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The Evolution of Reproductive Mode and Associated Familial Conflict in Livebearing Fishes

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The Evolution of Reproductive Mode and Associated Familial Conflict in Livebearing Fishes

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in

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by

Samantha Turner Levell

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Dedication

I dedicate my dissertation to my life partner, Samuel Bedgood, who has continued to be my confidant and best friend, who has not only supported my academic career, but also our passions outside of academia.

ABSTRACT OF THE DISSERTATION

The Evolution of Reproductive Mode and Associated Familial Conflict in Livebearing Fishes

by

Samantha Turner Levell

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, September 2022
Dr. David Reznick, Chairperson

Parent-offspring conflict arises because of the unequal patterns of relatedness among parents, offspring, and siblings. From an evolutionary perspective, parents optimize investment in each offspring to maximize their lifetime reproductive success. Females are equally related to all their offspring, so natural selection favors them allocating resources equally to each offspring. In contrast, natural selection will favor those offspring that acquire more resources, even at the expense of their mother and siblings. Recent thinking expands the potential influence of parent-offspring conflict from post-natal familial interactions to other facets of the organism's biology. The evolution of a livebearing reproductive mode, particularly involving a placenta, for example, is predicted to cause pre-natal mother-offspring conflict. In the livebearing fish family Poeciliidae, a placenta-like organ has evolved independently at least nine times. In my dissertation, I examine the potential for reproductive barriers in placental (*Heterandria formosa* and *Poeciliopsis prolifica*) and non-placental (*P. infans*) poeciliid species. I perform both natural and artificial insemination crosses between populations within three separate species of fish in the family Poeciliidae. In my first chapter, I find that both a placental and non-placental species exhibit reproductive incompatibility, but the location of the incompatibility is different. In my second and third chapters, I use artificial insemination to further investigate the effects of inter-population crosses on offspring size and number in two placental species, *H. formosa* and *P. prolifica*. In these species, natural differences in offspring size between populations of *H. formosa* are the source of conflict in offspring size. However, when females are mated to both their own and nonresident males, they produce intermediate offspring, discriminating against nonresident male's embryos. In *P. prolifica*, while there are no natural offspring size differences in the two populations, we find a similar pattern of incompatibility present in crosses that is mitigated when females are provided with sperm from her own, and a genetically distinct population. These results provide a new path for studying reproductive incompatibility and conflict in placental species, as well as evidence for cryptic female choice and discrimination against genetically different males.

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Introduction

Robert Trivers and the inception of parent-offspring conflict

Organisms are predicted to experience competition that results in conflict in virtually every aspect of their lives. Individuals must compete for finite resources such as food, mates, and shelter while avoiding environmental hazards (e.g. high winds, floods, extreme heat) and predation. Although resource competition and conflict are typically studied ecological contexts, conflict within families plays a significant role in the fitness of both parents and offspring. Familial conflict is not the same across taxa. Reproductive mode has a considerable influence on the timing, duration, location and resolution of conflict. In particular, familial conflict has special implications for placental reproduction, as mothers actively allocate resources to embryos that are simultaneously expressing paternally-derived genes (Haig 1997; Zeh and Zeh 2000; Crespi and Semeniuk 2004). Mammals are typically used to address placenta-focused questions. However, our ability to use mammals to answer questions about the evolution of placentation is limited because all living mammals inherited their placenta from a single common ancestor that lived approximately 180 million years ago (Meredith et al. 2011). I address such questions with species in the livebearing fish family Poeciliidae. Poeciliids have multiple, independent origins of placentas and, in three instances, have closely related sister taxa with and without placentas. In my dissertation, I investigate questions surrounding how placentation and associated characteristics (i.e. multiple mating by females) affect maternal investment and familial conflict, including intergenomic (intersexual and parent-offspring) conflict and sexual selection.

Observations of post-natal parental care inspired Robert Trivers to propose parent-offspring conflict in 1974. Trivers argued that the major driver of resource-driven conflict is relatedness. Mothers are equally related to all of their offspring, so all offspring are equal. An offspring is 100% related to itself, but only 50% related to its full siblings, and 25% related to its half siblings (those who have different fathers). The benefit an offspring gains from getting more resources from its mother is associated with the cost to its siblings, but that cost is decreased for full siblings compared to half siblings. These inequalities in relatedness, combined with inherent competition over limited maternal resources, are predicted to result in conflict because they predict that the quantity of resources that is in the best interest of each offspring to get from its mother is greater than what is in the best interest of the mother to give to the offspring. During periods of conflict, such as the end of the provisioning period (i.e. weaning), natural selection favors the termination of provisioning by the mother, and the continuation of resource solicitation by the offspring. The duration of conflict is predicted to be longer in broods of half- than of full-siblings due to lower coefficients of relatedness for half-siblings. These offspring are predicted to act more “selfishly” and try to solicit a greater quantity of resources than if they were all full siblings, extending the duration of conflict (Trivers 1974).

The path to, and the importance of the transition to viviparity

Trivers’ ideas have different implications dependent on an organism’s reproductive mode. For that reason, it is important to distinguish between viviparity and the subsequent classifications of lecithotrophy and matrotrophy. In his 1992 review, Daniel Blackburn proposes several possible paths to viviparity from oviparity. The transition from egg-laying first requires egg retention, which can result in viviparous lecithotrophy (“yolk-feeding”). Lecithotrophic females provision internally carried eggs prior to fertilization. Lecithotrophy is considered a weak form of placentation that is a precursor to matrotrophy (“mother-feeding”) (Crespi and Semeniuk, 2004). From here, the maternal-fetal interface may become fused as a placenta with the interdigitation of the embryonic trophoblast, leading to placentotrophy. Placentotrophy is the most intimate form of matrotrophy, offering a direct, physiological link between a mother and her offspring through which chemicals (i.e. hormones) and nutrients can be transmitted (R. Stewart and Blackburn 1988; Cross et al. 2003; Wildman et al. 2006). In poeciliids, the follicle forms the maternal-fetal connection. The follicle remains wrapped around the embryo throughout development rather than bursting during ovulation, like in mammals.

Differential provisioning of resources by lecithotrophs and matrotrophs also results in physiological developmental changes. Unfertilized eggs are small in matrotrophs (relative to lecithotrophs) because nearly all resource provisioning occurs during offspring development in matrotrophic species (**Fig. I.1**) (Mossman 1987). The degree of matrotrophy, measured as a “matrotrophy index”, is the ratio of a newborn offspring’s dry weight to the dry weight of a mature ova. This ratio describes how much mass an embryo gains or loses between fertilization and birth, a direct measure of maternal investment throughout development (Reznick et al. 2002). The mechanism of

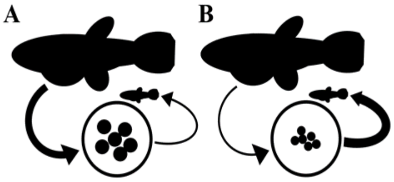

Fig. I.1 Lecithotrophic species (A) pre-provision large eggs, whereas matrotrophic species (B) have small eggs and invest resources into them throughout development.

matrotrophy indicates that females invest very little initially, and provisioning of resources by the mother to the embryo occur after fertilization. Because of this, interactions between the mother, offspring, and father acting through the embryo, and associated conflict over resources, are possible.

The intensity of pre-copulatory sexual conflict is predicted to weaken as a female gains the ability to internally provision her offspring post-fertilization, leading to decreased pre-copulatory divergence in male sexual characters in these species (Simmons 2005). The emphasis of sexual selection is predicted to shift towards post-copulation, where females can exert cryptic choice (Eberhard and Cordero 1995) through selective sperm use, or intrasexual sperm competition may occur. A transition from oviparity, to viviparity, to viviparous matrotrophy involve shifts not only in physiology and resource partitioning, but also in the considerations for familial conflict. In oviparous and lecithotrophic species, offspring cannot exert control over their size at birth, egg partitioning is entirely mother-controlled and predicted to be optimized (Janzen and Warner 2009; Jørgensen et al. 2011). Therefore, parent-offspring conflict should be absent in lecithotrophs.

The Viviparity-Driven Conflict Hypothesis

David and Jeanne Zeh expanded upon Trivers' hypotheses to argue that viviparity allows for internal parent-offspring conflict that can lead to the accelerated formation of post-zygotic reproductive barriers and lead to speciation. Predicted differences in speciation rate are due to antagonistic coevolution between maternal and paternal genomes, an emphasis on post-copulatory sexual selection (mediated by sperm competition and cryptic female choice), and genomic divergence due to independent resolutions of conflict in populations within a placental species leading to increased postzygotic reproductive isolation between them (Zeh and Zeh 2000, 2008; Crespi and Nosil 2013).

As mentioned before, viviparity and placentation have evolved repeatedly across vertebrate taxa (Blackburn 2015) through multiple, independent origins (Reznick et al., 2002) that appear to influence diversification within taxa (Helmstetter et al. 2016). Zeh and Zeh's Viviparity-Driven Conflict Hypothesis (VDCH) argues that the internal provisioning of embryos between fertilization and birth creates a novel arena for parent-offspring conflict to occur in mammals due to the intense and prolonged duration of maternal investment (Zeh and Zeh, 2000). Rather than post-birth solicitation cues like verbal calls, these interactions are presumably mediated through chemical communication (Trivers, 1974), such as through hormones (Haig, 1997, Crespi and Semeniuk, 2004).

The VDCH predicts that there will be exacerbated conflict in species that mate with multiple males (polyandrous) versus monogamous species. This is because the shift from pre- to post-copulatory reproductive incompatibility would be accompanied by increased mating rate. An increased mating rate would increase genetic diversity among sperm that females have the possibility to "choose" from. Vrana et al. use the interspecific crosses within the deer mouse genus *Peromyscus* to test this prediction. *P. maniculatus* and *P. polionotus* are two sister species that are promiscuous and monogamous, respectively. Intergenomic conflict is predicted to be more intense in polygamous species, so monogamous females mated to polygamous males should produce larger offspring because embryos sired by polygamous males are adapted to "pull" harder. The opposite would be expected in the reciprocal cross. When crossed, promiscuous *P. maniculatus*-sired offspring garner more resources and would be ~2.5 times larger at birth when born to monogamous *P. polionotus* mothers. However, due to the strength of the genetic incompatibility between them, most offspring die before birth. Reciprocal crosses produce smaller offspring than either pure species cross (Vrana et al. 1998, 2000). These results are due to paternal genomic imprinting producing offspring that are too large for the mother to support. The mother is unable to compensate for the accelerated growth of the offspring by either providing more resources or inhibiting growth, so they are stillborn. The *Peromyscus* system represents a classic example of intergenomic conflict resulting in genetic incompatibility. Though it is an interspecific cross, the reciprocal cross produces offspring that are stunted but viable, showing directionality in the incompatibility due to the father's genomic influence. As Vrana points out in a later paper, genomic imprinting seems to underlie speciation (through reproductive isolation), and may contribute to mammalian diversity (Vrana 2007).

Although the *Peromyscus* system presents a compelling argument for increased conflict due to polyandry, hybrid species crosses are problematic because there will always be underlying genetic incompatibilities when crossing distinct species. Making single-male single-female crosses alleviates issues of sperm competition and cryptic female choice but is not ecologically relevant to how *P. maniculatus* mates in nature. The *Peromyscus* system is appropriate for studying how mating system relates to reproductive isolation. However, it cannot be used to study the effect of placentation on reproductive isolation, because there are no non-placental *Peromyscus* species, or even mammals, to compare them to. To fully understand how placentation contributes to conflict and reproductive isolation, both placental, and non-placental species need to be included.

Applying the VDCH to poeciliids

The VDCH is proposed in an oviparous vs. viviparous context, but it can be extended to apply to varying degrees of viviparity from lecithotrophy (herein referred to as non-placental species) to matrotrophy (herein referred to as placental species) (Crespi and Semeniuk, 2004). Species in the livebearing fish family Poeciliidae represent an ideal framework for addressing the predictions of the VDCH and associated hypotheses. A vital feature of poeciliids for studying placentation-related conflict is that they have evolved follicular placentas, independently, at least nine times (Pollux et al. 2009). These placentation events have resulted in about 40 placental species (there are ~150 non-placental species). The presence of both placental and non-placental species allows us to make direct comparisons between closely related placental and non-placental sister species. Furthermore, all poeciliids are capable of storing sperm (Smith 2012), and many poeciliids have superfetation, which means they gestate multiple broods of offspring in different, distinct stages of development concurrently (Meredith et al., 2011).

Another trait of Poeciliidae is diversity in sexual characteristics that may influence how females provision their offspring based on their mate’s genotype. According to the VDCH, there will be a shift from pre- to post-copulatory mate choice as species evolve post-fertilization provisioning (e.g. matrotrophy). The intensity of pre-copulatory sexual conflict is predicted to weaken as a female gains the ability to internally provision her offspring post-fertilization, leading to decreased pre-copulatory divergence in male sexual characters (Simmons, 2005). The emphasis of sexual selection is predicted to shift towards post-copulation, where females can exert cryptic choice (Eberhard and Cordero, 1995) through selective sperm use or even differential resource provisioning. Intrasexual sperm competition may also occur. An analysis of the poeciliid phylogeny reveals that males lose secondary sexual characters, such as flashy colors and courtship behaviors, and instead forcibly copulate with females as a species becomes more matrotrophic (Pollux et al., 2014). Diminished pre-copulatory sexual traits with the evolution of matrotrophy suggests a post-copulatory shift in sexual selection towards sperm competition and cryptic female choice.

My dissertation addresses the aforementioned VDCH hypothesis regarding a shift in conflict from pre- to post-copulation in placental species in the first chapter. I utilize a pair of placental (*Poeciliopsis prolifica*) and non-placental (*Poeciliopsis infans*) poeciliid species and perform crosses between genetically distinct populations within each species to assess viability and fertility of the offspring produced by inter-population crosses. Based on the VDCH, we would expect to see negative effects on the placental species, but not the non-placental species. Preliminary evidence suggests post-zygotic reproductive barrier formation in the form of stunted offspring from crosses between populations of placental, but not several non-placental species in the genus *Poeciliopsis* (Morrison 2017).

In the second and third chapters, I assess the level of reproductive incompatibility between same-species populations of two placental poeciliids, *P. prolifica* and *Heterandria formosa*, using artificial insemination. Artificial insemination allows me to eliminate pre-copulatory cues, and reduce certain aspects of sperm competition, such as seminal fluid and sperm precedence. I can also control the number of spermatozeugmata, or sperm packages in my inseminations. In both species, I am performing crosses between genetically distinct populations.

In *P. prolifica*, reproductive incompatibility would present as reduced female reproductive fitness in terms of offspring size and number. I would not expect asymmetry in reproductive incompatibility based on any ecological characteristics of the populations, but it is possible based on the potential for Dobzhansky-Muller Incompatibilities (Orr and Turelli 2001). Inter-populational crosses of another species, *H. formosa*, result in one-way incompatibilities that can be explained by differences in offspring size (there are not significant differences in offspring size among *Poeciliopsis* populations). Congruent with the VDCH, when small-offspring females are crossed to large-offspring males, they are unable to carry the embryos to term and will abort a large portion of their brood (Schrader and Travis 2008, 2012). My experiment addresses these prior experiments with the addition of artificial insemination so that I can mate *H. formosa* females to males from both populations simultaneously and record the effects that the presence of both types of males has on offspring size and number.

Though some work has been published on the poeciliids, there is still much more to be done with regard to studying the consequences of placentation (Bassar et al. 2014).

In my dissertation, I use comparisons between three poeciliid species to examine how female resource allocation differs between placental and non-placental species. Within a species, genetic distance and/or population-specific life history traits (i.e. brood size, offspring size, and the level of polyandry) may influence genetic incompatibilities when males and females from different populations that have independent resolutions of conflict are crossed, which would be consistent with the VDCH.

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Chapter 1: Long-term Fitness Consequences of Inter-Population Crosses in a Placental and Non-placental Species Pair

Introduction

The Viviparity Driven Conflict Hypothesis (VDCH) predicts that placental species will exhibit faster speciation rates due to the diverse paths different populations take in resolving the never-ending conflict caused by placentation (Zeh and Zeh, 2008). The differences among populations in how conflict is resolved are predicted to accelerate the evolution of post-copulatory reproductive isolation. In the fish family Poeciliidae, pre-copulatory reproductive isolation appears to dominate post-copulatory reproductive isolation in governing the rate of speciation (Furness et al., 2019; Reznick et al., 2021). The unexpected consequence is that non-placental lineages have higher rates of speciation because they have more highly pronounced sexual selection and more stringent precopulatory mate choice. However, we still do not fully understand the reproductive consequences of placentation, or why it repeatedly evolves in Poeciliidae. Reproductive conflict caused by placentation may still contribute to accelerated evolution of post-copulatory reproductive isolation even if not also accelerating speciation rate. To assess this hypothesis, I compared rates of evolution of post-copulatory reproductive isolation among closely related placental and non-placental species.

Comparisons between placental- and non-placental species in the livebearing fish family Poeciliidae are an appropriate test to the VDCH because placentas have evolved repeatedly and we can sometimes find close relatives that either do or do not have placentas (Pollux et al., 2009). Mothers of non-placental species fully provision eggs prior to fertilization. The mature eggs of placental species are smaller than those of non-placental ones; mothers instead provision offspring during development (Schrader and Travis, 2008). Maternal provisioning can lead to greater conflict during development in placental species because it occurs while the paternal genome is active and potentially capable of influencing maternal provisioning. Previous experiments have demonstrated reproductive incompatibilities between populations of a placental poeciliid, *Heterandria formosa* in the form of aborted and under-developed embryos (Schrader and Travis, 2008). The existence of closely related species with and without placentas creates the opportunity for comparative studies that characterize the biological consequences of evolving a placenta (Bassar et al., 2014). Morrison (2017) provides preliminary evidence for such isolation in the fish genus *Poeciliopsis* by showing that crossing fish from different populations of a placental species causes a progressive decline offspring size as the genetic differences among populations increases. No such declines were seen in similar crosses among populations of two non-placental species. What is missing from this study is a complete evaluation of the F1 generation in terms of both their viability, and fertility when they reproduce.

Here, I build on this prior work by evaluating reproductive incompatibility in placental and non-placental species in the genus *Poeciliopsis*. *Poeciliopsis* is a predominantly Mexican genus that contains multiple pairs of placental-non-placental species (Reznick et al., 2002). There is no evidence for courtship in either species. Both species exhibit superfetation, in which they simultaneously carry multiple broods in different developmental stages. The two species I am using are *P. prolifica* (placental) and *P. infans* (non-placental). Both species live in river drainages on the Pacific side of Mexico, with *P. prolifica* living more North in warmer, coastal water, and *P. infans* living more inland and South at higher elevations, in cooler water. Matrotrophy index, a measure of the resources a female provisions to embryos as they develop, is greater than one for placental species and less than one for non-placental species. *P. prolifica* is moderately placental with an average matrotrophy index around eight. *P. infans* is very similar morphologically to *P. prolifica* and has a matrotrophy index of approximately 0.8 (Reznick et al., 2002; Pollux et al., 2014). *P. infans* is considered lecithotrophic, meaning that it fully provisions eggs before they are fertilized. Therefore, I predict greater incompatibility for reciprocal interpopulation crosses involving *P. prolifica*.

Potential sources of reproductive incompatibility in either species might include Dobzhansky-Muller incompatibilities (Muller, 1942; Unckless and Orr, 2009). Dobzhansky-Muller incompatibilities have been well-studied in other species like mice (Turner and Harr, 2014), flies (Coyne and Orr, 1989; Haerty and Singh, 2006), salamanders (Fitzpatrick, 2008), and rice (Yamamoto et al., 2010) in viability traits other than offspring size. While these incompatibilities are typically studied in relation to reproductive barriers between species in interspecific hybrids, I hypothesize that an interpopulation cross among genetically divergent populations could portray some of the same symptoms.

The populations in this experiment were selected based on genetic distance, measured by F_{ST} values generated from ddRAD sequencing data of individuals from four populations from each species (Morrison 2017). Here I utilize the two populations from each species that had the highest F_{ST}, indicating the most genetic differentiation between populations, in Morrison's (2017) experiment. There are no known ecological differences in either population pair; genetic differentiation is the only known factor that makes them distinct. Population pairwise F_{st} was similar between species: *P. infans*, $F_{st} = 0.255$; *P. prolifica*, Fst = 0.243 (Morrison, 2017). I performed interpopulation crosses across two generations to provide a more complete assessment of reproductive isolation than Morrison (2017).

When performing hybridization experiments, both pre- and post-copulatory measures of divergence are important. Experiments involving species hybrids in *Drosophila* create a template for studying pre- and post-copulatory divergence (Coyne and Orr, 1989; 1997). Here I only consider indices of post-copulatory reproductive isolation because the viviparity driven conflict hypothesis specifically predicts more rapid evolution of post-copulatory reproductive isolation in placental species

I recorded multiple potential measures for offspring viability and offspring fertility in this experiment. Offspring viability is reflected in dry mass at birth, and fertility is measured by the rate of offspring production by F1 individuals. Offspring size at birth is a measure of offspring quality to expand my assessment of reproductive fitness. In some placental poeciliids, larger offspring have a greater chance of survival and reproduction (Henrich, 1988; Reznick et al., 1996). Larger guppy offspring have a competitive advantage when food is scarce (Bashey, 2008). Another measure of offspring viability for the F1 fish is the size and age at the onset of maturity in F1 males.

Poeciliopsis do not exhibit clear signs of female maturity, so female maturation cannot be included. Fertility is measured as the rate of offspring production in the F1 individuals.

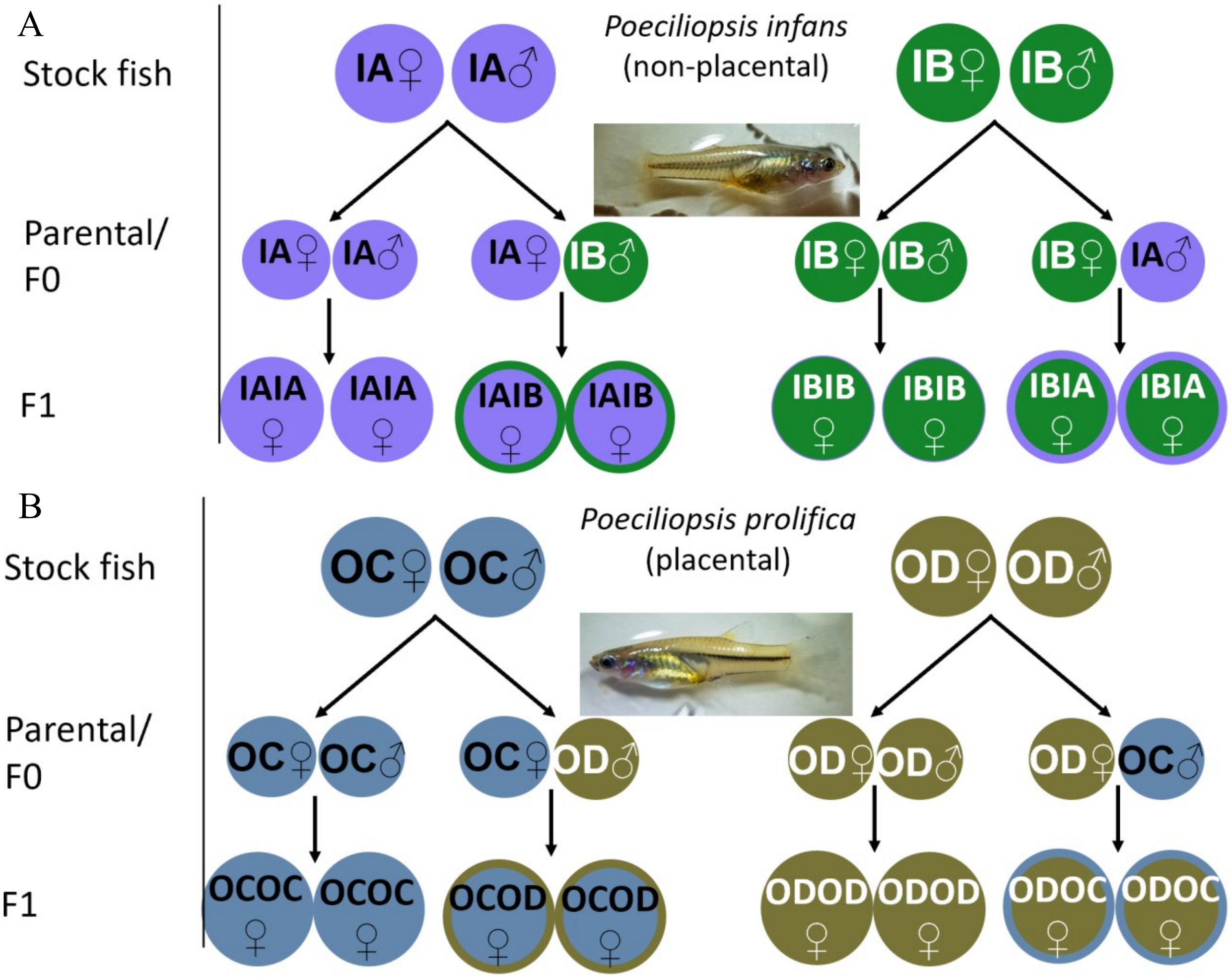

Figure 1.1. Cross and generation design for populations of A. *P. infans*; populations A (Rio Ameca) and B (Rio de la Pasion), and B. *P. prolifica*; populations C (Rio Piaxtla) and D (Rio Acaponeta).

To measure viability and fertility of F1 individuals, parental fish were set up in within population (intrapopulation) or between-population (interpopulation) crosses. The F1 individuals resulting from the initial crosses were mated to each other (avoiding inbreeding) as shown in **Fig. 1.1.** Coyne and Orr's assessment of fertility in interspecific *Drosophila* crosses (Coyne and Orr, 1989, 1997) corresponds to my performing a second generation of crosses with the hybrid F1 offspring. Then I compare the number of offspring produced by crosses among the hybrid offspring of the first generation versus the number produced by the control crosses. I expect interpopulation crosses of *P. prolifica* to exhibit signs of reproductive incompatibility in the form of inviable or smaller offspring from the parental crosses, and/or F1 offspring who have lower reproductive success. I may also see slower maturing, smaller F1 males. If *P. infans* exhibit any signs of incompatibility in interpopulation crosses, then I anticipate that they would be less severe than those seen in *P. prolifica* if the placentation does indeed accelerate the development of reproductive incompatibility per the Viviparity Driven Conflict Hypothesis.

Methods

I collected fish for this experiment in June 2018. The two *P. infans* populations are from Rio Ameca (“Population A”; 20.550655, -103.952146) and Rio de la Pasion (“Population B”; 20.160813, -103.039016). The two *P. prolifica* populations are from Rio Piaxtla (“Population C”; 22.461083, -105.373806) and Rio Acaponeta (“Population D”; 23.888083, -106.618611). The entirety of this experiment took place between May 2019 to March 2020 in the UC Riverside vivarium. All tanks were maintained in the same way; 20% of the water in the tanks was changed weekly, and fish were fed twice daily with a mixture of brine shrimp, Omega Fish Flakes, and daphnia. Both fish rooms had a 12:12 hour day:night cycle. *P. prolifica* were maintained in one room at 26.5°C, and *P. infans* were maintained in another room at 23.5°C, comparable to the temperatures each species experiences in nature.

Experimental Design

To generate fish for my parental crosses, I isolated females from stock tanks that were established in June 2018 for both *P. prolifica* and *P. infans*. Females were isolated in May 2019 in 9-liter tanks. Their offspring (the parental fish) were collected over the course of two weeks and moved into 19-liter block tanks containing broods from 1-3 females, with no more than 15 individuals. Males were removed from the block tanks as they matured (denoted by the visible formation of a gonopodium, or calcification of the anal fin), and moved into corresponding 19-liter tanks.

From the blocks of offspring established from the stock fish, random pairs were set up to ensure no inbreeding occurred. These crosses were set up in May 2019. Females were either mated to males from their same population (intrapopulation), or a genetically distinct one (interpopulation).

Offspring from the first two broods of the parental generation were reared in separate, 9-L sibling tanks, with no more than five individuals. These fish were used to produce the adult fish for the F1 generation treatments. Males were removed from the sibling tanks as they matured (denoted by the visible elongation of the anal fin) and moved into corresponding 19-liter tanks with other males from 1-3 females. Male tanks formed male blocks that were grouped to avoid inbreeding upon treatment cross setup. Treatments were maintained throughout the generations, such that an AxA cross's offspring would be mated to an AxA individual in the next generation (**Fig. 1.1**).

Offspring born beyond the second brood were immediately sacrificed at birth in a lethal MS-222 solution. Following sacrifice, offspring were measured and weighed and preserved in 5% formalin in a labeled 0.5 mL microcentrifuge tube.

F1 Generation

As F1 males matured, they were photographed for measurement. Males were removed from their same-brood sibling tank upon visual elongation of their anal fin, which metamorphoses into the gonopodium over a period of approximately 14-40 days for *P. prolifica,* and 35-65 days for *P. infans*. All photos were taken in a clear acrylic box filled to a 20 mm line with water. Photos were taken from 30cm height. I analyzed these photos using the program ImageJ. I chose to measure each male's standard length (measured from the front of the fish to the tip of the caudal peduncle, or the point where the fish's body meets its caudal fin), as it had the best visibility from the photos. After photographing, males finished maturing in 19-L tanks with males from 2-3 other blocks.

Upon all F1 individuals reaching maturity, random individuals were selected from within same-treatment blocks to create treatment groups for the F1 generation in December 2019. For fish from this generation, all offspring were sacrificed at birth in a lethal MS-222 solution. Following sacrifice, offspring were measured and weighed and preserved in 5% formalin in a labeled 0.5 mL microcentrifuge tube.

In both experimental generations, offspring mortality was recorded, but there was too little mortality to do a formal analysis. Furthermore, cross "success" was recorded to measure infertility of any crosses, but these data were also too similar across treatments (crosses were around 80% successful, on average). Once all offspring were collected for the experiment, I removed the formalin from each microcentrifuge tube and placed it in a 50°C drying oven for 24 hours. Upon removal from the drying oven, each individual fish was weighed on a Mettler AE 163 microbalance.

Statistical Analysis

My analysis focuses on characterizing offspring viability in terms of F1 dry mass at birth, and offspring fertility in terms of F1 rate of offspring production. I base my analyses on the first three to five broods of offspring for each female. A female was only included in the analysis if she produced at least three broods. Complete sample sizes are included in Appendix A. There was not sufficient offspring viability-at-birth data to analyze, so that will not be included in the results or discussion. Additionally, crosses were equally successful across treatments, so fertilization success is also not reported on.

For the dry mass of the F1 individuals at birth, I examine the interaction between maternal and paternal population. For *P. prolifica*, the MaternalxPaternal cross combinations are CxC, CxD, DxC, and DxD. For *P. infans*, the combinations are AxA, AxB, BxA, and BxB. For my assessment of F1 fertility, I examine fish origin in terms of cross type, which includes CC, CD, DC, DD for *P. prolifica* and AA, AB, BA, BB for *P. infans*. Analyses were selected based on the data distribution for each response variable. These distributions varied slightly for each species. I present these analyses by response variable and species:

F1 Offspring Viability

Offspring viability contains fertilization success, offspring mortality, offspring mass at birth, male size at maturity, and male duration to maturity. A key variable for determining offspring viability in poeciliids is mass at birth (Reznick et al., 1996), though the analyses and results for the other variables can be found in Appendix A.

For both species, the generalized linear mixed models have offspring dry mass at birth as the response variable, and the interaction between Maternal and Paternal population as the independent variable. For both species, models were fit to the data depending on its distribution, and mother ID was included as a random effect. Both models were analyzed using a Type III ANOVA, and I performed a post-hoc analysis of estimated marginal means when model components were statistically significant.

F1 Fertility

To measure the fertility of the F1 fish, I recorded litter size and inter-brood interval, measured as the number of days between each litter. These individual analyses are reported in Appendix A. To incorporate both of these variables to calculate a per-day average rate of offspring production, the total number of offspring produced in 3-5 litters was divided by the number of days that it took for a female to reach 3-5 litters, starting from the date that her first litter was born. Female reproduction was not truncated; females who produced three or four litters stopped reproducing well before the experiment was ended. Five females with per-day rates less than 0.10 were not considered in this analysis, because gaps in production were noted in the middle of the litter 1-5 range that can be attributed to adding a replacement male after the loss of the original male.

I used generalized linear models with Gamma inverse distributions to assess the daily rate of offspring production in both species, with cross type as the independent variable. Both models were analyzed using a Type II ANOVA, and a I performed a post-hoc analysis of estimated marginal means when model components were statistically significant.

Results

F1 mass-at-birth (viability), and offspring production rate (fertility) data were right skewed in both species, so I fit a generalized linear mixed models to an inverse Gamma distribution to these variables for both species. A post-hoc analysis of estimated marginal means was applied to viability for *P. prolifica* and fertility data for *P. infans* due to significant interactions between maternal and paternal populations in these respective models.

Reproductive incompatibility was present in both species, but the nature of the incompatibility was different. In *P. infans*, the fertility, but not viability of the F1 offspring were affected (**Fig 1.2**). F1 dry mass at birth was not affected by Maternal population ($\boldsymbol{X}^2$= 1.74, p= 0.19) or Paternal population ($\boldsymbol{X}^2$= 0.17, p= 0.68), alone or the interaction of maternal and paternal population ($\boldsymbol{X}^2$= 1.00, p= 0.32). The incompatibility is reflected in A-line intraspecific hybrid fertility; there was a significant effect of cross type on offspring production rate ($\boldsymbol{X}^2$= 23.04, $p<<0.001$). AA females produce about 30% more offspring daily than AB females (z= -3.03, p= 0.013). BB females produce slightly more offspring than BA females, but trend was not significant (z= 1.30, p= 0.57) (**Fig. 1.2**).

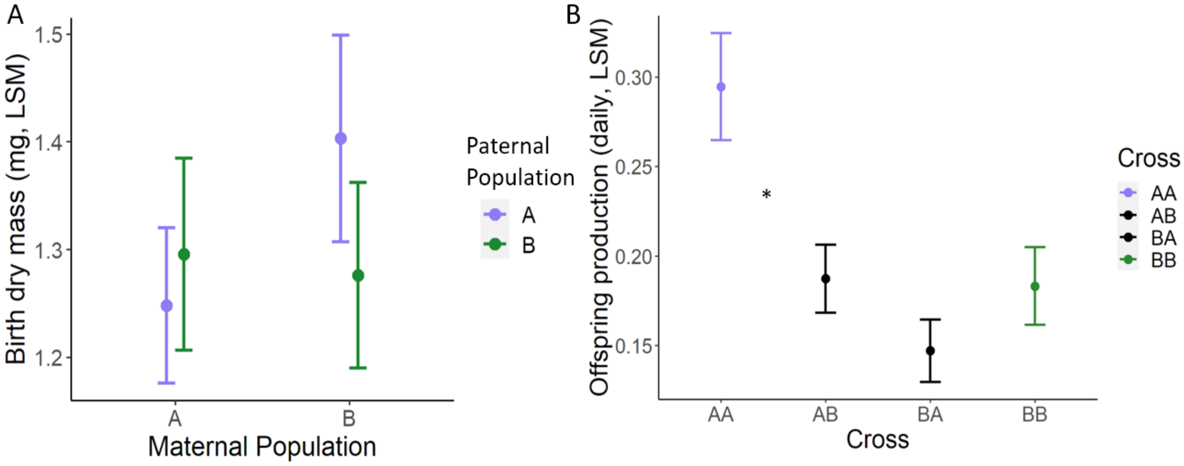

Figure 1.2. Least Squares Means (LSM) ± Standard Error of the LSM for A. Dry mass at birth (viability) and B. Offspring production rate (fertility) results for *P. infans*. Significant difference is denoted with "*".

The main driver of offspring production rate in *P. infans* was litter size, which was significant on its own and followed the same trend of AA females having 30% larger litters than AB females (t= 2.70, p= 0.0486). Inter-brood interval was not significant alone ($\boldsymbol{X}^2$= 2.05, p= 0.56). Both litter size and inter-brood interval are taken into account to produce the offspring production rate, which makes fertility significantly lower in A-line *P. infans* interpopulation hybrids.

For *P. prolifica*, the incompatibility lies in offspring viability, and there was no effect of interpopulation hybridization on F1 fertility (**Fig. 1.3**). There was no effect of cross on the rate of average daily offspring production ($\boldsymbol{X}^2$= 4.49, p= 0.21). However, for dry mass at birth, there was a significant interaction of Maternal population and Paternal population ($\boldsymbol{X}^2$= 7.73, p= 0.0054), as well as main effects of Maternal Population ($\boldsymbol{X}^2$= 13.71, p<0.001) and Paternal population ($\boldsymbol{X}^2$= 11.36, p<0.001). C females produce offspring that are about 10% larger when they are mated to C males than to D males (z= -3.37, p<0.001). D females show a similar trend in allocating fewer resources to C males than D males, but it was not significant (z= 0.59, p= 0.55). Furthermore, C males' offspring are smaller when their mother is a D female than a C female (z= -3.70, p<0.001). Interestingly, when these offspring are crossed to each other to produce the F2 generation, there are no differences in offspring size for CC, CD, DC, or DD crosses ($\boldsymbol{X}^2$= 0.26, p= 0.97).

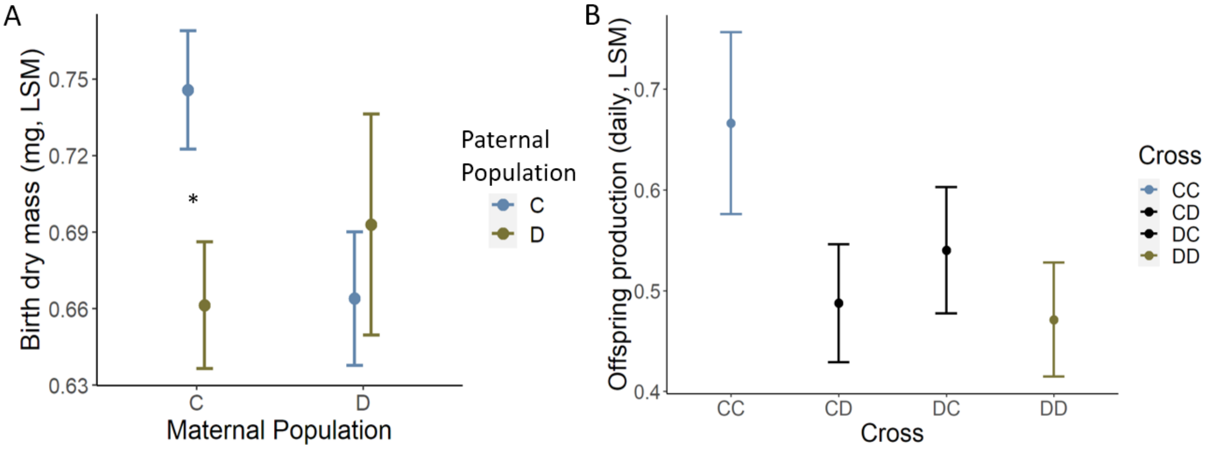

Figure 1.3. Least Squares Means (LSM) ± Standard Error of the LSM for A. Dry mass at birth (viability) and B. Offspring production rate (fertility) results for *P. prolifica.* Significant difference is denoted with "*".

Cross success was evenly distributed across treatments; 60-80% for *P. infans* and 75-100% for *P. prolifica.* The additional components of offspring viability, male time to and size at maturation, were not significant for either species and can be found in Appendix A. The individual components of fertility, inter-brood interval and litter size are also presented in Appendix A.

Discussion

In this experiment, I performed intraspecific, interpopulation crosses in two, closely related poeciliid species. Both species exhibited reproductive incompatibility, but the location of incompatibility was different. Hybrid F1 individuals from interspecific crosses in the non-placental *P. infans* produced fewer offspring, exhibiting reduced fertility. F1 *P. prolifica* (placental) interpopulation hybrid individuals were smaller at birth than non-hybrids, exhibiting reduced viability. In a similar experiment using these species where only the parental crosses were examined, offspring size (viability) was the only variable investigated that was found to be significant in *P. prolifica* (Morrison, 2017). My experiment was modeled after the assessment of fertility in interspecific *Drosophila* crosses (Coyne and Orr, 1989, 1997). I extended the analysis of reproductive incompatibility to examine additional fertility data from the F1 individuals.

Male maturation characteristics were also included in the experiment as a measure of offspring viability but were not found to be significant. Male-male interactions could be incorporated in future experiments but were not feasible in this experiment. Offspring mortality would have been another variable, but there was not enough data to analyze. Likewise, whether a cross was successful could be a measure of pre- or post-copulatory incompatibility, but there were no visible trends in these data.

Neither species show any significant trends in male maturation characters. The fact that time to maturity was the best predictor of size at maturation likely overpowered any ability I may have had to detect differences between treatments. It is also possible that reproductive incompatibility does not present itself in time to, or size at maturity in *Poeciliopsis*. In hybridization experiments between species of *Mytilus* mussels, maturation characteristics were not impacted by hybridization (Toro et al., 2002).

For the non-placental species, *P. infans*, there were no negative effects of interpopulation crosses on F1 mass at birth. When these individuals matured and were crossed to each other within the parental crosses, reproductive incompatibility was exhibited in the number of offspring in each litter; F1 offspring from AxB crosses exhibit decreased fertility. There is no such fertility deficit in BxA crosses, representing asymmetric incompatibility.

P. prolifica, shows signs of reproductive incompatibility in the viability of the F1 individuals as measure by size at birth. This was only present in C population females when they were mated to D males. The trend for DxD offspring to be smaller than DxD cross offspring is present as well, but it is not significant. Both were significant in previous work with smaller sample sizes (Morrison, 2017). Interestingly, there are no differences in the mass of the offspring born to F1 individuals. There is a significant increase in offspring size for all treatments, but it is noteworthy that the apparent reproductive incompatibility of C population females mated to D population males disappears. Maternal effects may buffer the detrimental effect of interpopulation crosses for *P. prolifica*. Offspring size has been shown to be a plastic trait in poeciliids (Bashey, 2008; Olivera-Tlahuel et al., 2015), and especially sensitive in placental poeciliids (Banet et al., 2010; Pollux and Reznick, 2011). Furthermore, adaptive maternal effects have been shown in species from insects to amphibians and birds (Fox et al., 1997; Pfennig and Martin, 2009; Giordano et al., 2014).

Interspecific crosses often reveal Dobzhansky-Muller Incompatibilities; accumulations of genetic incompatibility (Orr and Turelli, 2001). It is interesting that the incompatibility is asymmetric in both species; this trend has been shown in mice, insects, amphibians, fish, and plants (Bolnick et al., 2008; Good et al., 2008; Brothers and Delph, 2010; Brandvain et al., 2014; Gebiola et al., 2016). In another placental poeciliid, *Heterandria formosa*, there is an asymmetrical incompatibility, but it is explained by natural differences in offspring size between populations (Schrader et al., 2013). There are no documented life history differences between the *Poeciliopsis* populations. I cannot explain the mechanism that would contribute to the reproductive incompatibility displayed by these *Poeciliopsis* species. Nevertheless, the breakdown in both *Poeciliopsis* represents an example of isolation asymmetry, known as Darwin's Corollary to Haldane's rule, which are uniparentally inherited factors that differentially affect offspring produced from reciprocal F1 crosses (Turelli and Moyle 2007). Something about the maternal A and C maternal genotypes are incompatible with the B and D paternal genotypes, respectively, but we do not know the mechanism for this incompatibility. More likely, based on evidence from centrarchid fishes, this is a maternal mitochondria-associated incompatibility. In centrarchid fishes, maternal mitochondria has an accelerated rate of evolution, causing mito-nuclear incompatibilities (Bolnick et al., 2008). Reproductive characteristics are not the only ones that species hybrids may exhibit that represent signs of incompatibility based on Darwin's Corollary. In sunfish, for example, the muscle metabolic phenotype is affected between crosses of *Lepomis macrochirus* and *Lepomis gibbosus* (Davies et al., 2012). While my study focused on reproductive incompatibility, it would potentially be interesting to examine the other effects interpopulation crosses may have on interpopulation hybrid individuals from A and C population mothers, respectively.

One notable set of variables missing from my analysis of divergence in *Poeciliopsis* species in this experiment are pre-copulatory measures of incompatibility. These traits are not part of the VDCH but have been used by Coyne and Orr to assess reproductive incompatibility and divergence (Coyne and Orr, 1989, 1997). Most *Poeciliopsis* lack courtship, and lack of courtship in both *P. infans* and *P. prolifica* precluded this variable from my experiment.

A trend that cannot be ignored in these results is the significant differences in offspring size between the first and second generations. This trend is present in all treatments except one in *P. infans*, and all treatments for *P. prolifica*. It is important to consider why these differences occurred. Because the other variables measured do not reflect such an extreme difference, this result is a valid one. Still, there are a few things that could have contributed to it. One potential factor could be that the first generation of fish were reared and bred in early Spring, whereas generation two was produced and bred in the summer. The labs are in controlled settings in the vivarium of the university, so it is unlikely that there were environmental cues present to have an influence on offspring size. A more likely reason for the differences in offspring size is rearing condition. In the first generation, fish were reared in 19-L aquaria, with multiple female's (1-3, depending on how fecund the mother was) offspring being raised together in blocks. In the second generation, the first two litters of offspring from each female from the first generation were raised in separate, 9-L aquaria. F2 fish appeared to mature quickly, and I noted almost no dead individuals as they matured. These different rearing conditions were used for logistical purposes to avoid inbreeding in the experiment, but they likely influenced offspring size. Environmental factors can influence how mothers provision their offspring (Williams, 1966). Variation in maternal effects based on variation in food availability (Reznick et al., 1996; Banet et al., 2010) and social density (Leatherbury and Travis, 2019) have been examined in poeciliids, and, in general, placental species are more susceptible to negative maternal effects on offspring size. S Male traits can also be affected by rearing environment in poeciliids (RODD and SOKOLOWSKI, 1995; Lange et al., 2021). If this experiment were repeated, it would be important to control the rearing environment in both generations. Still, the result that offspring size was impacted by cross type in the first generation but not the second, is valid. Maternal effects are likely responsible for the buffered effect of cross type in the second generation.

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Chapter 2: Placental Fish "Weigh" Their Options When it Comes to Post-Copulatory Investment

Introduction

Females of matrotrophic species provide nutrients directly to their offspring throughout development, typically via a placenta. While this reproductive mode allows embryos to develop in a safe environment within the mother, inequalities in relatedness among offspring may turn the mother's womb into a battleground where developing offspring fight with each other and with their mother over allocation and acquisition of resources (Haig 2000). Haig postulates that there will be an eternal tug of war in which selection favors mothers that are able to control allocation to embryos at the same time that it favors fathers that acquire more resources.

Zeh and Zeh postulate that matrotrophic species will also experience a shift in sexual conflict from pre- to post-copulatory selection of mates and maternal provisioning during development (the viviparity drive conflict hypothesis (VDCH) Zeh and Zeh 2000). Because non-placental species fully provision eggs before mating, their best means for choosing who sires their young is to choose who they mate with. Matrotrophic species instead provide most resources to their young after mating and after the paternal genome is expressed in the developing embryo, so Zeh and Zeh predict a shift a shift towards cryptic female choice and differential allocation of maternal resources to embryos sired by different males. A predicted consequence of the resulting tug of war is that placental species will accumulate post-zygotic reproductive incompatibilities among populations faster than non-placental ones. These predictions have been investigated in terms of comparisons among vertebrate classes (Bush et al. 1977) and broad-scale speciation patterns (Barraclough et al. 1998; Hernández-Hernández et al. 2021), but rarely in terms of the maternal provisioning and interfertility among populations.

Post-copulatory sexual conflict can manifest via a female's ability to discriminate among sperm from different mates or to selectively provision offspring post-fertilization. Such abilities are often assumed in theory, but rarely empirically demonstrated. Differential success in siring offspring could happen either because of differences among sperm in their ability to fertilize eggs (sperm competition), cryptic female choice on the success of sperm in fertilizing eggs, or maternal discrimination among embryo genotypes in the allocation of resources. Reproductive incompatibility has been demonstrated in species hybrids of polyandrous and monandrous deer mice (Vrana et al. 2000; Vrana 2007), and matrotrophic fish populations of the same species that exhibit differences in offspring size (Schrader and Travis 2008). Investigators paired single males with single females from either the same or different populations/species and found one-way incompatibilities in offspring size and viability. In deer mice, males from the polyandrous species sired embryos that imposed an excess demand for resources on monandrous mothers. In fish, females from populations that normally produce small offspring suffered a loss of offspring when the sire was from a population that produces large offspring (Schrader and Travis 2005, 2008)

Plants serve as a foundation in studying conflict between mother, father, and offspring due to parent-specific gene expression, or genomic imprinting (Haig and Westoby 1989). Plants, like matrotrophic animals, provision seeds after fertilization via endosperm formation (Grossniklaus et al. 2001; Gehring and Satyaki 2017), and have the potential for post-copulatory sexual selection (Tonnabel et al. 2021). There is little evidence for sire identity affecting seed mass (Arnold 1994; Alleman and Doctor 2000; Bochenek and Eriksen 2011; Pélabon et al. 2015). Instead, pollination intensity (Christopher et al. 2020) and pollen priority (Conner et al. 1996) seem to be the main factors that influence siring success; current research concludes that there is little potential for differential provisioning in most angiosperms (Broyles and Wyatt 1990; Burkhardt et al. 2009; Pélabon et al. 2015). However, plants remain vital for studying conflict, as there is mounting evidence that imprinted genes that enhance conflict appear to drive reproductive incompatibility in hybrids in *Mimulus* plants (Garner et al. 2016; Coughlan et al. 2020; Sandstedt and Sweigart 2022).

The breadth of literature on siring success in livebearing species is more limited, having only been investigated in guppies. Artificial insemination studies have the virtue of controlling for pre-copulatory cues, sperm order, and some ejaculate components that could provide a competitive advantage to certain males. Such studies have been done; in guppies, more colorful males sire more offspring than less colorful males in an artificial insemination experiment (Evans et al. 2003). Guppies are part of the livebearing fish family Poeciliidae, whose members represent an ideal framework for addressing the predictions surrounding viviparity-driven conflict. A vital feature of poeciliids for studying maternal provisioning-related conflict is that they have evolved matrotrophy at least nine times (Pollux et al., 2009a). These events have resulted in the family having about 50 matrotrophic and 200+ lecithotrophic species. Male traits associated with pre-copulatory mate choice, like bright coloration, ornamentation, and courtship, exclusively evolve in lineages that lack placentas. Placentas evolve in lineages with and without such traits, but if these traits are present when the placenta evolves then they tend to be lost (Pollux et al. 2014; Furness et al. 2019). The loss of male traits associated with pre-copulatory mate choice in association with the evolution of matrotrophy suggests a shift away from pre-copulatory mate choice, perhaps associated with increasingly intense post-copulatory mate choice.

Here I report on an experiment in which I simultaneously artificially inseminate female *Poeciliopsis prolifica* with sperm from two males that are from either the same or a different population. *P. prolifica* is a placental poeciliid from the Sinaloa region in Mexico. *P. prolifica* has no courtship, and the females exhibit superfetation, allowing them to gestate multiple broods that are in different stages of development, simultaneously. There are no known differences in offspring size or fecundity between the populations used in this study, making this a good system in which to test the hypothesis that differences in conflict resolution in independent populations may result in incompatibilities when the populations are crossed to one another. The two populations utilized in this experiment were from Rio Piaxtla ("Population C"; 22.461083, -105.373806) and Rio Acaponeta ("Population D"; 23.888083, -106.618611), which are from separate drainages about 160 km apart. These populations have an F_{ST} (a measure of genetic differentiation between populations) of 0.243, indicating the populations are genetically distinct from one another (Morrison 2017).

Females from one population were mated to either two males from their own population, two males from the other population, or one male from each of their own and the other population. I quantified the success of each male in siring offspring and the mass at birth of the offspring, which will be a direct function of maternal provisioning. Because there are no natural differences in offspring size between populations, male genotype should not directly influence offspring mass. Differences in siring success could be attributable to either sperm competition or cryptic female choice. Differences in maternal provisioning could be attributable to either paternal or maternal influences, but some patterns of results can enable us to discriminate between the two. For example, if a mother allocates less to an embryo sired by a foreign male when offspring from both types of males are present than she does when both sires are nonresident, this implies maternal “choice” in allocation. Prior to this study, no one has artificially inseminated a placental species to determine if there are any biases in resource allocation by the female to different males. According to Zeh and Zeh, females may discriminate against males from different populations, and their embryos, to avoid supporting genetically defective offspring (Zeh and Zeh 2000). Conversely, males from a different population may “pull” more resources from the mother because she has not evolved the appropriate means for countering paternal influences on resource acquisition.

Methods

Fish Collection and Maintenance

This experiment took place from December 2018-April 2019. I collected the founders of my lab populations from populations C and D six months prior to the beginning of the experiment. The fish for this experiment were F1 individuals reared in a common laboratory setting. Each litter in a 20-liter aquarium. Males were removed as they matured in order to maintain virgin females for artificial insemination. Maturing males were identified based in the metamorphosis of the anal fin of males into a gonopodium, the intromittent organ (Fraser and Renton 1940). Immature males were moved into 20-liter male-only tanks separated by population and females were isolated in 9-liter tanks to grow and mature until I inseminated them, approximately 2 months later.

Artificial Insemination

I anaesthetized fish in a buffered 0.01% solution of MS-222. F1 male *P. prolifica* were selected at random from stock tanks then anaesthetized. Once the males began to turn belly-up in the water, they were placed on a bed of moist, sterile cotton under a dissecting scope. I 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, causing the male to ejaculate. The ejaculate consists of spermatozeugmata, each of which contains hundreds of sperm. I collected the spermatozeugmata with a micropipette tip attached to silicone tubing, then transferred them to a small drop of Hank's Balanced Salt Solution (HBSS). I then measured the length and weight of the male and placed him in a recovery container. The recovery solution included a drop of Stress Coat, which stimulates the production of the protective slime layer and facilitates the healing of any abrasions associated with handling.

During my artificial insemination pilot studies, sperm were viable in the HBSS for at least one hour. A maximum of 15 minutes passed from the time the first male was anesthetized to the time a given female was fully inseminated.

I inseminated females with ten spermatozeugmata each from two males, comprising three treatments Female x: 2C, 2D, or 1C+1D population males (**Fig 2.1**).

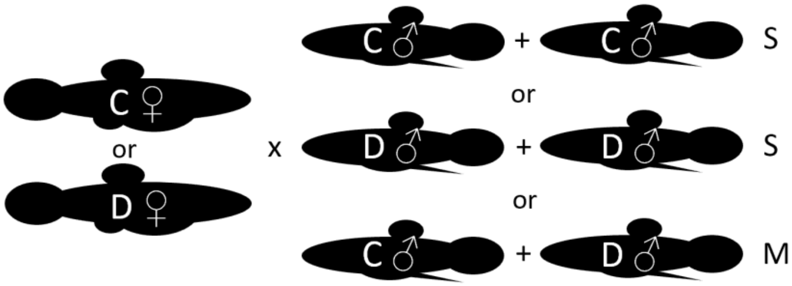

Figure 2.1. Experimental design for crosses between C or D females with C or D population males (Single, "S" crosses), or C and D population males simultaneously (Mixed, "M" crosses).

I anesthetized females in the same way as the males, except they were left in the MS-222 approximately 30 seconds longer until they were fully anesthetized. To transfer sperm, new drops of HBSS were formed for each female, and spermatozeugmata were carefully counted and siphoned from the male's source droplet and deposited into the female drop. Once both males' sperm were deposited in the female drop, the sperm were mixed by air agitation, re-counted and pipetted into the female's gonopore. Each female was measured and weighed, then allowed to recover in her experimental tank and treated with two drops of Stress Coat and a small dose of Maracin (an anti-bacterial fish medication) to limit the risk of infection from the procedure. As many females as possible were inseminated to maximize the ejaculate from each set of males.

Juvenile Collection and Preservation

The first female gave birth 30 days after insemination. After 30 days, each female's tank was checked daily for offspring to ensure that all offspring used in the analysis were no older than 24 hours. Newborn offspring were removed and placed immediately in a lethal, buffered 1% dose of MS-222. Each offspring was then transferred to a slide and placed under a dissecting scope where I measured total and standard length. I then weighed offspring to the nearest 0.0001 mg with an analytical balance and transferred them to a DNase/RNase-free, 0.6 mL microcentrifuge tube filled with 95%-100% ethanol. Tubes were labeled then stored in a -20ºC freezer.

Data Organization

The data associated with each offspring included: ethanol (lean) weight, days since insemination, number of broods per female, and number of offspring per brood. Female poeciliids invest fat stores into embryos late in development, and this final deposition is fairly consistent among embryos (Hagmayer et al. 2018). Any offspring born within a 48-hour interval were counted towards a single brood, as there is typically a 3-5-day interval between broods and two broods born within 48 hours is highly unlikely.

DNA Extraction, Sequencing

I extracted DNA from whole juveniles and the caudle peduncles of adult fish using a Qiagen DNEasy kit. After DNA was extracted, I used the Qiagen Multiplex PCR kit to prepare two mixes, each with three microsatellite loci for a total of six microsatellite loci. See the Supplement for primer information and cycling conditions. Amplicons were sequenced on a fragment analyzer at the University of Arizona

Genomics Core. I used the program Geneious to align the fragment data and assign genotypes.

Paternity Assignment & Analysis

Once all individuals were genotyped, I manually assigned paternity by comparing alleles between mothers, potential fathers, and offspring. I genotyped offspring from the 30 females (16 C, 14 D) that had at least four offspring in the Mixed population insemination treatment (See Appendix B for sample sizes in all treatments). I calculated a Beta Distribution for posterior probability using Matthew Bognar's online calculator (homepage.divms.uiowa.edu/~mbognar/applets/beta.html). I denote the sire with the apparent majority of offspring in a group as α, then denote the sum of the minority sire's offspring and any unassigned offspring as β. x was set to 0.5 and the P(X>x) was calculated. I performed this calculation for all females in the CD treatment. P represents the probability that one sire truly sired more offspring than the other, assuming all unassigned offspring belonged to the "other" male, which is the most conservative way of handling unassigned offspring. Because of the resulting small sample sizes (4 C females and 2 D females with sufficient offspring from both sires' populations), I did not perform a formal statistical analysis for paternity.

Statistical Analysis

Females in Mixed (M) insemination treatments were eliminated from the analysis if they did not have both possible sires represented among the offspring, and at least four offspring in total. From the 57 females (31 C, 26 D) that were inseminated with sperm from 22 males (13 C, 9 D) across the Single and Mixed population insemination treatments, 39 females gave birth (22 C, 17 D). While a paternity analysis to determine which males sired the most offspring was not feasible, I did perform a formal analysis for offspring size based on treatment and including paternity for the Mixed insemination treatment. The response variable for this model was offspring ethanol weight, and the independent variables were Dam Population, Sire Population, and Cross Type (i.e., whether the insemination was “Single Population” (S) or “Mixed Population” (M), with Dam as a random effect). The model was analyzed as a linear mixed model with a Type III ANOVA, followed by a pairwise post-hoc analysis with a Tukey adjustment, which calculated estimated marginal means (EMMeans). Offspring size was normally distributed, so the data were not transformed for the analysis.

Results

Offspring size data were normally distributed; thus, a linear mixed model was appropriate and a good fit to the data. Based on this model, there was a significant three-way interaction between Dam Population, Sire Population, and Cross Type (χ^2=5.57, p=0.0183). D population females provision

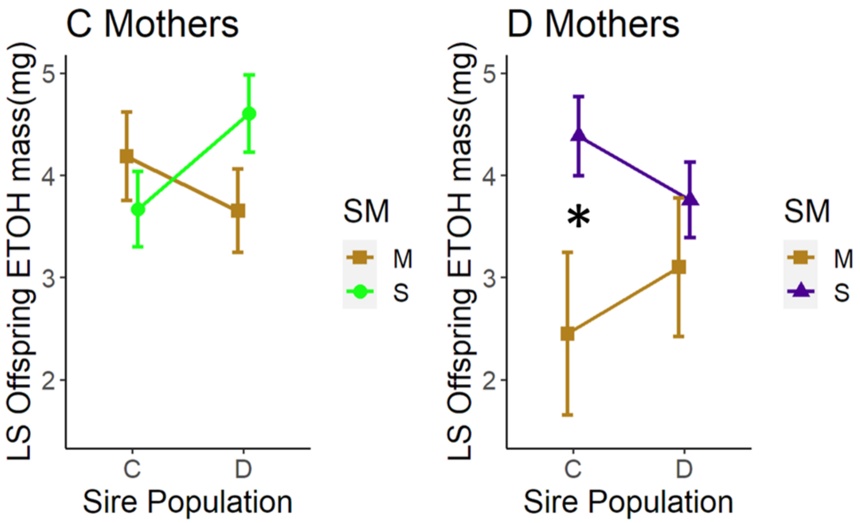

Figure 2.2. Least Squares Means (LSM) ± Standard Error of the LSM for of offspring ethanol mass results. Significant difference is denoted with “*”.

offspring differently depending on whether they are mated to C population males by themselves (Single population, or S treatment), or in combination with C and D population males simultaneously (Mixed population insemination, M treatment); D population females reduce allocation to D embryos when they are gestated with C male's embryos as opposed to only D male's embryos (t.ratio=-2.19, p=0.034). When born to D mothers, C-sired embryos are approximately 1.75 times larger in M treatments than in S treatments (**Fig. 2.2**).

In addition to the three-way, there are also two significant two-way interactions between Cross Type*Sire Population (χ^2=5.09, p=0.024) and Cross Type*Dam Population (χ^2=5.45, p=0.020). The interaction of Cross Type*Sire Population was not significant (χ^2=1.77, p=0.18). There are no significant main effects of Cross Type (χ^2=0.83, p=0.36), Dam Population (χ^2=3.68, p=0.055), or Sire Population (χ^2=1.95, p=0.16).

C females exhibit a similar trend of allocating fewer resources to offspring sired by D males, but it is not significant (t.ratio=-1.71, p=0.1009). There is also a trend in C females to over-allocate resources to non-resident male's offspring in S crosses, but it is not significant (t.ratio=-1.70, p=0.107). Based on the interaction in the main model, offspring size is based on a variety of factors that includes dam population, sire population, and whether females are supplied with sperm from one or both populations of males.

In the mixed insemination treatments, paternity data alone had samples sizes that were too small to be analyzed; only four C, and two D females had enough offspring sired by both population's sires. There is no obvious bias towards either sire. These data can be found in Appendix B.

Discussion

This study represents the first of its kind to demonstrate differential allocation of resources to different males' offspring when genetic distance is the only differentiating factor between males. In Single insemination treatments, there is no evidence for differential allocation between sires from different populations. However, when female *P. prolifica* females from population D are inseminated with both C and D males simultaneously, D population-sired embryos receive fewer resources than when only D population male's sperm was present. This means that *P. prolifica* females from population D discriminate against C population-sired offspring only when both C and D population males are present.

While the trend for C population females to reduce resource allocation to D population-sired offspring, it is only significant for D females. My ability to assess both offspring size because of paternity, and paternity in general in this experiment was limited. Artificial insemination has a notoriously low success rate and that, coupled with unequal sire success, impacted this experiment's sample size. Due to these impacts, it is difficult to interpret the results.

While this study cannot define conflict at the cause of incompatibility, it is noteworthy that females fully provision the offspring of nonresident males when they are the only developing offspring present. It is only when females are carrying offspring sired by both resident and non-resident males that they decreased the size of the non-resident males. Females were inseminated by sperm from two males in all treatments, so this discrimination against offspring sired by nonresident males is not merely a consequence of having more competition between males. What remains to be shown is whether this discrimination against offspring sired by nonresident males is mediated by the female or a consequence of the offspring of resident males having a competitive advantage.

There is some evidence for female sperm discrimination in the semelparous marsupial genus, *Antechinus.* Female *A. agilis* benefit from polyandry, and discriminate among males as the breeding season progresses (Fisher et al. 2006b). *A. stuartii* have similar reproduction, and genetically variable sires improve female fitness (Fisher et al. 2006a). There is no incompatibility because of polyandry reported for *Antechinus* females, and females did not discriminate against male's embryos in terms of provisioning; only selective sperm use. Females only mated with males that were in their local population. Polyandry has been proposed as a mechanism to reduce the potential for incompatibility to reduce female fitness, as mating with genetically variable sires can help to create genetically variable offspring (Simmons 2005).

My results did not produce any indication of differences in the fertilization success of males from different populations in any of the treatments. While my sample size diminishes my ability to detect differences if they were present, I do not have good cause to expect such differences, given no noted life history differences in either population that would result in either population's males having different sperm characters. Very little is currently known about poeciliid sperm, seminal fluid or ovarian fluid and how they might affect the dynamics of fertilization. The seminal fluid in poeciliids is outside of the spermatozeugmata in the ejaculate, but there are likely additional fluids within the spermatozeugmata that may play a role in sperm activation. In other organisms, like *Drosophila melanogaster* (fruit flies) accessory gland proteins in male sperm can influence traits in females such as egg production, sperm storage, mating behaviors, and female longevity (Clark et al. 1995; Bertram et al. 1996; Wolfner 1997; Chapman et al. 2000).

Most poeciliids rely on forced copulation. Prior studies on sperm and ovarian fluid characteristics of poeciliid fishes are dominated by work on guppies, which are non-placental and have male courtship. Colorful males have faster and more viable sperm (Locatello et al. 2006), and there may be incompatibility between populations of guppies driven by ovarian fluid composition (Gasparini and Evans 2013). Female receptivity and diet appear to also have an influence on male mating success, perhaps mediated by ovarian fluid (Gasparini et al. 2012; Cardozo and Pilastro 2018). There is a potential for male characters such as seminal fluid proteins, to play a role in these interactions.

Overall, my experiment does appear to support the Viviparity Driven Conflict Hypothesis, which postulates that there is higher conflict in placental species. As shown in this experiment, D population females are in conflict with C population males only when both C and D population male’s sperm is present. It is imperative that more studies like this one be done in other matrotrophic species. We are only beginning to understand how mothers allocate resources to their offspring, and this study implies there may be differential investment based on male characters. In non-placental species like guppies, females allocate all resources to their offspring before fertilization, so there is no influence of the sire on offspring size. Offspring size is an important determining factor in early offspring survival and fitness for the placental *H. formosa* (Henrich 1988). Understanding how females make investment decisions has implications for their offsprings' entire life.

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Chapter 3: Can Females Differentially Allocate Resources to Offspring Sired by Different Males?

Introduction

Observations of post-natal parental care inspired Robert Trivers (1974) to propose the existence of parent-offspring conflict. Trivers argued that the major driver of resource-driven conflict is relatedness. Mothers are equally related to each of their offspring. An offspring is 100% related to itself, but only 50% or 25% related to its siblings, depending on whether they have the same or different fathers, respectively. These inequalities in relatedness, combined with inherent competition over limited maternal resources, are predicted to result in conflict because what one offspring gains by commanding more parental resources is paired with a cost that is discounted by that offspring's relatedness to its siblings. During periods of conflict, natural selection favors the termination of provisioning by the mother, and the continuation of resource solicitation by the offspring. The intensity of conflict is predicted to be greater in broods of half- than of full-siblings due to lower coefficients of relatedness for half-siblings because the reduction in relatedness from 0.5 to 0.25 reduces the cost associated with selfishness. Half-siblings are therefore predicted to act more "selfishly" and try to solicit a greater quantity of resources than if they were all full siblings, extending the duration of conflict (Trivers, 1974).

The Viviparity Driven Conflict Hypothesis (VDCH) postulates that matrotrophic species will experience a shift in conflict from pre- to post-copulatory sexual selection. This shift results from a change in the venue of conflict from fertilization in species without matrotrophy to which maternal allocation of resources with matrotrophy. In mammals, the battlefront of conflict is the placenta. In non-mammalian matrotrophic species, tissues analogous to the placenta provide the same direct, physical connection between mother and offspring.

Matrotrophic species are predicted to accumulate post-zygotic reproductive incompatibilities more rapidly than species without matrotrophy because of differences among populations in how conflict is resolved (Zeh and Zeh, 2000; 2001; Crespi and Nosil, 2013). In my previous chapter, I showed that *Poeciliopsis prolifica* exhibits reciprocal incompatibility in the absence of any apparent differences among populations in life history traits like fecundity or offspring size. In this study, I perform a similar experiment on *Heterandria formosa,* a species with populations that are adapted to high versus low population densities. Females from localities with high population densities produce substantially larger offspring that those from populations with low population densities. Because egg sizes are the same in both populations, mothers from populations that produce large offspring must provide more resources to their offspring during development. If conflict results in a balance between maternal control over resource allocation and offspring (paternal) control over resource acquisition, then the hybridization of a male and female from populations that differ in offspring size will create a mismatch between the control of allocation by mothers and acquisition by offspring (fathers). Offspring size presents a clear source of incompatibility for *H. formosa*. I aim to address whether it also presents an opportunity for conflict over resources to occur when females are provided with sperm from localities that demand different levels of resources from mothers.

Angiosperms present a foundational model for studying conflict between mothers, fathers, and offspring. Most of this work relates to the study of genomic imprinting, in which genes from each parent will be differentially expressed in offspring, leading to conflict between the maternal and paternal genomes (Haig and Westoby 1989). There is mounting evidence that imprinted genes that enhance conflict appear to drive reproductive incompatibility in hybrids in *Mimulus* plants (Garner et al. 2016; Coughlan et al. 2020; Sandstedt and Sweigart 2022).

In this experiment, I use artificial insemination to test whether females carrying offspring sired by different males can discriminate among offspring in the allocation of resources. Artificial insemination allows me to remove any premating cues that a female could experience and has been used to demonstrate a preference for colorful males in guppies (Evans et al., 2003). *H. formosa* does not naturally exhibit any courtship behavior, but the possibility of paternal influences on embryos post-copulation has not yet been studied in this way. Here I aim to address the relative ability of offspring sired by different fathers to compete for maternal resources during development. Females were mated to males from their own versus different populations. I ask whether paternity is biased towards the mother's population and whether she discriminates against offspring sired by foreign males when she is mated to males from both populations simultaneously. I predict that females will discriminate against offspring sired by foreign males. I test this hypothesis by performing crosses between populations of the highly matrotrophic poeciliid, *Heterandria formosa*. *H. formosa* is a small, promiscuous poeciliid found in the southeast United States. A unique feature of this species is that there are large differences among populations in average offspring size corresponding to their matrotrophy index, which ranges from 40 in populations that produce small offspring to 67 in populations that produce large offspring (Schrader and Travis, 2009). Differences in offspring size may contribute to reproductive incompatibility between populations. F_{ST}, a measure of genetic distance between populations that explains how genetically differentiated they are, is likely to be significant for these two populations of *H. formosa*. Baer (1998) shows that Wacissa River and Trout Pond (a locality similar to Lake Jackson) has an F_{ST} of 0.30, which means they are genetically distinct and have relatively high genetic differentiation.

Previous research indicates that females from Trout Pond and Moore Lake, which typically produce many, small offspring, often abort offspring sired by Wakulla Springs and Wacissa River (WR) males, two populations that typically produce few, large offspring (Schrader and Travis, 2008; 2009; Schrader et al., 2013). In contrast, when females from large offspring producing populations are mated to males from populations that produce small offspring, the females produced relatively large offspring and suffered no loss of offspring viability. However, these crosses were set up with natural mating between one male and one female. Here I instead artificially inseminated females with sperm from multiple males, enabling me to assess how females allocate resources to embryos sired by males from their own versus a genetically distinct population when both are present at the same time. Specifically, I asked whether females can differentially allocate resources to offspring as a function of the offspring's genotype.

Methods

Fish Collection and Maintenance

Stocks of wild-caught *H. formosa* for each population were established six months prior to 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 prior to maturity ensuring that I had virgin females for artificial insemination. I discriminate between males and females based in the metamorphosis of the anal fin of males into a gonopodium, the intromittent organ (Fraser and Renton, 1940). Immature males were moved into 20-liter male-only tanks separated by population and females were isolated in 7.5-liter tanks to grow and mature until I inseminated them, ~2 months later.

Inseminations

I anaesthetized fish in a 0.01% solution of MS-222. F1 male *H. formosa* were selected at random from stock tanks then anaesthetized. Once the males began to turn in the water (after approximately 15s), they were placed on a bed of moist, sterile cotton. I 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, causing the male ejaculate. The ejaculate consists of spermatozeugmata, each of which contains hundreds of sperm. I collected the spermatozeugmata with a micropipette tip attached to silicone tubing, then transferred them to a small drop of Hank's Balanced Salt Solution (HBSS). I then measured the length and weight of the male and returned to container to recovery. The recovery solution included a drop of Stress Coat, which stimulated the production of the protective slime layer and facilitates the healing of any abrasions associated with handling. It takes approximately two minutes to extract sperm from and measure each male, and another four minutes to prepare an insemination droplet and inseminate a female.

Sperm was observed to be viable in the HBSS for at least 15 minutes. In poeciliids, sperm are packaged in structures called "spermatozeugmata", each of which contains thousands of individual sperm cells (Liu et al., 2018). I performed a pilot study before beginning the experiment to see if there were detectable differences in the number of spermatozeugmata per male, or sperm per spermatozeugmata. While this was a small pilot study (I looked at three males from one population of *H. formosa*), there were no significant differences in sperm between this small subset of males. From the published literature, there is no evidence that *H. formosa* exhibit differences in testis mass between populations (Schrader et al., 2012).

I inseminated females with spermatozeugmata from either two or four males, comprising four treatments: A: female x 2 WR males, B: female x 2 LJ males, C: female x 1 WR male + 1 LJ male, D: female x 2 WR males + 2 LJ males (**Fig 3.1**). Equal amounts of spermatozeugmata from each male were used for each treatment regardless of how many males were included, totaling 40 spermatozeugmata transferred to 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.

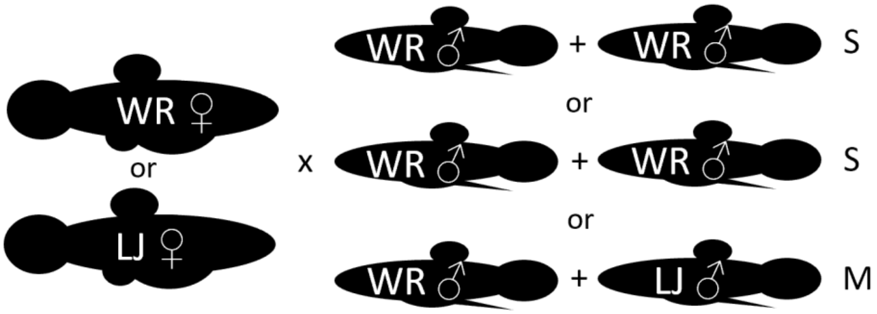

Figure 3.1. Experimental design for crosses between WR or LJ females with WR or LJ population males (Single, "S" crosses), or WR and LJ population males simultaneously (Mixed, "M" crosses).

I anesthetized females in the same way as the males, though they were left in the MS-222 approximately 30 seconds longer until they were fully anesthetized. Sperm was collected in a single drop and inserted into the female's gonopore. Each female was allowed to recover in her tank and treated with two drops of Stress Coat and a small dose of Maracin (an anti-bacterial fish medication) to limit the risk of infection from the procedure. As many females as possible were inseminated to maximize the ejaculate from each set of males, and males were stripped repeatedly over the course of the insemination period (approximately two weeks).

Female *H. formosa* were inseminated in two phases in Fall 2017 (WR) and Spring 2018 (LJ). A total of 54 WR females were inseminated, but five died before they gave birth. 38 (79%) of surviving females produced offspring. A total of 51 LJ females were inseminated, and while no females died, four females were removed from the experiment due to illness. 38 (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. 30 WR and LJ males were used to inseminate LJ females across all treatments, and 25 successfully sired offspring.

Juvenile Collection and Preservation

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 hours. I captured newborn fish and placed them immediately in a lethal, 1% dose of MS-222. Each offspring was then transferred to a slide and placed under a dissecting scope where I measured total and standard length. I then weighed offspring to the nearest 0.0001 mg with an analytical balance and transferred them to a DNase/RNase-free, 0.6 mL microcentrifuge tube filled with 95%-100% ethanol. Tubes were labeled then stored in a -20^{o}C freezer.

DNA Extraction & Sequencing

I extracted DNA from whole juveniles and the caudle peduncles of adult fish using a Qiagen DNEasy kit. After DNA was extracted, I used Qiagen Multiplex PCR kit to prepare two mixes, each with three microsatellite loci for a total of six microsatellite loci. I sourced the microsatellite loci from previous poeciliid studies (see supplement).

901 individuals were sequenced, comprised of the following WR/LJ females in each treatment: A: 5/3, B: 9/5, C: 10/8 D: 3/7. Of these, four WR C and one LJ D females were removed because I could not conclusively assign mixed paternity to their litters. Furthermore, one LJ B female was eliminated as 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 D: 3/6.

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

Data Organization & Analysis

Because the females from the two populations were inseminated at different times, I analyzed the populations separately and report on the results as such. Furthermore, because there were no significant differences found between the C and D treatments (in which one male from each population or two males from each population were used to inseminate a single female), I re-grouped my 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-hour interval were counted towards a single brood, as there is typically a 3-to-5-day interval between broods and two broods born within 48 hours is highly unlikely.

Statistical Analysis

Paternity

Some offspring could not be assigned to any of the potential fathers. These instances were due to shared alleles between dams and potential sires, as well as possible sequencing errors. In some cases, the assignment rate of a female's offspring to a single male was lower than 50%. It would not be appropriate to analyze the raw paternity data given the lack of confidence in the proportion of offspring sired by a particular male or population. I took a Bayesian approach to paternity assignment. Using Matthew Bognar's Beta Distribution [for posterior probability] Calculator (homepage.divms.uiowa.edu/~mbognar/applets/beta.html), I denote the sire commanding the apparent majority of offspring in a group as α, then denote the sum of the minority sire's offspring and any unassigned offspring as β. x was made to be 0.5 and the $P(X>x)$ was calculated. P represents the probability that one sire truly sired more offspring than the other, assuming all unassigned offspring belonged to the "other" male, which is the most conservative way of handling unassigned offspring. Once this was calculated for all females, I retained those for which $P>0.85$, or an 85% confidence level that a particular sire sired the majority of the offspring birthed by a particular female. I created a summary dataset in which each female became a single datum with the proportion of offspring sired by a "LJ" male.

Offspring size as a result of Cross Type and Paternity

The models for offspring wet weight for each population were analyzed as a linear mixed model with a Type III ANOVA, followed by a pairwise post-hoc analysis with a Tukey adjustment, which calculated estimated marginal means (EMMeans). The independent variables were Sire Population, and Cross Type, with the two cross type categories being whether the insemination was “Single” or “Mixed” population, as well as 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, 2012). Each population was analyzed separately.

Results

LJ females produce significantly smaller WR-sired offspring in Mixed, than in Single insemination treatments. LJ females, who are from the population that typically produces small offspring, exhibit a significant interaction between Sire Population*Cross Type ($\boldsymbol{X}^2$=7.06, p=0.00789). The Sire Population*Cross Type interaction occurred because LJ females produce offspring that are 1.5x as large when sired by WR

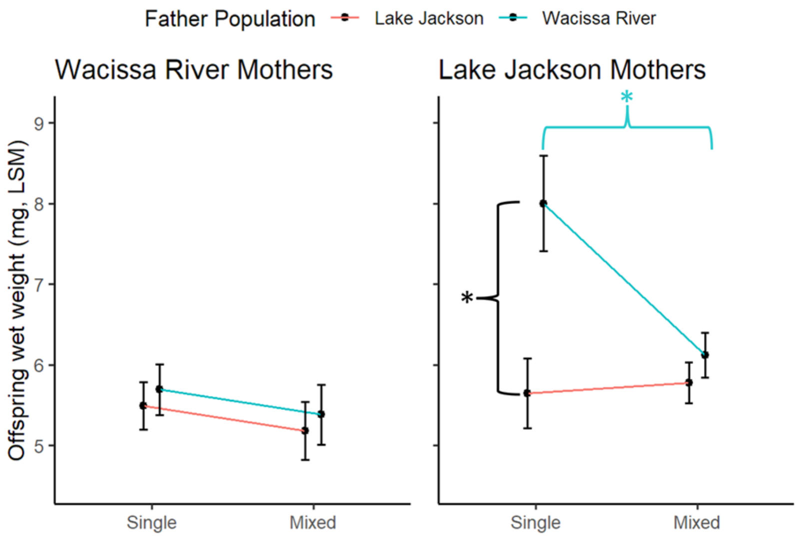

Fig. 3.2. Least Squares Means (LSM) ± Standard Error of the LSM for Offspring wet weight based on treatment for Wacissa River and Lake Jackson. Significant differences are indicated with a black * for differences between sire population and a blue * for differences between Single sire population and Mixed sire population inseminations.

males than LJ males in Single insemination treatments (t.ratio = -3.21, p = 0.0039), but there was no difference in offspring size sired by either LJ or WR males produced by females inseminated with sperm of males from both localities in Mixed insemination treatments (t.ratio = -1.77, p = 0.0776) (**Fig. 3.2**). There is also a significant effect of brood number as a covariate for LJ ($\boldsymbol{X}^2$=17.47, p<0.001), due to a decrease in offspring size in later broods.

WR females, who are from the population that produces large offspring, exhibit no differences in offspring size based on sire population ($\boldsymbol{X}^2$=0.16, p=0.6933). There is no interaction between Sire Population*Cross Type ($\boldsymbol{X}^2$=1.81, p=0.1791). There is no significant effect of brood as a covariate for WR ($\boldsymbol{X}^2$=0.57, p=0.4522).

Regarding paternity assignment alone, there were 11 females (6 WR, 5 LJ) with significant (>0.85 confidence) probabilities from the Beta Distribution (detailed in Supplement). Offspring of 4/6 WR females are significantly biased towards LJ males, and offspring of 4/5 LJ females are significantly biased towards LJ males. Though I cannot perform a formal statistical analysis, the trend in these results suggests a bias in paternity in favor of "LJ" males in the mixed insemination treatments.

Discussion

I demonstrate again that females carrying broods of mixed population paternity can allocate resources differently than when females carry broods from a single population of males. This study differs from the previous one because the two populations differ in offspring size, which potentially exacerbates their reproductive incompatibility because of differences between them in how conflict is resolved. There was no effect of using two versus four males on offspring size, but the sample sizes in each of those two treatments alone were small. While the paternity assignment using microsatellite markers was not accurate enough for us to compare fertilization success, I was still able to analyze how females provision offspring when they are mated to males from their own population, a different one, or their own and a different one in combination.

Wacissa River females, who produce fewer, larger offspring in nature, produced offspring of a similar size regardless of the mix of sperm they received. The consistency in offspring size seen by Wacissa River females is not new; Schrader and Travis have done similar experiments in Wacissa River and Wakulla Springs *H. formosa*. Both populations produce larger offspring in nature. When paired Trout Pond and Moore Lake *H. formosa,* respectively, they observed asymmetrical incompatibility similar to what I report here. 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). I documented a very few aborted embryos in this experiment, and there was no trend in treatment or population in the frequency of aborted embryos. A likely reason for reduced abortion rate is the large size of the Lake Jackson females used in this experiment. The standard lengths of *H. formosa* in the Schrader and Travis experiment ranged from 17mm to 23mm, and they found a positive association between female size and offspring size. In my experiment, I did not find a correlation between maternal and offspring size, but female standard length (at the time of sacrifice) in this experiment ranged from 22mm to 32mm. Furthermore, their experiment showed that larger Lake Jackson females were able to support larger embryos, which supports the ability for Lake Jackson females in this experiment to successfully produce such large offspring (Schrader and Travis, 2008). Larger Lake Jackson females can also carry more simultaneous broods of offspring at a time (Schrader and Travis, 2012).

Despite the differences in female and offspring size for Lake Jackson, there is still evidence of offspring size incompatibility when these females are mated to Wacissa River males. Given no other choice in male's sperm to use, Wacissa River-sired offspring in Single insemination treatments were over-sized compared to any other treatment. When Trout Pond *H. formosa* females were crossed with Wakulla Springs males, over-sized offspring were also reported, along with a high number of aborted embryos (Schrader and Travis, 2008). Had these Lake Jackson females been a smaller size, they may also have aborted these larger embryos. I extended this result by including a Mixed insemination treatment. Wacissa River-sired offspring in the Lake Jackson Mixed insemination treatment are smaller than in the Wacissa River-sired Single insemination treatment. For the first time, I provide evidence that, when given a choice, female *H. formosa* can resist these high resource-demanding embryos if there are also Lake Jackson-sired embryos present.

The VDCH predicts that there will be accelerated evolution of post-copulatory reproductive isolation among populations of placental, compared to non-placental species. I predicted that this incompatibility will be visible when I compare matings among populations within closely related placental and non-placental species. Individuals from different populations of the placental species should display greater degrees of reproductive incompatibility than those from non-placental species. Previous work by Keenan Morrison shows that placental poeciliids experience reductions in offspring size that are correlated with increasing genetic distance (Morrison, 2017). Similar crosses among populations of non-placental species showed no such changes in offspring size at birth. My first chapter reinforces these results. Here again, offspring size is the main source of evidence for reproductive incompatibility.

While this study cannot distinguish conflict from incompatibility, it is worth noting that females inseminated only with sperm from non-resident males do not discriminate against offspring from non-resident males. It is only when females are provided with sperm from both resident and non-resident males that they decreased the size of offspring sired by the non-resident males. In all the treatments, females were given sperm from two males, so this result is not merely a consequence of having more competition between males.

Even though I cannot distinguish conflict from incompatibility, this study does present a compelling argument for cryptic female choice. The field of female began with William Eberhard’s 1996 book on sexual conflict (Eberhard, 1996). As with this study, it is difficult to prove that a female is exhibiting preference or discrimination irrespective of the male’s sperm traits or imprinted genes. Because this field is so young, gene expression in developing embryos from different fathers has not yet been examined. There has been some improvement in showing that several species can discriminate against a particular male's sperm (Firman et al., 2017), but there have not been any studies that pair cryptic female choice in fertilization success with differential maternal investment in offspring sired by different males.

I cannot at present comment on how these females partition resources as they do. *H. formosa* provision their embryos via a follicular placenta. Embryos are retained in the follicle until birth and the follicles are in turn retained within the ovary (Uribe and Grier, 2011; Carmen Uribe and Grier, 2018). However, I cannot discriminate between the influence of the maternal versus the paternal genome. Wacissa River sire's embryos are more demanding than those sired by Lake Jackson males and are more successful in soliciting extra resources from Lake Jackson females when only offspring sire by Wacissa River males are present. When females are instead provided with sperm from both populations, offspring sired by males from the Wacissa River are no longer oversized. Females instead allocate resources equally to all offspring. Whether it be discrimination or strategic allocation by these females, it is still remarkable to see the differences in allocation of resources between treatments. It is important to do similar research in other matrotrophic species to determine if they are capable of differential allocation and begin to investigate the mechanisms by which matrotrophic females allocate resources to their offspring. Furthermore, more advanced genetic techniques should be applied (such as ddRADseq) to increase the accuracy of paternity assignment, which will allow us to draw conclusions about paternity and male-male post-copulatory sexual selection. Poeciliid male sperm characters have only been investigated extensively in guppies. Understanding matrotrophic species' sperm characters may provide insights into post-copulatory sexual selection. There are many facets to incompatibility, and we are only beginning to understand them in poeciliids.

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Conclusion

I have shown how matrotrophic poeciliids experience reproductive incompatibility when females from one population are mated to males from another. In my first chapter, female of the placental species *P. prolifica* from the C population produced smaller offspring when they were mated to males from D population than when mated to males from the C population. While D females did not replicate this result significantly, the same trend was present. There were no similar occurrences for the non-placental *P. infans* in similar reciprocal crosses among two populations that were as genetically different from one another as were the two *P. prolifica* populations.

In my second chapter, female *P. prolifica* from populations C and D were artificially inseminated with sperm from C, D or C and D males simultaneously. These females were not exposed to any pre-copulatory cues from their mates. The results were different from the first experiment, in which males and females were housed together for the duration of the experiment and in which there was never the potential for direct competition among sperm from two different males nor the potential for there to be contemporary developing offspring sired by different fathers. In both the C and D females, there was a trend to over-allocate resources to offspring sired by nonresident males when fertilized with only foreign sperm, but this result was only significant in D females. In mixed insemination treatments, in which females were fertilized with C and D male's sperm, C females allocated significantly less resources to D-sired embryos than when only D males were present. This is an interesting distinction, because in chapter one, we only found reduced offspring sizes when C females were mated to D males. In chapter one, females were housed with the males, so the presence of the male before and/or during copulation could influence how a female provisions her offspring. Furthermore, it implies that females can discriminate among offspring in the allocation of resources. Alternatively, there might be competition among embryos that favors the mother's population. Through artificial insemination, all cues from the male are removed, and seminal fluid is reduced through the mixing of the spermatozeugmata from multiple males. Sperm characteristics are often mediated through seminal fluid, but sperm competition can still occur post-insemination. The role of seminal fluid versus sperm characteristic in shaping maternal investment would be an interesting avenue of research to pursue considering these results.

My third chapter replicates the methods of Chapter 2 in an experiment performed on a second matrotrophic species. Doing so with *Heterandria formosa* added another element of complexity to maternal investment. In *P. prolifica*, there is no notable difference in the two populations other than genetic distance. Populations of *H. formosa* give birth to small or large offspring depending on whether they experience high or low predation. Previous experiments show that females that typically produce small offspring struggle to support embryos sired by males that typically produce large offspring. Earlier research showed that Wacissa River (WR) females produce similarly-sized offspring regardless of who they are mated to. Lake Jackson (LJ) females over-allocate resources to offspring sired by WR males. However, they allocate significantly less resources to offspring sired by WR males when embryos from LJ males are also present. The apparent discrimination against WR males by LJ females opens the possibility of a few forms of sexual conflict. It could be a product of male-male sperm competition, accessory gland proteins (which has been shown in fruit flies) (Gillott 2003), female choice, or a form of communication between mother and embryo resulting in differential allocation of resources. While my experiment cannot answer any of these questions, it creates an avenue of research for future experiments utilizing artificial insemination and sperm characterization in matrotrophic poeciliids.

All three experiments were based on low sample size. These experiments consisted of many treatments that were logistically challenging. Particularly for the artificial insemination experiments, success rate of the insemination has been historically low. In my experiments, I achieved around 70-80% success which was relatively high. However, this resulted in consistent sample sizes less than 10 individuals per treatment, and these numbers were further reduced when considering that individuals could only be retained in mixed insemination treatments if sires from both populations sired at least one offspring. Detection of paternity was also a challenge, as I utilized six microsatellite loci. Using microsatellites was practical considering the large numbers of offspring produced in these experiments, but the success rate of siring offspring was sometimes impossible due to lack of allelic diversity between males.

My research advances the study of evolution by presenting a novel avenue for the study of sexual selection and conflict. Before these experiments, it was assumed that females would allocate resources to their offspring based on sire identity (Schrader and Travis, 2008, 2009). Artificial insemination with sperm from multiple males adds a new dimension to the evaluation of maternal investment. Evans and Magurran (2001) used artificial insemination to show that colorful male guppies had more competitive sperm. Guppies are non-placental so their experiment cannot address whether male genotype or population would influence maternal provisioning. There is now clear evidence that female *H. formosa* can discriminate against demanding embryos when there are other embryos present from males whose offspring are less demanding of resources. While the mechanics of the interaction are still unknown, it provides a novel avenue of research for cryptic female choice.

In 1996, William Eberhard published a book titled “Female Control: Sexual Selection by Cryptic Female Choice”. It had traditionally been perceived that female organisms could not discriminate against particular males. However, the ability for a female to influence mate choice and offspring provisioning would be highly adaptive under certain circumstances. Mate recognition is important not only to avoid hybridizing species, but also to avoid genetically inferior, or genetically related mates. Many species have evolved sensory cues to identify and discriminate against mates before copulation (Higgie et al., 2000), but we do not know the extent to which most species do this. In 1996, Eberhard commented that it was not useful to synthesize the entire field, because it was too young. This statement is still true, as cryptic female choice is difficult to assess and there are many other factors that must be taken into consideration to claim that females are exhibiting preference or discrimination (Birkhead, 1998).

The field of cryptic female choice and discrimination has grown in the last 26 years. Firman et al. (2017) summarized advances in cryptic female choice literature since 1996. It is now known that post-mating sperm discrimination may allow a female to avoid inbreeding in arthropods, fish, mice and birds (Ala-Honkola et al., 2010; Firman and Simmons, 2015; Løvlie et al., 2013; Tuni et al., 2013; Welke and Schneider, 2009), though it can also act to favor more genetically similar males in dung beetles (Ward, 2000). Likewise, females can discriminate against the sperm of closely related species that would result in offspring with reduced fitness (Cramer et al., 2016). Furthermore, female *Drosophila melanogaster* flies can eject sperm from males expressing sex-distorter genes (Angelard et al., 2008). Across all of these studies, there is evidence for a female influence on the copulatory success of males.

Cryptic female choice is clearly possible, but it is still not well understood. It is important to extend research beyond fertilization success and further investigate the role of the female provisioning of developing young. Many of the groups of organisms that cryptic female choice has been investigated in do not exhibit matrotrophy, so offspring size is not often taken into consideration. Using poeciliids to investigate questions regarding cryptic female choice and discrimination will elucidate how female organisms not only discriminate against male's sperm, but also shape their developing young. Applying more advanced physiological and genomic techniques can reveal the underlying mechanisms.

Overall, my dissertation contributes to the VDCH by providing evidence of reproductive incompatibility in two placental poecilid species. Furthermore, it exposes F2 breakdown in interpopulation crosses of a non-placental poeciliid in brood size, which had not been previously recorded. Whether it be cryptic female choice, male competition, or Dobzhansky-Muller Incompatibilities, it provides a path for study using poeciliids as a model. While I was not able to apply advanced genomic techniques in my dissertation, there clear opportunity to use these tools in future research.

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Appendix A

In this appendix, I include sample sizes for each generation and species, as well as individual measures of offspring viability and fertility for the experiment in Chapter 1.

Table A.1a. Replication for *P. infans* crosses in the parental generation.

	A male	B male
A Female	8	6
B Female	7	6

Table A.1b. Replication for *P. infans* crosses in the F1 generation.

Cross	Sample size
AA	12
BB	11
BA	12
BB	9

Table A.1c. Replication for *P. prolifica* crosses in the parental generation.

	C male	D male
C Female	8	10
D Female	9	10

Table A.1d. Replication for *P. prolifica* crosses in the F1 generation.

Cross	Sample size
CC	12
CD	14
DC	16
DD	14

The following figures detail individual measures of viability and fertility:

<u>Offspring viability</u>

There was not sufficient data on offspring mortality of cross success to analyze. Most offspring survived, and most crosses were successful. The additional variables for offspring viability I present here are male maturation characters, neither of which are dependent on experimental treatment for either species. First, there is no effect of treatment on time to the start of male maturation in either *P. infans* ($\boldsymbol{X}^2$= 0.53, p= 0.47) or

P. prolifica ($\boldsymbol{X}^2$= 0.67, p= 0.41) (**Fig. A.1**). The main determinant of size at maturity is how old the male was in both *P. infans* ($\boldsymbol{X}^2$= 22.12, p<<<0.001) and *P. prolifica* ($\boldsymbol{X}^2$= 90.92, p<<<0.001)). There is no effect of treatment on male maturation size in either *P. infans* ($\boldsymbol{X}^2$= 0.0024, p= 0.96) or *P. prolifica* ($\boldsymbol{X}^2$= 0.96, p= 0.33) (**Fig. A.2**).

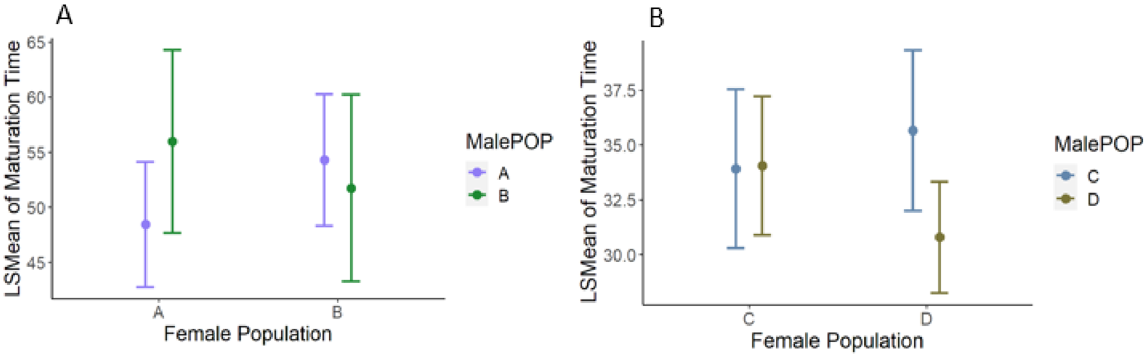

Figure A.1: Least-Square Means + Standard Error bars for Male time to the start of maturation results for A. *P. infans* and B. *P. prolifica.*

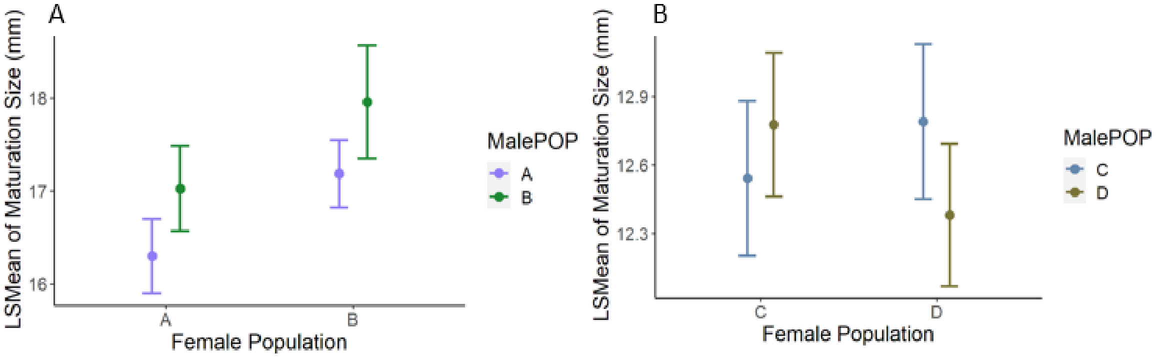

Figure A.2: Least-Square Means + Standard Error bars for Maturation size results for A. *P. infans* and B. *P. prolifica.*

Offspring fertility

There was a significant effect of cross on litter size for *P. infans* ($\boldsymbol{X}^2$= 23.04, p<<0.001) (Fig. A.4). AA females had approximately 30% larger litter sizes than AB females (z= -3.03, p= 0.013). The reciprocal crosses of BA and BB origin fish did not exhibit the same trend (z= 1.23, p = 0.57) (Fig. A.3). Inter-brood interval was not significant for *P. infans* ($\boldsymbol{X}^2$= 1.00, p= 0.32), so litter size is the main factor for rate of offspring production in *P. infans* in this experiment.

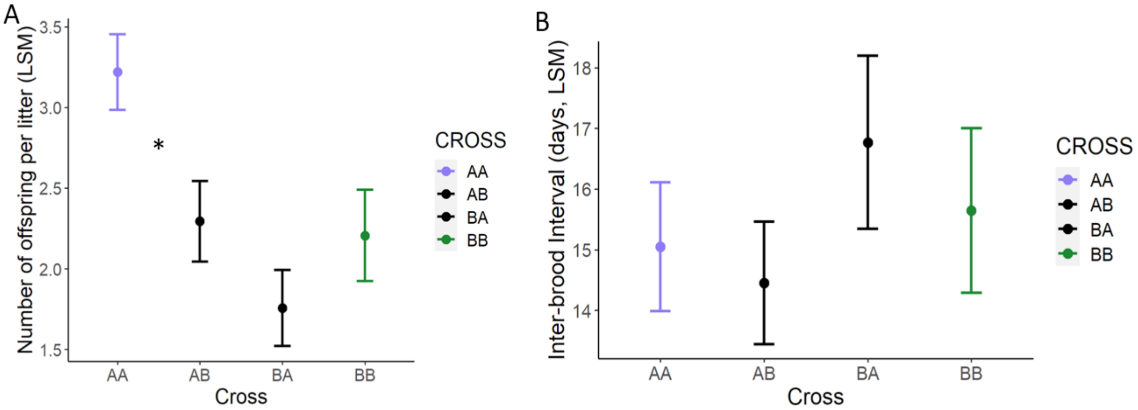

Figure A.3: Least-Square Means + Standard Error bars for A. Litter size and B. Inter-brood interval results for *P. infans*. Significant result is denoted with “*”.

In *P. prolifica,* there was a trend for longer intervals in CD compared to CC crosses, and for DD females to have longer inter-brood intervals than CC females. However, the overall effect of cross is not significant ($\boldsymbol{X}^2$= 6.58, p= 0.086). Furthermore, there are no trends in average litter size ($\boldsymbol{X}^2$= 2.15, p= 0.54) (**Fig. A.4**). As shown in the rate of offspring production in the main body of this chapter, the apparently longer inter-brood intervals did not affect the overall offspring production rate.

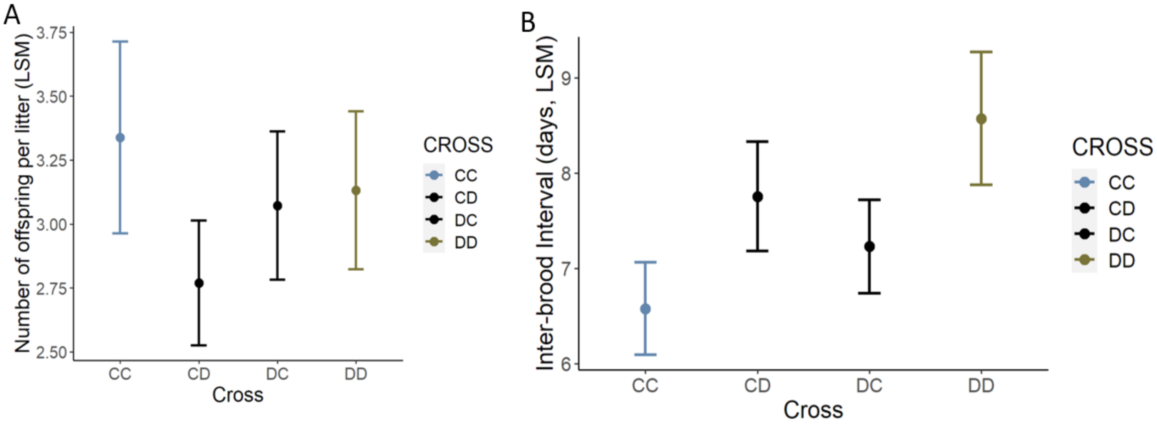

Figure A.4: Least-Square Means + Standard Error bars for A. Litter size and B. Inter-brood interval results for *P. prolifica*.

Appendix B

These tables detail sample sizes, statistics, microsatellite information, and paternity data from the experiment in Chapter 2.

Table B.1. Females represented in each treatment after filtering the data.

Treatment	Population C	Population D
CC (Single C)	6	5
DD (Single D)	5	5
CD (Mixed)	5	3

Table B.2. Offspring size model results

	ChiSq Value	df	p-value
SM	0.8330	1	0.36140
Mother Population	3.6844	1	0.05492
Sire Population	1.9527	1	0.16229
SM*Mother Population	5.4544	1	**0.01952**
SM*Sire Population	5.0910	1	**0.02405**
Mother Population*Sire Population	1.7734	1	0.18296
SM*Mother Population*Sire Population	5.5674	1	**0.01830**

Table B.3. Microsatellite primers used for paternity assignment (Soucy and Travis 2003; Ala-Honkola et al. 2009):

Primer	Sequence (5' to 3')
TSS005 F:	CTT TAA TAC CCA ATC AGT GG
TSS006 R:	GTTT CAA CTG GAA GAG GAG TTG TC
HETF05 F:	CATAGATTCTGCAGGCAGTG
HETF05 R:	GTTTCTCAGTGACTATAAGGCCAAC
HETF04 F:	CACTACACTGGCAAACCCATC
HETF04 R:	GTTTTCAAACCTCCTAGTATGACAA
TSS013 F:	TCA TCT GGA GCA GGC ACA TG
TSS014 R:	GTTT GCG TTT GGT TTC CTA CTG AC
TSS051 F:	CGC CGC TTA CCA GAA CTT AAT
TSS052 R:	GTTT TCA GGC TCT CTG TTT GTC CA
SLS045 F:	TG GAA ATT GTA AAT CTG TGT TC
SLS046 R:	GTTT CC GGG AAC TTC ATT GTC AGT

Table B.4.a Cycling conditions for HETF05, TSS013-14, TSS051-52

95ºC			15 mins
	28 cycles		
		94ºC	30s
		59ºC	90s
		72ºC	60s
60ºC			20 mins

Table B.4.b Cycling conditions for TSS005-6, HETF04, SLS045-46

95ºC			15 mins
	28 cycles		
		94ºC	30s
		55ºC	90s
		72ºC	60s
60ºC			20 mins

Table B.5 Beta distribution table for certainty in paternity assignment. Values above 0.85, or 85% certainty that the "Majority Sire" sired more than 50% of the offspring born to a given female.

Dam ID	Dam population	n Genotyped	n C (actual, NAs)	n D (actual, NAs)	Majority Sire	P(X>x)
2	D	5	3	0,2	C	0.6875
10	D	**4**	**3**	**1**	**C**	**0.875**
13	D	**14**	**1,2**	**12**	**D**	**0.99829**
12	C	**7**	**1,2**	**5**	**D**	**0.89062**
39	C	**17**	**4,5**	**12**	**D**	**0.96159**
41	C	**9**	**2,3**	**6**	**D**	**0.85555**
42	C	**9**	**6**	**2,3**	**C**	**0.85555**
59	C	11	6	4,5	C	0.62305

Appendix C

These tables detail sample sizes, statistics, microsatellite information, and paternity data from the experiment in Chapter 3.

Table C.1. Females represented in each treatment after filtering the data

Treatment	Population WR	Population LJ
WR only	3	2
LJ only	5	3
1WR+1LJ*	10	8
2WR+2LJ*	3	7

**These treatments were not statistically significant from each other and were combined into the "Mixed" insemination group.*

Table C.2a. Model results for Wacissa River offspring size

Wacissa River	ChiSq Value		df	p-value
Sire Population	0.1555		1	0.6933
SM	0		1	0.9970
Brood	0.5651		1	0.4522
Sire Population*SM	1.8049		1	0.1791

Table C.2b. Model results for Lake Jackson offspring size

Lake Jackson	ChiSq Value	df	p-value
Sire Population	3.1558	1	0.07566
SM	0.0672	1	0.79544
Brood	17.4713	1	**2.917e-05**
Sire Population*SM	7.0584	1	**0.00789**

Table C.3. Microsatellite primers used for paternity assignment (Soucy and Travis, 2003; Ala-Honkola et al., 2009):

Primer	Sequence (5’ to 3’)
TSS005 F:	CTT TAA TAC CCA ATC AGT GG
TSS006 R:	GTTT CAA CTG GAA GAG GAG TTG TC
HETF05 F:	CATAGATTCTGCAGGCAGTG
HETF05 R:	GTTTCTCAGTGACTATAAGGCCAAC
HETF04 F:	CACTACACTGGCAAACCCATC
HETF04 R:	GTTTTCAAACCTCCTAGTATGACAA
TSS013 F:	TCA TCT GGA GCA GGC ACA TG
TSS014 R:	GTTT GCG TTT GGT TTC CTA CTG AC
TSS051 F:	CGC CGC TTA CCA GAA CTT AAT
TSS052 R:	GTTT TCA GGC TCT CTG TTT GTC CA
SLS045 F:	TG GAA ATT GTA AAT CTG TGT TC
SLS046 R:	GTTT CC GGG AAC TTC ATT GTC AGT

Table C.4.a Cycling conditions for HETF05, TSS013-14, TSS051-52

95ºC				15 mins
		28 cycles		
			94ºC	30s
			59ºC	90s
			72ºC	60s
60ºC				20 mins

Table C.4.b Cycling conditions for TSS005-6, HETF04, SLS045-46

95ºC			15 mins
	28 cycles		
		94ºC	30s
		55ºC	90s
		72ºC	60s
60ºC			20 mins

Table C.5. Beta distribution table for certainty in paternity assignment. Values above 0.85, or 85% certainty that the “Majority Sire” sired more than 50% of the offspring born to a given female.

Dam ID	Dam population	n Genotyped	n LJ (actual, NAs)	n WR (actual, NAs)	Majority Sire	P(X>x)
10	WR	20	8	3,12	LJ	0.17964
12	WR	11	4	1,7	LJ	0.17188
16	WR	21	8,12	9	WR	0.25172
25	WR	18	2	0,16	LJ	0.00014
30	WR	13	9	3,4	LJ	0.927
31	WR	14	1	13	WR	0.99988
32	WR	9	6	0,3	LJ	0.85547
33	WR	19	9	4,10	LJ	0.40726
35	WR	20	13	6,7	LJ	0.91647
104	LJ	42	22	14,20	LJ	0.62239
105	LJ	43	32	4,11	LJ	0.99953
106	LJ	14	5	3,9	LJ	0.13342
115	LJ	22	12	10	LJ	0.66819
124	LJ	21	18	3	LJ	0.9998
126	LJ	39	37	1,2	LJ	1
133	LJ	14	7	1,7	LJ	0.5
136	LJ	20	10	5,10	LJ	0.5
137	LJ	20	11	5,9	LJ	0.6762
139	LJ	12	5,6	6	WR	0.5
140	LJ	20	16	1,4	LJ	0.99779
141	LJ	15	8	2,7	LJ	0.60474
145	LJ	24	6,15	9	WR	0.10502
148	LJ	20	5,9	11	WR	0.6762
149	LJ	21	6,7	14	WR	0.94234
152	WR	14	4	10	WR	0.95386
154	WR	20	13	7	LJ	0.91647