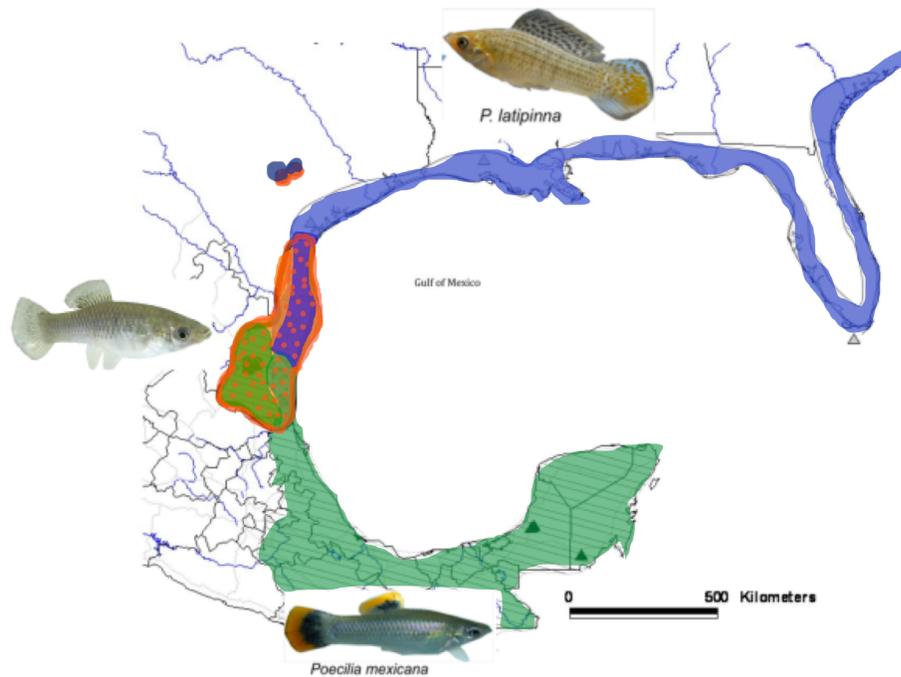


Figure 2. Distribution map of *P. fomosa* (orange), *P. latipinna* (blue) and *P. mexicana* (green).

The maternal species of *P. formosa* is the Atlantic molly, *P. mexicana limantouri* (Avisé et al. 1991; Scharl et al. 1995). The range of *P. mexicana* expands from northern Atlantic slope of Mexico to the Yucatan peninsula; the subspecies *Poecilia m. limantouri* is found in the northern part of the range as far north as Tampico, Tamaulipas (Fig. 2). *Poecilia mexicana* belongs to the “shortfin mollies” subgroup of the subgenus *Mollinesia* (Ptacek & Breden 1998) and, contrary to males of the sailfin complex, males of the shortfin complex do not use courtship displays in mating (Ptacek 1998). Ryan et al. (1996) and Schlupp & Plath (2005) showed that *P. mexicana* males preferred to mate with conspecific females and Schlupp & Plath (2005) showed that they transferred more sperm to conspecific females, suggesting that they can discriminate between conspecific and heterospecific females that are morphologically very similar. However, Plath et al.

(2007a) found that male *P. mexicana* prefer larger females, but this preference was not retained when audience males were present, suggesting that the presence of competitors may affect the choice of males.

The sailfin molly, *Poecilia latipinna* has been proposed to be the paternal species of *P. formosa*, although this conclusion is mainly based on inferences and no actual molecular data support it with a high level of confidence (Schartl et al. 1995; Stock et al. 2010). *Poecilia latipinna* lives in brackish habitats, although it can survive in fresh water habitats as well (Brown 1953), and is sympatric with *P. formosa* in the southern part of its range (Fig. 2). Male *P. latipinna* are more colorful than females, and they possess an enlarged dorsal fin that they sometime use during courtship displays. Male size varies continuously within a population (Trexler & Travis 1990) but once maturity is reached, males do not grow much further (Travis 1994). It takes as little as 30 days for a small male to reach maturity and more than 60 days for larger males. Larger males court more than smaller ones, and smaller males tend to sneak-copulate (or force copulate) with females without courting and displaying (Farr et al. 1986; Travis & Woodward 1989; Swanbrow Becker et al. 2012). These differences between males of different class sizes are not supported by the findings of Ptacek & Travis (1996) though, who suggested that behaviors vary between populations and are not limited to the differences in the males' body size.

When male *P. latipinna* from populations sympatric with *P. formosa* are given a choice of mating with a conspecific or a heterospecific female, and the females are size matched, the males show a greater mating preference for conspecific females (Ryan et al. 1996; Gabor & Ryan 2001; Gabor & Aspbury 2008; Robinson et al. 2008; Aspbury et al.

2010; Gabor & Grober 2010). These results suggest that males discriminate between potential mates; however, Gumm & Gabor (2005) demonstrated that this male mating preference is weaker when *P. formosa* are larger than female *P. latipinna* suggesting a possible conflict between species and mate-quality recognition. There is no support for the hypothesis that males use independent visual traits to recognize mates (Gumm et al. 2006) and currently there is no support for the hypothesis that males use only chemical cues to discriminate between conspecific and heterospecific females (Aspbury et al. 2010). However, Gumm et al. (2006) showed that males prefer to associate with conspecifics over heterospecifics based on visual cues alone. Additionally, Aspbury & Gabor (2004) showed that males produce more sperm when presented with conspecific rather than heterospecific females. These results support the hypothesis that the males can indeed discriminate between the two species when the females are size matched, although it is still not clear if males use a specific combination of visual traits to select their mates, and consequently, which particular traits heterospecific females may be mimicking/sharing. However, it is difficult to make general conclusions about male mate preference in *P. latipinna*, as males from different populations act differently and sometimes show opposite behaviors (Gabor et al. 2010) and reproductive investment (Robinson et al. 2011), and males are not always consistent with their preference (Gabor & Aspbury 2008).

Woodhead & Armstrong (1985) suggested that smaller male sailfin mollies tend to mate with the heterospecific *P. formosa* more than larger males, but no data collected in the laboratory from Gabor & Ryan (2001) and Gumm & Gabor (2005) support this prediction. However it is possible that in a more natural environment the prediction of

Woodhead & Armstrong (1985) may be upheld. Larger males prevent smaller males from mating with preferred larger females (Travis & Woodward 1989) and females prefer larger males (Ptacek & Travis 1997; Gabor 1999), which may result in smaller males possibly being more prone to mating with heterospecific females (such as a best-of-a bad-job scenario).

Population genetics of the three species

Although this particular unisexual-bisexual mating complex has been known for many decades, we still know very little about the historical biogeography of the complex as a whole and of each species that is part of it. However, several population genetics studies have been focused on understanding the distribution of genetic variation within the three species. The following paragraphs summarize the information gathered until present.

Poecilia mexicana: Only one study has been published regarding the population genetics of *P. mexicana*. Tobler et al. (2008) compared populations of *P. mexicana* found in the region of Tabasco in Mexico, using 10 msat loci and 1 mtDNA locus, and found no indication of isolation by distance (from now on IBD), but did find high genetic differentiation between populations that occupy different habitats. In this region of Mexico, some populations of *P. mexicana* are found to inhabit waters with high sulfur levels. The results of Tobler et al. (2008) suggest that populations inhabiting sulfuric habitats were more similar to one another, than to geographically close populations inhabiting water without sulfur. No other information is available regarding the

distribution of genetic variation in *P. mexicana* throughout its range, or about its historical biogeography.

Poecilia latipinna: Of the two parental species of *P. formosa*, more population genetics studies have focused on *P. latipinna*. Trexler (1988) compared genetic variation in population of *P. latipinna* found at the northern limits of the species range. The author compared populations from Georgia, northern Florida and south Florida by using 29 allozyme loci and found that most of the genetic variation was explained by individual variation within populations rather than variation between populations within regions or variation between regions. Populations in south Florida showed lower population-level heterozygosity and lower levels of within population genetic variation than populations in either Georgia or North Florida. However, all three regions show high levels of migration and no evidence of isolation by distance (IBD).

In a second population genetics study, Gabor et al. (2005) looked at the genetic variation of populations of *P. latipinna* sympatric with *P. formosa* (Mexico and introduced populations in central TX) and allopatric to *P. formosa* (Louisiana and Florida) using 21 allozyme loci. The results of Gabor et al. (2005) are in agreement with the results of Trexler (1988). Most of the variation (virtually all of it) was explained by within population variation and the authors found no evidence of IBD. The goal of Gabor et al. (2005) was to examine whether there was a correlation between behavioral traits and genetic isolation between populations in allopatry and sympatry with *P. formosa*. Gabor & Ryan (2001) found that males in allopatric populations have a weaker strength of preference for conspecific females than males from sympatric populations, providing evidence for the presence of behavioral character displacement in this species. However,

Gabor et al. (2005) found no correlation between the males' behavior and genetic differentiation between populations. Both the population genetic studies mentioned here point to a recent divergence among the populations and panmixia. Trexler (1988) further suggests that *P. latipinna* might have expanded its range following the coastline of the Gulf of Mexico in a north-east direction. However, a complete phylogeographic study on the species has not been performed yet. My work presented in chapter four will shed further light on the historical biogeography of this species.

Poecilia formosa: *Poecilia formosa* is composed by both triploid and diploid lineages. Whereas multiple origins of triploid lineages have been recorded in the Río Purificación basin and the connected Río Soto la Marina river system (Lampert et al. 2005, 2006), no strong evidence points to either a single or multiple origin of the diploid lineage(s) (Stock et al. 2011). All the studies performed so far on the diploid lineages support the hypothesis of *P. formosa* being the product of hybridization due to the high heterozygosity found in this fish (Abramoff et al. 1968; Turner et al. 1980; Tiedemann et al. 2005; Lampert et al. 2009). The work presented in chapter three is an investigation of the genetic composition of *P. formosa* and of how variation within the species is distributed among populations. By looking at the hybrid index of individual *P. formosa*, we were able to test the hypothesis of a single hybrid origin, as well as determine whether *P. formosa* is a simple F1 hybrid.

The era of high throughput sequencing

In the past two decades or so, we have made giant steps forward in the development of new sequencing techniques, which allow us to answer questions at a level of resolution

that we could only dream about in the late 1990s. The development of next-generation sequencing platforms has provided us with a relatively cheap tool to obtain an immense amount of information about the genetic structure of an individual, or individuals within a population, or populations within a species. Although next-generation sequencing was originally developed to sequence entire genomes (REF), evolutionary biologists and population geneticists are starting to use this new technique to perform population genomics studies (Gompert & Buerkle 2009; Gompert & Buerkle 2011; Gompert et al. 2012; Nice et al. submitted). The use of next-generation sequencing is extremely attractive because it allows us to obtain millions of reads for each individual at a relatively low cost, compared to the previously used Sanger sequencing method. However, the organization and analysis of the data present a non-trivial computational challenge to population geneticists. Luckily, new assembly software and population genomics models are continuously being published and the field of population genomics is rapidly evolving (Gompert et al. 2010a; Gompert et al. 2010b; Forister et al. 2010).

The work presented in Chapter 2 is one of a handful of studies performed using a genome complexity reduction technique, which allowed us to perform analyses in a population genomic framework. The work herein is the first investigation of genomic variation across the range of a unisexual species.

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CHAPTER II

DIFFERENCES IN RESOURCE ASSIMILATION BETWEEN A SPERM-DEPENDENT SPECIES AND ITS SEXUAL HOST¹

Abstract

Unisexual sperm-dependent species are characterized by their dependence on a sexual host, and they must live in sympatry with their sperm donor species. If niche overlap between the unisexual species and its host is substantial, the intrinsic faster population growth of the unisexual species over the sexual species can cause the sperm parasite to competitively exclude its host from resources, causing its own demise. However, theoretical models show that coexistence between the two species is possible even if niche differentiation has not occurred, if the unisexual species is a poorer competitor than the sexual host and if the effect that the unisexual species has on the exploitative abilities of the sexual species is smaller than the effect that the sexually reproducing individuals have among themselves. We tested the predictions of these models in the unisexual-bisexual mating complex consisting of the unisexual *Poecilia formosa*, and one of its sexual hosts, *P. latipinna*. Fishes were housed from parturition for 76 days with both conspecific and heterospecific individuals under both limited and *ad libitum* food regimes

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Only one of the predictions of the models was met: intraspecific competition for *P. latipinna* was higher than interspecific competition. However, this difference in competitive pressure on *P. latipinna* did not translate in higher mortality rates for this species.

Introduction

Unisexual-bisexual mating complexes are found whenever a unisexual species reproduces by a form of modified parthenogenesis in which the unisexual females rely on mating with males of a bisexual species to produce offspring (Dawley 1989).

Gynogenesis is a form of modified parthenogenesis where females produce diploid eggs ameiotically, but must mate with males of closely related species (see Choleva et al. 2008 for exceptions) to initiate embryogenesis (Dawley 1989; Niemeitz et al. 2002; Schlupp 2005). However, the male's genetic information does not enter the egg (but see Scharl et al. 1995a), and, as a result, gynogenetic females have a strictly clonal inheritance, are sperm-dependent, and considered sexual parasites. Due to their mode of reproduction, gynogenetic species must live in sympatry with their sexual host species, giving rise to unisexual-bisexual mating complexes (Dawley and Bogart 1989 and references within).

Understanding the evolutionary maintenance of gynogenetic species is of interest because multiple selective pressures act in concert hindering their persistence. Gynogenetic species reproduce clonally and, therefore, lack mechanisms to produce novel genetic variation (Maynard Smith 1968) as well as to purge deleterious mutations (Muller 1964; Kondrashov 1988). As a result, these asexual lineages are predicted to be susceptible to extinction and suffer from higher mutational loads than sexually reproducing species

(Muller 1964; Kondrashov 1984). Furthermore, by only producing females, gynogenetic species have a faster intrinsic population growth than their sexually reproducing host species (Maynard Smith 1971; Lively and Lloyd 1990). Consequently, in habitats inhabited by both gynogens and their sexual hosts, the relative frequency of gynogens should rise rapidly. Assuming all but the reproductive mode is equal between asexual and sympatric sexual lineages, population growth in asexuals should lead to the displacement of sexual individuals and, subsequently, the extinction of gynogens, due to the lack of sperm donors (Schlupp 2005).

A stable maintenance of unisexual-bisexual mating complexes requires the two-fold advantage of asexuals in terms of population growth rate to be mitigated. Ecologically, such a reduction of the asexuals' short-term advantage may be mediated through differential competitive abilities between reproductive forms, as competition has been widely documented to affect species coexistence and community structure in a wide variety of taxa (e.g., *Paramecium*: Gause 1934; flour beetles, *Tribolium* spp: Park 1948; barnacles: Connell 1961; and plants: Tilman 1982). Higher competitive ability in terms of resource exploitation in sexuals could effectively reduce the reproductive success of asexual sperm-parasites and could therefore contribute to mediating stable maintenance of unisexual-bisexual mating complexes. Several hypotheses based on competitive interactions have been proposed to explain the maintenance of unisexual-bisexual mating complexes. The Frozen Niche Variation hypothesis (Vrijenhoek 1978) provides a mechanism by which competition between an asexual sperm-parasite and its host is reduced. The presence of multiple, independently evolved clonal lines of the unisexual species in a population, as in the case of hybridogenetic species (sperm-dependent

unisexual species that reproduce semi-clonally), facilitates coexistence between the sexual parasite and its host because the microhabitat is partitioned among the clonal lineages and the host, and competition is reduced (Vrijenhoek 1978). However, when only one clonal lineage is present, as in the case of gynogenetic species, and niche overlap between the gynogens and their sexual hosts is significant, coexistence should be maintained only if the gynogenetic species is a poorer competitor than the sexual species. In other words, the effect of unisexuals on the ability of the sexual species to acquire resources must be smaller than the effect that the sexually reproducing individuals have among themselves. When the effects of intraspecific competition are greater than the effects of interspecific competition in the sexual species, coexistence between the asexual and sexual species is predicted (Doncaster et al. 2000; Schley et al. 2004).

A well-known unisexual-bisexual mating complex is found in the livebearing fish family, Poeciliidae. *Poecilia formosa* (Amazon molly) is a gynogenetic fish that likely arose from a hybridization event between a female *P. mexicana* (Atlantic molly) and a male *P. latipinna* (sailfin molly) (Awise et al. 1991; Schartl et al. 1995b). *Poecilia latipinna* and *P. formosa* are commonly found in mixed species shoals in the wild (Schlupp and Ryan 1996). *Poecilia formosa* sexually parasitizes both male *P. mexicana* and *P. latipinna* depending on the geographic region, and no significant trophic niche differentiation has been demonstrated between the three species (Scharnweber et al. 2011b). Consequently, niche differentiation likely does not facilitate coexistence between *P. formosa* and its sexual hosts. However, *P. formosa* has a reduced foraging rate in the presence of *P. latipinna*, whereas the foraging rate of the sexual species is not affected by the presence of gynogenetic individuals (Alberici da Barbiano et al. 2010). These results

suggest that the unisexual species is a poorer competitor than its sexual host, which could contribute to mediating stability of the unisexual-sexual mating system (Schley et al. 2004). Because Alberici da Barbiano et al. (2010) only focused on the behavioral responses of adult subjects during 10 min foraging bouts in the presence of a heterospecific or a conspecific fish, the present study investigated whether differences in competitive abilities between *P. formosa* and *P. latipinna* from birth to a few months of age differentially affect the body condition of the two species. We tested this hypothesis using two treatments of food availability (*ad libitum* and limited) and two competitive treatments (intraspecific and interspecific). This approach allowed us to address several a priori predictions: (1) Under *ad libitum* food regimes, no difference in body condition should be found between the two species, whether they are reared with conspecifics or heterospecifics, because competitive effects should be low, and individuals should be able to equally invest in body condition. (2) Under limited food regimes, when competitive interactions are exacerbated (Schoener 1971), the body condition of individuals and the mortality rates of either species should differ depending on whether they are reared with conspecifics or heterospecifics. In particular, following the predictions of the Doncaster et al. (2000) and Schley et al. (2004) models, we predicted that *P. formosa* would have a lower body condition and a higher mortality rate than *P. latipinna*, when fish are reared with heterospecific individuals (interspecific competition). Additionally, *P. latipinna* should have a lower body condition and higher mortality rates when tested with conspecifics (intraspecific competition) than when tested with heterospecific individuals (interspecific competition).

Materials and Methods

Fishes were collected at the headwaters of the San Marcos River in San Marcos, TX (29° 89' N; 97° 82' W) in May 2011, and the experiment continued until September 2011.

Each female was placed in a 19L tank and isolated from other females to facilitate parturition of fry. Whenever a female *P. latipinna* and a *P. formosa* gave birth to offspring within 24h of each other, we initiated one full set of experimental trials.

Offspring of both species were assigned to 19L aquaria that were fed either (a) *ad libitum* or (b) limited diet. Within each feeding regime, and for both species, the fish were housed in an intraspecific competition treatment consisting of conspecifics ($N = 6$ conspecific individuals per aquarium) or in an interspecific competition treatment consisting of both species ($N = 3$ heterospecifics and $N = 3$ conspecifics per aquarium) where only one species served as the focal species. As a result there were eight experimental treatments across the feeding regime and competition treatments in a fully factorial design.

Each pair of field-caught *P. latipinna* ($N=13$) and *P. formosa* ($N=13$) provided enough offspring for all eight treatments. Fishes were fed both live brine shrimp and 75mg food pellets (200 Purina AquaMax) daily in the *ad libitum* treatments, whereas we only fed 25mg of food pellets to limited food treatments, with one weekly supplement of live brine shrimp. We measured the standard length (SL) of all fish at 76 days, when they were euthanized using a high dose of ms-222 and fixed in a 5% formaldehyde solution.

To assess body condition in experimental fish, we conducted a storage fat content analysis. We air-dried specimens at 65° C for 5 days, at which time we determined the dry weight (g) of each individual. Each fish was then placed in 3 consecutive 24h petroleum ether baths, which extracts all soluble nonstructural lipids from carcasses

(Tobler 2008). We then weighed the fish again and calculated the relative fat content in each fish by subtracting the post-extraction mass from the pre-extraction mass and dividing by the pre-extraction mass (Tobler 2008). We log-transformed the data for the analysis. For the conspecific treatments, we analyzed the average of the body condition of all fish alive at 76 days. For the heterospecific treatments, we analyzed the average of the body condition of the focal species of fish alive at 76 days. We also calculated the average mortality (# of dead individuals/ # of fish at the beginning of the testing period) for each species in each treatment at 76 days (data were square root transformed).

We used an ANCOVA to examine the effects of food regime (*ad libitum* or limited), species (*P. latipinna* or *P. formosa*), and competitive regime (reared with conspecifics or heterospecifics) on body condition (relative fats; log transformed) with SL as a covariate. Given that no significant interactions were found between SL and any of the other main effects on body condition ($F \leq 2.8$, $P \geq 0.1$), the final model excluded the interaction terms that included the covariate. We also used a generalized linear model to examine the effects of food regime, species and competitive regime on mortality after 76 days (square root transformed). Given the experimental design, all replicates were independent of each other. All analyses were performed using JMP v. 9.0, and $\alpha = 0.05$.

Results

There was a significant three-way interaction between the food regime (*ad libitum* vs. limited), species (*P. latipinna* vs. *P. formosa*), and competition treatments (conspecific vs. heterospecific) on body condition (Table 1; Figure 3). *Poecilia latipinna*, in the

intraspecific competition treatments, had significantly lower body condition than fish tested in any other treatment and food regime (Tukey's HSD; Figure 3).

Table 1. Effect tests for ANCOVA on body condition (pre-extraction mass – post-extraction mass /pre-extraction mass), across food regimes, species and the competition treatments, and standard length (SL) as covariate. Significant *P* values are in bold type

Response Variable	Main Effects	d.f.	<i>F</i>	<i>P</i>
Body condition	Food regime	1	5.89	0.020
	Species	1	11.40	0.001
	Treatment	1	7.76	0.007
	Standard Length (SL)	1	0.98	0.320
	Food regime × species	1	3.42	0.070
	Food regime × treatment	1	3.49	0.070
	Treatment × species	1	3.91	0.052
	Food regime × treatment × species	1	4.07	0.048

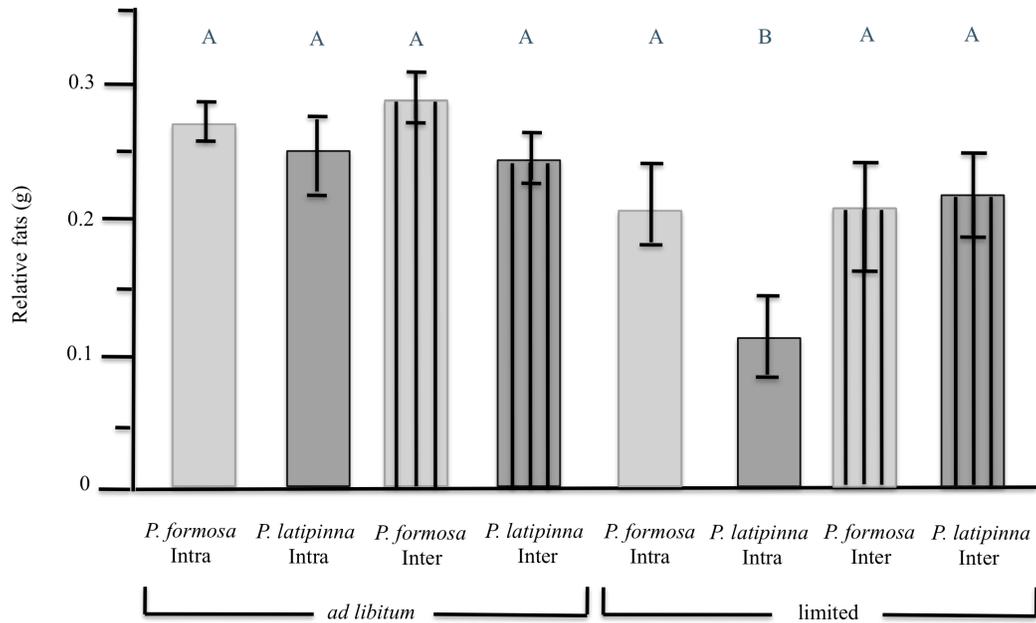


Figure 3. Average body condition (pre-extraction mass-post-extraction mass/pre-extraction mass \pm s.e.) of *P. latipinna* (dark grey) and *P. formosa* (light grey) across food regimes and competition treatments (striped = interspecific; solid = intraspecific). Letters summarize post-hoc comparisons; different letters indicate significant differences ($P < 0.05$) between the treatments.

Mortality (defined in methods section) differed between the food regimes (ad libitum mean \pm s.e. = 0.35 ± 0.05 ; limited mean \pm s.e. = 0.62 ± 0.06 ; GLM: $F_{1,97} = 11.9$, $P = 0.001$) and also between treatments (intraspecific mean \pm s.e. = 0.59 ± 0.05 ; interspecific mean \pm s.e. = 0.38 ± 0.05 ; GLM: $F_{2,97} = 7.49$, $P = 0.007$). There was no significant interaction between species, treatment and food regime on mortality (GLM: $F_{2,97} = 0.03$, $P = 0.95$; Table 2).

Table 2. Morality (mean \pm s.e) of *P. latipinna* and *P. formosa* across food regimes

and treatments. There was no significant interaction between species, treatment and food regime on mortality (# of dead individuals at 76 days / # of individuals at the beginning of the tests) (GLM: $F_{2,97} = 0.03$, $P = 0.95$).

Species	Treatment	Mean \pm s.e.
<i>P. latipinna</i>	<i>Ad libitum</i> Intraspecific	0.50 \pm 0.10
	<i>Ad libitum</i> Interspecific	0.29 \pm 0.11
	Limited Intraspecific	0.71 \pm 0.11
	Limited Interspecific	0.43 \pm 0.13
<i>P. formosa</i>	<i>Ad libitum</i> Intraspecific	0.37 \pm 0.11
	<i>Ad libitum</i> Interspecific	0.24 \pm 0.09
	Limited Intraspecific	0.79 \pm 0.09
	Limited Interspecific	0.57 \pm 0.13

Discussion

The present study was designed to empirically test the predictions of the Doncaster et al. (2000) and Schley et al. (2004) models, which predict that, in order for coexistence to be maintained between a unisexual and a sexually reproducing species, the unisexual species needs to be a poorer competitor than the sexually reproducing species, and intraspecific competition in the sexually reproducing species must be higher than interspecific competition. The effects of intraspecific competition on body condition in *P. latipinna* were higher than the effects of interspecific competition on the body condition of *P.*

latipinna. As a result, one of the requirements for coexistence between a sperm-dependent species and its host was met. However, *P. formosa* did not appear to be a poorer competitor to *P. latipinna*. Neither food regime, nor intra or inter-specific competition, appear to have an impact on *P. formosa*'s ability to allocate food resources to fat storage.

These results are in agreement with the findings of Scharnweber et al. (2011a), where *P. formosa* was not found to be less efficient in feeding than *P. latipinna*. However, Alberici da Barbiano et al. (2010), found a decrease in the foraging behavior of adult *P. formosa* when foraging with heterospecifics than when feeding with conspecifics during brief foraging trials (10 min). If this same behavioral difference in foraging occurred over the long-term period of the present study, then we would predict that *P. formosa* would have lower body condition when in interspecific competition treatments than when in intraspecific competition treatments. We found no support for this prediction. It is possible that gynogenetic individuals resume foraging efforts after being in the presence of heterospecifics for an extended period of time (as in the present study). In addition, in the current study we tested juvenile fishes, rather than adult fishes. It is possible that there are ontogenetic changes in foraging and nutrient sequestering in *P. formosa*, with younger individuals investing more in foraging and resource assimilation than older individuals. Additionally, behavioral differences in foraging ultimately might not be the only variables affecting growth and storage. For example, differential assimilation, allocation, and expenditure of resources could lead to similar body conditions between species.

Together with body condition, we considered mortality as an additional variable

to test the predictions of coexistence models empirically (Doncaster 2000; Schley et al. 2004). In limited food conditions, there was higher mortality than in *ad libitum* food conditions, suggesting that the food limitation was sufficient to affect individual survival. In addition, there was higher mortality when fishes were in the intraspecific competition treatments than when in the interspecific treatments. This result suggests that, for both species, intraspecific competition has stronger effects on mortality than does interspecific competition. However, when this result is considered with the results on body condition, it appears that mortality and body condition are not related to each other. If that were the case, we would expect fish tested in treatments with high mortality to also have better body conditions, given that the surviving fish would have to compete with fewer individuals for the same amount of resources compared to fish tested in treatments where mortality was low. However, we found no interaction between treatment, species and food regime on mortality.

The two-fold population growth advantage that asexual species have over sexually reproducing species presents a cost, rather than a benefit, to gynogenetic individuals. We were able to find support for one of the predictions of coexistence models in the unisexual-bisexual complex of *P. formosa* and *P. latipinna*, however, the second condition for coexistence was not met. Intraspecific competition in *P. latipinna* was higher than interspecific competition, but *P. formosa* was not a poorer competitor than *P. latipinna*. It is apparent that competition alone, is insufficient to explain the maintenance of this unisexual-bisexual mating complex, and several mechanisms might be working in concert to limit the population growth of the gynogenetic *P. formosa*. Other ecological factors, such as a low tolerance to extreme temperatures have been

shown to affect *P. formosa* significantly (Fisher and Schlupp 2009). Mating behavior might be playing a role as well. Male *P. latipinna* from many populations sympatric to *P. formosa* prefer mating with conspecifics and, therefore, limit the reproductive output of *P. formosa* (Gabor and Ryan 2001). Alberici da Barbiano et al. (2011) found that *P. formosa* does not have a two-fold reproductive advantage over host-species females based on the number of female embryos produced per female. However, Schlupp et al. (2010) found the opposite result as *P. formosa*, in the population tested in that paper, showed a two-fold reproductive advantage over *P. latipinna*. Additionally, Alberici da Barbiano et al. (2011) found no evidence of frequency-dependent male mate choice, yet, using a different population, Riesch et al. (2012) did find evidence of frequency-dependent male mate choice. These results suggest that, perhaps, different dynamics might be occurring concurrently in different populations.

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CHAPTER III

COEXISTENCE OF UNISEXUAL SPERM-DEPENDENT SPECIES AND THEIR HOSTS IN A METAPOPOPULATION FRAMEWORK USING A SIMULATION MODEL¹

Abstract

The two-fold reproductive advantage of asexual reproduction may become a potential disadvantage for sperm-dependent unisexual species. The sperm-dependent asexual species may competitively exclude its host from resources, eventually causing the extinction of the host, which will be followed by the demise of the asexual species. We tested the hypothesis that the ability of a sperm dependent unisexual individuals to occupy a patch by themselves for one generation in a metapopulation, can aid the maintenance of vertebrate unisexual lineages even when niche differentiation has not occurred. We used a probabilistic model to capture the inherent stochasticity in extinction-colonization dynamics. We simulated coexistence between asexual and sexual species by changing the extinction and colonization probabilities of both species within and between patches. We performed two sets of simulations for each probability changed: one set where no asexual-only patches were allowed to persist, and one

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set where these type of patches could persist for one generation. Allowing for the presence of asexual-only patches decreased the probability of extinction of mixed patches, allowing both sperm-dependent individuals and their sexual hosts to coexist with higher extinction rates and lower colonization rates than when asexual-only patches were not present in the metapopulation.

Introduction

The maintenance of sex presents a conundrum for evolutionary biology because the costs of sexual reproduction (cost of producing males, energy expenditure to find a mate, exposure to diseases, segregation of alleles) appear to be immediate and substantial whereas its benefits (facilitation of adaptations, elimination of deleterious mutations) are postponed (reviewed in Avise 2008). However, explaining the long term maintenance of asexuality is also problematic because despite the advantages of asexual reproduction (no cost of producing males that leads to rapid population growth), the long-term costs are substantial (accumulation of deleterious mutations and lack of genetic variation to respond to environmental changes, but see Lutes et al. 2010 for a possible mechanism for how asexual individuals can maintain heterozygosity). Because of these costs, asexually reproducing species are predicted to be short-lived (Muller 1964; Maynard Smith 1968; Beukeboom and Vrijenhoek 1998). Unisexual vertebrate species (which reproduce asexually) are extremely rare, and constitute only 0.1% of all extant vertebrate species (Avise 2008). Even more rare are unisexual-bisexual mating systems that consist of a unisexual (i.e., all female) species that requires sperm from a closely related bisexual species for reproduction (Dawley 1989). Some unisexual species reproduce by gynogenesis in which an unreduced, unrecombined egg that requires sperm to start

embryogenesis is produced and no genetic information from the sperm cell is inherited by the embryo. Therefore the individuals are all clones of one another (Dawley 1989). Other sperm-dependent species reproduce via hybridogenesis in which an unrecombined haploid egg is produced that requires sperm to be fertilized, but no recombination between the two genomes takes place. The paternal genes are expressed by the hybrid offspring but the paternal chromosomes are discarded during meiosis; therefore inheritance is exclusively maternal and individuals within a lineage are all hemiclones of one another (Vrijenhoek 1984).

Given their dependence on the host species, the maintenance of gynogenetic or hybridogenetic species is even more difficult to explain than the maintenance of a parthenogenetic species. Sperm-dependent unisexual lineages experience a combination of some of the costs of both sexual and asexual reproduction: the cost of finding a mate, exposure to sexually transmitted diseases, accumulation of deleterious mutations and lack of genetic recombination. Additionally, because male sperm donors do not gain any fitness advantages (but see Schlupp et al. 1994), the sperm-dependent unisexual individuals are considered sexual parasites, and as a result, selection should favor males that avoid mating with them.

One of the proposed advantages of asexually reproducing species, the lack of the cost of producing males, may be a disadvantage for sperm-dependent lineages. The two-fold reproductive advantage allows populations of the unisexual species to grow at a faster rate and it can become a hindrance to the sperm-dependent species, rather than an advantage, because the parasite can potentially drive its host to extinction and cause its own demise via competitive exclusion when niche differentiation has not occurred. All

known vertebrate gynogenetic or hybridogenetic lineages arose from hybridization events and most of them between very closely related species (Avisé 2008 and references within). Generally, these lineages depend on their parental species for reproduction, but some exceptions are known (Niemeitz et al. 2002; Choleva et al. 2008;). Regardless of how closely or distantly related the unisexual species are to the sperm donor, most sperm parasites must live in sympatry with the host species and cannot colonize new habitats like other parthenoforms (Dawley 1989; Vrijenhoek 1998; Choleva et al. 2008). In a recent model Janko and Eisner (2009) demonstrated that the presence of sperm-dependent lineages can have a long-term negative effect on the sexual host by hindering or even stopping the spatial expansion (and therefore limiting the biogeographic distribution) of the host species.

Models suggest that coexistence between unisexual sperm dependent lineages is possible under numerous circumstances. Schley et al. (2004) showed that coexistence between unisexual sperm-dependent lineages and their hosts is possible when the competitive abilities of the two species differ so that there is a trade-off between growth capacity of the unisexual species and its efficiency as a competitor. Persistence of the sperm dependent parasite was never possible when sexual and asexual populations had equal competitive abilities and carrying capacities (Schley et al. 2003). Doncaster et al. (2000) proposed that coexistence between a parthenogen and a sexually reproducing species with overlapping niches is possible if the effect that the asexual species has on the exploitative abilities (ability to consume the resource of interest) of the sexual species is smaller than the effect that the sexually reproducing individuals have among themselves. More recently, Kokko et al. (2008) demonstrated that coexistence between a gynogenetic

species and a sexual host is possible if the two species live in a metapopulation as long as colonization rates are high enough to counteract the local extinction rates. In this scenario, coexistence is maintained despite local extinctions, because the extinction/colonization dynamics of the metapopulation help to recover extinct populations of the host species and the parasite.

One assumption of the Kokko et al. (2008) model is that the extinction of the sexual host in a patch is immediately followed by the extinction of the parasite and, therefore, no asexual-only patches are considered. Our model examines how asexual-only patches would affect the dynamics of the system. Allowing for the presence of asexual-only patches could have two outcomes. First, the asexual population size within the metapopulation may increase too quickly given that immigration from asexual-only patches is possible together with immigration from mixed patches (patches occupied by both species). As a result, competition between the two species may become more intense and coexistence is favored only under stricter conditions than previously modeled (i.e., higher extinction probabilities for the asexuals). Second, given that the presence of asexual-only patches allows for asexual individuals to also emigrate from (and not just immigrate to) mixed patches it can potentially decrease interspecific competition in these patches. As a result the condition under which maintenance is reached should be more relaxed than when asexual-only patches are not considered (i.e. lower extinction probabilities).

To explore these alternative hypotheses, we developed a stochastic model to determine whether allowing the sperm parasite to persist for one generation in asexual-only patches will facilitate its maintenance in a metapopulation framework. We use a

probabilistic model because it better captures the inherent stochasticity in extinction—colonization dynamics. We found that allowing for the presence of asexual-only patches does decrease the probability of extinction of mixed patches allowing both species to coexist with higher extinction rates and lower colonization rates than when asexual-only patches are not present in the metapopulation.

Materials and Methods

We developed a stochastic metapopulation model to explore how introducing asexual-only patches affect the coexistence of a sexual host species and its asexual parasite. The model was built using R (r-project). Our model was not spatially complex as we assumed that all patches were equally connected. Additionally, we were concerned with dynamics at the patch level, not the individual level (Fig. 4).

We simulated 100 patches of four types: (a) patches with sexual host species only (P_s), (b) patches with the sexual host and unisexual parasite (i.e. mixed patches, P_m), (c) patches with the unisexual parasite species (P_a), and (d) empty patches (P_e). We started all simulations with $P_s = 30$, $P_m = 30$, $P_a = 0$, and $P_e = 40$ and ran the model for 5000 iterations equal to 5000 generations. We chose 5000 iterations because running the model for more iterations did not provide any additional information. For each model iteration, species went extinct or colonized new patches probabilistically given the patch extinction and colonization probabilities as defined in Table 3 (Fig. 4).

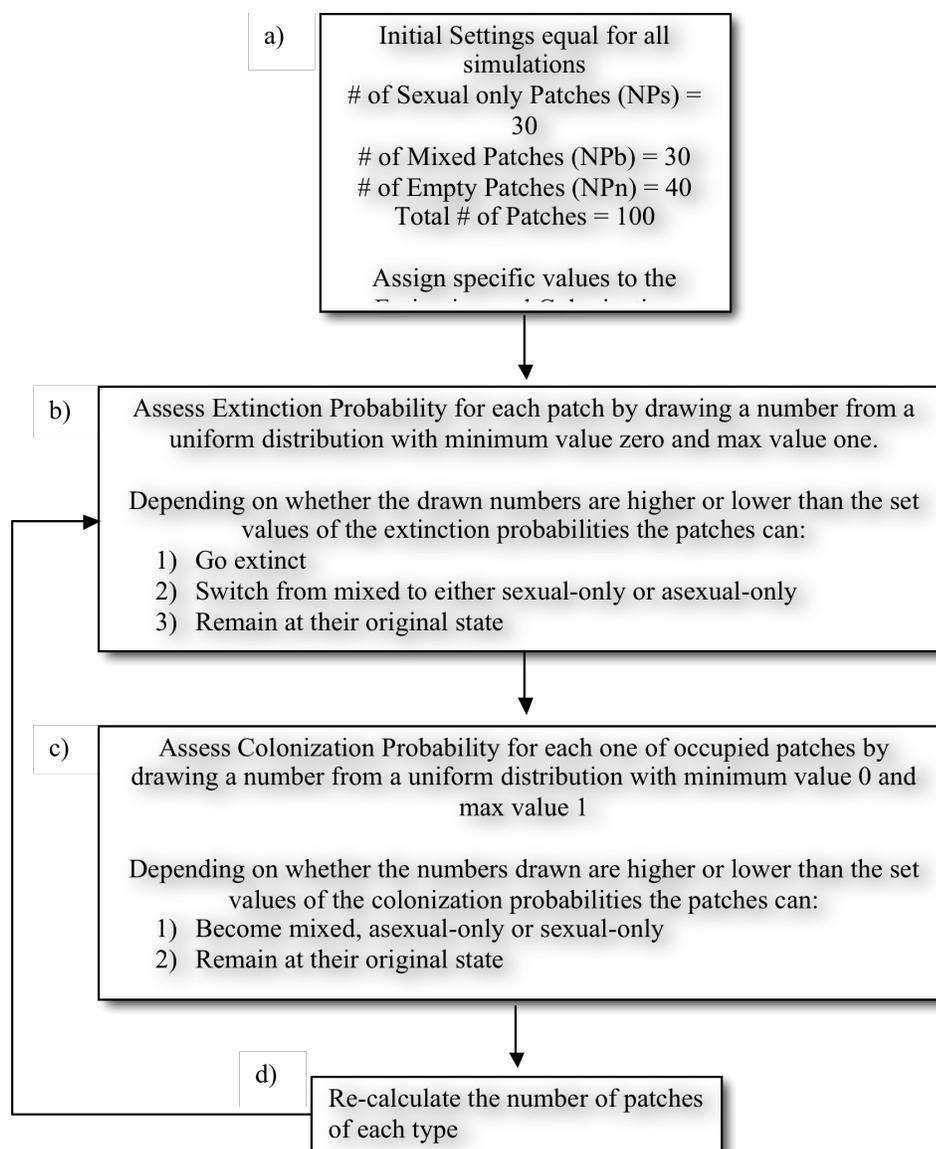


Figure 4. Outline of one model iteration. (a) Initial step: programmer sets values of the simulation parameters. (b) Model assesses extinction probabilities of each patch. (c) Model assesses colonization probabilities of each patch. (d) “state” of each patch is re-assessed and the number of patches of each type (summarized in Table 3) is re-calculated.

Table 3. Model Variables and their Definitions.

Variable	Definition
E_s	Extinction prob. for sexuals alone
$E_{s,m}$	Extinction prob. for sexuals in mixed patches
E_a	Extinction prob. for asexuals alone
$E_{a,m}$	Extinction prob. for asexuals in mixed patches
C_s	Colonization prob. for sexuals
$C_{s,a}$	Colonization prob. for sexuals with asexuals present
C_a	Colonization prob. for asexual
$C_{a,s}$	Colonization prob. for asexuals with sexuals present
P	Number of patches (total)
P_s	Number of patches with sexual species only
P_a	Number of patches with asexual species only
P_b	Number of mixed patches
P_e	Number of empty patches
Waiting period	Unisexuals occupy asexual-only patches for one iteration

The main difference between the Kokko et al. (2008) model and the one described herein is that we allowed a patch to be occupied by only unisexual females for one iteration (i.e, one generation); whereas in the Kokko et al. (2008) model, the extinction of the sexual species in a patch was immediately followed by the extinction of the asexual species. In our model, when the patch was not re-colonized by the sexual host within one generation, the patch would go extinct, but if re-colonization took place, then further mating could occur and the parasite would be maintained/rescued locally. For this reason the extinction probability of the asexual individuals when in asexual-only patches (E_a) was always assigned a value of 1 so that if a patch inhabited by only the asexual lineage was not colonized by the host within a single iteration, then that patch would go extinct. The extinction probability of the host when in mixed patches ($E_{s,m}$) was always assigned a

higher value than the extinction probability of the host when in sexual-only patches (E_s) to account for and mimic the costs of coexistence.

We varied colonization and extinction probabilities and the ability of the unisexual lineage to occupy asexual-only patches for one iteration (refer to Table 3 and Fig. 5, 6). For each combination of parameters that we examined, we conducted 20 replicate simulations (performing more simulations did not yield different results). We first found the lowest probability of extinction that did not result in coexistence and then examined how increasing colonization probabilities affected coexistence. Each colonization and extinction parameter combination was explored with and without the ability of the asexuals to wait for the host for one iteration. We used unpaired t-tests to determine whether the proportion of mixed patches (P_m) differed between treatments with and without a waiting period. T-tests were done for each value of the parameter we examined. No multiple comparisons were performed.

Results

The ability to persist for one generation in asexual-only patches significantly increased the proportion of mixed patches present in a metapopulation, directly affecting coexistence between the parasite and its host species (Fig. 5, 6). This effect occurred over a range of values associated with different rates of extinction and colonization.

An increased probability of extinction of the host in mixed patches ($E_{s,m}$) and of the asexual individuals in mixed patches ($E_{a,m}$) both resulted in a decreased proportion of mixed patches regardless of the ability of the unisexual individuals to wait for the sexual host for one generation. However, when the extinction probability $E_{s,m}$ was set to 0.12 and asexuals were not allowed to persist for one generation without the host, then 100%

of the mixed patches became sexual-only. When asexual-only patches were present in the simulations, only 16% of the mixed patches changed state. When the extinction probability $E_{a,m}$ was lowered to 0.065, 100% of the mixed patches became sexual-only when asexuals could not wait one generation, whereas only 50% of the mixed patches went extinct when the condition was met.

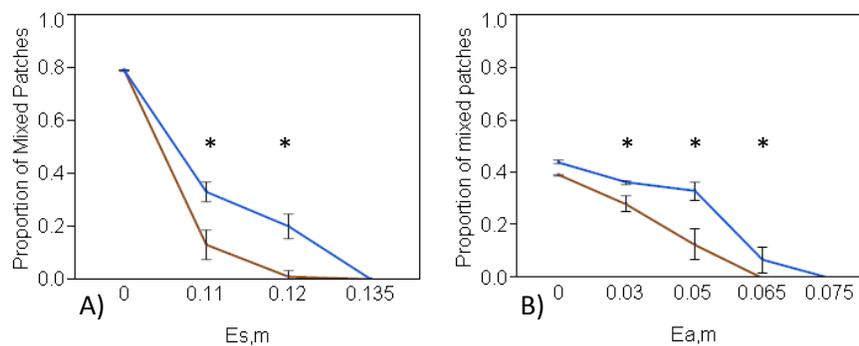


Figure 5. Proportion of occupied mixed patches relative to extinction rates. Except where stated otherwise, we used the following values for model parameters: $E_a = 1$, $E_{s,m} = 0.11$, $E_s = 0.1$, $E_{a,m} = 0.05$, $C_s = 0.003$, $C_a = 0.003$, $C_{s,a} = 0.0028$ and $C_{a,s} = 0.0023$. The following parameters were altered for individual sets of simulations: (a) $E_{s,m}$ and (b) $E_{a,m}$. The asterisks indicate a $P < 0.05$ (unpaired t-test). The blue line indicates simulations where a waiting period of one generation was allowed while the brown line indicates simulations where the condition was not met.

Increasing the colonization probabilities of the asexual or sexual species increased the proportion of mixed patches regardless of the values we used for the extinction

parameters (Fig. 6). For all extinction parameters, the ability of the unisexual to wait for the host did not have a detectable effect when we varied the colonization probability of the sexual species (C_s ; Fig. 6A, E) or the probability of the parasite to colonize patches where the sexual species was present ($C_{a,s}$; Fig. 6D, H). The ability of the unisexual lineage to persist for one generation without hosts affected the model results when we varied the probability of the sexual host to colonize patches where the asexual species was present ($C_{s,a}$; Fig. 6B, F) or the colonization probability of the asexual (C_a ; Fig. 6C, G). The effect of the waiting period was particularly pronounced when the probabilities of the sexual host to colonize patches with the parasite ($C_{s,a}$) and the colonization probability of the parasite (C_a) were low. When we set the colonization probability $C_{s,a}$ to 0.005 and the extinction probability $E_{s,m}$ to 0.135 and a waiting period was allowed, after 5000 iterations less than 0.05% of patches went extinct while 50% of the patches went extinct if the condition was not met (Fig. 6B). A similar result was found when looking at the colonization probability $C_{a,s}$ (Fig. 6C). The most striking difference was found when the probability of colonization of the parasite (C_a) was set to a low 0.005 and the extinction parameter $E_{a,m}$ was lowered to 0.075. In this instance the probability of mixed patches to become sexual-only patches was 0.03% when a waiting period was allowed and close to 100% when the condition was not met (Fig. 6G).

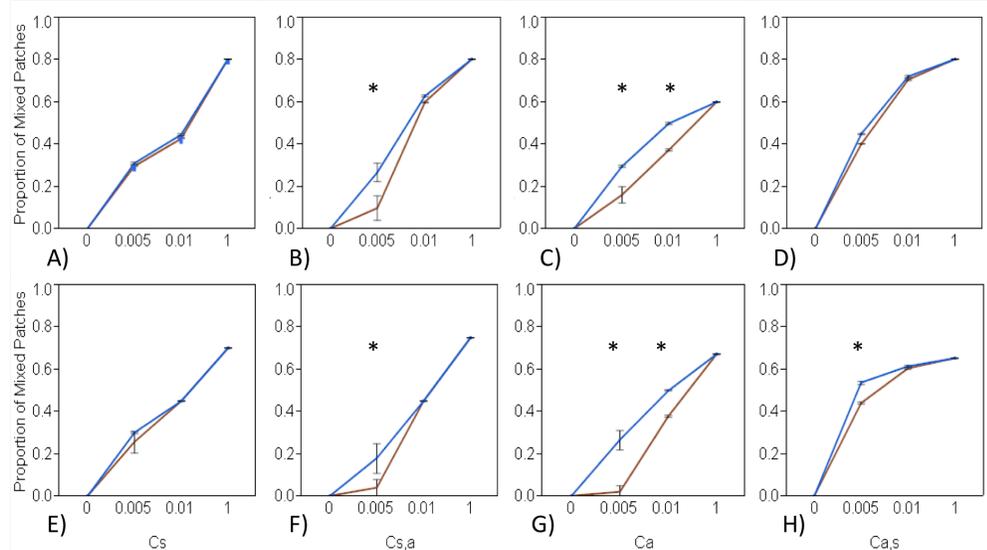


Figure 6. Proportion of occupied mixed patches relative to colonization rates. (a-d)

$E_{s,m} = 0.135$, while (e-h), $E_{a,m} = 0.075$ and only one colonization rate was changed in each graph, while all the other parameters were assigned the same values as for graph in Fig. 1. Asterisks indicate a $P < 0.05$ (unpaired t-test) in the difference of occupied mixed patches. The blue line indicates simulations where a waiting period of one generation was allowed while the brown line indicates simulations where the condition was not met.

Discussion

In situations in which the host is not an efficient colonizer of patches already inhabited by the unisexual species (low $C_{s,a}$) or the sperm-dependent individuals are not good colonizers of patches (low C_a), then the ability wait for the sexual host for one generation significantly increases the probability of persistence of the unisexual species by providing the time lag necessary for colonization to occur and counteract the local extinction.

The two-fold reproductive advantage that unisexual species have over sexually reproducing species, due to the benefit of not producing males, can potentially become a disadvantage when the unisexual species is a sexual parasite. This is because the sexual parasites depend closely on the sexual hosts for reproduction and can drive the hosts to extinction by competitive exclusion when niche overlap is substantial (Vrijenhoek 1984; Doncaster et al. 2000; Schley et al. 2004). This potential problem may be faced by many unisexual species in a mixed-mating complex, although some species have been found to not be as dependent on the hosts as others (Graff and Polls Pelaz 1989).

Sperm-dependent unisexual species can compete with their hosts for resources, space or possibly mates and it is likely that multiple mechanisms facilitate coexistence. Furthermore, different mechanisms may have greater influences in different unisexual-bisexual mating complexes. Higher mortality rates as well as a lower tolerance to stressful conditions in the unisexual species counteract the effects of the two fold reproductive advantage of the parasites over the hosts. For example, in the *Cobitis* complex (*C. elongatoides* – *C. taenia* – *C. tanaitica*), gynogenetic triploids have a lower fecundity than their sexual counterparts (Juchno and Boron 2010). The gynogenetic *Poecilia formosa* (*P. latipianna* – *P. formosa* – *P. mexicana* complex) was also found to not have a two-fold reproductive advantage over its host *P. latipinna* (Alberici da Barbiano et al. 2011). Frequency-dependent male mate choice is another mechanism by which the unisexual population can be prevented from growing exponentially and therefore facilitating coexistence. In the *Poeciliopsis monacha-lucida* complex, for example, frequency-dependent male mate choice can cause fluctuations in the unisexual population, thus preventing the unisexual species from outcompeting their sexual hosts

over time (Moore and McKay 1971). However, this mechanism doesn't seem to explain the maintenance of the *P. latipinna-formosa* complex, where at least 30% of *P. formosa* are inseminated regardless of their frequency (Alberici da Barbiano et al. 2011). Thus, it is hard to establish one general mechanism influencing coexistence that applies to all known unisexual-bisexual mating complexes. In some systems, the unisexual species have been able to expand their ranges beyond the ones of the parental species (Choleva et al. 2008), can use multiple hosts (Niemeitz et al. 2002), or can even establish asexual-only populations (Dawley 1989 and references within).

Although sexual selection is likely a driving force behind the maintenance of unisexual-bisexual mating complexes, Kokko et al. (2008) showed that metapopulation dynamics alone, without considering male mate choice or fertilization rates, can be enough to explain the maintenance of unisexual-bisexual mating complexes, when both species are good colonizers. With the present model we corroborated Kokko et al. (2008) results and showed that allowing the asexual species to wait for the host species for one generation in asexual-only patches can facilitate persistence by compensating for relatively lower colonization probabilities and higher extinction probability than previously modeled.

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CHAPTER IV

THE IMPACT OF SOCIAL CONTEXT ON MALE PREFERENCE IN A UNISEXUAL-BISEXUAL MATING COMPLEX¹

Abstract

Male sailfin mollies *Poecilia latipinna* were tested in five different treatments that varied in the relative frequency of heterospecific gynogens (Amazon molly *Poecilia formosa*) to conspecific females to determine whether social interactions among males within a population causes some males to mate with heterospecific females. Male *P. latipinna* inseminated a significantly higher proportion of conspecific females and fertilized a significantly higher number of conspecific eggs regardless of the treatment. Nonetheless, preference for conspecific females was not exclusive as a range of 20 to 50% of heterospecific females were fertilized. Social interactions among males may best explain the results and may therefore play an important role in the maintenance of unisexual–bisexual mating complexes.

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Introduction

Selection often favors individuals that evolve a mate preference for conspecific mates (Coyne and Orr 2004). Mating with heterospecifics can result in a lowering of an individual's fitness because hybrid offspring may be non-viable, sterile or, in the particular instance of a unisexual–bisexual mating complex where the unisexual species reproduces by gynogenesis, the offspring will not inherit the paternal genes.

Unisexual–bisexual mating systems are found when an all-female species depends on the sperm of a closely related species for reproduction (Dawley and Bogart 1989).

Gynogenesis is a form of asexual reproduction used by several unisexual species that requires sperm from heterospecific males to trigger embryogenesis but no syngamy occurs and no genetic information of the male is inherited by the offspring (although some exceptions are known: Graf and Polls Pelaz 1989; Scharl et al. 1995a).

Gynogenetic females are thus clonal and must live in sympatry with the sperm donor species (sexually reproducing host species).

Here, the focus is on the Amazon molly *Poecilia formosa* (Girard 1859) and Sailfin molly *Poecilia latipinna* (LeSueur 1821) unisexual–bisexual mating system to examine the effect of male–male interactions on the frequency of heterospecific mating. *Poecilia formosa* is an internally fertilizing ovoviviparous fish that lacks parental care. *Poecilia formosa* arose from a hybridization event between an Atlantic molly *Poecilia mexicana* Steindachner 1863 and *P. latipinna* c. 100 000 years ago (Avisé et al. 1991; Scharl et al. 1995b); although there is some controversy about this time period (Dries 2003). The gynogenetic *P. formosa* must mate with males of its parental species to trigger embryogenesis, however, males of the parental species exhibit a stronger preference for

conspecific females over heterospecific mates (Ryan et al. 1996; Gabor and Ryan 2001). Given the lack of benefits from mating with gynogenetic females (see Schlupp et al. 1994 for exceptions), mating mistakes on the part of males in this and other unisexual–bisexual complexes are fundamental in maintaining the unisexual sperm-dependent species over time. Nonetheless, sometimes it may be advantageous for males to mate indiscriminately with both conspecific and heterospecific females depending on the cost of maintaining a strong mate preference (Heubel et al. 2009).

Ecological or behavioral mechanisms must be in place to prevent unisexual species from outcompeting their sexual hosts given the two-fold reproductive advantage asexual species have over sexually reproducing species (Maynard Smith 1968). Recently, Schlupp et al. (2010) found that *P. formosa* has equal fecundity to its host *P. latipinna* and suggested that ecological factors, such as higher mortality among juvenile *P. formosa*, prevent *P. formosa* populations from growing exponentially as predicted by theory (Schlupp et al. 2010). An alternative, non-mutually exclusive hypothesis is that frequency-dependent male mate choice can cause fluctuations in the unisexual population, thus preventing the unisexual species from outcompeting their sexual hosts over time (Moore and McKay 1971).

There is little knowledge about the effect of the composition of potential mating partners (*e.g.* the frequency of conspecific and heterospecific females in a shoal) on the plasticity of mate choice (Alonzo and Sinervo 2001). Males may express frequency-dependent mate choice depending on social interactions with other males. For example, competition for conspecific mates may become more pronounced when conspecific females are less frequent in a population, therefore subordinate males may mate more

with heterospecific females, because they have limited or no access to conspecific mates. In the unisexual–bisexual complex of *Poeciliopsis monacha-lucida* Miller 1960, Moore and McKay (1971) found that when only one male or multiple males of the same age, not organized in strict hierarchical scale, were given access to both conspecific and heterospecific females, only the eggs of conspecific females were fertilized. When males were of different ages (and therefore organized in a strict hierarchical scale), some heterospecific females were fertilized as well as conspecific ones (Moore and McKay 1971). Kawecki (1988) showed that males that are subordinate tend to mate with heterospecific females because they have a shorter assessment time available, given that part of their mate searching time is limited by constant aggressive interactions with dominant males. When assessment time is limited, indiscriminate mate choice is more probable (Schmeller et al. 2005).

Mate choice involves two processes: species recognition and mate quality recognition (Ryan and Rand 1993). The two processes may interfere with each other when heterospecific females resemble high-quality conspecific females (Pfennig 1998). In the *P. formosa* and *P. latipinna* mating system, the hybrid *P. formosa* closely resembles females of their parent species. If males are not given enough time to assess the potential mates, then they may incur a mate quality-species recognition conflict. Indeed, male *P. latipinna* from some sympatric populations show a conflict between species and mate quality recognition when heterospecific females are larger and thus resembled high-quality conspecifics (Gumm and Gabor 2005). In this system, large female size is preferred by males (Ptacek and Travis 1997; Gabor 1999), and larger females are more fecund (Farr 1989).

In the *P. formosa* and *P. latipinna* system, male *P. latipinna* do not organize themselves in complex social dominance hierarchies, but large males have been observed to be dominant over smaller males (Baird 1968; Woodhead and Armstrong 1985; Travis and Woodward 1989) and both sperm-dependent and host-species females prefer to mate and associate with larger males (Marler and Ryan 1997; Ptacek and Travis 1997; Gabor 1999). Additionally, smaller males tend to resort to sneak copulations when large males are present and are chased away by larger males, who tend to show courtship behaviors towards females (Travis and Woodward 1989). These aggressive interactions cause smaller males to have a shorter assessment time, which may result in smaller males mating with smaller females or even heterospecifics.

Most mate-choice studies performed on mollies have presented a male with a choice of two females and were not aimed at analyzing the interactions among males (apart from Plath et al. 2008). In the present study, whether social context influences male mate choice was tested. This question was approached by manipulating the relative frequency of conspecific and heterospecific females in mixed shoals of *P. formosa* and *P. latipinna* to assess whether the relative availability of conspecific females affected male–male interactions, potentially resulting in some males mismating more often and a higher proportion of heterospecific females being inseminated. The proportion of fertilized females of each species within each replicate was analyzed as the response variable. If females of the unisexual species are very frequent in a population while conspecific females are rare, male–male competition for high-quality mates (*i.e.* conspecifics) will be high and larger host-species males should be more likely to secure those matings while smaller males may be more prone to mate with heterospecific females.

Materials and Methods

Fishes were collected during the summer 2006 and 2008 from a locality in northern Mexico (25° 18' 0" N; 97° 51' 36" W) where *P. formosa* and *P. latipinna* occur in sympatry. Fishes were transported to San Marcos, TX, U.S.A. and housed in 2100 l outdoor tanks at the Biology Department greenhouse. Experiments were conducted during September to October 2006, March to October 2007, March to April 2008 and April 2010. Fish smaller than 20 mm standard length (*LS*) were brought into the laboratory and reared indoors and kept on a 14L:10D cycle. Males were removed to make sure that all tested females were virgins. Poeciliid males develop a gonopodium *via* fusion of the anal fin rays when they become sexually mature, making it easy to identify and remove males from the rearing tanks before they are fully matured. Poeciliid females can store sperm up to several months (Constantz 1989); therefore, virgin females were used to ensure that any inseminations took place during the trials. Fishes were fed once daily with brine shrimp *Artemia* sp. (Bio-Marine; www.aquafauna.com) and Spirulina Flakes mixed with Freshwater Flake food (Ocean Star International Inc.; www.osishipping.com).

The experiment consisted of five treatments each replicated six times in 378.5 l outdoor mesocosms. Six males of different *LS* were tested in each replicate: two small males (22–28 mm), two medium males (28–35 mm) and two large males (>35 mm). Testing males of different sizes mimicked more closely the composition of natural shoals and allowed for social interactions to occur. In each replicate, the six males were tested with 18 females to reproduce the 3:1 sex ratio that is found in natural populations (Hubbs, 1964). The treatments were as follows: (1) all *P. latipinna* females: 18 conspecific

females (control 1), (2) high *P. latipinna* females: 15 *P. latipinna* females and three *P. formosa* females, (3) equal ratio: nine *P. latipinna* and nine *P. formosa* females, (4) high *P. formosa*: three *P. latipinna* females and 15 *P. formosa* females; and (5) all *P. formosa* females: 18 *P. formosa* females (control 2). By keeping female densities constant, but varying the frequencies of gynogenetic females, the effects of changing frequency of the gynogenetic species on the reproductive success of females of both species were analyzed. A total of 540 females and 180 males were tested for a total of six replicates per treatment, but fewer individuals were included in the analyses because the last replicates of treatments 1 and 5 had to be discarded due to severe weather conditions that decimated the fish population in both trials. An attempt to match female sizes in treatments with both species was made, but sperm-dependent females were larger on average than host-species females (Table 4). Fishes were maintained in the mesocosms for 28 days. A mesh screen was placed on top of the testing tanks to prevent predation. Fishes were fed once daily, and apart from feeding, they were left undisturbed until the conclusion of the testing period. Fishes were removed on the 28th day (the average brood cycle is 30 days; Constantz 1989), to retrieve the embryos before the females dropped their broods, though this may have resulted in an underestimate of fertilization rates. This effect, however, should be spread equally across all treatments as the two species do not have significantly different brood cycles (Hubbs and Dries 2002).

Table 4. Female standard length (L_s) \pm SE differences between species at the beginning (L_{si}) and the end (L_{sf}) of the testing period. ΔL_s indicates the change in L_s during the 28 days testing period. Wilcoxon sums-rank tests, were performed whenever the variances associated with the measurements were significantly different between species, otherwise t-tests were performed.

Treatment	<i>P. latipinna</i>	<i>P. formosa</i>	Z or t	P
(2) High <i>P. latipinna</i> females L_{si}	31.2 \pm 0.4	33.7 \pm 1.9	Z = 1.38	0.17
(2) High <i>P. latipinna</i> females L_{sf}	38.7 \pm 0.4	43.4 \pm 1.1	t = -3.99	<0.01*
(2) High <i>P. latipinna</i> females ΔL_s	8.13 \pm 0.8	8.15 \pm 1.6	t = -0.01	0.99
(3) Equal ratio L_{si}	29.4 \pm 0.8	33.7 \pm 1.4	Z = 2.42	0.01*
(3) Equal ratio L_{sf}	37.3 \pm 0.4	41.6 \pm 0.6	Z = 5.74	<0.001*
(3) Equal ratio ΔL_s	7.55 \pm 0.9	7.55 \pm 1.3	t = -0.001	0.99
(4) High <i>P. formosa</i> L_{si}	29.9 \pm 0.6	38.1 \pm 1.2	Z = -2.53	<0.05*
(4) High <i>P. formosa</i> L_{sf}	38.4 \pm 1.0	43.3 \pm 0.3	Z = -4.65	<0.001*
(4) High <i>P. formosa</i> ΔL_s	6.82 \pm 2.0	5.43 \pm 1.2	t = 0.63	0.54
(1) All sexuals vs (5) all asexuals L_{si}	31.9 \pm 0.6	35.6 \pm 0.6	t = -5.08	<0.001*
(1) All sexuals vs (5) all asexuals L_{sf}	40.9 \pm 0.4	41.5 \pm 0.5	t = -0.91	0.30
(1) All sexuals vs (5) all asexuals ΔL_s	10.4 \pm 0.9	5.0 \pm 0.7	t = 4.73	<0.001*

* Indicates significant values

At the end of the experiment, females were euthanized using four drops of clove oil in 40 ml of water and dissected. The number of fertilized eggs as well as the proportion of fertilized females in each treatment was recorded. Females' LS was

measured both at the beginning (L_{si}) and at the end (L_{sf}) of the testing period.

Statistical analyses

To determine if the relative frequency of conspecific to heterospecific females affected the proportion of fertilized females of either species, a generalized linear model on the arcsine-transformed proportion of fertilized females with main effects of treatment, species and season was performed. The season effect was a nominal fixed effect that takes into consideration the time within the breeding season (early = March to May; middle = June to August; late = September to October). The control treatments (1 and 5) were excluded from this analysis. A Levene's test for equality of variances was performed including all treatments to determine if the variances around the mean proportion of fertilized females varied depending on the treatment. A Wilcoxon rank-sum test was performed between treatments 1 and 5 to test whether conspecific and heterospecific females were fertilized at different proportions when they were the only species of females available for males to mate. To test whether the proportion of fertilized females in each treatment translated into different fitness consequences for either conspecific or heterospecific females, a mixed factor ANCOVA was performed on the number of fertilized eggs with female final LS (L_{sf}) as a covariate and treatment, species and season as effects. A last set of analyses was conducted to compare the fecundity of the two species relative to each other and to the findings of Schlupp et al. (2010). The ratio of female *P. formosa* embryos to female *P. latipinna* embryos was calculated by dividing the total number of fertilized eggs for each female by the mother's L_{sf} to control for the fact that larger females produce more eggs and had more fertilized

eggs (*Lsf* was a significant effect in the ANCOVA performed in the present study). The value obtained for *P. latipinna* was then divided by two to obtain the number of female embryos following Snelson and Wetherington (1980) who found that poeciliids show a 1:1 male:female embryo ratio. For *P. formosa*, the total number of fertilized eggs was only divided by the mother's *Lsf* because they only produce females. A Wilcoxon signed-rank test was used to compare the number of female embryos of each species.

Results

The relative frequency of heterospecific to conspecific females did not have an effect on the proportion of females fertilized. There was no significant difference in the arcsine-transformed proportion of fertilized females between the treatments that had both female species present, whereas there was a significant effect of species and season (Table 5). A significantly higher proportion of conspecific females were fertilized (*P. latipinna*: mean \pm s.e. = 0.65 ± 0.08 ; *P. formosa*: 0.35 ± 0.08 ; Wilcoxon signed rank, $W_{1,18} = 45.5$, $P < 0.001$). Although the time of the breeding season had a significant effect (Table 5), the lack of a significant interaction with either treatment or species suggests that both species were affected similarly. Finally, there was a significantly higher proportion of conspecific females with fertilized eggs when the two control treatments (1 and 5), where males had no choice of species, were compared (*P. latipinna*: mean \pm s.e. = 0.80 ± 0.03 , *P. formosa*: 0.34 ± 0.13 , Wilcoxon rank-sums $Z_{1,10} = 2.44$, $P < 0.01$).

Table 5. Effect tests for the generalized linear model on the proportion of fertilized females across treatments, seasons and species.

Main Effects	d.f.	Chi Square	<i>P</i>
Treatment	2	0	1.00
Species	1	7.51	< 0.01*
Season	2	9.32	< 0.01*
Treatment*Species	2	3.04	0.24
Treatment*Season	4	4.52	0.34
Species*Season	2	0	1.00
Treatment*Season*Species	4	0.04	0.99

*Indicates significant values

Although the frequency of heterospecific gynogenetic females did not have an effect on the proportion of fertilized females of either species across treatments, it had an effect on the variance associated with the proportion of fertilized conspecific females. When the variances around the mean of each treatment (including 1 and 5) were compared for each species, there was a significant difference for the host species (Levene's test, $P < 0.001$) but not for the unisexual *P. formosa* (Levene's test, $P > 0.05$). The variance associated with treatments 1, 2 and 3 for *P. latipinna* (host) were significantly smaller than the variances associated with treatment 4 (high unisexual females; Fig. 7).

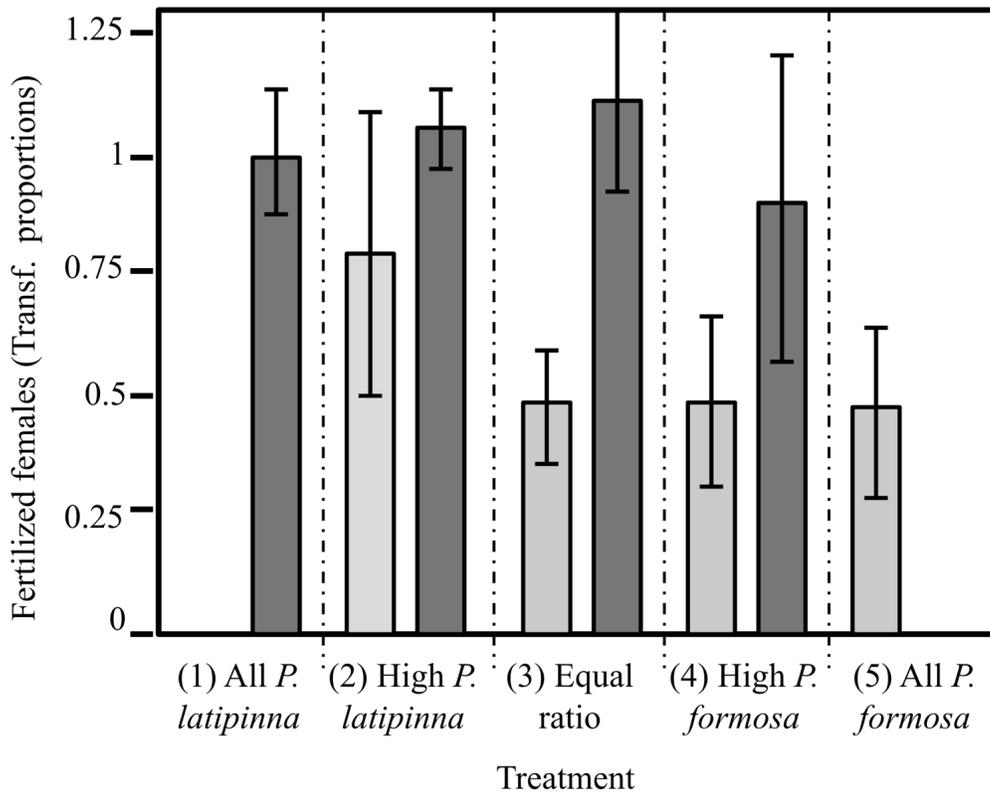


Figure 7. Proportion of fertilized females (arcsine transformed) by species (Host species *Poecilia latipinna* in dark grey, sperm-dependent species *P. formosa* in light grey) within treatments. The treatments were as follows: (1) All *P. latipinna* females: 18 conspecific females (control 1), (2) High *P. latipinna* females: 15 *P. latipinna* females and 3 *P. formosa* females, (3) Equal ratio: 9 *P. latipinna* and 9 *P. formosa* females, (4) High *P. formosa*: 3 *P. latipinna* females and 15 *P. formosa* females, and (5) All *P. formosa* females: 18 *P. formosa* females (control 2). The variance associated with treatments (1), (2) and (3) for *P. latipinna* were significantly smaller than the variances associated with treatment (4) (Levene's test, $P < 0.01$).

Male *P. latipinna* prefer to associate with larger females (Ptacek and Travis 1997; Gabor 1999), but a higher proportion of conspecific females were fertilized across treatments even though they were on average smaller than heterospecific *P. formosa* (Table 1). Although an attempt to size match females at the beginning of the testing period was made, heterospecific females were significantly larger than conspecific females in all but treatment 1 (Table 1). Larger females produce more eggs and have higher fitness (Travis et al. 1990). Despite being smaller, however, *P. latipinna* had a larger number of fertilized eggs across all treatments (*P. latipinna*: mean \pm s.e. = 13.1 ± 0.8 ; *P. formosa*: 5.2 ± 0.6 ; Wilcoxon rank-sums, $Z_{1,489} = -8.3$; $P < 0.001$).

The ANCOVA performed using treatments 2, 3 and 4 indicated significant effects for the covariate *Lsf*, the main effects species and season, as well as a weak interaction between species and *Lsf* on the number of fertilized eggs per female (Table 6). Given the lack of an interaction between season and species, no further analyses were performed. *Poecilia latipinna* produced more female embryos than *P. formosa* (*P. latipinna*: mean \pm s.e. = 0.1 ± 0.1 ; *P. formosa*: 0.12 ± 0.02 Wilcoxon rank sums, $Z_{1,311} = -4.3$, $P < 0.001$).

Table 6. Effect and covariate tests for ANCOVA on the number of fertilized eggs across treatments, species and seasons. Sl_f is defined as the standard length of the females at the end of the 28-day trials. Only treatments (2), (3) and (4) were used for this analysis

Main Effects	d.f.	<i>F</i>	<i>P</i>
Species	1	11.3	< 0.01*
Treatment	2	0.14	0.87
L_{sf}	1	3.96	< 0.05*
Season	2	3.17	< 0.05*
Species*treatment	2	1.62	0.20
Species* L_{sf}	1	4.00	< 0.05*
Species*season	2	2.13	0.12
Treatment* L_{sf}	2	0.63	0.53
Treatment*season	4	1.92	0.10
L_{sf} *season	2	1.49	0.23
Species*treatment* L_{sf}	2	0.67	0.51
Species*treatment*season	4	2.39	0.05
Species* L_{sf} *season	2	0.11	0.89
Species*treatment* L_{sf} *season	4	0.27	0.89
Treatment* L_{sf} *season	4	1.8	0.13

Discussion

Male *P. latipinna* fertilized a higher proportion of sexually reproducing conspecific females than gynogenetic heterospecific females across the breeding season regardless of the relative frequency of sexual to asexual females. The variance around the mean proportion of fertilized conspecific females across treatments increased as the number of unisexuals increased, suggesting that perhaps social interaction among males affects their mate choice. When conspecific females were abundant (treatments 1, 2 and 3; Fig. 7), there was little variance associated with the mean proportion of fertilized conspecific females and the variances were not statistically different between treatments, suggesting that males behaved similarly across the treatments. The variance for the mean number of fertilized conspecific females associated with treatment 4, when unisexuals were more abundant, was significantly larger than the ones associated with treatments 1, 2 and 3 for *P. latipinna* (Fig. 7). These results suggest that as the availability of conspecific females decreases and, therefore, male–male competition increases, then males are not as consistent in their mate preference as when conspecific females are abundant. The variances for the mean proportion of fertilized unisexuals were not statistically different across treatments, suggesting that host-species males behaved similarly towards unisexuals regardless of their frequency.

Males may be more or less choosy depending on the context. When gynogenetic females constituted >50% of the female population c. 30% had developing embryos, while when they comprised 16% of the female population 50% had developing embryos. These results are in agreement with the findings of Moore & McKay (1971), who recorded that in natural populations of *Poeciliopsis* where unisexuals constituted >90%

of the female population, only 5% were fertilized, whereas in populations where the unisexuals accounted for just 65% of the female population then 95% of them were fertilized. The results are also concordant with the findings of Heubel et al. (2009) who suggested that it might not always be beneficial for males to be choosy and discriminate against heterospecific matings despite the current population composition.

The preference for *P. latipinna* females was not exclusive, as several *P. formosa* were fertilized in most replicates. These mating events may have been due to the mistakes of smaller males, or unisexuals might have been inseminated after the males had mated with all available conspecific females. Differentiating between these two hypotheses is impossible with the data at hand. Additionally, when males were presented with only *P. formosa* (treatment 5), they mated with *c.* 30% of the females. Therefore, it can be inferred that, on average, at least *c.* 30% of heterospecific females are inseminated by male *P. latipinna* regardless of the population composition. Considering these results in the long term, 30% may still be enough inseminated females for the gynogenetic species to have enough offspring present in the next generation and explain the maintenance of this unisexual population. The results for treatment 5 support the claim by Hubbs (1964) that males attempt to court and mate with heterospecific females if conspecifics are not available. Nonetheless, it is obvious that such a situation in nature would not persist for long given the life span of the males and the fact that without the host males the sperm-dependent species cannot reproduce. A recent theoretical model, however, has shown how metapopulation dynamics with recurrent colonization events (Kokko et al., 2008) can be sufficient to explain the maintenance of unisexual–bisexual mating complexes when local extinction of populations occurs. Therefore, male

permissiveness may be playing an important role in the maintenance of the unisexual species.

Variation in mating behavior among male *P. latipinna* across populations has been recorded (Ptacek and Travis 1997; Gabor and Ryan 2001; Gumm and Gabor 2005; Gabor et al. 2010) as well as seasonal plasticity (Heubel and Schlupp 2008), but males from the specific population tested in the present study have shown a relatively constant preference for conspecific females. The preference for conspecific females was not lost even when heterospecific females were larger (Gumm and Gabor 2005). Additionally, a preference for mid-sized females and not larger females was recorded for the males of the population used in the present study (Gabor et al. 2010). These previous findings are supported with the present results. In the experimental treatments (2–4), growth was not different between species (Table 4) and heterospecific females were larger than conspecific females at the end of all these treatments. Female *Poecilia formosa* were significantly larger than *P. latipinna* females but, despite this, no evidence was found of a species-mate quality recognition conflict. On the contrary, male *P. latipinna* fertilized more conspecific females than heterospecific females in all treatments. These results, coupled with the results of previous work, suggest that perhaps males in this particular population have evolved a strong preference for conspecific females and have overcome the mate quality-species recognition conflict as previously suggested by Gumm & Gabor (2005).

Asexually reproducing organisms are predicted to have a two-fold reproductive advantage over sexually reproducing ones because they do not incur the cost of producing males (Maynard Smith, 1968). In the present study, this theoretical prediction

was not supported. Not only was a higher proportion of conspecific females fertilized regardless of the frequency of the gynogenetic heterospecific females but also a higher number of conspecific eggs. Sexually reproducing *P. latipinna* females on average had a higher number of fertilized eggs than gynogenetic *P. formosa*. *Poecilia formosa* did not have a two-fold reproductive advantage over host-species females based on the number of female embryos produced per female whereas Schlupp et al. (2010) found the opposite results. The results reported here are more in agreement with the findings of Riesch et al. (2008).

Both the present results and Riesch et al. (2008) suggest that host-species males inseminate more conspecific females than heterospecific ones. Heubel and Schlupp (2008) did not find support for this conclusion. Although Heubel and Schlupp (2008) did not find evidence for any frequency-dependent regulating mechanisms, which comports with the present study, Heubel and Schlupp (2008) found a significant interaction between season and species on the association preference of host-species males, whereas no effect of an interaction on the proportion of fertilized females of either species was detected in the present study. The present results may be different from those of Heubel and Schlupp (2008) because association preference in a controlled setting was not the response variable of interest, instead male preference was extrapolated from the proportion of females they inseminated and fertilized in both species in a mesocosm setting. Overall, in the present study, the presence of the sperm parasite *P. formosa* does not appear to have a two-fold advantage over the host species and male *P. latipinna* show a clear preference for conspecific over heterospecific females independent of season. The frequency of unisexual sperm-dependent individuals varies in time and space within

a unisexual–bisexual mating system (Hubbs 1964; Booij and Guldemond 1984; Vrijenhoek 1994; Heubel et al. 2009), but although population dynamics were not tested over an extensive period of time, the present findings may still have a long-term predictive significance. The lack of frequency-dependent mating preference on the part of males and the constant higher fecundity of host-species females regardless of the frequency of heterospecific unisexual females suggest that this variation in frequency over time is most probably due to ecological mechanisms such as lower tolerance to stressful food or temperature conditions or competition with the host species for resources (Alberici da Barbiano et al. 2010; Tobler and Schlupp 2010).

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CHAPTER V

ORIGINS AND POPULATION GENETICS OF *POECILIA FORMOSA*

Abstract

Unisexual sperm-dependent vertebrates are of hybrid origins, extremely rare, and are predicted to be short-lived because several selective pressures act upon them at the same time. Firstly, unisexual vertebrates are predicted to lack genetic recombination, which allows for the evolution of adaptations and novel traits. Additionally, the lack of recombination causes the accumulation of deleterious mutations, which, over time, will cause the extinction of the unisexual species. Secondly, the two-fold reproductive advantage that asexual species have over sexual species becomes a disadvantage for unisexual sperm-dependent species. Their modes of reproduction causes them to be dependent on a sexual host, and, if niche differentiation doesn't occur, they can potentially outnumber the sexual population and outcompete it from resources, eventually causing their own extinction. Thirdly, unisexual sperm-dependent females, must compete with both conspecific and heterospecific females to access mates. Additionally, selection should favor males of the host species that avoid mating with unisexual females, given that these interspecific matings do not provide any fitness advantage to the males. The Frozen Niche Variation Hypothesis was proposed to provide a mechanism to explain the maintenance of unisexual bisexual mating complexes over long periods of time.

If multiple clonal lines of the unisexual species exist in a population, different clones will use a different sub-niche of the host species (in most case its parental species), and coexistence between the two species will be maintained because competition between the clones and the sexual host will be reduced. We did an in-depth analysis of the genome of the species found in the *Poecilia formosa* – *P. latipinna* and *P. mexicana* unisexual-bisexual mating complex to test whether the frozen niche variation applies to this system. We found that the main assumption of the frozen niche variation hypotheses, the lack of genetic recombination in the hybrid, did not hold true, because a substantial amount of recombination has occurred in *P. formosa*. However, this extensive recombination has provided the unisexual *P. formosa* with a substantial amount of genetic recombination, which might explain its maintenance over time.

Introduction

The maintenance of sex presents a conundrum for evolutionary biology because the costs of sexual reproduction (cost of producing males, energy expenditure to find a mate, exposure to diseases, segregation of alleles) appear to be immediate and substantial while its benefits (facilitation of adaptations, elimination of deleterious mutations) are postponed (reviewed in Avise 2009a). However, the long-term maintenance of unisexual organisms is of interest to evolutionary biologists as well, because the advantages of asexual reproduction are all immediate (no cost of producing males and therefore exponential growth), but the long-term costs are substantial (accumulation of deleterious mutations and lack of genetic recombination to respond to environmental changes). Unisexual species are therefore predicted to be short-lived when compared to sexually

reproducing species (Muller 1964; Maynard-Smith 1968; Beukeboom & Vrijenhoek 1998). Unisexual vertebrate species are extremely rare, and constitute only 0.1% of all extant vertebrate species (Avisé 2009a). Although rare, several divergent vertebrate taxa exhibit some form of unisexuality (Dawley 1989; Avisé 2009a). Unisexual vertebrates are products of hybridization events between two or more sexually reproducing species (Dawley and Bogard 1989, and references within), and reproduce by different forms of parthenogenesis.

Parthenogenesis is a form of asexual reproduction where embryo development occurs without insemination and fertilization (Dawley 1989). Hybridogens, on the other hand, produce haploid eggs and are inseminated by males, making them sperm-dependent unisexual species, however, no syngamy between the paternal and maternal genomes occurs, and the paternal chromosomes are discarded once oogenesis starts (Dawley 1989). Paternal genes, however, are expressed in hybridogenetic individuals, but not inherited by their offspring. Gynogenesis is a third type of unisexual reproduction where females must mate with males of a closely related species (but refer to Choleva et al. 2008 for exceptions), but the non-recombinant embryos do not inherit any genetic information from the sperm donor (Dawley 1989). Because gynogens require sperm to initiate development of offspring, but no paternal genes are expressed, they are considered sexual parasites.

The maintenance of a gynogenetic species is even more paradoxical than the maintenance of a simple parthenogenetic species because gynogens need to face the costs of both sexual and asexual reproduction: the cost of finding a mate, exposure to diseases, accumulation of deleterious mutations and lack of genetic recombination to evolve

adaptations. Additionally, because male sperm donors do not gain any fitness advantage, selection should favor males that avoid mating with them.

Unisexual vertebrates provide interesting study systems because they can allow us to understand the maintenance of asexual reproduction as well as how selection and drift shape hybrid genomes. Depending on the reproductive mechanism, their genomic architecture may differ greatly. Parthenogens, for example, are full clones of one another. The clonal nature of parthenogens is due to the fact that their reproductive mode allows them to reproduce fully asexually (Dawley 1989). However, hybridogens are hemiclinal on the maternal line because paternal chromosomes are discarded, and multiple clonal lineages may be found in one population. Lastly, gynogenetic individuals are full clones of one another because there is no inclusion of paternal genes.

Whenever a unisexual species is sperm-dependent, as in the case of hybridogenetic and gynogenetic species, it must rely on males of another species for reproduction, and, as a result, must live in sympatry with the sexual hosts. This dependence on a host may present a problem whenever niche differentiation has not occurred between the sperm-parasite and the sexually reproducing species because of the two-fold reproductive advantage that asexual species have over sexually reproducing species (Maynard Smith 1968). If niche differentiation between the two or more species found in a unisexual-bisexual mating complex has not occurred, the fast increase of the asexual population size will cause inter-specific competition to increase rapidly, and, as a result, one or more of the species might be competitively excluded from resources. In particular, if the asexual sperm-dependent species excludes its host from resources, it will cause its own extinction, because males will no longer be available. However,

coexistence between a unisexual sperm-dependent species and its host can be achieved and maintained if multiple lineages of the unisexual species are present in a population. Vrijenhoek (1978) proposed that given their hybrid nature, hybridogenetic individuals freeze the genotypic divergence present in the parent population, and, as a result, they can use a sub-portion of the resource space used by the sexually reproducing parent species (Frozen Niche Variation Hypothesis). According to the Frozen Niche Variation hypothesis (FNV), selection will then act on the clonal lineages that overlap greatly with each other, or with the parent species' mean, and only lineages that have minimal overlap with each other or the parent species will be found in a locality (reviewed in Vrijenhoek and Parker 2009). Schenck and Vrijenhoek (1986, 1989) found that there are strong dietary differences between different hemiclones of the hybridogenetic *Poeciliopsis monacha-lucida*. Additionally, field surveys in this system have revealed that unisexual females outnumber sexual females in populations where the hybridogens are multi-clonal, whereas the opposite happens in populations where the unisexuals are monoclonal (Vrijenhoek 1979), suggesting that the presence of multiple clonal lineages in a population is advantageous for the hybridogenetic species. The FNV hypothesis has never been tested in a gynogenetic species, and this hypothesis could only be plausible if the gynogenetic species arose from multiple hybridization events yielding multiple frozen clonal lineages and, therefore, limiting competition between the hybrids and the parent species. If the frozen niche variation is the underlying mechanism that allows for the persistence of a unisexual-bisexual mating complex, then we would predict that multiple clonal lineages should be present in the asexual population and, as a result, we would detect genetic variation within the asexual lineage.

Population genetics tools can be very powerful to test hypotheses such as the FNV hypothesis. The genomic architecture of an F1 hybrid is different from that of more complicated hybrids. If individuals are F1 hybrids, then they should be heterozygous at each locus because they are intermediate between the parent species, and, therefore, have an admixture proportion of 0.5. If any amount of backcrossing has occurred, then the admixture proportion should be skewed towards either 0 or 1, because more than half of the hybrid's genome has ancestry from one parent over the other. Population genetics tools can also allow us to distinguish between the presence of a single F1 clonal line vs. multiple F1 clonal lines in a population. If multiple F1 clonal lines are present in a population, within population structure should be pronounced and there should be evidence of distinct genotypes. Additionally, assignment of ancestry would differ between groups of individuals (clonal lineages) at the same locus. Thinking about it in a visual framework, individuals should be grouped into as many genetic clusters as there are clonal lines, whereas, if only one clonal line exist, then individuals will all be part of the same genetic cluster.

One of the main assumptions of the FNV hypothesis is that there is no recombination within the various hybrid lineages. Population genetic tools allow us to test this assumption. If an F1 hybrid reproduces clonally, it should show high linkage disequilibrium when compared to sexually reproducing species. Hybrids have high linkage disequilibrium due to the admixture of the parent genomes, however, if recombination occurs, this admixture linkage disequilibrium decays over time (Gay et al. 2008). However, if the hybrid reproduces asexually, as in the case of a gynogenetic

species, then linkage disequilibrium will not decay, and should remain higher than that found in sexually reproducing species (Gay et al. 2008).

The Amazon molly (*Poecilia formosa*) is a perfect candidate to explore some of the assumptions and requirements of the frozen niche variation hypothesis, as well as understanding the organization of the genome of a gynogenetic species. *Poecilia formosa* was the first vertebrate recognized as asexual (Hubbs and Hubbs 1932) and is a gynogenetic species that uses *P. mexicana* (Atlantic molly), *P. latipinna* (Sailfin molly) and *P. latipuncata* (Tamesi molly) as sexual hosts (Niemeitz et al. 2002). Like every other known unisexual vertebrate, *P. formosa* is thought to be a hybrid (Hubbs and Hubbs 1932; Abramoff et al. 1968; Avise et al. 1991; Schartl et al. 1995; Tiedemann et al. 2005). *Poecilia mexicana* was recognized to be the maternal species of *P. formosa* (Avise et al. 1991; Schartl et al. 1995), whereas *P. latipinna* (or an extinct ancestor of *P. latipinna*) is the putative parental species (Avise et al. 1991). Although recent studies suggest that *P. formosa* is an F1 hybrid (Tiedemann et al. 2005, Stoek et al. 2010), there is not strong evidence supporting this claim. Additionally, it is still not clear whether *P. formosa* is the product of a single or multiple hybridization events. This last aspect of the identity of *P. formosa* is important to understand if we are interested in knowing how unisexual-bisexual mating complexes are maintained over time and in knowing whether the frozen niche variation hypothesis can be applied to this system.

In the present study we investigated the genomic composition of *P. formosa* and tested the predictions of the FNV hypothesis as a potential mechanism for the maintenance of this unisexual-bisexual mating complex. We generated thousands of DNA sequence markers for *P. formosa* and its parental species using a next-generation

sequencing population genetics framework to answer the following questions: 1) Is *P. formosa* a hybrid species? 2) If *P. formosa* is a hybrid, is it an F1 hybrid or is its genomic composition indicative of a more complicated hybrid origin? 3) Is *P. formosa* the product of a single or multiple hybridization origins, and therefore, do multiple clonal lineages exist?

Materials and Methods

We generated DNA sequence data for 200 fish: 42 *P. formosa* (five localities where the fish is sympatric with *P. mexicana* and six localities where the fish is sympatric with *P. latipinna*), 81 *P. latipinna* (from 22 localities across LA, TX, and Mexico), and 67 *P. mexicana* (from 13 localities across Mexico and Honduras).

We isolated and purified DNA from caudal fin clips following the protocol of Brookes et al. (1997). We fragmented the genome using restriction enzymes (EcoR1 and MSE) following the methods of Gompert et al. (2010; 2012) to generate a library for each individual. Individuals were labeled with unique 10 bp-long identification sequence. We amplified the barcode-adapted fragments with two amplifications from which PCR products were pooled. We then separated the amplified sequences on a 2% agarose gel, and isolated sequences between 250 and 500bp in length by cutting the gel. We used the Qiagen's Qiaquick Gel Extraction 15 Kit (Cat. No. 28706; Qiagen Inc., Valencia, CA, USA) to purify the fragments. DNA sequencing of the libraries was performed by the National Center for Genome Research (Santa Fe, NM, USA) using the Illumina GAI platform.

We used SeqMan NGen 3.0.4 (DNASTAR) to perform a de novo assembly using a

subset of sequences (8 million) and generated a partial reference sequence. This reference was produced by using We used a match size of 71 base pairs (bp), a minimum match percentage of 92%, a match score of 10, a mismatch penalty of 15, and a gap penalty of 30. We then removed low quality contigs: contigs that included complemented reads, contigs that were shorter than 88bp or longer than 96 (these values indicated poor alignment given that the expected contig length was 92b). With the remaining sequences we generated a partial reference genome that contained a total of 237,473 consensus sequences. Lastly, we assembled the full dataset (43 million sequences) to this partial reference genome by using a minimum match percentage of 90%.

We used custom **Perl** scripts (available from the authors) together with **samtools** and **bcftools** (Li et al., 2009) to identify variable sites. We only called variant sites for data that were present in at least 50% of the individuals, and if the probability of the data assuming all samples were homozygous for the reference allele was less than 0.01. We additionally removed all loci for which the allele counts for heterozygotes were unlikely given a binomial distribution. With these pruning, we identified 32,492 variable sites. Because of the stochasticity inherent in next-generation sequencing, we incorporated genotype uncertainty into our analyses rather than attempting to assign genotypes to individuals (see the following section).

Due to the low numbers of individuals for each locality, we pooled localities into geographical regions to obtain adequate samples sizes to perform all of our analyses (Figure 1). Regional grouping included 3 geographical regions for *P. latpinna*: North (FL, LA, north TX), central (Populations that were introduced in central TX from FL in the 1950s), and South (South TX and North Mexico), *P. formosa*; 2 regions for *P.*

formosa: North (sympatric with *P. latipinna*; these also included populations found in Central TX where individuals of *P. formosa* were introduced from Brownsville, TX), South (sympatric with *P. mexicana*); and 3 regions for *P. mexicana*: North (North Mexico), Central (Central Mexico), South (South Mexico, Yucatan Peninsula and Honduras; Figure 8).

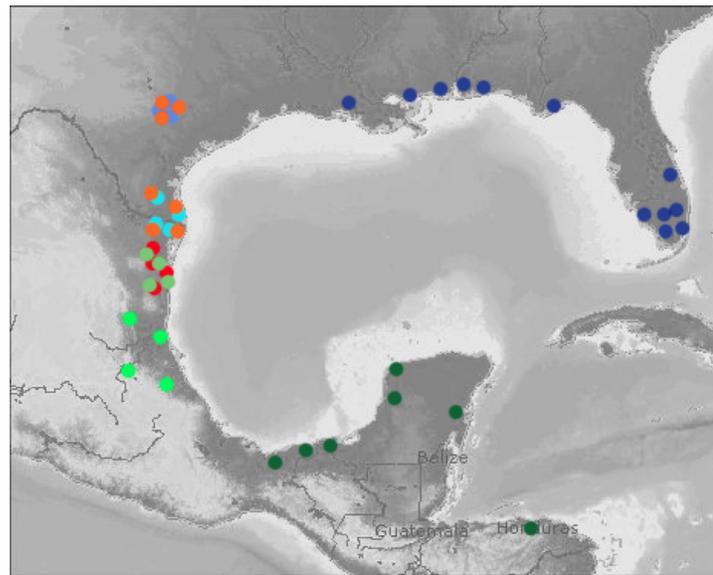


Figure 8. Sample populations pooled into geographic regions. Dark blue (North *P. latipinna*), blue (Central *P. latipinna*), light blue (South *P. latipinna*), orange (North *P. formosa* sympatric with *P. latipinna*), red (South *P. formosa* sympatric with *P. mexicana*), light green (North *P. mexicana*), green (Central *P. mexicana*), dark green (South *P. mexicana*).

Population genetics analyses

We used Bayesian models to calculate allele frequencies for each locus based on the observed data by using the allele frequency Bayesian model presented in Gompert et al. (2012), which is similar to the models used by Pritchard et al. (2000), Gillespie (2004) and Hedrick (2005). Two assumptions of the model are that 1) the data do not contain errors (and, given our stringent post-assembly parameters, this is a reasonable assumption) and 2) sequences are sampled stochastically and have a limited coverage for each nucleotide. The model treats the genotypes of individuals as an unknown variable, and is calculated from the allele frequencies obtained from the sequenced data (for more details on the model, see Gompert et al. 2012). The allele frequency model was written by ZG and relies on the GNU scientific Library (Galassi et al. 2009). The posterior probabilities for parameter estimates (genotype probabilities) were obtained using Markov Chain Monte Carlo (MCMC) of 20,000 steps and we recorded samples every 10th step.

We summarized population genetic structure at the individual level via a Principal Component Analysis (PCA) by using the genotype probabilities for the three genotypes as variables ($3 \times 26,313 = 78,939$). We used the covariance matrix to produce the PCA in **R** (using the *rcomp* function in the *composition* package) to center but not scale the genotype probabilities. We also calculated pairwise G_{st} summary statistics and summarized them using a nonmetric multidimensional scaling plot (using the *MASS* package in R) to display genetic structure at the population level.

Hybrid index

We used the Bayesian genomic cline model (Gompert and Buerkle 2009; Gompert and Buerkle 2011) to calculate the hybrid index of the 42 *P. formosa* given their putative parental populations as a prior. We set populations of *P. latipinna* found in the southern part of its range, and populations of *P. mexicana* found in the northern part of its range, as our putative parent populations. It is not known exactly where *P. formosa* originated, but genetic evidence points to the region of Tampico (corresponding to the southern portion of the range of ... and the northern part of the range of southern range of *P. latipinna* and northern range of *P. mexicana*; Schart et al. 1995). The cline parameter h_i is the probability of ancestry of an individual given two parent populations and is equivalent to an estimate of admixture proportion (Buerkle 2005; Gompert and Buerkle 2011).

We were specifically interested in determining whether Pf is a frozen frozen F1 hybrid or a more complicated hybrid. Cline parameter α , a component of the Bayesian genomic cline model, denotes an increase or decrease in the probability of parent 1 ancestry relative to a null expectation based on the hybrid index (Gompert and Buerkle 2011; Gompert et al. 2012a). Given a hybrid index, if there is excess contribution from either parent species, then the α index will be significantly different than 0.

Linkage disequilibrium

One of the core assumptions of the Frozen Niche Variation hypothesis is that the hybrid individuals freeze the genetic variation present in the parent populations because they lack genetic recombination (Vrijenhoek 1979). To test whether the FNV hypothesis can be applied to *P. formosa*, it was necessary to determine if this hybrids do indeed lack genetic recombination. Given the hybrid origin of *P. formosa*, and the presumed lack of recombination in this asexual species, we predicted substantially higher linkage disequilibrium in this species compared to the parental species.

We, therefore, calculated Burrow's composite measure of linkage disequilibrium (Δ) between all pairs of variable sites (Weir 1979; Gompert et al. 2012b). We calculated Δ between each pair of loci (Δ_{ii}) iteratively for 75 times using the estimated genotype posterior probabilities. We then averaged the 75 iterations to obtain a mean LD for each pair for a total of 6.9×10^8 pairs (scripts available from the authors). For each geographic region, we calculated the average linkage disequilibrium across all pairs.

Results

We limited data to only those markers with a minimum of 5 reads/ marker/ region (population grouping) which produced 26,313 SNP. Our first question was whether we could confirm results from previous studies regarding the hybrid status of *P. formosa*. Results of the PCA analysis suggest that *P. formosa* is genetically intermediate between *P. latipinna* and *P. mexicana*. PC1 and 2 collectively explained 63% of the variation and

divides the three species into three distinct clusters, with *P. formosa* individuals located between the parental species, consistent with the hypothesis of a hybrid origin for *P. formosa* (Figure 9a). This first result supports the hypothesis of the hybrid origin of *P. formosa*. PC3 explained 5.7% of the variation, and separated the populations of *P. mexicana* into three groups, which corresponded to the three geographic regions (Figure 9b). PC4 explained 4.0% of the variation and divided *P. latipinna* into two geographic regions: North + Central and South (Figure 9c). This clustering of populations comports with the history of the region. Pops in TX were introduced in the 1950s from Florida and Louisiana.

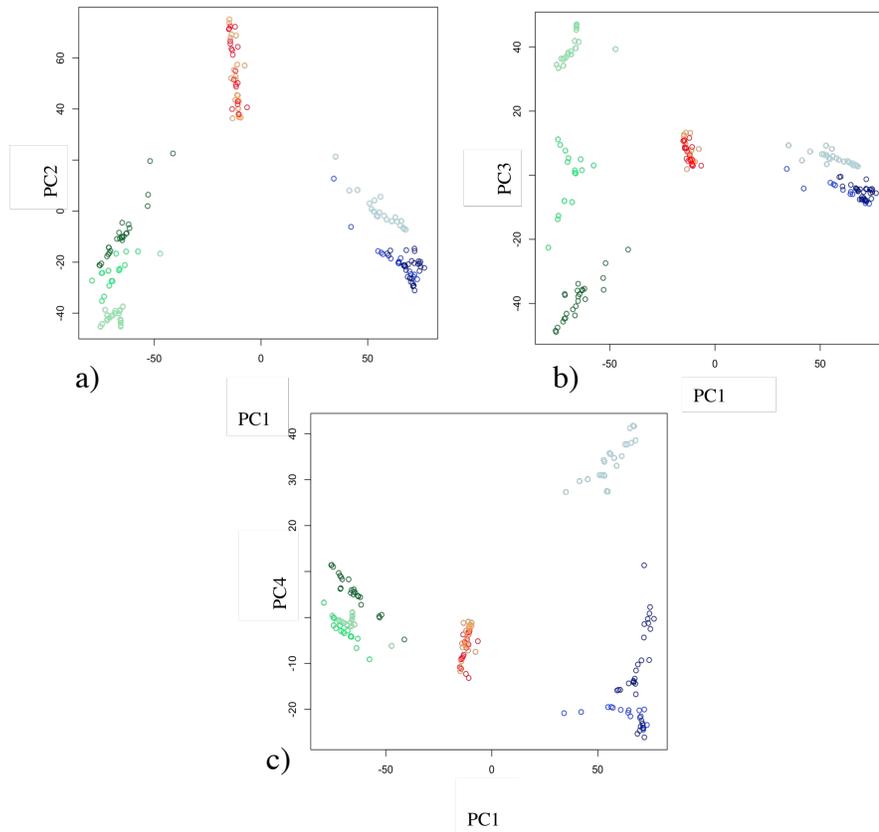


Figure 9. PC plots for the 200 individuals, based on the three genotype probabilities at each locus. Dark blue (North *P. latipinna*), blue (Central *P. latipinna*), light blue (South *P. latipinna*), orange (North *P. formosa* sympatric with *P. latipinna*), red (South *P. formosa* sympatric with *P. mexicana*), light green (North *P. mexicana*), green (Central *P. mexicana*), dark green (South *P. mexicana*).

Calculation of pairwise G_{st} also confirmed the genomic intermediacy of *P. formosa*, confirming once more the hybrid origin of *P. formosa* (figure 10). G_{st} between populations of *P. latipinna* and *P. mexicana* ranged from 0.341 to 0.380 (mean = 0.361), whereas G_{st} between *P. formosa* and *P. latipinna* spanned from 0.144 to 0.170 (mean = 0.163), and G_{st} between *P. formosa* and *P. mexicana* spanned from 0.132 to 0.180 (mean

= 0.155). G_{st} within *P. latipinna* ranged from 0.043 and 0.081 (mean = 0.06), and G_{st} estimated within *P. mexicana* ranged from 0.077 to 0.125 (mean = 0.096). The G_{st} estimate between the two populations (regions) of *P. formosa* was 0.028, and while this value was smaller than other within-species estimates, it was not zero (Confidence Intervals = 0.0278, 0.0288).

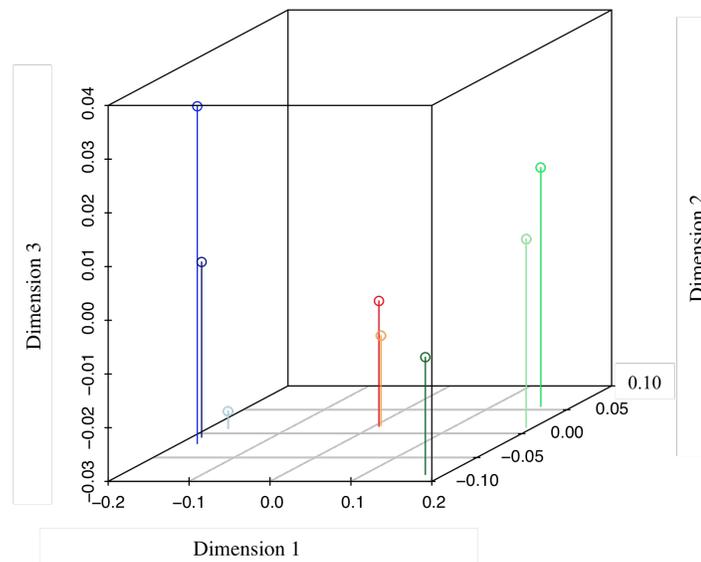


Figure 10. Non-metric multidimensional scaling of pairwise G_{st} between all populations with stress $k=3$. Dark blue (North *P. latipinna*), blue (Central *P. latipinna*), light blue (South *P. latipinna*), orange (North *P. formosa* sympatric with *P. latipinna*), red (South *P. formosa* sympatric with *P. mexicana*), light green (North *P. mexicana*), green (Central *P. mexicana*), dark green (South *P. mexicana*).

The hybrid index estimates supported the results of both the PCA and G_{st} calculations, and corroborated the hypothesis that *P. formosa* is a hybrid between *P. mexicana* and *P. latipinna*. The hybrid indices of the 42 *P. formosa* ranged from 0.37 to 0.56 (mean = 0.49; Figure 11).

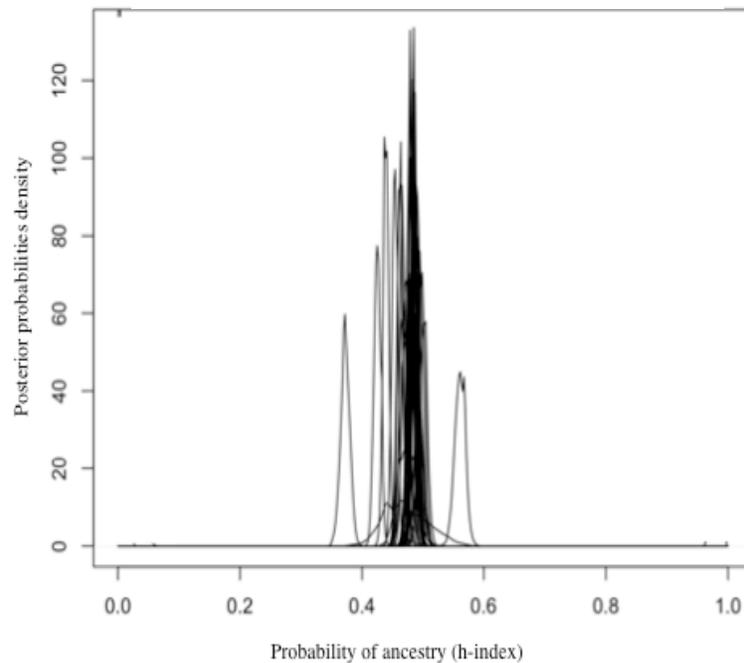


Figure 11. Posterior probability estimates of hybrid index for the 42 *P. formosa* used in this study. The assigned putative parent populations were *P. mexicana* found in the northern part of its range, and *P. latipinna* found in the southern part of its range.

We also used the Bayesian gen cline model to examine the organization of the hybrid genome. The distribution of the α index across loci, revealed that not all loci found in *P. formosa* are heterozygous, but about 28% of the loci had excess contribution from either parental species. Interestingly, the excess contribution appears to be symmetrical, with each parent contributing excessively to about 12% of the loci (Figure 12). Thus, Pf is clearly not an F1 in the strict sense of being heterozygous at all loci.

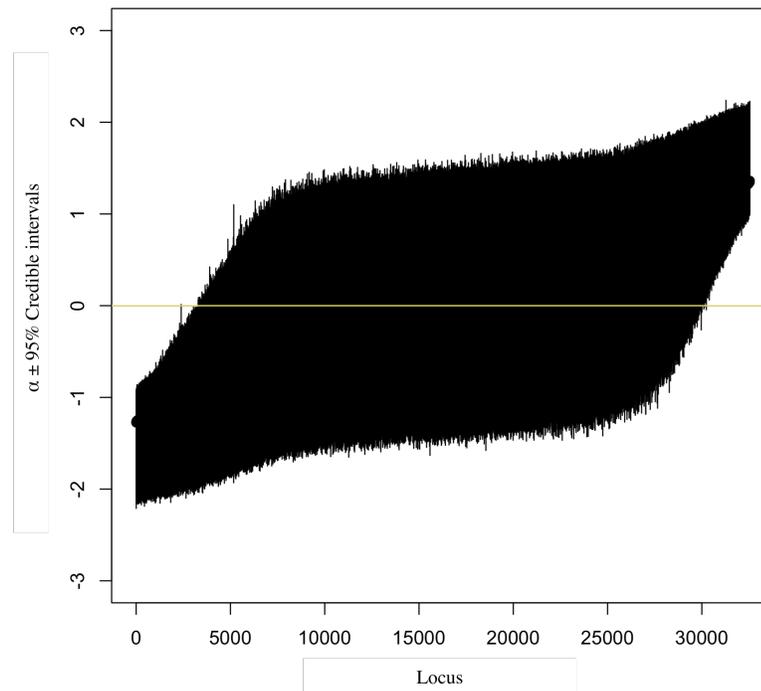


Figure 12. Plot of α index \pm 95% confidence intervals for each locus. The yellow abline displays the expected α index given the hybrid index of the individuals.

We additionally calculated the average linkage disequilibrium between pairs of SNPs across the genomes of each species to confirm whether the frozen niche variation hypothesis can be applied to the *P. formosa* – *P. latipinna* – *P. mexicana* mating complex. The distribution of linkage disequilibria across pairs of SNPs of the two geographic regions of *P. formosa*, overlapped with the distributions of *P. latipinna* and *P. mexicana* (Figure 13). The linkage disequilibrium in *P. formosa* was not high, as expected for a hybrid and clonal species, but, on the contrary, was quite similar to the linkage disequilibrium found in the sexually reproducing parent populations. This result,

coupled with the results obtained from the calculation of the α index, suggest that *P. formosa* has undergone, or is undergoing, genetic recombination.

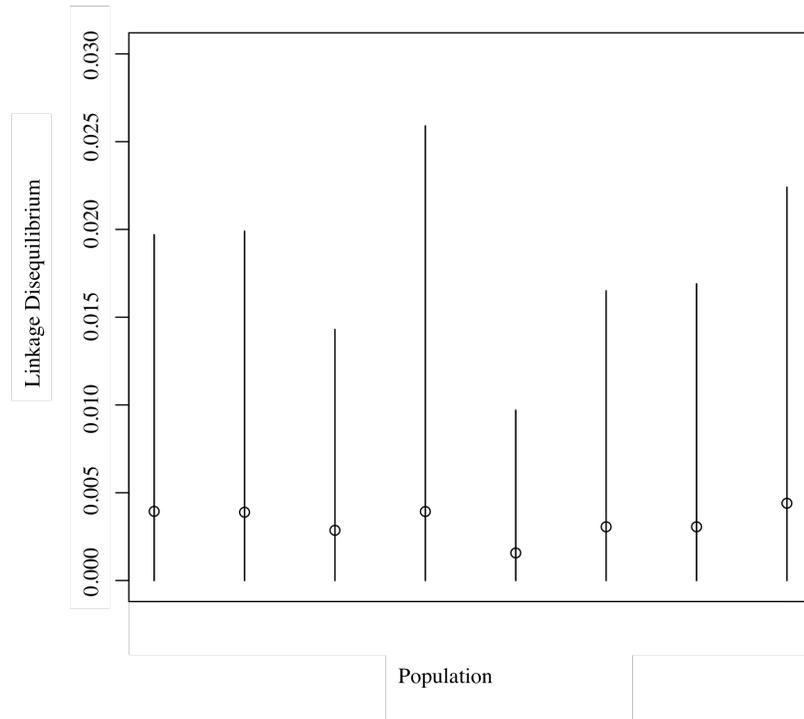


Figure 13. Mean linkage disequilibrium and upper and lower quantiles of each geographic region (population). From left to right North *P. formosa* (sympatric with *P. latipinna*), South *P. formosa* (sympatric with *P. mexicana*), North *P. latipinna*, Central *P. latipinna*, South *P. latipinna*, North *P. mexicana*, Central *P. mexicana* and South *P. mexicana*.

Discussion

The Frozen Niche Variation hypothesis (Vrijenhoek 1979) is a plausible mechanism for the persistence of the gynogenetic hybrid *P. formosa* only certain conditions are met. Specifically, the FNV hypothesis would be applicable if multiple, discrete asexual clonal lineages exist within *P. formosa*. We used a next-generation sequencing population genomics approach to collect information on variation from across the genomes of *P. formosa*, *P. latipinna* and *P. mexicana* and to understand the genomic architecture of the gynogenetic species. The methodology used herein allowed us to obtain genotype information from thousands of variable sites dispersed across the entire genome of the fish, with which we achieved a higher level of resolution than previous studies of these species.

Our results are in agreement with the findings of several papers published in the past: *P. formosa* is a hybrid between *P. mexicana* and *P. latipinna* (Abramoff et al. 1968; Turner et al. 1980; Avise et al. 1991; Scharl et al. 1995; Tiedemann et al. 2005; Stock et al. 2010). Our PCA, the calculation of pairwise G_{st} , and the hybrid index for each of the *P. formosa*, all suggest that *P. formosa* has an intermediate genotype between *P. latipinna* and *P. mexicana*. Interestingly, the genotypic variation within *P. formosa* was higher than expected.

The high genotypic diversity we found in *P. formosa* is in agreement with previously published results (Turner et al. 1990; Schaschl et al. 2008; Stock et al. 2010), which all found that *P. formosa* was genotypically variable; however, these previous works all suggest that the high diversity in *P. formosa* is due to the accumulation of mutations that are then maintained by selection, rather than because of the presence of

multiple clonal lineages due to multiple hybridization events. Stock et al. (2010) suggest that the high genetic diversity in *P. formosa* is due to high mutation rates because the phylogenetic analyses done in that study (using mtDNA) suggested a monophyletic origin of *P. formosa*. Turner et al. (1990) also suggested that high mutation rates were more probable than multiple hybrid origins because even in populations of *P. formosa* where *P. latipinna* is not present there was indication of high clonal differences (by fingerprinting). However, it is also true that some of the *P. formosa* studied in Turner et al. (1990) were collected in areas where triploid individuals are present, and, therefore, the high clonal diversity found in the Rio Purification population might have been caused by the presence of triploids. Our results do not fully support the conclusions set forth by these previous studies. If *P. formosa* were indeed a frozen F1 hybrid that only accumulated mutations over time, then one would predict high linkage disequilibrium within *P. formosa* because of the high admixture linkage disequilibrium present in hybrids and maintained by clonal reproduction. Our results, however, do not support this prediction. The distribution of linkage disequilibria in the regional samples of *P. formosa* are not different than the distribution of linkage disequilibria in either *P. latipinna* or *P. mexicana*. This result suggests that some amount of genetic recombination has occurred historically, or is currently occurring in *P. formosa*.

One possible explanation for the low linkage disequilibrium found in *P. formosa* could be that, before the onset of gynogenesis, some recombination of the genome of this species occurred. The ancestral *P. formosa* might have been a sexually reproducing hybrid for some time before becoming gynogenetic. This possibility could explain why no one has been able to reproduce *P. formosa* in the laboratory. Turner et al. (1980)

extensively tried to synthetically reproduce *P. formosa*, but were unable to do so. These authors suggested that the onset of gynogenesis might have been due to the action of a few alleles placed in a novel genetic environment via hybridization.

An alternative explanation for the low linkage disequilibrium and high genetic diversity found within *P. formosa* could be that the hybrid is currently still undergoing some amount of genetic recombination. This conclusion suggests that the high genotypic diversity of *P. formosa* is due to an ongoing, substantial amount of genetic recombination, and, therefore, *P. formosa* does not appear to be a frozen F1 hybrid. The results from the calculation of the α index support the conclusion that *P. formosa* is not a frozen F1 hybrid. If that were the case, we would predict that, given its hybrid index of 0.50, all loci would have roughly equal probability of ancestry from either parent species. However, an hybrid index of 0.50 can be obtained in multiple ways. For example, if a hybrid is fixed for each parent allele at the same number of loci, then it would still have a hybrid index of roughly 0.5. The α index is a genomic cline parameter that allows us to distinguish between the various genomic compositions that would produce a specific hybrid index. The α index allows us to determine the probability of ancestry of each locus, given the two parent populations and the hybrid index of individuals (Gompert and Buerkle 2011; Gompert et al. 2012a). As shown in figure 4, most loci in *P. formosa* are heterozygous, as it would be expected in an F1 hybrid, however, about 12% of the loci, have excess ancestry from either parent species, suggesting that (combined with the linkage disequilibrium results) genetic recombination has occurred, making the genetic composition of *P. formosa* more complex than that of a frozen F1 hybrid.

A possible mechanism by which asexual hybrids lose heterozygosity at each locus is pre-miotic endoreplication followed by coupling and crossing-over of homologous chromosomes, instead of sister chromosomes (Lutes et al. 2010; more refs). However, Rasch et al. (1982), ruled out the hypothesis of pre-miotic endoreplication in *P. formosa*, and suggested that this gynogen produces eggs ameiotically because no doubling of DNA was detected before meiosis. Tiedemann et al. (2005) also found some loci in *P. formosa* that were homozygous for one of the parent species, and suggested that mitotic gene conversion might explain the pattern. Mitotic gene conversion could potentially explain our results. When this particular type of recombination occurs, some loci will become homozygous for one of the alleles (Chen et al. 2007), causing a loss of heterozygosity. The probability of the occurrence and success of gene conversion varies across the genome (Jeffreys and May 2004), and only conversions that do not cause a selective disadvantage to individuals will be maintained over time. This mechanism will cause genomes to vary among individuals, and will cause a decay of the admixture linkage disequilibrium because recombination between parent genomes occurs, and will cause heterozygosity to be lost. Mitotic gene conversion could potentially explain the high genotypic diversity in *P. formosa*.

Overall, *P. formosa* does not appear to be a frozen F1 hybrid between *P. latipinna* and *P. formosa*, and given its complicated genomic structure, at the moment it is impossible to determine if it is the product of a single or multiple hybridization events. As a result, it is impossible to know if one or multiple hybrid lineages exist. However, we can conclude that the frozen niche variation *sensu stricto* does not apply to *P. formosa*. Vrijenhoek (1979) proposed the frozen niche variation hypothesis as a possible

mechanism that allows the coexistence between a sperm-dependent unisexual species and its sexual host by reducing the amount of competition between the two species. One of the main assumptions of the frozen niche variation hypothesis is that no genetic recombination occurs in the hybrid (Vrijenhoek 1979), however, we found that a substantial amount of genetic recombination has occurred (or is occurring) in *P. formosa*. Nonetheless, the general concept of the frozen niche variation (*sensu lato*) can be applied. As stated by Vrijenhoek and Parker (2009), when hybrid clones arise, each clonal line will consume a portion of the resource space used by the sexual species. Over time, however, natural selection will get rid of those clonal lines that overlap with each other, and overlap greatly with the mean of the sexual population. As a result, after some generations, only clonal lineages that do not overlap in their resource use and minimize overlap with the sexual species will be maintained. However, if the rate of clonal formation is too high, natural selection might not have enough time to act and the sexual host might go extinct (Vrijenhoek and Parker 2009). If we consider the high genotypic variation found in *P. formosa*, it is possible that different genotypes can code for phenotypes that allow the hybrids to occupy different portion of the resource space of the parent species. Over time, only phenotypes that do not overlap greatly with one another, and with the mean phenotype of the parent species, will be maintained over time. Consequently, only the genotypes that cause non-overlapping phenotypes will be maintained, whereas all other genotypes will be selected against and go extinct.

The persistence of *P. formosa* might not be as paradoxical as previously thought. The presence of some genetic recombination (by whichever mechanism) might allow individuals to use a slightly different resource space from one another, and different than

the bulk of the individuals of the parent species present in the population. This genetic recombination, might also allow for a reduction of the accumulation of deleterious mutations (Muller 1964), allowing *P. formosa* to not go extinct as fast as it is predicted by theoretical models (Loewe and Lamatsch 2008).

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