MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 4140–4151 doi: 10.1111/j.1365-294X.2011.05264.x

Do density-driven mating system differences explain reproductive incompatibilities between populations of a placental fish?

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Abstract

Matrotrophy, the provisioning of embryos between fertilization and birth, creates the potential for conflict between mothers and embryos over the level of maternal investment. This conflict is predicted to drive the evolution of reproductive isolation between populations with different mating systems. In this study, we examine whether density-driven mating system differences explain the patterns of asymmetric reproductive isolation observed in previous studies involving four populations of the matrotrophic least killifish, *Heterandria formosa*. Minimum sire number reconstructions suggested that two populations characterized by low densities had lower levels of concurrent multiple paternity than two populations characterized by high densities. However, low levels of genetic variation in the low-density populations greatly reduced our probability of detecting multiple mating in them. Once we took the lower level of genetic variation into account in our estimations, high levels of multiple paternity appeared the rule in all four populations. In the population where we had the greatest power of detecting multiple mating, we found that multiple paternity in *H. formosa* typically involves multiple sires contributing to offspring within the same brood instead of different fathers contributing to distinct, simultaneously provisioned broods. Paternity was often skewed towards one sire. Our results suggest that differences between *H. formosa* populations in the levels of multiple paternity are not sufficient to explain the reproductive isolation seen in previous studies. We suggest that other influences on maternal–foetal conflict may contribute to the pattern of reproductive isolation observed previously. Alternatively, the asymmetric reproductive isolation seen in previous studies might reflect the disruption of maternal–foetal coadaptation.

Keywords: conflict, mating system, reproductive isolation, viviparity

Received 4 March 2011; revision received 27 July 2011; accepted 5 August 2011

Introduction

In organisms that provision young beyond fertilization, parents and offspring are likely to be in conflict over the level of parental investment (Trivers 1974; Parker *et al.* 2002). This conflict occurs because parents are equally related to all of their offspring whereas each individual offspring is more closely related to itself than its siblings. As a result of this genetic asymmetry, the optimal level of parental investment is expected to be greater from the offspring's perspective than from the parent's (Trivers 1974). Parent–offspring conflict has been studied most extensively in animals with postnatal parental care. However, this conflict is also expected to be important in organisms that provision young between fertilization and birth, a mode of reproduction referred to as matrotrophy (Zeh & Zeh 2000, 2008). While this mode of reproduction is most often associated with mammals, it has evolved in several taxa,

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perhaps most notably the poeciliid fish, a group in which it has several independent origins (Reznick *et al.* 2002; Pollux *et al.* 2009; Pires *et al.* 2010). Conflict between mothers and their embryos over prenatal maternal investment is expected to influence the level of maternal investment within a population (Parker *et al.* 2002; Long 2005) and has been suggested as an important force in the evolution of genomic imprinting (Haig 2000) and the evolution of placentation itself from more rudimentary forms of viviparity (Crespi & Semeniuk 2004).

It has also been argued that parent–offspring conflict over prenatal maternal investment will play an important role in speciation. Specifically, Zeh & Zeh (2000, 2008) have suggested that parent–offspring conflict will accelerate the rate at which postzygotic reproductive isolation evolves in matrotrophic taxa. In formulating this viviparity-driven conflict hypothesis (VDCH), Zeh & Zeh (2000) argued that the internal provisioning of embryos between fertilization and birth in matrotrophic species creates an arena for maternal–foetal conflict. The magnitude of this conflict will depend in part on the genetic mating system of the population. In polyandrous populations, offspring are less constrained by kin selection. Here, selection favours the evolution of traits that aid individual offspring in coercing higher levels of maternal investment, while females are expected to evolve traits that allow them to resist this coercion. The two forces reach an equilibrium within a population at which a high level of offspring demand is matched by a high level of maternal resistance. In monandrous populations, offspring of a single mother are more closely related to one another and there is less conflict between mothers and individual offspring over maternal investment. Here, the equilibrium between demand and resistance is attained at lower levels of each force. Zeh & Zeh (2000) argued that crosses between populations with different mating systems will disrupt this conflict-driven equilibrium between offspring demand and maternal resistance, resulting in reduced fitness in hybrids (Zeh & Zeh 2000, 2008; see also Brandvain & Haig 2005). Moreover, Zeh and Zeh suggested that a specific asymmetry in reproductive isolation would be apparent in crosses between populations with different mating systems. In crosses between a female from a monandrous population and a male from a polyandrous population, high levels of offspring demand will be met with relatively weak maternal resistance. This mismatch is predicted to increase the rate of spontaneous abortions and maternal death during pregnancy, but surviving offspring are predicted to be relatively large. In the reciprocal hybrid cross, weak offspring demand will be met with strong maternal resistance. Although these crosses are expected to have a higher success rate than the reciprocal cross, they are expected to result in embryonic depravation and the production of relatively small newborns (Zeh & Zeh 2000).

In an earlier study, Schrader & Travis (2008) exploited previously described mating system differences (Soucy & Travis 2003) between two populations of the highly matrotrophic least killifish, *Heterandria formosa*, to test the predictions of the VDCH. Two aspects of *H. formosa's* reproductive biology suggest that maternal–foetal conflict can be an important evolutionary force. First, this species is highly matrotrophic, with embryos displaying a 30- to 50-fold increase in dry mass between fertilization and birth (Schrader & Travis 2005, 2009). The resources used by embryos during development are transferred from the mother through a maternal–foetal interface composed of the ovarian follicle and the offspring's pericardial sac (Grove & Wourms 1991, 1994), and embryos influence the level of maternal investment they receive (Schrader & Travis 2009). Second, *H. formosa* exhibits a high level of superfetation, with females simultaneously carrying up to six broods of young at different stages of development (Travis *et al.* 1987). The presence of superfetation and the fact that most broods consist of multiple embryos creates the potential for both inter- and intrabrood conflict between embryos for maternally supplied resources (Schrader & Travis in press). The results of crosses between *H. formosa* populations with different mating systems were consistent with one of the major predictions of the VDCH. Specifically, crosses between females from a relatively monandrous population [Trout Pond (TP)] and males from a relatively polyandrous population [Wacissa River (WR)] had a higher rate of aborted embryos than the reciprocal cross and both within population crosses (Schrader & Travis 2008).

While these results are tantalizing, there are two reasons to hesitate in drawing conclusions about the power of the VDCH to account for them. First, the VDCH requires consistent differences between populations in their level of polyandry to drive offspring demand and maternal resistance to different equilibria. While some studies have found variation among local populations in components of the mating system measured at a single point in time (Trexler *et al.* 1997; Soucy & Travis 2003; Mobley & Jones 2007; Neff *et al.* 2008), there are very few studies that have resolved whether these differences are consistent over time, and thus could serve as an evolutionary force, or whether they are the immediate consequences of temporally fluctuating aspects of local ecology and behaviour. Among the few studies, the results are inconsistent. Mobley & Jones (2007) found that mating system parameters in a population of the dusky pipefish, *Syngnathus floridae*, exhibited very

little seasonal or annual variation. In contrast, a recent study of the mating system of the cichlid fish, *Ctenochromis hoeri,* found that multiple paternity was much more common in the rainy season (100% of sampled broods) than in the dry season (14% of sampled broods; Sefc *et al.* 2009). Similar temporal variation in the mating system has been demonstrated in *Poecilia latipinna,* where Trexler *et al.* (1997) found distinct seasonal variation in the level of multiple paternity in one of the four populations they studied, and most recently in sand lizards, *Lacerta agillis*, where characteristics of the mating system are sensitive to annual variation in operational sex ratio and temperature (Olsson *et al.* 2011). Levels of multiple paternity in *H. formosa* may vary temporally in some populations. Soucy & Travis (2003) suggested that the lack of courtship in this species will cause mating rates and levels of multiple paternity to vary with male–female encounter rates which likely increase with population density; some, although not all, populations of *H. formosa* experience significant temporal variation in density (Leips & Travis 1999; Richardson *et al.* 2006).

Second, the mating system differences between the WR and TP populations reported by Soucy & Travis (2003) are confounded with many other differences between them. The TP and WR populations experience very different environmental conditions and exhibit striking differences in their life histories (Leips & Travis 1999; Schrader & Travis 2005; Richardson *et al.* 2006). An ideal test of the VDCH would require crossing replicate monandrous and polyandrous populations and asking whether crosses between females from monandrous populations and males from polyandrous populations have consistently higher abortion rates than the reciprocal crosses or within mating system crosses. Interestingly, crosses between two other *H. formosa* populations [Moore Lake (ML) and Wakulla Springs (WS)] show an asymmetry in the viability of hybrid crosses that is similar to the pattern observed in crosses between TP and WR. Crosses between ML females and WS males resulted in more aborted embryos than the reciprocal hybrid cross and both within population crosses (Schrader & Travis 2009). The levels of multiple mating in ML and WS have not been described; were they to resemble the distinctions described between the TP and the WR populations (Soucy & Travis 2003) and were all of these distinctions temporally consistent; the results in hand would offer convincing support for the VDCH.

In this study, we combine data on population densities and levels of multiple paternity to examine the temporal consistency of the mating systems of the four *H. formosa* populations that were the subject of previous laboratory crosses (ML, TP, WR, and WS) and the potential for one ecological variable, population density, to influence the mating system. Our objectives are (i) to determine whether the level of concurrent multiple paternity in these populations is relatively stable from year to year; (ii) to address whether any spatial or temporal variation in the level of concurrent multiple paternity follows directly from variation in population density; and (iii) to determine whether the ML and WS populations exhibit consistent mating system differences that might explain the variation in abortion rate observed previously (Schrader & Travis 2009) and match the previously reported patterns in the TP–WR results (Schrader & Travis 2008).

Materials and methods

Heterandria formosa is a small poeciliid fish found in freshwater throughout the coastal plain of the southeastern United States. The *H. formosa* populations examined in this study, ML, TP, WR and WS, display consistent and substantial differences in life-history traits, population densities and abiotic conditions. ML (27 ha) and TP (5 ha) are both located in the Apalachicola National Forest, Leon County, Florida. Females from these populations produce large broods of relatively small offspring (Leips & Travis 1999; Schrader & Travis 2005, 2009). WR and WS are spring-fed rivers originating in Jefferson and Wakulla County, Florida, respectively. Females from these populations produce small broods of relatively large offspring (Leips & Travis 1999; Schrader & Travis 2005, 2009). These four populations also differ in several aspects of their ecology. First, in ML and TP, the dominant species of submerged vegetation is *Myriophyllum laxum,* while in WR and WS, it is the naturalized exotic *Hydrilla verticulata*. Second, WR and WS have a more moderate thermal regime, higher pH and higher conductivity than ML and TP (Leips & Travis 1999; Northwest Florida Water Management District, unpublished data). Finally, *H. formosa* densities are on average much lower in TP and ML than in WR and WS (see Results later).

We estimated *H. formosa* densities at each population in late April or early May for 10 years (2000–2009). Densities were estimated using a 0.5-m^2 throw trap as described in Leips & Travis (1999) and Richardson *et al.* (2006). At each site, in each year, we threw the trap three times in microhabitat likely to contain *H. formosa* (i.e. shallow water with vegetative cover). The number of females, males and juveniles in each trap was recorded. We tested whether populations differed in their average adult density across the 10 years they were sampled using a two-way ANOVA. In this analysis, we used log (adult density + 1) as the response variable to meet the assumptions of ANOVA. The suitability of the standard two-way ANOVA rests on the assumption that

census data taken 1 year apart are independent. This assumption is reasonable because the short lifespan of these fish makes it very unlikely that successive censuses are counting any of the same fish (J. Travis unpublished data).

During the last three censuses (2007–2009), we collected pregnant females from each population to estimate the level of concurrent multiple paternity (CMP; the per cent of females carrying broods sired by >1 sire). We attempted to collect at least 20 females from each population for the paternity analysis. We used females caught in the throw trap whenever possible. When we collected fewer than 20 females in the throw trap, we used dipnets to collect additional females. TP nearly dried in 2008, and we were unable to collect any females for paternity analysis. All females were euthanized in the field with an overdose of anaesthetic (MS-222) and were preserved in 95% ethanol. Females were later measured (standard length in mm) and dissected to remove developing embryos. For each female, we removed all of the eyed embryos (Travis *et al.* 1987) for genotyping. Previous collections from these populations indicate that eyed embryos represent the majority of embryos in the ovary of females collected during our spring census (e.g. 77% of the embryos carried by ML females and 89% of the embryos carried by WS females; Schrader unpublished data). Ova and un-eyed embryos were discarded. We examined the variation among populations in female body size via an ANOVA on log-transformed standard lengths and examined size-adjusted fecundity among populations with ANCOVA on log-transformed fecundity, using the log-transformed standard length as the covariate.

After dissection, females and individual embryos were digested in 400 μL of sarkosyl urea. DNA was extracted from 50 μL of each sample with 8 μL Sprintprep Activator (magnetic beads) and 80 μL of 100% isopropanol (Levitan 2008). Extracted DNA was diluted to a concentration of 5 ng per μL. We used three to six microsatellite markers to genotype mothers and their embryos. All samples from all populations were genotyped at three loci: *Hetfor5*, *TSS013* and *TSS051* (Soucy & Travis 2003; Athrey *et al.* 2007). We used three additional loci *Hetfor4*, *TSS005* and *SLS45* (Nakamura 2001, Soucy & Travis 2003; Athrey *et al.* 2007) to genotype samples from the WR collections. We did not use these additional loci in all populations because simulations using the program *PrDM* (Neff & Pitcher 2002) indicated that the addition of these loci only improved our ability to detect multiple paternity in the WR (see Results). These simulations were run using genetic data from a sample from each population that were genotyped at all six loci.

Microsatellites were amplified in 12 μL multiplexed polymerase chain reactions (PCR) using Qiagen Multiplex PCR master mix according to the manufacturers protocol. The cycling conditions for *Hetfor5*, *TSS013* and *TSS051* were the following: 95 °C for 15 min, 35 cycles of 30 s at 94 °C, 90 s at 59 °C, 60 s at 72 °C and a final elongation at 72 °C for 30 min. The cycling conditions for *Hetfor4*, *TSS005* and *SLS45* were identical except the annealing temperature for these loci was 55 °C. Primers were labelled with a fluorescent dye, and fragments were sized on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Fragment analyses were performed using GENEMAPPER (Applied Biosystems), and all allele calls were confirmed visually. We checked each progeny array for the presence of unusual genotypes (i.e. genotypes that were not consistent with the genotype of the known mother). Samples with unusual genotypes and those that failed to amplify at a given locus were rerun. We used GENEPOP on the web to test for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium (Fisher's exact tests).

We assessed the probability of detecting multiple paternity (as indicated by the presence of three of more paternal alleles in a group of siblings) within each collection using the program *PrDM* (Neff & Pitcher 2002). This program uses a Monte Carlo simulation to calculate the probability of detecting multiple mating (when it occurs) within a population. The Monte Carlo simulation implemented in *PrDM* incorporates the effects of genetic variables (number of loci, number of alleles, allele frequencies), brood size, number of sires and the reproductive skew among sires. For each population, we assessed the probability of detecting multiple paternity assuming that two males sired offspring within a brood. We ran simulations assuming either no skew in paternity (each male sired 50% of the progeny) or skewed paternity (the most successful male sired 75% of the progeny). All simulations were run using the average fecundity for a given collection.

We estimated the level of concurrent multiple paternity (CMP, the proportion of females carrying multiply sired broods) in each population using two different approaches. First, for each female's offspring, we used GERUD 2.0 (Jones 2001, 2005) to reconstruct paternal genotypes from the array of sib genotypes and the maternal genotype. This allowed us to estimate the minimum number of sires contributing to a female's offspring. Second, we used the program *fmm* to estimate levels of CMP in each population (Neff *et al.* 2002). This program uses a Bayesian approach to estimate the frequency of multiple mating and 95% confidence intervals, given limited genetic information. For these analyses, we used a uniform prior distribution as advocated by Neff *et al.* (2002) for cases where there are no

other biological data available to use in specifying the prior probability distribution (using alternative prior distributions provided nearly identical estimates of the frequency of multiple paternity). We ran these simulations assuming multiply mated females mated with two sires who shared paternity evenly (each male sired 50% of the progeny) or that paternity was skewed towards one male (the most successful male sired 75% of the progeny).

For each collection, we report the level of CMP as the per cent of sampled females carrying multiply sired broods. The level of CMP is obviously an incomplete measure of mating system variation. Ideally, we would estimate the average number of sires per female and per brood as well as the reproductive skew among sires for each population. Likelihood methods, such as those implemented in the program COLONY (Wang 2004; Jones & Wang 2010) can be used for these purposes (e.g. Neff *et al.* 2008). However, there are two reasons that these methods are likely to give inaccurate estimates of sire numbers in most of our populations. First, likelihood approaches require highly polymorphic loci to correctly infer monogamy when it occurs and identify the actual number of sires. When markers provide limited information, these approaches often overestimate rates of multiple paternity and the number of sires (Sefc and Koblmüller 2009). The levels of genetic variation in ML and TP are such that likelihood-based paternity assignment is likely to be error prone in these populations (see Results). Moreover, adding more, low-diversity loci does not appreciably improve the performance of the likelihood method implemented in COLONY (Sefc and Koblmüller 2009). Second, the relatively low fecundity of *H. formosa* females also limits our ability to correctly estimate sire numbers using likelihood approaches. This is especially problematic in WR where females have the lowest fecundity (see Results). A few studies have assessed the effects of sibship size on the performance of likelihood-based paternity assignment approaches (Wang 2004, Sefc and Koblmuller 2009). However, these studies have considered sibling groups of >25 individuals. Thus, it is unclear whether these methods perform well in species with low fecundity.

The combination of low genetic variation in some populations and low fecundity in others make comparisons of sire number difficult in our system. In WS, however, there are both high levels of genetic variation and fairly high fecundity (see Results), which make estimates of sire number based on likelihood methods more reliable. For this population, we used COLONY version 2.0 (Wang 2004) to estimate the number of sires contributing to each female's offspring. This program uses a maximum-likelihood method to partition offspring into full-sibling groups using the offspring genotypes, the allele frequencies in the population and the maternal genotype. We used the sibship assignments from COLONY to estimate the average number of sires per female and per brood in each WS collection and the reproductive skew among sires. Reproductive skew was quantified using Nonacs' binomial skew index (B; Nonacs 2000). This index is based on the observed variance in male success corrected by the expected variance if each male had an equal probability of siring offspring. Negative B values indicate an even distribution of paternity, values near 0 indicate random paternity and positive values indicate that paternity is skewed towards one sire. Significance levels for B were estimated using simulations with 10 000 permutations. Skew values were calculated and analysed using the program SKEW CALCULATOR 2003 (http://www.eeb.ucla.edu/Faculty/Nonacs/shareware.htm).

Results

Across the 10 years of our field surveys, the adult densities of WR and WS were consistently higher than those of ML and TP (Fig. 1). The magnitude of these differences varied among years. However, averaged across years, the adult density of WS was nine times higher than the adult density of ML (33.4 and 3.6 adults per 0.5 m^2, respectively), and the adult density of WR was seven times higher than the adult density of TP (39.4 and 5.5 adults per 0.5 m^2, respectively). Statistical analysis of these data revealed a significant effect of population ($F_{3,107} = 18.91$, $P < 0.001$) and no effect of year ($F_{3,107} = 1.06$, $P = 0.399$) on population density. Post hoc comparisons revealed that WR and WS had significantly higher adult densities than ML and TP ($P < 0.05$ in all comparisons after Bonferroni correction).

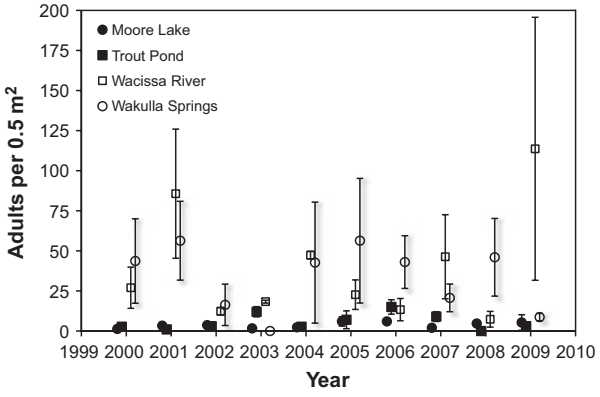

Fig. 1 Average adult density per 0.5 m^2 (±1 standard error) in Moore Lake, Trout Pond, Wacissa River and Wakulla Springs over 10 consecutive years of sampling. In each year, density estimates are spread along the horizontal axis for clarity.

In contrast, there were no significant differences between the average adult densities of WR and WS or ML and TP ($P > 0.05$ in both comparisons after Bonferroni correction). These results indicate that if population density influences the mating system, then density differences between populations are sufficiently consistent to promote consistent differences in mating system.

Female size and fecundity varied considerably within populations (Table 1). The largest female collected from a population was 25–85% larger than the smallest female. Fecundity varied even more widely, with some females carrying over ten times the number of embryos carried by others. As has been reported in other work (Leips & Travis 1999; Schrader & Travis 2009), larger females carried more embryos (correlation between female size and number of eyed embryos: ML, $r = 0.77$, $P < 0.0001$, $N = 57$; TP, $r = 0.87$, $P < 0.0001$, $N = 34$; WR, $r = 0.71$, $P < 0.0001$, $N = 73$; WS, $r = 0.74$, $P < 0.0001$; $N = 54$). Populations did not differ in the relationship between female size and fecundity (ANCOVA, test for heterogeneity of slopes, $F_{3,210} = 1.7$, $P = 0.16$).

Despite the wide variation within populations, there were significant differences between populations in both average female size and average fecundity adjusted for female size. The differences in average female size were quite significant (ANOVA, $F_{3,214} = 31.61$, $P < 0.0001$). Post hoc comparisons revealed that TP females were significantly larger and WR females significantly smaller than females from the other populations ($P < 0.05$ after Bonferroni correction). ML and WS females were intermediate in size and did not differ significantly from each other ($P > 0.05$ after Bonferroni correction). These differences in average female size were driven by the increased frequencies of larger females in some populations because the smallest females in each population were similar in body size (Table 1). The average size-adjusted fecundity varied as much as two-fold among populations (effect of female size, $F_{1,213} = 304.9$, $P < 0.0001$; effect of population, $F_{3,213} = 60.3$, $P < 0.0001$). Post hoc comparisons of least-square means indicated that average size-adjusted fecundity was highest in ML (LS mean = 14.44 embryos) and TP (LS mean = 13.33 embryos), lowest in WR (LS mean = 7.46 embryos) and intermediate in WS (LS mean = 9.78 embryos). These patterns match those reported in previous studies of these populations (Leips & Travis 1999; Schrader & Travis 2009), which indicates considerable long-term consistency in life-history differences in addition to the consistency in population densities.

We genotyped a total of 227 adults (ML = 59; TP = 35; WR = 75; WS = 58) and 2594 embryos (ML = 858; TP = 710; WR = 442; WS = 584). A total of nine genotyped females were not carrying eyed embryos. The number of embryos genotyped (2594) was slightly less than the total number of embryos removed (2759). This discrepancy was because of our inability to obtain high-quality DNA from some embryos. There was substantial variation among loci and populations in the total number of alleles present at the microsatellite loci (Table 2). In most collections, *TSS051* had the greatest number of alleles and *SLS45* had the fewest. At each locus, ML and TP had fewer alleles and lower heterozygosity than WR and WS

Table 1 The number of pregnant females sampled (*N*), mean female standard length (SL in mm), the range of female SL, mean fecundity (number of embryos in stages 2–5), the range of fecundities, estimated levels of concurrent multiple paternity (CMP) and the probability of detecting multiple paternity (*PrDM*) in multiple collections from four *Heterandria formosa* populations. Parentage analysis was based on genotypes at 3 microsatellite loci (*Hetfor5*, *TSS013* and *TSS051*) in all collections except Wacissa River in which parentage analysis was based on 6 loci (*Hetfor5*, *TSS013*, *TSS051*, *Hetfor4*, *TSS005* and *SLS45*). CMP values are the per cent of females in a given collection with three or more paternal alleles present in the sample of eyed embryos. *PrDM* values are for simulations assuming skewed and even paternity, respectively

Collection	*N*	Mean Female SL	Range	Mean fecundity	Range	CMP (%)	*PrDM*
Moore Lake 2007	18	18.6	14.7–20.2	13	5–21	44	0.53, 0.58
Moore Lake 2008	19	20.1	16.8–25.7	15.3	6–26	32	0.43, 0.46
Moore Lake 2009	20	20.1	17.0–25.5	18.8	6–45	50	0.50, 0.52
Trout Pond 2007	20	22.7	18.3–27.4	24.8	8–48	20	0.37, 0.39
Trout Pond 2009	14	21.3	14.2–26.1	18.2	3–32	29	0.48, 0.51
Wacissa River 2007	18	17.3	14.2–21.8	5.3	3–8	61	0.69, 0.83
Wacissa River 2008	35	18.5	15.6–22.3	6.9	2–17	60	0.85, 0.93
Wacissa River 2009	20	17.9	16.1–20.3	5.8	3–14	40	0.85, 0.94
Wakulla Springs 2007	20	20.3	17.1–23.6	12.6	4–26	70	0.97, 0.99
Wakulla Springs 2008	19	20.2	16.6–22.9	10.6	3–22	63	0.94, 0.98
Wakulla Springs 2009	15	19.7	17.1–24.2	12	7–29	80	0.98, 0.99

Table 2 The characteristics of the microsatellite loci used to genotype mothers and their embryos. For each locus, we report the total number of alleles (*A*) observed in the population and the observed heterozygosity (H_o) in the female sample. Only a subset of individuals from Moore Lake (n = 20), Trout Pond (n = 20) and Wakulla Springs (n = 54) were screened at *Hetfor4*, *TSS005*, and *SLS45*

Locus												
	Hetfor5		*TSS013*		*TSS051*		*Hetfor4*		*TSS005*		*SLS45*	
Population	*A*	H_o	*A*	H_o	*A*	H_o	*A*	H_o	*A*	H_o	*A*	H_o
Moore Lake	3	0.26	6	0.38	5	0.72	4	0.46	2	0.36	1	0
Trout Pond	1	0	3	0.38	3	0.82	3	0.76	2	0.5	1	0
Wacissa River	7	0.47	11	0.84	14	0.83	13	0.60	6	0.61	3	0.54
Wakulla Springs	10	0.52	15	0.88	16	0.86	8	0.68	5	0.56	3	0.64

(Table 2). These differences were slight for *SLS45* but considerable for the other loci, in which WR and WS had 2–5 times the number of alleles and twice the heterozygosity as ML and TP. This is consistent with the positive correlation between population density and average heterozygosity observed in a previous study of seven *H. formosa* populations (Soucy & Travis 2003). There were no significant deviations from Hardy–Weinberg equilibrium at any locus in any population ($P > 0.05$ in all cases), although the data suggested an excess of heterozygotes at *TSS051* in TP (P = 0.075). There were no significant deviations from linkage equilibrium among the loci ($P > 0.05$ in all cases).

We detected a null allele at *TSS051* in the 2009 WS collection. The null allele was indicated by the presence of a single homozygous female carrying several embryos with a different homozygous genotype. We estimated the frequency of this null allele as the number of inferred null heterozygotes in the female sample (1) divided by the total number of alleles in the adult female sample (108). The frequency of this null allele was <1% and is not likely to affect our estimates of multiple paternity.

Estimates of CMP were based on genotypes at three loci (*Hetfor5*, *TSS013* and *TSS051*) in all populations except WR where CMP was based on genotypes at all six loci (the original three loci plus *Hetfor4*, *TSS005* and *SLS45*). This decision was based upon whether the use of all six loci improved our probability of detecting multiple mating. In ML and TP, the additional loci exhibited low variability (e.g. two of the three additional loci had only one and two alleles, respectively) and *PrDM* indicated that adding these loci did not improve the probability of detecting multiple mating. For example, in the 2007 ML collection, the probability of detecting multiple paternity with the first set of three loci was 0.53 and 0.58 assuming skewed and even paternity, respectively. With all six loci, these probabilities were nearly identical: 0.53 and 0.59. The same was true for the 2007 TP collection. The probability of detecting multiple paternity with the first set of three loci was 0.37 and 0.39 assuming skewed and even paternity, respectively. With all six loci, these probabilities were still quite low: 0.47 and 0.50. In WR and WS, the additional loci were more variable. Adding these loci increased the probability of detecting multiple mating in WR. For example, in the 2007 WR collection, the probability of detecting multiple paternity with the first set of three loci was 0.59 and 0.67 assuming skewed and even paternity, respectively. These probabilities with all six loci were 0.69 and 0.83. In WS, the probability of detecting multiple paternity was quite high with the first set of loci and adding the additional loci offered little improvement. For example, in the 2007 WS collection, the probability of detecting multiple paternity was 0.97 and 0.99 assuming skewed and even paternity, respectively. These probabilities with all six loci were 0.98 and 1.00.

Across all collections, the detected levels of CMP using GERUD varied fourfold, between 20% and 80% (Table 1). There was no evidence for significant differences between years in the estimated level of CMP in any of the four populations (ML, χ^2 = 1.42, P = 0.50; TP, χ^2 = 0.34, P = 0.56; WR, χ^2 = 2.09 P = 0.35; WS, χ^2 = 1.14, P = 0.57) so we pooled years for further analyses. With years pooled, the estimated level of CMP using GERUD was highest in WS (70%) and lowest in TP (24%; Fig. 2). The significant difference between the estimated level of CMP in the WR (53%) and that in TP (24%) (χ^2 = 8.42, P = 0.004) was consistent with previous results (Soucy & Travis 2003). Similarly, the overall level of CMP in WS (70%) was significantly greater than the level in ML (42%; χ^2 = 8.98, P = 0.003). Examined broadly, these differences between populations appear associated with the consistent, order-of-magnitude differences between them in their densities.

These results suggest substantial differences between these four populations in their levels of CMP that would align the results of the interpopulation crosses with the predictions of the VDCH. However, these esti-

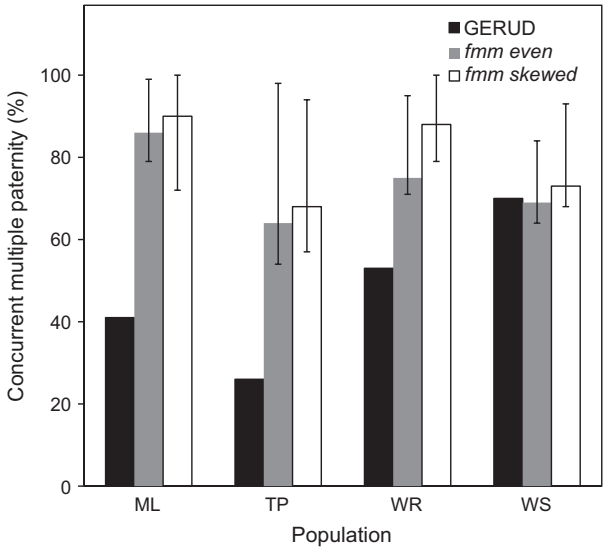

Fig. 2 Estimates of concurrent multiple paternity (the per cent of females carrying multiply sired broods) in Moore Lake (ML), Trout Pond (TP), Wacissa River (WR) and Wakulla Springs (WS). For each population, we present the estimated level of CMP based on the minimum sire number reconstructions from GERUD (black bars) and the expected level of CMP and 95% confidence intervals from simulations run using *fmm* assuming even (grey bars) and skewed (open bars) paternity.

mated differences do not take into account the fact that the probability of detecting multiple mating was not the same in each population. Across all loci, WR and WS display substantially more genetic variability than do ML and TP, which directly affects the ability to detect multiple sires represented in a female's offspring. The probability of detecting multiple mating was much lower in the low-density populations (ML and TP), despite their higher fecundities, than in the high-density populations (WR and WS; see Table 1). In fact, the level of genetic variation in ML and TP provided as little as half the power to detect multiple paternity in those populations when compared to detection probabilities in WR and WS. The low power of these loci to detect multiple mating in ML and TP suggest that the earlier estimates of CMP severely underestimate the true level of CMP in these populations.

Using *fmm* (Neff *et al.* 2002), we estimated the levels of CMP to be between 64% and 86% assuming even paternity and between 68% and 90% assuming skewed paternity (Fig. 2). The levels of multiple paternity in ML, TP and the WR estimated using *fmm* were considerably higher than the minimum estimates provided by GERUD. In contrast, estimates of multiple paternity in WS were relatively unaffected by the method of estimation. Given the large overlap in the 95% confidence intervals of these estimates, there is little convincing evidence for variation among populations in their levels of CMP nor is there convincing evidence that a difference in density of an order of magnitude is associated with variation in the level of CMP.

Table 3 COLONY estimates of CMP (the % of females carrying multiply sired broods), average number of sires per female and the average number of sires per brood in each Wakulla Springs collection. Estimates of sires per brood are for multiply sired broods only

Year	CMP (%)	Sires per female	Sires per brood
2007	80	2.5 (1–5)	1.84
2008	74	3.05 (1–6)	2.17
2009	93	2.5 (1–5)	2.02

In each WS collection, COLONY inferred higher levels of multiple paternity than GERUD (compare Tables 1 and 3). Across all collections, the number of sires ranged from 1 to 6 and was positively correlated with fecundity ($r = 0.42$, $P = 0.0015$). The average number of sires per female for each collection was between 2.5 and 3.05, and the average number of sires per brood in multiply mated females was between 1.84 and 2.17 (Table 3). The average number of sires per brood was >1 in 39 of the 40 multiply mated females. This indicates that multiple paternity in *H. formosa* typically involves multiple sires contributing to offspring within the same brood instead of different fathers contributing to distinct broods. *B* values ranged from −0.083 to 0.35, and paternity was significantly ($P < 0.05$) skewed in 12 of the 40 multiply sired females with significant *B* values ranging from 0.095 (the most successful of three males sired 57% of the offspring) to 0.35 (the most successful of two males sired 94% of the offspring).

Discussion

In this study, we examined whether there are consistent, density-driven mating system differences between four *H. formosa* populations that could explain the asymmetry in the fitness of hybrid crosses observed in two previous studies (Schrader & Travis 2008, 2009). We found consistent and dramatic differences between populations in density, an ecological variable that is expected to influence the level of multiple paternity in *H. formosa* (Soucy & Travis 2003). Despite differences in density, we found mixed evidence for mating system differences. Estimates of multiple paternity based on the minimum number of reconstructed sires indicated higher levels of CMP in WS and WR than in ML and TP. However, the probability of detecting multiple paternity in ML and TP was much lower than in WR and WS. An alternative analysis that accounts for these

differences suggested high levels of CMP in all four populations (Fig. 2).

Our field censuses revealed that WR and WS have consistently higher densities of *H. formosa* than ML and TP (Fig. 1). These results agree with other studies involving two (Richardson *et al.* 2006) or three (Leips & Travis 1999) of these populations. The density differences we report here are associated with previously documented differences in *H. formosa* life-history traits. Specifically, the low-density populations (ML and TP) have higher fecundity (Leips & Travis 1999; Schrader & Travis 2009) and smaller offspring (Leips & Travis 1999; Schrader & Travis 2005, 2009) than the high-density populations (WR and WS). These results further support previous suggestions that population density is an important force shaping patterns of life-history variation in *H. formosa* (Leips & Travis 1999; Leips *et al.* 2009). There was also a clear association between population density and measures of genetic variation in these populations. Across all loci, the high-density populations exhibited more alleles and higher levels of heterozygosity than the low-density populations (Table 2). This pattern agrees with the positive correlation between population density and heterozygosity observed by Soucy & Travis (2003) in a study of seven *H. formosa* populations.

Despite consistent density variation among populations (Fig. 1), we found little compelling evidence for an association between levels of density and CMP. In addition, despite temporal variation in density across an order of magnitude within the WR and WS populations (where we had the highest power to detect multiple mating), we found no concordant temporal variation in the level of CMP. In fact, between 2008 and 2009, the change in CMP in WR and WS was opposite that expected if increasing density increased CMP. Specifically, between 2008 and 2009, CMP estimated in GERUD increased from 63% to 80% in WS as density decreased nearly fivefold (Fig. 1, Table 1). The change in CMP estimated using COLONY was in the same direction: from 74% to 93%; (Table 3). During this same period, CMP estimated using GERUD decreased from 60% to 40% and density increased 20-fold in the WR (Fig. 1, Table 1). Although we found no evidence for a positive effect of population density on levels of CMP, we cannot rule out the possibility that density affects other parameters of the mating system such as the average number of sires per brood. In fact, there is some data suggestive of an increase in the number of sires per female and sires per brood with density in WS. Specifically, the COLONY analyses for WS indicated that the average number of sires per female and sires per brood for multiply mated females were highest in the 2008 collection, which corresponded to the collection with the highest density (see Fig. 1 and Table 3).

In a previous study, Soucy & Travis (2003) found that the level of CMP was significantly higher in WR than in TP (66% vs. 15%). The levels of CMP that we observed based upon minimum sire number reconstruction in WR (44–70%) and TP (25–29%) were similar to those reported by Soucy & Travis (2003). However, the low levels of genetic variation in TP greatly reduced our power of detecting multiple mating, an issue not taken into account by Soucy & Travis (2003), who estimated a low probability of nondetection for the level of genetic variation displayed in their data. When we accounted for the different levels of genetic variation in these populations, we estimated high levels of CMP in both of them.

The difference between our estimates of CMP in TP and the estimate from Soucy & Travis (2003) might indicate a large increase in the level of CMP between 2002 and 2007 if the probability of detecting multiple paternity was much higher in the earlier sample. We used the allele frequencies from Soucy & Travis (2003) to calculate the probability of detecting multiple paternity in their sample using *PrDM* (Neff & Pitcher 2002). The results of this analysis indicate that there was a low probability of detecting multiple paternity in these data (0.48) and that the level of CMP estimated for TP by Soucy & Travis (2003) is an underestimate of the true level of CMP in this population. These considerations argue against a temporal change in the level of CMP in TP.

Schrader & Travis (2008) argued previously that the mating system differences between TP and the WR described by Soucy & Travis (2003) could explain the asymmetry in hybrid fitness we observed in crosses between these populations in a manner consistent with the VDCH (Schrader & Travis 2008). The results here suggest a strict application of the VDCH may not account for the results of these crosses nor of crosses between the ML and WS populations, which show the same patterns (Schrader & Travis 2009). We suggest four other hypotheses for the asymmetries in hybrid fitness we documented in previous studies. These hypotheses are all rooted in the disruptive effects of hybridization on maternal–foetal interactions but are based on different underlying forces shaping this interaction.

First, theory suggests that maternal–foetal conflict can drive the evolution of reproductive isolation between populations even in the absence of mating system differences. Specifically, Kondoh & Higashi (2000) developed a model of reproductive isolation driven by intragenomic conflict that incorporates mating system variation and the costs and benefits associated with the production of foetal growth enhancers (GE) and foetal growth suppressors (GS). Kondoh & Higashi (2000) found that con-

flict combined with differences between populations in the costs of producing GE and GS can drive populations with the same mating system to different equilibrium levels of GE and GS. Crosses between populations will disrupt the balance between GE and GS and prevent the normal development of hybrid embryos. Our finding that levels of CMP are likely to be uniformly high certainly supports a hypothesis of substantial conflict between the interests of individual embryos and their mothers in *H. formosa*. Prior work documenting a variety of ecological differences between these populations, especially in conditions leading to different growth rates and levels of resource limitation (Leips & Travis 1999; Richardson *et al.* 2006), suggests that the costs and benefits of GE and GS production can differ between them. We note, however, that this model assumes that growth enhancers are paternally expressed and growth suppressors are maternally expressed (Kondoh & Higashi 2000). We know little about the presence of genomic imprinting in *H. formosa* except that *IGF2*, which is imprinted in mammals, is biallelically expressed in the placenta of *H. formosa* (Lawton *et al.* 2005). Whether maternal–foetal conflict can drive the evolution of reproductive isolation between allopatric populations in species without genomic imprinting has not been examined explicitly.

Second, it is possible that a modified version of the VDCH that incorporates mating system differences and population size explains the results of our previous population crosses. In formulating the VDCH, Zeh & Zeh (2000) argued that the magnitude of maternal–foetal conflict will depend on the genetic mating system of the population. While the importance of antagonistic co-evolution between mothers and embryos will certainly be affected by the mating system, population size is also likely to play a critical role. For example, theory predicts sexual conflict can lead to the rapid evolution of reproductive isolation between large allopatric populations (Gavrilets 2000). However, antagonistic co-evolution driven by sexual conflict is less likely in small populations because they may experience less intense sexual conflict, have lower levels of standing genetic variation and have fewer mutations (Gavrilets 2000; Gavrilets & Hayashi 2005; Gay *et al.* 2009). Recent empirical work supports the prediction that antagonistic co-evolution is less likely in small populations (Gay *et al.* 2011) and that sexual conflict can lead to incipient reproductive isolation between large but not small populations (Gay *et al.* 2009). High levels of multiple paternity and large population sizes indicate that antagonistic co-evolution between mothers and embryos is likely to be important in WR and WS. However, despite high levels of polyandry, the genetic consequences of consistently low population sizes may reduce the potential for antagonistic co-evolution between mothers and embryos in ML and TP.

A third, related hypothesis is that high levels of inbreeding in the low-density populations have reduced the intensity of maternal–foetal conflict compared with the high-density populations despite high levels of multiple paternity in all populations. This hypothesis is similar Brandvain & Haig's (2005) weak-inbreeder–strong-outbreeder hypothesis which argues that the strength of sexual conflict over fertilization and seed provisioning in angiosperms is greater in outcrossing populations than in selfing populations because outbreeding produces a lower average level of relatedness among offspring. The much lower levels of genetic variation (Table 2) and lower densities (Fig. 1) in ML and TP compared with WR and WS are certainly consistent with a greater potential for inbreeding in these populations. How quantitative variation in the level of inbreeding (e.g. from selfing to full- and half-sib mating) might affect the outcome of maternal–foetal conflict deserves further investigation.

Our final hypothesis is that hybridization between populations disrupts maternal–foetal coadaptation driven by selection for different sized offspring in different populations. Theory suggests that even in the absence of maternal–foetal conflict, selection on offspring traits (e.g. size at birth) can lead to maternal–foetal coadaptation (Wolf & Brodie 1998). Such coadaptation within a population can lead to divergence between populations and may play an important role in speciation (Wolf & Brodie 1998). In both previous studies involving these populations (Schrader & Travis 2008, 2009), crosses between a female from a population characterized by a low level of matrotrophy (ML and TP) and a male from a population characterized by a high level of matrotrophy (WR and WS) resulted in a higher rate of aborted embryos than the reciprocal cross or either within population cross. We suggest that in matrotrophic species such as *H. formosa*, the disruption of maternal–foetal coadaptation produced by hybridization has more dire consequences for offspring fitness when the maternal parent is from a low-matrotrophy population. In contrast, differences in maternal investment into individual offspring (i.e. egg size) do not appear to predict patterns of reproductive isolation in oviparous fish. For example, in Centrarchid fishes, differences between species in egg size do not predict the direction of asymmetry in F1 hybrid crosses (Bolnick *et al.* 2006, 2008). Instead the direction of asymmetry is predicted by the rate of mitochondrial evolution of the maternal parent relative to the paternal parent as well as body size differences between the parents (Bolnick *et al.* 2008). Our hypothesis, that differences in the level of matrotrophy predict patterns of hybrid inviability in matrotrophic species, predicts that crosses between

allopatric populations with similar levels of matrotrophy (e.g. WS × WR and ML × TP) should not result in high abortion rates in either direction of the hybrid cross. We are currently testing this prediction.

Our estimates of sire number in WS suggest that multiple paternity within in broods is very common in *H. formosa* and that there is often reproductive skew among sires. These results are similar to those of other studies of poeciliid fish. For example, in a study of several natural populations of the guppy, *Poecilia reticulata*, Neff *et al.* (2008) found that 95% of broods exhibited multiple paternity and that paternity within a brood was often skewed towards one sire. In *H. formosa*, the frequent occurrence of multiple paternity within broods increases the opportunity for intrabrood conflict between sibling embryos for maternal resources. In species with uniparental care, this type of conflict is expected to increase the level of overt competition between siblings relative to interbrood conflict (Mock & Parker 1997). The manifestation and consequences of competition between sibling embryos of matrotrophic poeciliids such as *H. formosa* are intriguing areas for future study (Schrader & Travis in press).

In conclusion, our results indicate that extreme variation in population density, within and among populations, does not necessarily translate into differences in frequency of multiple paternity. Thus, differences between *H. formosa* populations in the frequency of multiple paternity are not sufficient to explain the asymmetric reproductive isolation seen in previous studies (Schrader & Travis 2008, 2009). We suggest that other influences on maternal–foetal conflict may be important in explaining these differences. Alternatively, the asymmetric reproductive isolation seen in previous studies might reflect the disruption of maternal–foetal coadaptation.

Acknowledgements

We thank K. McGhee, M. Adreani, M. Aresco, M. Gunzburger, R. Hale and P. Hill for help in field and J. Biesiadecki, L. Noa and M. Lowenberg for help in the laboratory. D. Levitan kindly provided laboratory space during the beginning of this project. E. Berdan, G. Kozack, K. McGhee and several anonymous reviewers provided helpful comments on a previous version of this manuscript. This work was performed under Florida State University's ACUC Protocol 9321 and was supported by US National Science Foundation award DEB 08-22547 to J. Travis and M. Schrader and a Florida State University Dissertation Research Grant to M. Schrader.

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Data accessibility

Population density data, female fecundity and paternity data, and genotypes of mothers and embryos: DRYAD entry: doi:10.5061/dryad.p21g8.