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Original Article

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Can females differentially allocate resources to offspring sired by different males?

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ABSTRACT

The viviparity-driven conflict hypothesis postulates that the evolution of matrotrophy (postfertilization maternal provisioning) will result in a shift from a pre- to postcopulatory mate choice and thus accelerate the evolution of postcopulatory reproductive isolation. Here, we perform artificial insemination experiments on *Heterandria formosa*, a matrotrophic poeciliid fish, to probe for evidence of postcopulatory female choice. We established laboratory populations from Wacissa River (WR) and Lake Jackson (LJ). The WR females normally produce larger offspring than the LJ females. We artificially inseminated females with sperm from each population or from both populations simultaneously. When LJ females were inseminated with sperm from WR and LJ males, they allocated fewer resources to WR-sired offspring than when they were inseminated with WR sperm alone. The LJ females carrying developing offspring sired by males from different populations were thus able to discriminate against non-resident males when allocating resources to developing young. The WR females, which normally produce larger offspring than LJ females, did not discriminate among males from different localities. These findings provide insights into the ability of females from one population to exercise a form of postcopulatory mate selection.

Keywords: mate choice; sexual selection; maternal provisioning; matrotrophy; evolution; reproductive isolation; *Heterandria formosa*; fish; artificial insemination

INTRODUCTION

Zeh and Zeh (2000, 2001) offered the viviparity-driven conflict hypothesis (VDCH) to explain why postmating reproductive isolation has emerged more quickly in matrotrophic than in non-matrotrophic species. In matrotrophic species, the mother continues to provision offspring during development, whereas in non-matrotrophs, maternal provisioning is completed before fertilization. In the VDCH, parent–offspring and sibling conflicts create the conditions that promote the rapid evolution of postmating isolation.

Mothers and offspring are in conflict because offspring are selected to solicit more maternal resources than a mother is selected to provide (Trivers 1974). This inequality is a byproduct of their relatedness to each other and of the costs and benefits associated with maternal allocation and embryonic acquisition of resources. Mothers are equally related to all offspring because they share half of their genes with all of them. The benefit they gain by increasing the allocation of resources to one baby is matched by the equal cost of the necessary withholding of resources from another baby. Offspring share 100% of their genes with themselves, but only 50% of their genes with full siblings. The consequence is that any benefit they gain from acquiring more resources from their mother is associated with a cost of reduced allocation to their siblings that is discounted by half. If the litter contains offspring sired by different fathers, then the cost of reduced allocation to a half-sibling is only one-quarter of the benefit an individual gains from acquiring more resources from the mother. It is these differences in the benefits and costs associated with maternal allocation and offspring acquisition that are the root of the predicted conflict between mothers and offspring.

The VDCH posits that matrotrophic females evolve polyandry as a method of hedging their bets against mating with genetically incompatible males; however, the presence of offspring from diverse sires increases maternal–fetal conflict because any

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benefit one baby gains from acquiring more parental resources is associated with the reduced cost of denying resources to half-siblings (Trivers 1974). Mothers can reconcile this heightened conflict with the virtue of mating with multiple males if they can discriminate against offspring of less desirable males, shifting their allocations of resources from them to the offspring of more desirable males. This postcopulatory mate choice can extend to discriminating against offspring of non-native males, thereby accelerating reinforcement between populations and, eventually, incipient species, as demonstrated in angiosperms (Garner *et al.* 2016, Coughlan 2020, 2023, Sandstedt and Sweigart 2022).

Many of the elements of the VDCH are well established. Polyandry can function as insurance against genetic incompatibilities between males and females (Engqvist 2006, Rodriguez-Munoz and Tregenza 2009, Arbuthnott *et al.* 2015). There is ample evidence for premating discrimination by females against non-native males across taxa (Pillay 2000, 2006, Svensson *et al.* 2006, Galipaud *et al.* 2015, Guo *et al.* 2020). Matrotrophic females in polyandrous matings are capable of choosing among offspring with different sires via biased resource allocation against those offspring (Engelhardt *et al.* 2006, Lindholm *et al.* 2013). To our knowledge, there are no data on whether matrotrophic females can accelerate reinforcement between populations by postmating discrimination against non-native males.

The matrotrophic poeciliid species *Heterandria formosa* is well suited for examining this problem. There are well-studied populations in North Florida with extensive variation in life histories, much of it associated with variation in population density (Leips *et al.* 2000, Schrader and Travis 2012a). Offspring size at parturition is especially variable among populations; females from localities with high population densities produce substantially larger offspring than those from populations with low population densities. Differences between populations in offspring size have a genetic basis (Baer 1998a, Leips *et al.* 2000). Because initial ovum sizes are the same in both populations, mothers from populations that produce large offspring do so by providing more resources to their offspring during development (Leips *et al.* 2000, Schrader and Travis 2005).

Offspring size is a battleground for the type of conflict posited by the VDCH. Extensive polyandry occurs in these populations (Soucy and Travis 2003). Offspring from different populations differ in their level of coercion for maternal resources (Schrader and Travis 2009, Schrader *et al.* 2013) and, during gestation, they compete with one another within and among litters (Schrader and Travis 2012b). Larger offspring are more likely to survive than smaller offspring (Henrich and Travis 1988, Leips *et al.* 2013, Felmy *et al.* 2022), placing a premium on offspring coercive ability and forcing mothers that are carrying numerous offspring simultaneously to allocate resources strategically.

Pairs of *H. formosa* populations that differ in offspring size display an asymmetric pattern of postmating isolation (Schrader *et al.* 2013). When females from a population in which offspring are small are mated with males from a population in which offspring are large, there is a mismatch between the amount of resources that mothers are accustomed to providing and the amount of resources that offspring are accustomed to receiving. This conflict generates a high rate of stillborn offspring; crosses in the reverse direction produce viable offspring of intermediate size (Schrader and Travis 2008). No such effects are observed when crosses are made between males and females from different populations with similar average offspring sizes.

We used two populations of *H. formosa* that typically produce fewer, larger [Wacissa River (WR)] or many, smaller [Lake Jackson (LJ)] offspring. These habitats differ in several abiotic and biotic features, especially predation risk on *H. formosa* and population density. The Wacissa River has higher *H. formosa* population densities and lower predation risks (MacRae and Travis 2014). Prior work has shown that differences in offspring size among north Florida populations, including these two, are best explained as responses to different regimens of population density (Schrader and Travis 2012a); high population densities favour the evolution of larger offspring size.

Here, we demonstrate that *H. formosa* females discriminate against offspring sired by non-native males by reducing resource allocation to them. We used artificial insemination of females with sperm from two males to remove any premating cues about male identity. When sperm are mixed in a balanced, factorial design, we can discriminate between the roles of sires and mothers in determining offspring size and provide a direct evaluation of postcopulatory mate selection via discriminatory resource allocation.

MATERIALS AND METHODS

Heterandria formosa study system

Heterandria formosa is a small, promiscuous, highly matrotrophic poeciliid found in the southeast USA. A unique feature of this species is that there are large differences among populations in average offspring size corresponding to their matrotrophy index, which is the estimated dry mass of offspring at birth divided by the dry mass of the egg at fertilization (Reznick *et al.* 2002). Their matrotrophy index ranges from 40 in populations that produce small offspring to 67 in populations that produce large offspring (Schrader and Travis 2012b). Differences in offspring size contribute to reproductive incompatibility between populations (Schrader and Travis 2008, 2009, Schrader *et al.* 2013).

Local populations in north Florida tend to be genetically distinct at near-neutral markers. Soucy and Travis (2003) showed that allele frequency distributions at three loci are almost always significantly different between several pairs of *H. formosa* populations. Baer (1998b) found high levels of pairwise F_{ST}, a measure of genetic distance between populations, among *H. formosa* populations, but there is no calculated or estimated F_{ST} that exists for WR and LJ. Bagley *et al.* (2013) showed that WR and LJ have different mitochondrial cytochrome *b* haplotypes.

Fish collection and maintenance

Stocks of wild-caught *H. formosa* for each population were established 6 months before the beginning of the experiment. The fish used in this experiment were F1 individuals grown in a common laboratory setting. Males and females were separated before maturity, ensuring that we had virgin females for artificial insemination. We discriminate between males and females based on the metamorphosis of the anal fin of males into a gonopodium, the intromittent organ (Fraser and Renton 1940). Immature males were moved into 20 L male-only tanks separated by population, and females were isolated in 7.5 L tanks to grow and mature until

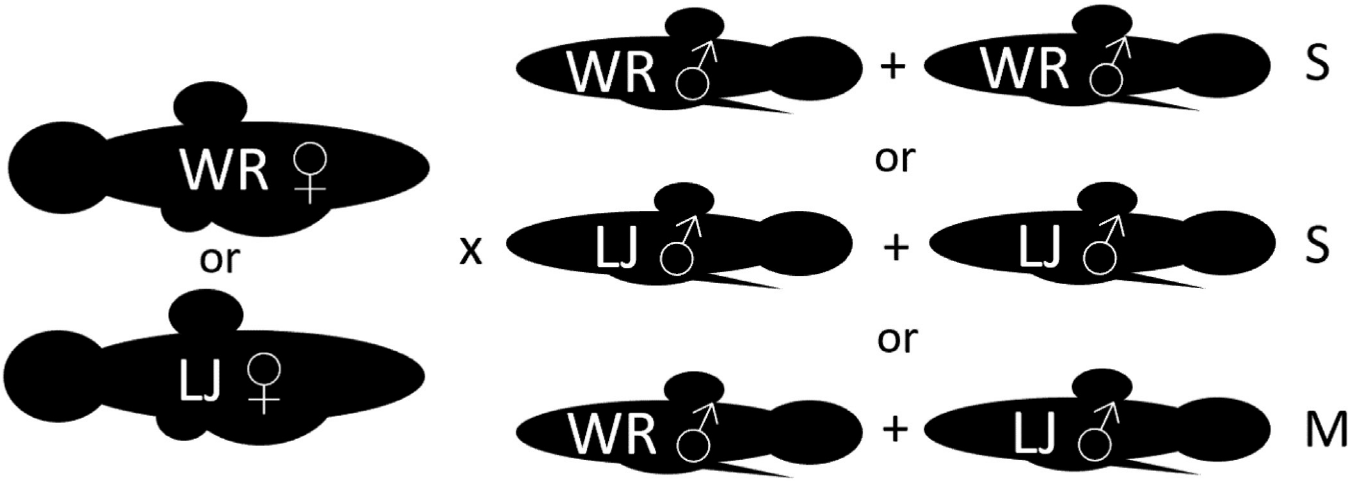

Figure 1. Experimental design for crosses between Wacissa River (WR) or Lake Jackson (LJ) population females with WR or LJ population males (Single, 'S' crosses), or WR and LJ population males simultaneously (Mixed, 'M' crosses).

we inseminated them, ~2 months later. All individually housed female *H. formosa* were fed one pipette drop of a mix of live baby brine shrimp and Tetramin flakes, twice per day.

Artificial insemination and experimental design

We artificially inseminated females with sperm from multiple males from their own population and the genetically distinct population. To do so, F1 male *H. formosa* were selected at random from stock tanks. We anaesthetized them in a .01% solution of buffered MS-222. Once the males began to turn in the water (after ~15 s), they were placed on a bed of moist, sterile cotton. We positioned the male with pieces of sterile cotton placed on either side of the head and caudal fin, moved the gonopodium anteriorly, then pressed lightly on the abdomen with a blunt-tipped probe coated in silicone, expressing the ejaculate. The ejaculate consists of spermatozeugmata, each of which contains thousands of sperm (Liu *et al.* 2018). We collected the spermatozeugmata with a micropipette tip attached to silicone tubing, then transferred them to a small drop of Hank's balanced salt solution. We then measured the length and weight of the male and returned him to the recovery container. The recovery solution included one drop of Stress Coat, which stimulates the production of the protective slime layer and facilitates the healing of any abrasions associated with handling. It takes ~2 min to extract sperm from and measure each male and further 4 min to prepare an insemination droplet and inseminate a female.

Sperm was observed to be viable in the Hank's balanced salt solution for ≥15 min. We performed a pilot study before beginning the experiment to see whether there were detectable differences in the number of spermatozeugmata per male or sperm per spermatozeugmata. Although this was a small pilot study (we looked at three males from one population of *H. formosa*), there were no significant differences among males in the number of sperm per spermatozeugmata.

We inseminated females with spermatozeugmata from either two or four males, comprising four treatments: (A) female × 2 WR males; (B) female × 2 LJ males; (C) female × 1 WR male + 1 LJ male; and (D) female × 2 WR males + 2 LJ males. These treatments were later combined into 'Single' male population and 'Mixed' male population insemination treatments (Fig. 1; see the '*Data organization and analysis*' section).

We anaesthetized females in the same way as the males, although they were left in the MS-222 for ~30 s longer to ensure that they were fully anaesthetized given their larger body size. Twenty spermatozeugmata from each male were inserted into the gonopore (gonadal opening) of each female. Spermatozeugmata were counted as they were pipetted out of the male's drop and into the mixed drop. The pipette tip was replaced between males, and only intact spermatozeugmata were transferred to a female. Each female was allowed to recover in her tank and treated with two drops of Stress Coat and a small dose of Maracin (an antibacterial fish medication) to limit the risk of infection from the procedure. As many females as possible were inseminated with the ejaculate from each pair of males. Males were stripped repeatedly over the course of the insemination period (~2 weeks), with ≥4 days in between inseminations.

Female *H. formosa* were inseminated in two phases, in autumn 2017 (WR) and spring 2018 (LJ). A total of 54 WR females were inseminated, but five died before they gave birth. Thirty-eight (79%) of surviving females produced offspring. A total of 51 LJ females were inseminated, and although no females died, four females were removed from the experiment owing to illness. Thirty-eight (81%) of surviving females produced offspring. A total of 36 WR and LJ males were used to inseminate WR females across all treatments, and 21 successfully sired offspring. Thirty WR and LJ males were used to inseminate LJ females across all treatments, and 25 successfully sired offspring.

Collection and preservation of juveniles

After 30 days, the normal duration of embryonic development, each female's tank was checked daily for offspring to ensure that all offspring used in the analysis were no older than 24 h. We captured newborn fish and placed them immediately in a lethal, 1% solution of MS-222. Each offspring was then transferred to a slide and placed under a dissecting scope, where we measured total and standard length. We then weighed offspring to the nearest .1 mg with an analytical balance and transferred them to a Dnase/Rnase-free, .6 mL microcentrifuge tube filled with 95%–100% ethanol. Tubes were labelled, then stored in a −20°C freezer.

DNA extraction and sequencing

We extracted DNA from whole juveniles and the caudle peduncles of adult fish using a Qiagen DNEasy kit. After DNA was extracted, we used a Qiagen Multiplex PCR kit to prepare two mixes, each with three microsatellite loci, for a total of six microsatellite loci. We sourced the microsatellite loci from previous poeciliid studies (Supporting Information, Table S1, S2). For fish in the mixed population insemination treatments, the six loci contained between 3 and 12 unique alleles. Nine hundred and one individuals were sequenced, consisting of the following WR/LJ females in each treatment: (A) 5/3; (B) 9/5; (C) 10/8; and (D) 3/7. Of these, four WR treatment C and one LJ treatment D females were removed because we could not assign mixed paternity conclusively to their litters. Furthermore, one LJ treatment B female was eliminated because she only gave birth to a single offspring. These eliminations resulted in the following number of WR/LJ remaining females in each treatment: (A) 5/3; (B; 9/4; (C) 6/8; and (D) 3/6.

Amplicons were sequenced on a fragment analyser at the University of Arizona Genomics Core. We used the program GENEIOUS to align the fragment data and assign genotypes. Once all individuals were genotyped, we assigned paternity manually by comparing alleles between mothers, potential fathers, and offspring.

Data organization and analysis

Because the females from the two populations were inseminated at different times, we analyse and report results for each population separately (Supporting Information, Table S3). Furthermore, because there were no significant differences found between treatments C and D (in which one male from each population or two males from each population were used to inseminate a single female), we regrouped our treatments into 'Single' population insemination (treatments A and B) or 'Mixed' population insemination (treatments C and D).

The data associated with each offspring included: days since insemination, number of broods per female, and number of offspring per brood. Any offspring born within a 48 h interval were counted towards a single brood, because there is typically a 3- to 5-day interval between broods, and two broods born within 48 h is highly unlikely.

Statistical analysis

Paternity

We analysed data only from broods with a paternity assignment rate of >50%. Some offspring could not be assigned to any potential father because of shared alleles between dams and potential sires, in addition to possible sequencing errors. We used a Bayesian method for assessing the posterior probability that one male was the sire of >50% of the offspring. To do this, we assumed, as a null hypothesis, that each male would sire 50% of all offspring, on average. For each cross in the analysis, we designated the male to whom we assigned the majority of offspring in that cross as our focal male and considered all remaining offspring, whether assigned to the other male or unassigned, as if they had been assigned to that other male. We then used the beta distribution to calculate the posterior probability that our focal male indeed sired more than his fair share, meaning >50%, using the calculator provided by Matthew Bognar (https://homepage.divms.uiowa.edu/~mbognar/applets/beta.html). We concluded that paternity was biased in a particular cross if the posterior probability exceeded .85, an admittedly arbitrary threshold but not an unreasonable one.

Offspring size as a result of cross type and paternity

We retained all offspring with assignable sires for the analysis of offspring size based on cross type and sire population. The models for offspring wet weight for each population were analysed as a linear mixed model with a type III ANOVA. We used Tukey's method for pairwise contrasts when there was a significant interaction, in order to diagnose which treatment combinations were significantly different from each other. We did this by calculating estimated marginal means (EMMeans) with a Tukey adjustment. The independent variables were sire population and cross type, with the two cross type categories being whether the insemination was 'Single' or 'Mixed' population, in addition to the interaction between sire population and cross type. Dam was a random effect. Brood number, meaning which in a sequence of broods an offspring was born in, was included as a covariate, because offspring size decreases with brood number in *H. formosa* (Schrader and Travis 2012a). Each population was analysed separately.

RESULTS

Paternity

The LJ males appeared to sire more offspring than WR males in the Mixed insemination treatments. There were 11 females (6 WR and 5 LJ) for which the probability of biased paternity by LJ males was high (>.85 confidence; Supporting Information, Table S5). Offspring of four of six WR females were biased towards LJ males, and offspring of four of five LJ females were biased towards LJ males.

Offspring size as a result of cross type and paternity

The LJ females, which were from the population that produces smaller offspring in the wild, produced significantly smaller WR-sired offspring in Mixed than in Single insemination treatments (Fig. 2; Supporting Information Table S4). The WR males sired offspring that were ~35% larger than offspring sired by LJ males when only their sperm were present. When sperm from both LJ and WR males were present, the size of the offspring sired by WR males were only 6% larger and were not significantly different from those sired by LJ males. The offspring sired by LJ males in LJ females were the same size regardless of the identity of the other male.

These results were supported by the statistical analyses. There was a significant interaction between sire population and cross type ($F = 7.01$, $P = .014$). The post hoc contrast showed that in the Single insemination treatment, the WR-sired offspring were larger than the LJ-sired offspring (t.ratio = −3.21, $P = .0039$). The *t*-ratio is the ratio of the difference between the sample

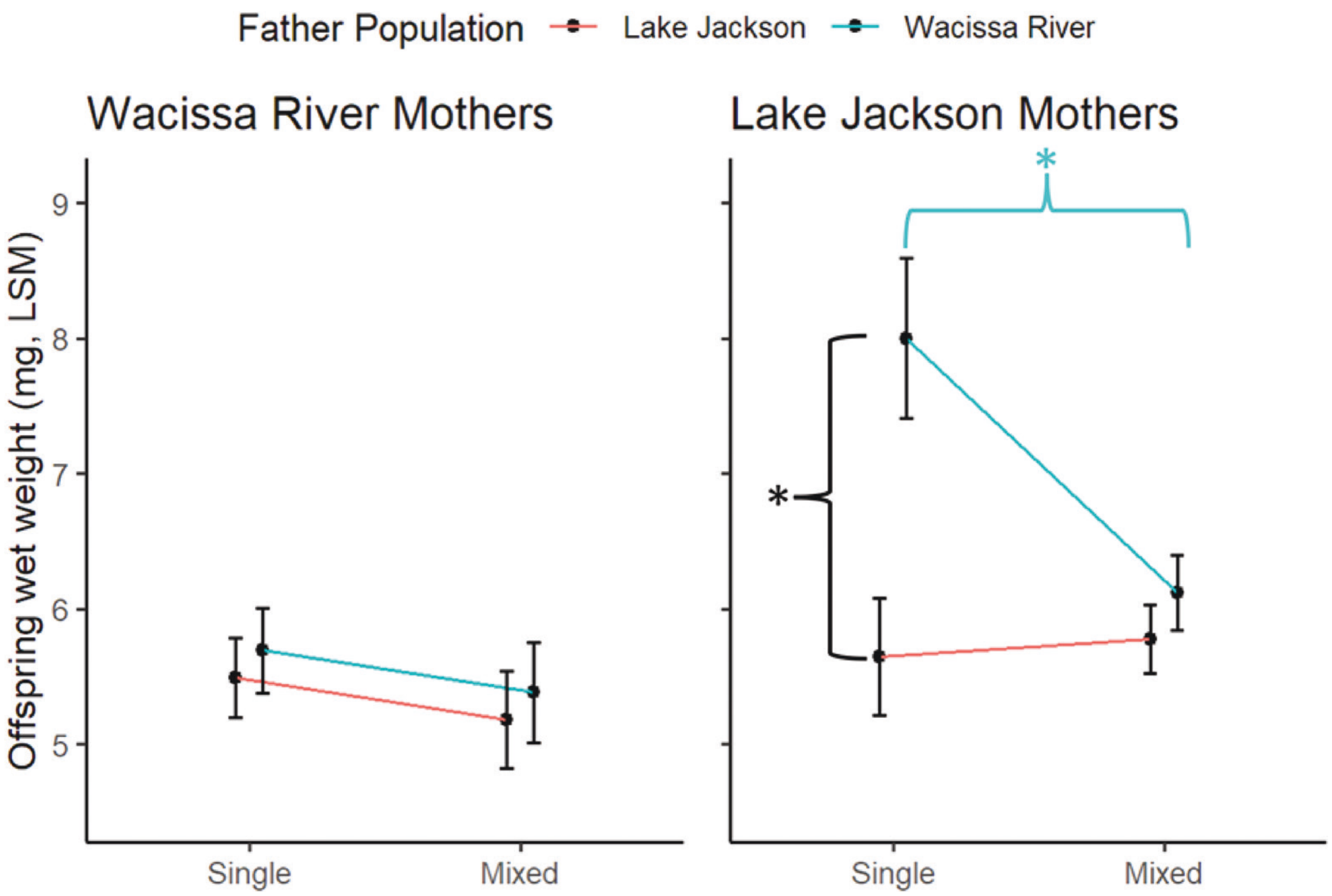

Figure 2. Least squares means (LSM) ± SE of the LSM for offspring wet weight based on Single or Mixed insemination treatment for Wacissa River and Lake Jackson. Significant differences are indicated with a black asterisk for differences between sire population and a blue asterisk for differences between Single sire population and Mixed sire population inseminations.

mean and the population mean to the standard error of the mean. In the Mixed insemination treatment, there was no significant difference in size between offspring sired by a WR male and offspring sired by an LJ male (t.ratio = −1.77, *P* = .0776; Fig. 2). There was also a significant effect of brood number as a covariate (*F* = 17.33, *P* < .001); offspring sizes were smaller in later broods.

The patterns for WR females, which were from the population that produces large offspring, were different (Fig. 2). Offspring sired by WR males were slightly larger than those sired by LJ males, regardless of treatment, but there were no significant differences among any of the four combinations of sire identity and mating treatment. The main effect of sire population was not significant (*F* = .15, *P* = .69), nor was there an interaction between sire population and cross type (*F* = 1.80, *P* = .19). There was no significant effect of brood as a covariate for WR (*F* = .56, *P* = .45).

DISCUSSION

Females from Lake Jackson carrying broods of mixed population paternity can limit allocation to offspring sired by Wacissa River males. Females from Wacissa River make no such adjustments to their allocation of resources to developing offspring. In all the treatments, females were given sperm from at least two males; therefore, this result is not merely a consequence of the relative intensity of male–male competition.

Wacissa River females, which produce fewer, larger offspring in nature, produced offspring of a similar size regardless of the mix of sperm they received. Schrader *et al.* (2013) obtained similar results when they mated fish from Wacissa River to ones from Trout Pond, which, like Lake Jackson, has females that typically produce smaller offspring. Offspring produced from the Wacissa River × Trout Pond crosses exhibited asymmetrical incompatibility. Females from populations that produce small offspring aborted a high number of offspring when they were inseminated by males from populations that produce large offspring (Schrader and Travis 2008, 2009, Schrader *et al.* 2013). Our results differed from those of (Schrader and Travis 2008, 2009) because we documented few aborted embryos but, as seen by Schrader and Travis, LJ mothers produced larger offspring when sired by WR males.

A possible reason for the virtual absence of aborted embryos when Lake Jackson females were mated with WR males is that the females in this study were considerably larger than the average size of females in the earlier papers by Schrader and Travis. The standard lengths of *H. formosa* in the experiment conducted by Schrader and Travis ranged from 17 to 23 mm. They found that larger females aborted a smaller proportion of their offspring (see Schader and Travis 2008: fig. 1B). The abortion rate for females of >19 mm was 0%–20%. In our experiment, our smallest females were larger than those depicted by Schader and Travis (2008: fig. 1B); therefore, the absence of embryo abortion might be attributable to the larger sizes of the females in our experiment.

A second unexpected feature of our results is that WR females did not produce larger offspring than the LJ females (Fig. 2). In all prior studies they produced larger offspring. We note that experiments with LJ females were done at a different time from the experiment on WR females, hence our choice to analyse them separately. It is possible that some confounding environmental influence lies behind the unexpectedly small offspring produced by the WR females. Although food level was not a factor in our experiment, it represents one such environmental variable that has been associated with decreased offspring size in several matrotrophic poeciliid species (Reznick 1996, Banet and Reznick 2008, Banet *et al.* 2010, Pollux 2011).

The LJ males have greater success in siring offspring than WR males, regardless of which females they are mated with. The LJ females typically produce more offspring in each brood than WR females (Schrader and Travis 2012a), which might favour the evolution of more competitive sperm or more sperm per ejaculate than WR males. Schrader *et al.* (2012) showed that LJ males do not seem to have extraordinarily large testes for their body size (dry mass = .166 mg in Lake Jackson compared with .12 mg in Wacissa River); therefore, any male effects are likely to be in properties of the sperm or the number of sperm per ejaculate. This is an interesting future direction to pursue among *H. formosa* populations, especially considering different density regimens in each population. Colorful male guppies command higher paternity (Evans *et al.* 2003) and have faster and more viable sperm (Locatello *et al.* 2006); therefore, there might be life history-based differences in sperm characters among *H. formosa* males from different populations. It is also important to note that females might exert control over male seminal products (Eberhard and Cordero 1995).

Although we cannot, at present, comment on how, physiologically, *H. formosa* females can selectively partition resources to offspring as a function of their genotype, we can at least conclude that they can distinguish among offspring with different genotypes when allocating resources. This is consistent with Zeh and Zeh's (2000) prediction that there will be a shift in the arena of sexual selection from precopulation to postcopulation associated with the evolution of placentation. *Heterandria formosa* offers a unique perspective on female choice, because there is no male courtship or any evidence for precopulatory female choice in this species.

Our key result is the differential investment patterns of LJ females to offspring sired by local and non-resident males. Why do we not also see WR females discriminating against offspring sired by LJ males? One possibility is that the difference between populations in offspring size creates an asymmetry in conflict. Because WR offspring are bigger, we expect them to be adapted to demand more resources from the mother, but the LJ females are adapted to deliver less. The consequence is that offspring demands can be damaging, especially to the half-siblings sired by an LJ male. When a WR female is carrying a baby sired by an LJ male, we expect instead that the offspring are adapted to demand less than the mother is prepared to deliver. The offspring demands thus pose no threat to either the mother or to half-siblings sired by WR males. These results are analogous to those obtained for interspecific hybridizations between the deermouse species *Peromyscis polyonotus*, which is monogamous, and *Peromyscus maniculatus*, which is polygamous (Vrana *et al.* 2000). Polygamy is analogous to large offspring because each condition is predicted to intensify the expected demand for maternal resources by the developing offspring. In mice, the pairing of a female from the monogamous species with a male from the polygamous species means pairing a more demanding offspring with a mother less well adapted to meeting that demand. Such pairings are associated with reduced offspring fitness in comparison to the reciprocal cross between a female from the polygamous species with a male from the monogamous species.

Examples of within-population postcopulatory female choice have been demonstrated in mice (Lindholm *et al.* 2013) and primates (Maestripieri and Roney 2005, Engelhardt *et al.* 2006, Setchell *et al.* 2013). Factors such as sperm competition can muddy results, as in *Anthechinus* marsupials, in which large males dominate paternity in the wild, but in the laboratory the females prefer genetically dissimilar males regardless of size (Parrott *et al.* 2015). However, the female-mediated consequences of population crosses have not been examined in matrotrophic species until now.

Our study contributes insights about the way females of matrotrophic species provision their offspring and are capable of discriminating between offspring sired by different fathers. Previous studies show female preference towards males owing to precopulatory cues, whereas ours is the first study to demonstrate this in a species without female-driven precopulatory mate choice. Our results also provide support for the viviparity-driven conflict hypothesis by demonstrating a shift in the arena of conflict from pre- to postcopulation. Future studies can expand on the ways that matrotrophic females discriminate against males on a physiological level and the capability for females from certain populations to provision resources differentially among males.

SUPPLEMENTARY DATA

Supplementary data is available at *Biological Journal of the Linnean Society* online.

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AUTHOR CONTRIBUTIONS

S.T.L., D.N.R., and J.T. contributed equally to the experimental design. S.T.L., S.A.B., and J.T. contributed equally to the analysis of the data. S.T.L. wrote the paper and all authors contributed equally to the interpretation of the data and editing of the manuscript.

CONFLICT OF INTEREST

None declared.

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SHARED DATA

Data deposited in the Dryad digital repository https://doi.org/10.5061/dryad.h18931ztf.

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