Postmating, Prezygotic Isolation among species of the *Drosophila virilis* subgroup

By

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ABSTRACT

Reproductive barriers between sexually reproducing organisms prevent interbreeding and gene flow between species. Early studies of reproductive isolation focused on prezygotic and postzygotic isolating mechanisms, yet postmating, prezygotic isolation (PPI) barriers have not been fully explored. In this thesis, I characterized the phenotypic and the evolutionary process of postmating prezygotic isolation among heterosepcific matings in Drosophila. Using species of the *Drosophila virilis* subgroup and microscopic approaches, I initially examined egg laying, egg hatchability, egg fertilization and sperm storage and retention in *D. virilis* females' reproductive tract mated with *D. novamexicana* males. I found that *D. virilis* females laid similar numbers of eggs compared to a conspecific mating. However, the number of eggs hatched was significantly lower in heterospecific than conspecific crosses. Furthermore, unhatched eggs were unfertilized. In spite of the large number of sperm transferred to female's storage, few sperm were retained in storage shortly after mating. I further scored egg laying and hatchability between other heterospecific and conspecific crosses and found that PPI evolved during the diversification of the *D. novamexiana – D. americana* clade. Finally, eggs laying in heterospecific crosses and the reduction in egg hatchability in heterospecific crosses suggest that females exert cryptic control of the heterospecific ejaculate and influence the process of sperm usage during the fertilization of eggs.

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Table of Contents

List of Tables

List of Figures

List of Abbreviation

CYAM	—	Cornmeal- Yeast-Agar- Molasses
mm	—	Millimeter
ºC	—	Degree Celsius
♂	—	Male
♀	—	Female
ml	—	Milliliter
PBS	—	Phosphate–buffered saline
nM	—	Nanomolar
DAPI	—	4',6-diamidino-2-phenylindole
DNA	—	Deoxyribonucleic acid
µl	—	Microliter
χ^2	—	Chi square

List of Appendices

1.0 INTRODUCTION

1.1 Species and speciation:

Among sexually, asexually and dually reproducing organisms, species are always regarded as fundamental units in evolution and biodiversity. Although evolutionary biologists generally concur that species are real and objective, there is considerable controversy over the way in which species should be defined. Accordingly, several species concepts have been proposed to deal with the so called "species problem". The biological species concept (BSC), originally proposed by Mayr and Dobzhansky states that species in sexually reproducing organisms are groups of interbreeding natural populations that are reproductively isolated from other such groups (Coyne and Orr 2004). In addition, Dobzhansky contributed to the BSC by elaborating on types of barriers or isolating mechanisms that can keep two closely related species distinct (Coyne and Orr 2004).

Isolation between diverging populations or incipient species occurs by means of prezygotic and postzygotic barriers. Prezygotic barriers are mechanisms that prevent the process of zygote formation between two heterospecific species. Furthermore, prezygotic isolation can be divided into premating and postmating isolation barriers. Premating isolation can be effectively maintained by behavioural and ecological differences between species, and mechanical incompatibilities between reproductive structures.

Among animals, elaborate courtship behaviours not only serve as recognition signals between males and females, but also prevent mating with members of other species. Signals that differ from species to species reduce attraction and create behavioural isolation during the breeding period (Andersson 1994). Behavioural isolation caused by differences in contact pheromones, can be seen for example in sea snakes. The species *Laticauda colubrine* and *L.*

frontalis are morphologically identical, sympatric and breed at the same time of the year, but there is no evidence of hybridization production between these two species. Differences in the sex pheromones (skin lipids) in adult females of *L. Colubrine* and *L. frontalis* allow males to recognize conspecific females for mating over heterospecific females (Shine et al. 2002).

Ecological isolation can occur when different species occupy different habitats (habitat isolation) or breed in different seasons (temporal isolation). Habitat isolation has been well explored in frogs, *Rana blairi* and *R. pipiens*. *R. blairi* resides and breeds in turbid waters (silty streams), whereas *R. pipiens* resides in clear streams. Occasionally, these species hybridize when they encounter each other in intermediate habitats (Lynch 1978). Temporal isolation has been found between two sympatric species of Atlantic corals, *Montastraea annularis* and *M. franksi*. Although species discharge their gametes at the same time, difference in spawning period (which is non-overlapping) reduce the potential of earlier spawning sperm to fertilize eggs of later spawning species (Knowlton et al. 1997). Geographical isolation, although occasionally linked to ecological differentiation, is more likely to help maintain isolation between diverged species rather than to be a cause of speciation (Coyne and Orr 2004). A textbook example of geographical isolation exists in the Hawaiian Drosophila clade that formed from adaptive radiation associated with the formation of the Hawaiian Islands (Carson 1982).

Mechanical incompatibility due to differences in genital morphology in animals with internal fertilization, leads to a mismatch that foils mating attempts among different species. A clear example of changes in genital morphology occurs in mating attempts between two species of the Carabid beetles, *Carabus maiyasansn* males and *C. iwakianus* females. During copulation, females suffer mortality due to the mismatch between male genitalia (anapophysi) and female

genitalia (vaginal pouch). This incompatibility tears the female reproductive tract and ultimately causes her death (Sota and Kubota 1998).

All the isolation barriers mentioned above describe premating, prezygotic forms of reproductive isolation. Postmating prezygotic isolation, where the isolation occurs after mating between heterospecific species but prior to fertilization, is less explored. As my study focuses on postmating prezygotic isolation barriers, I describe them in detail under Section 1.1.1.

Postzygotic isolation barriers yield inviable and sterile hybrid progenies after mating between different species, thus the interspecific hybrids are unfit and selected out rather than being successful progenitors for new species to evolve. A well known example of hybrid inviability involves the crosses between *D. melanogaster* females and *D. simulans* males. While hybrid daughters are viable, hybrids males die during the development in the transition from larva to pupa (Sturtevant 1920; Sawamura 2000). In 1936, Dozhansky reported F1 hybrid male sterility between the sibling species *D. persimilis* and *D. pseudoobscura* when crossed reciprocally. In both crosses, testes of hybrid males vary considerably in size, indicating a disruption of the process of spermatogenesis (Dobzhansky 1970).

1.1.1 Postmating, prezygotic isolation or gametic isolation

For a potential isolation mechanism to be efficient, it should prevent gene flow between diverging populations or incipient species. More recently, attention has been given to "Gametic isolation" in which gene flow is reduced after mating has taken place but prior to zygote formation. Two forms of gametic isolating barriers have been recognized: competitive and noncompetitive (Coyne and Orr 2004). Competitive isolation barriers are also known as "conspecific sperm precedence" and takes place in females' reproductive tract. When females

mate to both conspecific and heterospecific males, sperm of conspecific males outcompete sperm of heterospecific males (Howard 1999; Coyne and Orr 2004). Conspecific sperm precedence is a widespread phenomenon that occurs in many insects, and in animals with internal fertilization. In ground crickets, conspecific sperm precedence occurs between the sister species *Allonemobius fasciatus* and *A. socius.* Females of either species inseminated by both conspecific and heterospecific males, produced offspring mostly sired by the conspecific male, independent of the order of mating (Gregory and Howard 1994). Similarly, in Drosophila, when females of *D. simulans* mate sequentially to conspecific males and to either heterospecific males of *D. mauritiana* or *D. sechellia*, the conspecific males fathered a larger proportion of progeny than the heterospecific males (Price 1997).

In contrast, noncompetitive isolation does not involve male - male competition. This type of isolation emerges at any stage between copulation and fertilization to disrupt the sperm from fertilizing an egg in the heterospecific females' reproductive system. Noncompetitive gametic isolation falls into many forms, ranging from poor transfer and storage of sperm to the inability of sperm to fertilize an egg (Coyne and Orr 2004). For instance, in birds there are many barriers that exist between insemination and fertilization (Birkhead and Brillard 2007). Mating between females of the Mallard duck *Anas platyrhynchos* and males of Muscovy drakes *Cairina moschata*, results in a high proportion of infertility. Infertility has been shown to be a consequence of ineffective storage of sperm in sperm storage tubules (SSTs) as well as inability of the sperm to penetrate the perivitelline layer (PVL) of the egg (Sellier et al. 2005). The phenomenon of insemination reaction is an example of ejaculate inviability in the heterospecific reproductive tract. It was studied in numerous species in Drosophila by Wheeler (1947). A large mass forms in the female's uterus after the transfer of heterospecific sperm, which obstructs

ovulation and ultimately fertilization (Patterson 1946). Females of different geographic populations of *D. mojavensis* mated to a closely sister species of *D. arizonae* males, produced significantly fewer offspring than conspecifics. This is due to the insemination mass found in females' uterus after mating that obstructs oviposition and further fertilization (Kelleher and Markow 2007). Another example of noncompetitive isolation in *Drosophila* is a reduction in sperm transfer during copulation with a heterospecific female. *D. simulans* females copulate for longer with *D. sechellia* males than with *D. simulans* males. However, low numbers of sperm are transferred during copulation and very low numbers of offspring are produced (Price et al. 2001).

1.2 *Drosophila virilis* subgroup

The *Drososphila virilis* subgroup consists of five species; *D. virilis, D. lummei*, *D. novamexicana, D. americana texana* and *D. americana americana*. These species are holarctic in distribution. *D. virilis* and *D. lummei* species are endemic to the Palearctic region (Northern Europe, Africa and Asia), while *D. novamexicana, D. americana texana* and *D. americana americana* species are endemic to the Nearctic region (North America) (Throckmorton 1982). *D. virilis* is a cosmopolitan domestic species and its wide range of distribution is due to human transportation (Throckmorton 1982). Moreover, *D. virilis* are known for their high thermotolerance and high tolerance for ethanol, suggesting that the rapid expansion of *D. virilis* in habitats that have not been occupied by related species is due to their ability to survive in different kinds of environments (reviewed in Mirol et al. 2008). *D. virilis* has been reported in breweries and timberyards and *D. lummei* was collected from the borders of lakes and streams (Throckmorton 1982). In North America, *D. novamexicana* resides in the drier habitat of lower

river valleys of New Mexico and the surrounding states, whereas *D. a. texana* is found in eastern United States of America (Figure 1) (Throckmorton 1982).

While phylogenetic studies have established *Drosophila virilis* as the most ancestral species within the subgroup, the clade of *D. a. americana, D. a. texana* and *D. novamexicana* (for now or referred to as *D. novamexicana* - *D. americana* clade) remains unresolved. Considerable morphological differences are found among the *Drosophila virilis* subgroup. There is a noticeable difference in body colour. *D. novamexicana* has the lightest colour of all and *D. a. texana* has the darkest colour of all (Patterson and Stone 1952; Throckmorton 1982; Spicer 1991) Moreover, the species can be recognized based on differences in the phallic part of the male genitalia (Watabe and Higuchi 1979; kulikov et al. 2004).

Chromosome examination of species in the *Drosophila virilis* subgroup show that all species have a karyotype that is derived from an ancestral five pairs of autosomes (2, 3, 4, 5, and 6) and a pair of sex chromosomes (X and Y) (Throckmorton 1982; Orr and Coyne 1989). The karyotype shows no differences between *D. virilis, D. lummei* and *D. novamexicana* species, while the karyotype of *D. a. americana* and *D. a. texana* species is different due to a centromeric fusion of the 2nd and 3rd chromosomes when compared to the chromosomes of the other species in the subgroup. Additionally, the karyotype of *D. a. americana* has a centromeric fusion of X and the 4th chromosomes differs from *D. a. texana* (Figure 2) (Throckmorton 1982; Caletka and McAllister 2004; Morales-Hojas et al. 2008). At the molecular level, sequences of the *Cytochrome b* and *Cytochrome c oxidase* mitochondrial genes are useful for studying the phylogenetic relationship among closely related species. A recent study found extensive sequence divergence of these two mitochondrial genes among species of the *virilis* subgroup. While all strains of *D. novamexicana* were monophyletic, *D. a. americana* and *D. a. texana*

strains could not be separated as two distinct species (Figure 3) (Caletka and McAllister 2004). Using microsatellites, a study showed a closer relationship between *D. novamexicana* and *D. a. texana* rather than *D. a. americana* with *D. a. texana* despite the fact that they share the centromeric fusion of the 2nd and 3rd chromosomes and *D. novamexicana* lacks this fusion (Figure 4) (Orsini et al. 2004). Another microsatellite analysis showed no significant genetic differentiation between *D. a. americana* and *D. a. texana* (Schafer et al. 2006). In summary, studies using molecular data have so far been unable to resolve the phylogenetic relationship of *D. novamexicana* - *D. americana* clade.

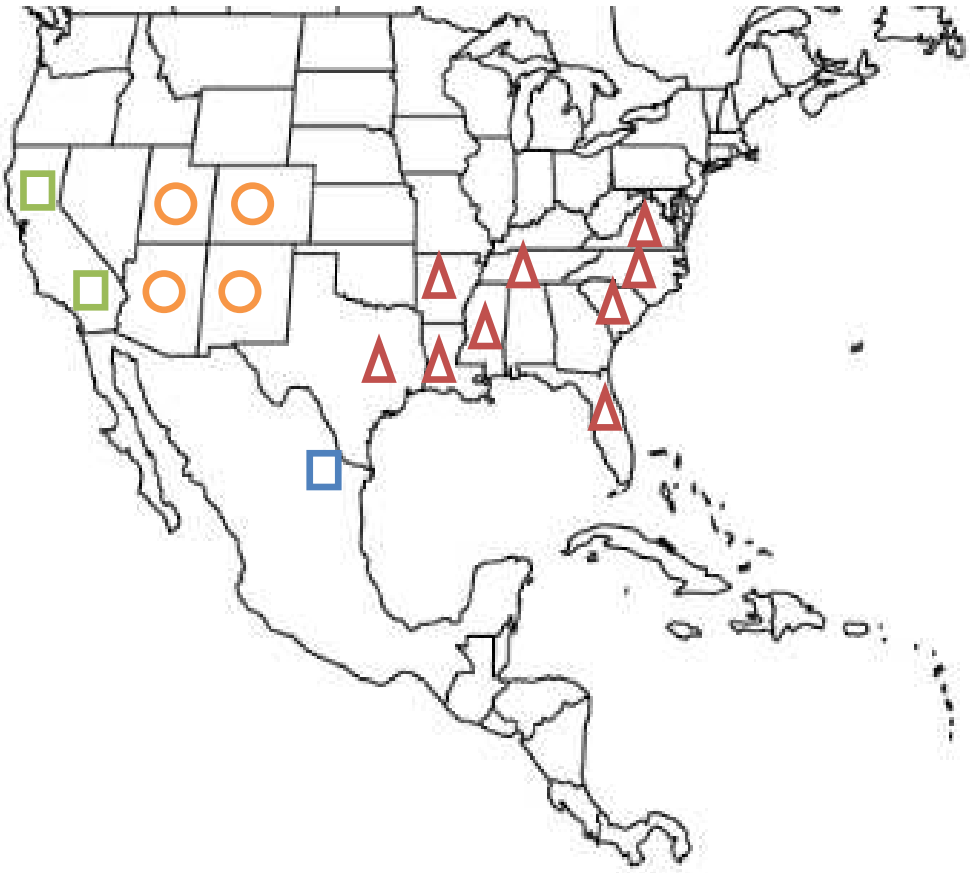

Figure 1. Generalized range and distribution of *D. virilis*, *D. novamexicana* and *D. a. texana* in the United States of America and Mexico. Information about the collection locations is from the UC San Diego Drosophila Stock Center.

=*D. virilis* (Pasadena and Truckee, California)

= *D. virilis* (Puebla, Mexico)

=*D. novamexicana* (Utah, Colorado, Arizona and New Mexico)

=*D. a. texana* (Texas, Louisiana, Arkansas, Mississippi, Tennessee, North Carolina, South Carolina, Virginia and Florida)

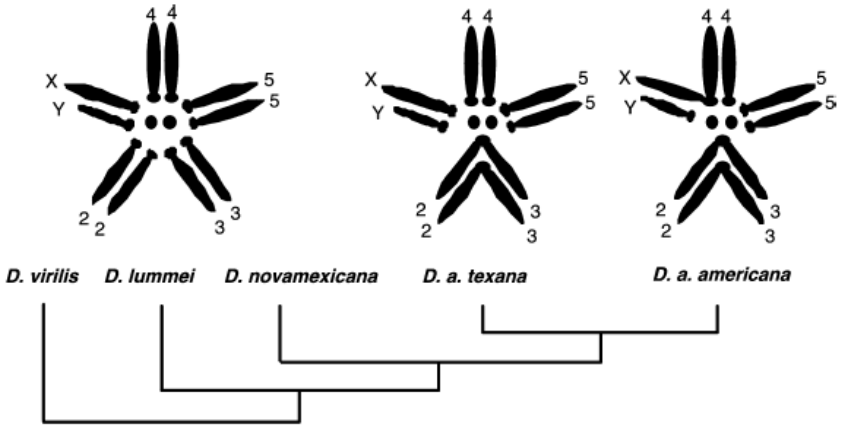

Figure 2. Chromosomal arrangements and phylogeny for the *Drosophila virilis* subgroup as proposed by Throckmorton and modified by Caletka and McAllister (2004).

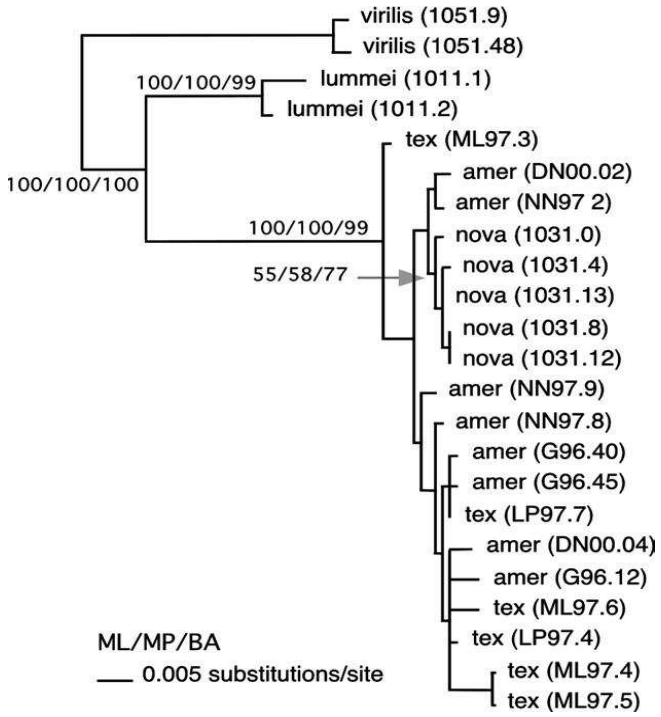

Figure 3. Phylogenetic tree based on sequences of the *Cytochrome b* and *Cytochrome c oxidase subunit II* mitochondrial regions in *Drosophila virilis* subgroup (Caletka and McAllister 2004). Numbers on the nodes symbolize support based on maximum likelihood (ML), maximum parsimony (MP), and Bayesian (BA) analyses.

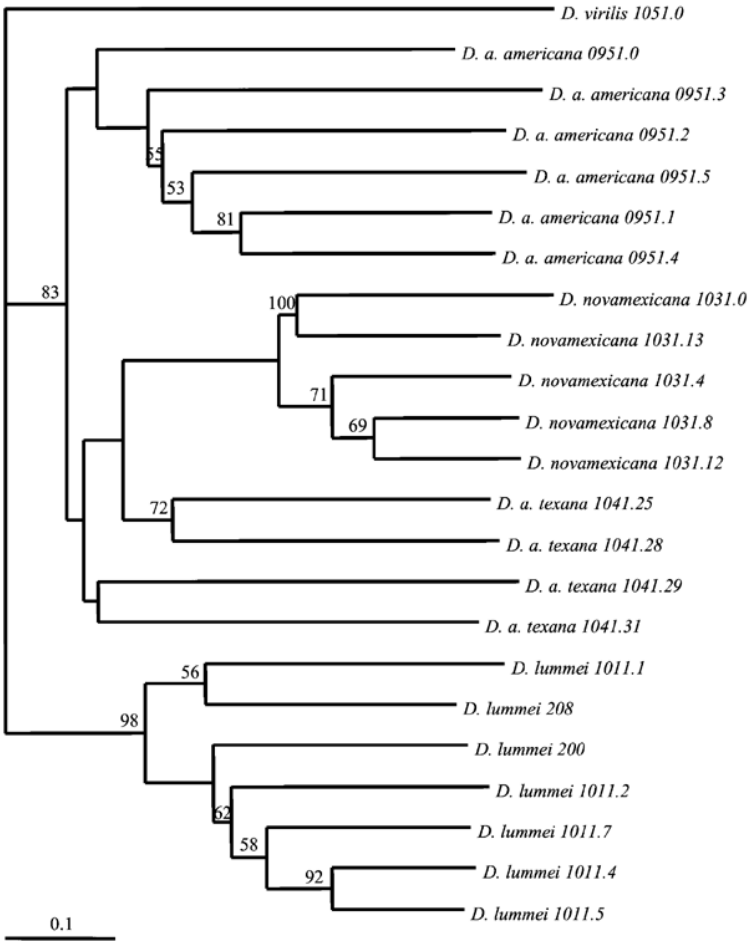

Figure 4. Phylogenetic tree of *Drosophila virilis* subgroup based on shared alleles of microsatellite loci between the species (Orsini et al. 2004). Bootstrap values higher than 50% are shown over tree branches.

1.3 Reproductive isolation (Pre and postzygotic barriers) in the *Drosophila virilis* subgroup

1.3.1 Premating, prezytotic isolating barriers

Females of *Drosophila virilis* species have the highest crossability with all heterospecific males of the subgroup, whereas, *D. virilis* males show the strongest courtship discrimination towards all heterospecific females with the result that very low numbers of hybrids are produced (Throckmorton 1982). Recent work has shown that when females of *D. novamexicana* were exposed for 2 weeks to *D. virilis* males, only 14% of females produced some progeny (Nickel and Civetta 2009). Moreover*, D. virilis* males were able to recognize and walk away from heterospecific females after tapping them, suggesting the possibility that males can sample and recognize species-specific cuticular hydrocarbon female profiles (Nickel and Civetta, 2009). However, in the reciprocal cross, preliminary observations in our lab have indicated that *D. novamexicana* males court and copulate with *D. virilis* females but produce few offspring (Nickel 2008). *D. a. texana* also shows courtship discrimination against heterospecific females, but after a long-term exposure copulation occurs (Throckmorton 1982; Nickel and Civetta 2009).

1.3.2 Postmating, prezygotic isolating barriers

In the early 1940's, crosses between *D. virilis* females and *D. a. americana* and *D. a. texana* males were used to assess the effect of sexual isolating barriers and sperm problems that might prevent fertilization. *D. virilis* females produced few offspring when mated to *D. a. americana* and *D. a. texana* males (Patterson and Stone 1952). Moreover, this study found sperm immobility in the seminal receptacle of the females' reproductive tract within 24 hours of insemination (Patterson and Stone 1952). Additionally, a recent study has supported the previous findings by showing a low rate of egg hatchability when *D. virilis* females are mated to *D. a.*

americana males. Furthermore, most of the eggs laid were not fertilized, suggesting a fertilization incompatibility and thus a strong postmating, prezygotic isolation (Sweigart 2010).

1.3.3 Postzygotic isolating barriers

In crosses between *D. virilis* females and *D. novamexicana* males, all females produce hybrids but 93% of the male offspring are sterile, indicating a strong postzygotic isolation (Orr and Coyne 1989). Phylogenetic analysis of species of the *Drosophila virilis* subgroup shows D. *lummei* as a species closely related to *D. virilis* (Figure 4). The cross between *D. virilis* females and *D. lummei* males produces 95% fertile hybrid males (Lumme and Heikkinen 1990). However, 47% percent of the hybrid offspring died before pupariation and 25% failed to emerge from the pupal case, indicating a postzygotic isolation barrier (Lumme and Heikkinen 1990). Furthermore, in this heterospecifc cross, the production of progeny considerably varies according to the strain of *D. lummei* used. In fact, depending on the strain of *D. lummei*, females can produce 10% to 50% as many progeny as conspecific females (Throckmorton 1982).

Mating between *D. virilis* females and *D. a. texana* males produce hybrid offspring, among which one-third of the male progeny are sterile (Patterson and Stone 1952; Orr and Coyne1989; Laminissou et al. 1996). When these F1 males were backcrossed with females of either species, the percentage of sterile hybrid males increased with successive backcrosses which could be due to the incompatibility between the Y chromosome of *D. texana* and the autosomes of *D. virilis* females (Laminissou et al. 1996). In Table 1, I summarize reproductive isolating barriers that have been identified in crosses among species of the *Drosophila virilis* subgroup.

Table 1. Reproductive isolating barriers among species of the *Drosophila virilis* subgroup

Crosses	Isolating barriers	Category	Description	References
D. novamexicana ♀× *D. virilis* ♂	Prezygotic Isolation	Premating, prezygotic isolating barriers	Mate discrimination (mate after long period of exposure, 2 weeks)	Nickel & Civetta 2009
D. virilis ♀ × *D. novamexicana* ♂	Prezygotic isolation Postzygotic isolation	Postmating, prezygotic isolating barriers Intrinsic postzygotic isolating barriers	a)Low production of progeny b)Fertilization incompatibility Male hybrid sterility	a) Nickel 2009 Orr & Coyne 1989
D. virilis ♀ × *D. a. americana* ♂	Prezygotic isolation	Postmating, prezygotic isolating barriers	Low production of progeny Fertilization incompatibility	Patterson & Stone 1952; Sweigart 2010
D. virilis ♀ × *D. a. texana* ♂	Prezygotic isolation Postzygotic isolation	postmating, prezygotic isolating barriers Intrinsic postzygotic isolating barriers	Low production of progeny Male hybrid sterility	Patterson & Stone 1952; Orr & Coyne 1989; Laminissou et al. 1996
D. virilis ♀ × *D. lummei* ♂	Postzygotic isolation	Intrinsic postzygotic isolating barriers	Hybrid inviability	Lumme and Heikkinen 1990
D. lummei ♀× *D. a. americana* ♂	Postzygotic isolation	Intrinsic postzygotic isolating barriers	Hybrid sterility	Throckmorton 1982
D. a. americana ♀× *D. virilis* ♂	Prezygotic isolation	Premating, prezygotic isolating barriers	Mate discrimination	Throckmorton 1982
D. a. texana ♀× *D. virilis* ♂	Prezygotic isolation	Premating, prezygotic isolating barriers	Mate discrimination	Throckmorton 1982

1.4 Objectives

The primary objective of my thesis was to characterize the isolating barriers to fertilization that play a role in preventing the production of hybrids among species of the *Drosophila virilis* subgroup. I specifically tested:

1. Egg hatchability in crosses between different *D. virilis* females and *D. novamexicana* males
2. Whether any reduction in egg hatchability is due to zygote mortality (postzygotic barrier) or unfertilized eggs (postmating, prezygotic barrier).
3. What is the fate of sperm within the reproductive tract of *D. virilis* females?
4. When did postmating isolating barriers evolve among species of the *Drosophila virilis* subgroup?

2.0 MATERIALS AND METHODS

2.1 *Drosophila* species and maintenance

Four species of the *Drosophila virilis* subgroup, *D. virilis, D. lummei*, *D. novamexicana, D. americana texana*, were reared and maintained during the completion of this thesis. For each species, geographically diverse strains were obtained from the San Diego Drosophila Stock Center; *D. virilis* (Argentina 1051.49, California 1051.00, Japan 1051.09, Mexico 1051.48 and Russia 1051.52); *D. lummei* (Japan 1011.08); *D. novamexicana* (New Mexico 1301.08 and Utah 1301.08) and *D. americana texana* (wild type 1041.16) An additional outbred population was created for *D. virilis* and *D. novamexicana* by mixing equal numbers of individuals from all the different strains. Flies were reared in round-bottom bottles (64 ×130 mm) containing standard cornmeal-yeast-agar-molasses medium (CYAM) (Appendix I). Bottles were kept in a 12:12 light: dark cycle and at 18-20ºC. For stock maintenance, flies were allowed to freely mate and laid eggs in fresh media, the parental generation were discarded after eighteen days and the new generation of adults transferred to fresh medium.

2.2 Establishment of crosses for experimental testing

The goal of this thesis was to characterize postmating isolation barriers, either prezygotic or postzygotic, that contribute to reproductive isolation between species of the *Drosophila virilis* subgroup. Therefore, crosses were performed between species (heterospecifics). Individuals of the *Drosophila virilis* subgroup are known to remain virgin for at least 10 days after eclosion (Markow and O'Grady 2007), so bottles from each species stock were emptied and inspected daily for new adult emergence. Newly emerged flies were lightly anesthetized using CO_2 gas flow through an acrylic frame with a porous polyethylene pad. Virgin females and males were separated by sex and placed in cylindrical vials (28.5 × 95mm) containing CYAM medium. Males and females were held for 10-12 days before setting up crosses to ensure sexual maturity (Markow and O'Grady 2007). In order to properly characterize reproductive barriers between heterospecifics, same conspecific crosses were tested as controls. Conspecific and heterospecific crosses (Table 2 and 3) were set up using a single pair of sexually mature flies. For the different crosses listed in Tables 2 and 3, counts were obtained of eggs laid by females and the proportion of eggs hatched. The proportion of fertilized eggs was calculated for the crosses between outbred strains of *D. virilis* and *D. novamexicana*.

Table 2. Number of conspecific and heterospecific crosses performed between *D. virilis* and *D. novamexicana*

♀ / ♂		***D. virilis***						***D. novamexicana***	
Strains		Japan	Argentina	Russia	Mexico	California	Outbred	Utah	Outbred
D. virilis	Japan	24	—	—	—	—	—	—	—
	Argentina	—	29	—	—	—	—	—	—
	Russia	—	—	38	—	—	—	—	—
	Mexico	—	—	—	10	—	—	—	—
	California	—	—	—	—	13	—	—	—
	Outbred	—	—	—	—	—	30	—	21
D. novamexicana	Utah	25	25	39	15	14	—	45	—
	Outbred	—	—	—	—	—	21	—	29

Table 3. Number of conspecific and heterospecific crosses performed among species of the *Drosophila virilis* subgroup

♂ \ ♀	***D. virilis*** (outbred)	***D. lummei*** Japan (1011.08)	***D. a. texana*** wild type (1041.16)	***D. novamexicana*** (outbred)
D. virilis (outbred)	20	—	—	—
D. lummei Japan (1011.08)	20	13	8	15
D. a. texana wild type (1041.16)	20	4	18	15
D. novamexicana (outbred)	30	19	35	31

2.3 Egg Hatchability

A single female and male pair were placed in an egg-laying chamber made using a polystyrene petri dish (60 ×15 mm) containing fresh CYAM medium attached to a 100 ml graduated polypropylene beaker (VWR – catalogue # 25384-152). Every 24 hours, flies were slightly anesthetized using the CO_2 pad, the petri dish was removed and a new dish with fresh CYAM medium was attached to the chamber. The replacement of dishes continued for five consecutive days (Figure 5). Using a Nikon (SMZ645) light microscope, the eggs laid were counted eachday and 48 hours later hatched eggs were scored. Unhatched eggs can be recognized as a white compact shape containing cytoplasmic mass and hatched eggs can be seen as an empty outer chorion membrane due to larval emergence.

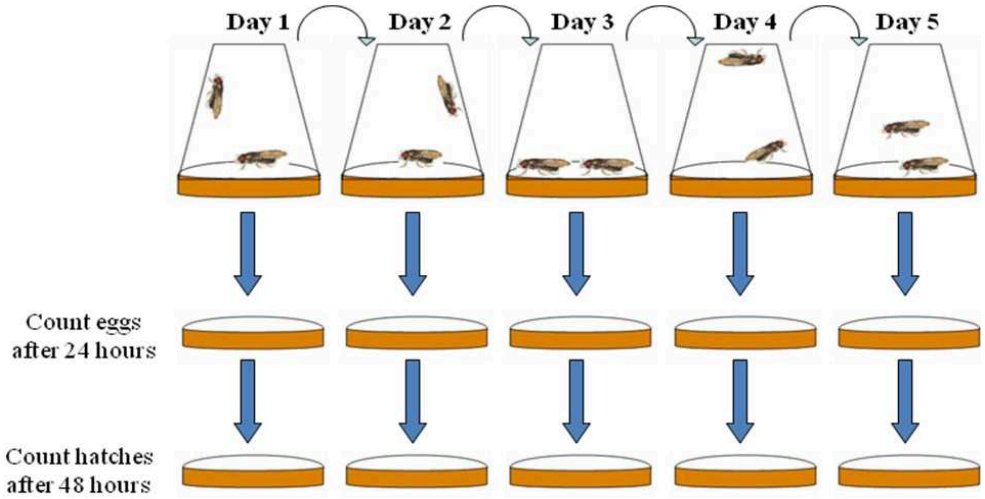

Figure 5. Experimental design for egg laying and hatchability test

2.4 Fertilization of the eggs

Fertilization of the eggs was examined in crosses between outbred strains of *D. virilis* and *D. novamexicana*. A single female and male pair of conspecifics and heterospecifics (*D. virilis* female and *D. novamexicana* male) was established. Each pair was placed in a single cylindrical polyethylene vial (25 × 95 mm) containing CYAM fresh medium. Courtship behaviour was observed for 6 hours and copulation duration was recorded. Mated females were individually transferred into a fresh egg-laying chamber using a fly pooter (made of a thin plastic pipe with two ends, one end attached to a 1 ml pipette tip and the other also attached to a 1ml pipette tip with a small piece of cloth). Each day, females were transferred to fresh egg-laying dishes, and the number of eggs laid was counted: 48 hours after eggs were laid, hatched eggs were scored. Under a Nikon (SMZ1500) dissecting microscope, unhatched eggs were collected from the media with a wooden handle dissecting pin and placed on a clean microscope slide. A drop of 1X

PBS (phosphate buffered saline) (Appendix Π) was added to prevent adhesion to the slide surface. Eggs were manually dechorionated using minutien pins (0.1 mm diameter). The dorsal appendage was removed and by gentle press at the posterior pole using the minutien pin, eggs were pried loose from the chorion. The inner vitelline membrane was removed by immersing the dechorionated eggs in a small tube containing a 1:1 solution of heptane and 90% methanol. The eggs were dropped to the layer between heptane and methanol and slowly descended to the bottom of the tube when their waxy layer was lost (Warn and Warn, 1986). Eggs without a vitelline membrane are almost transparent and easily damaged. Therefore, intact eggs were collected by pouring the haptane-methanol solution on a small piece of dark cloth. Within one minute the solution evaporated and the eggs were visible on the cloth surface. A couple of drops of 1X PBS were added on the eggs using a glass pasteur pipette to prevent eggs from adhesion to the cloth surface and desiccation. The eggs were then gently picked up with a 0.25 mm diameter insect pin and placed on a drop of 1X PBS on a clean microscope slide. Eggs were tested for fertilization by adding 1 μl (300 nM) of DAPI (Molecular Probes, D3571) nucleic acid stain (see section 2.5 Preparation of DAPI). DAPI binds to the DNA of cell nuclei staining them fluorescent blue. Eggs were incubated in a dark room for 30 minutes and then examined under an Olympus (BX60F) or a Nikon Eclipse (E400) fluorescence microscope for evidence of fertilization.

2.5 Preparation of DAPI

To make DAPI stock solution, dissolve 10 mg of DAPI (Molecular Probes, D3571) in 2 mL of distilled water. A first dilution was prepared by adding 1 µl of DAPI stock solution into 1000 µl of 1X PBS (Table 5). Dissolve 5 µl of the first dilution in 170 µl of 1X PBS to get 300 nM working DAPI solution.

2.6 Tracking of sperm within female storage organs

This test was performed by using *D. virilis* and *D. novamexicana* outbred strains. A single female and male were placed in a cylindrical polyethylene vial (25 × 95 mm) containing fresh CYAM medium. Mating was observed and copulation duration was recorded for hererospecific and conspecific pairs. At intervals after mating of 0, 24 and 48 hours, inseminated females were transferred using the fly pooter into vials with fresh CYAM media. Females were flash frozen by submerging the vials in liquid nitrogen, and then transferred to small tubes and stored in a freezer at -70°C. Under a Nikon (SMZ1500) dissecting microscope and on a clean microscope slide, a frozen female fly was placed on its side. A drop of 1X PBS was added to facilitate dissection. Using a wooden handle dissecting pin in one hand and a pair of forceps (Dumont #5) (Fine Science Tools) in the other, the females' reproductive tract was dissected. The pin was used to poke the thorax and keep a tight grip on the fly, whereas the pair of forceps was used to tear the lateral side of the abdomen allowing the content to spread. Sperm storage organs; uterus, pair of spermatheca and seminal receptacle (Figure 6) were separated and each placed in a fresh drop of 1X PBS on a single clean microscope slide. These slides were dried in an oven set at 60°C for 5 minutes, fixed in 3:1 methanol: glacial acetic acid for 5 minutes and washed three times with1X PBS (Price et al. 2001). Organs were stained using 1 µl of DAPI

(300 nM), and incubated in the dark for 30 minutes. Slides were examined under an Olympus (BX60F) or a Nikon Eclipse (E400) fluorescence microscopes and the presence of sperm was determined (Figure 7).

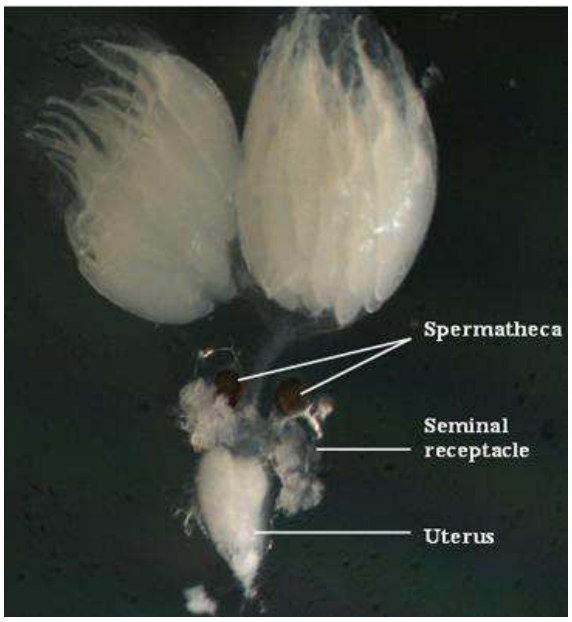

Figure 6. Sperm storage organs in the female reproductive tract viewed under a dissecting microscope (400X)

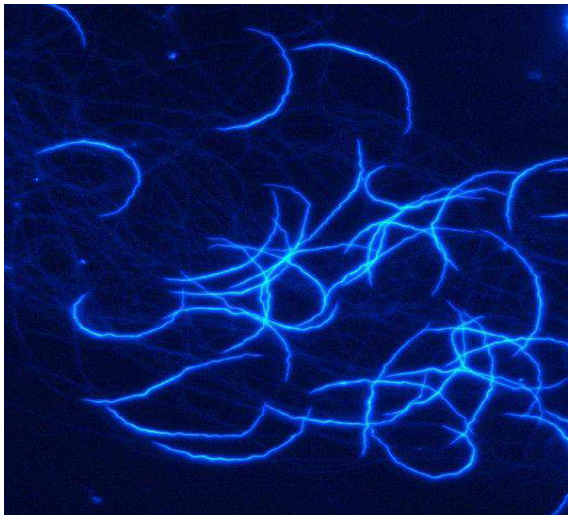

Figure 7. DAPI-stained sperm heads in the female seminal receptacle viewed under a fluorescence microscope (400X)

2.7 Data analysis

I compared the mean number of eggs laid, the proportion of hatched eggs, and the proportion of fertilized eggs for different crosses using a one-way analysis of variance (ANOVA). The cross groups were used as the treatment. When significant differences were found among groups, an *a posteriori* Tukey test was run to find which means were significantly different from one another. All statistical tests were conducted in SPSS (version 12.0).

Comparisons of number of females with sperm in storage were done using a 2 × 3 Chi Square test as well as Fisher Exact test.

3.0 RESULTS

3.1 Results from crosses between *D. virilis* females and *D. novamexicana* males

3.1.1 *D. virilis* females lay similar numbers of eggs after conspecific and to heterospecific matings

I analyzed the number of eggs laid by *D. virilis* and *D. novamexicana* females mated to conspecific males and *D. virilis* females mated to *D. novamexicana* males. Five different strains of *D. virilis* were used in the crosses and I found that the average number of eggs laid by females mated to heterospecific males was not significantly different than the number laid by *D. virilis* and *D. novamexicana* females mated to conspecific males. Only crosses involving two *D. virilis* strains, Russia ($F_{2,101}$= 28.60; P< 0.001) and Japan ($F_{2,91}$= 22.23; P< 0.001) showed significant differences in numbers of eggs laid, with *D. novamexicana* females laying significantly more of eggs than *D. virilis* females (Appendix III and Figure 8).

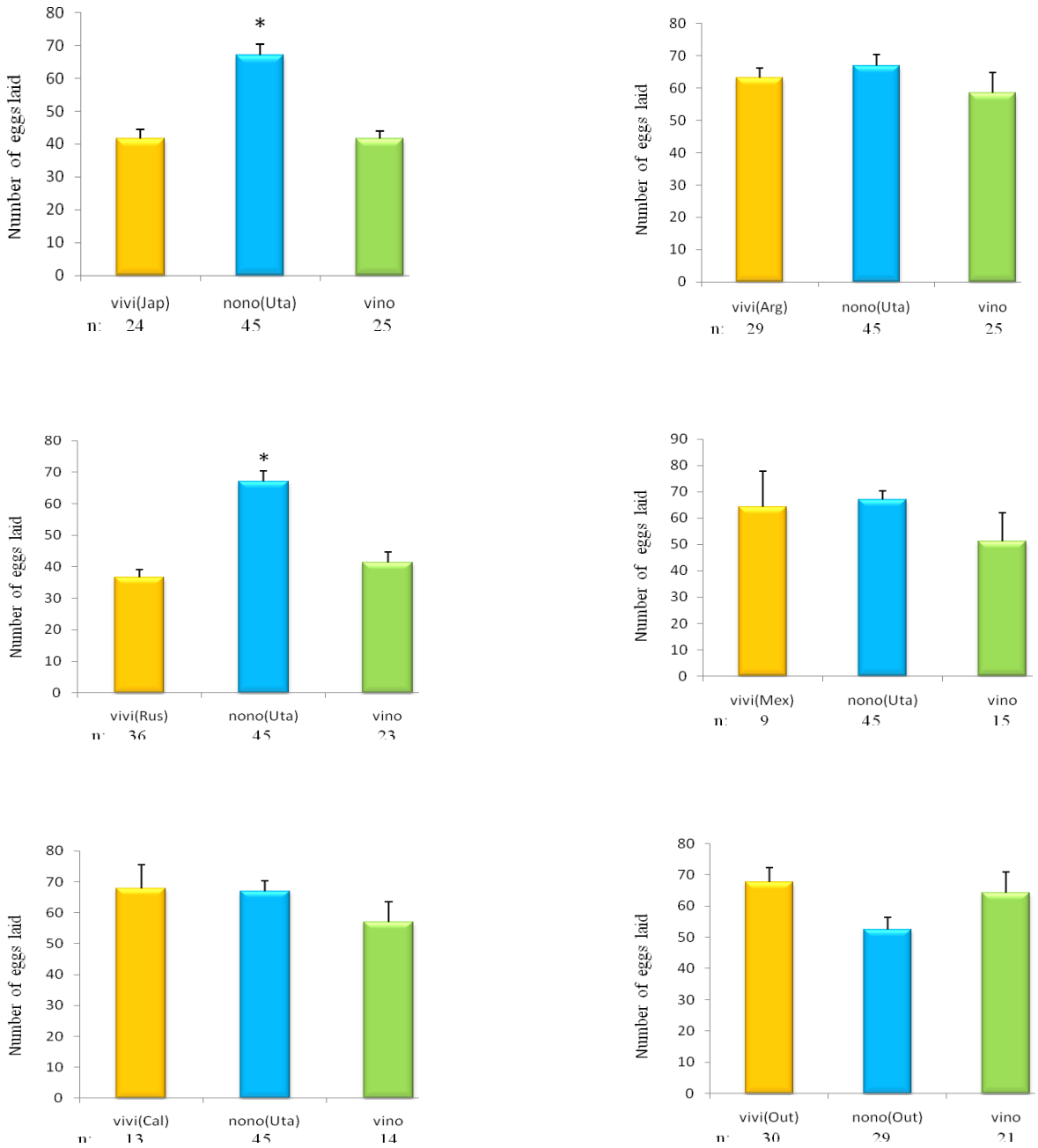

Figure 8. The average number and standard error of eggs laid by *D. virilis* and *D. novamexicana* females. vivi (*D. virilis* ♀× *D. virilis* ♂), nono (*D. novamexicana* ♀×*D. novamexicana* ♂), vino (*D. virilis* ♀× *D. novamexicana* ♂), Jap (Japan), Uta (Utah), Arg (Argentina), Rus (Russia), Mex (Mexico), Cal (California), Out (Outbred), n (number of crosses), significant differences are denoted with an asterisk (*).

3.1.2 *D. virilis* females mated to *D. novamexicana* males hatch a lower proportion of eggs than females mated to conspecifc males.

The proportion of eggs hatched from the heterospecific cross was always significantly lower than the proportion of hatches from both of the conspecific crosses (Appendix IV). Depending on the *D. virilis* strain used, the proportion of unhatched eggs ranged from 0.85 to 0.96 (Figure 9). Because the number of eggs laid and the proportion of eggs hatched can be affected by inbreeding in laboratory strains of Drosophila, I also tested an outbred population of *D. novamexicana* and *D. virilis* that were established in the lab by mixing males and females of different strains (see materials and methods). Only 6% of the eggs laid by *D. virilis* females mated with *D. novamexicana* males successfully hatched (Figure 9).

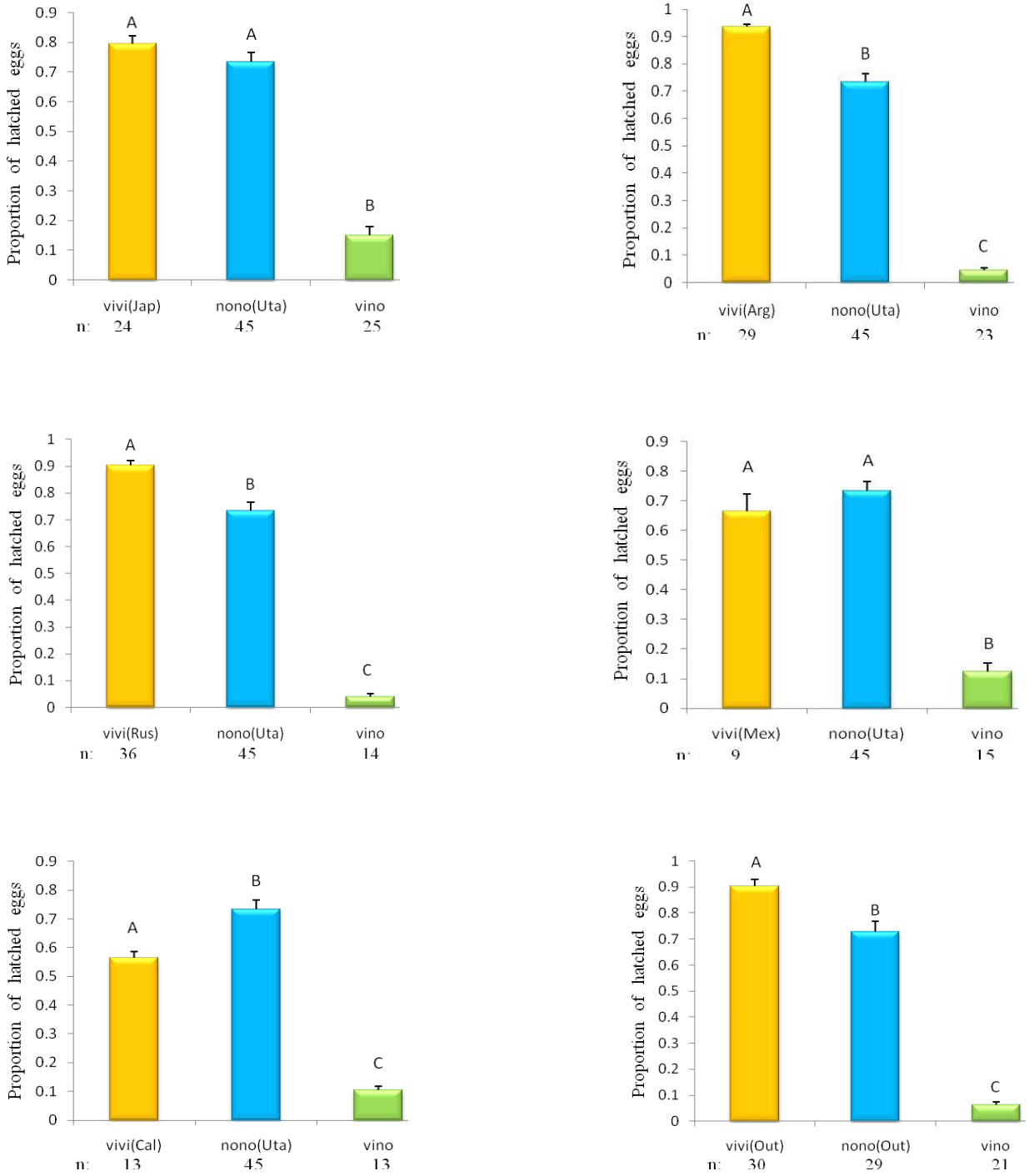

Figure 9. The average proportion and standard error of hatched eggs laid by *D. virilis* and *D. novamexicana* females. The labels are as in Figure 8. Proportion of hatched eggs that are not significantly different are labeled with the same letter. Proportion of hatched eggs that are significantly different are denoted with different letters.

3.1.3 Unhatched eggs are unfertilized

Unhatched eggs could result either from fertilized eggs that fail to develop or unfertilized eggs. I stained unhatched eggs without their chorion and vitelline layers with DAPI to test for nuclear division. I counted the number of eggs hatched as fertilized and tested unhatched eggs laid from both conspecific and heterospecific crosses for fertilization. No evidence of cell division was found among unhatched eggs (Figure 10A). I found significant differences in the proportion of fertilized eggs (Figure 10B) ($F_{2,68}$= 173.42; P< 0.001) due to a significantly lower proportion of 5% of eggs fertilized by *D. novamexicana* males that mated with *D. virilis* females (Tukey post-hoc test: P < 0.001) (Appendix V and Figure 11). The results indicate that while there may be some partial postzygotic isolation (Orr and Coyne 1989), the vast majority of unhatched eggs in heterospecific crosses between *D. virilis* females and *D. novamexicana* males are the result of some form of postmating prezygotic isolation.

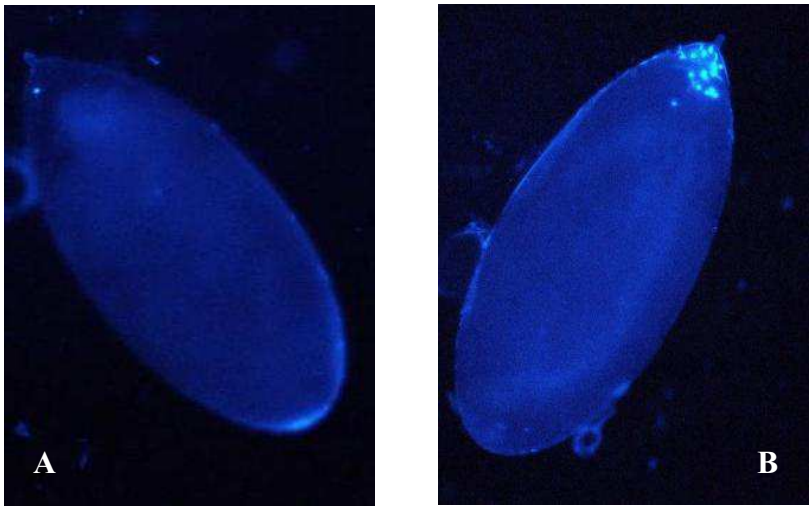

Figure 10. DAPI staining of Drosophila embryos. (A) no nuclear divisoin in an unhatched egg at 48 hours after egg laying. (B) Cluster of dividing nuclei in the preblastoderm stage at 2 hours after egg laying by a female mated to a conspecific male.

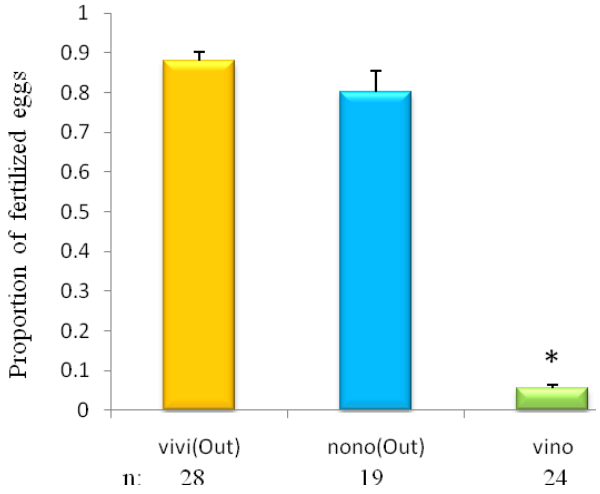

Figure 11. The average proportion and standard error of fertilized eggs laid by *D. virilis* and *D. novamexicana* females. The labels are as in Figure 8. Proportions of fertilized eggs are significantly lower in the heterospecific cross compared to the conspecific crosses.

3.1.4 The sperm of heterospecifc males are not stored in females

Postmating prezygotic isolation can result from problems in sperm transfer during copulation, problems with sperm storage or the inability of sperm to fertilize heterospecific eggs. I used the *D. virilis* and *D. novamexicana* outbred populations to test whether sperm transfer and/or storage was affected in the heterospecific cross. I did this by observing copulations and dissecting females immediately after mating (0 hour) and at two intervals of 24 and 48 hours after mating. Immediately after mating, large numbers of sperm transferred to the females were found in the uterus in both conspecific and heterospecific crosses. Because of these large numbers and the fact that the sperm head is a needle-like structure (Figure7) that sometimes only faintly stains with DAPI, I tested differences between crosses by scoring the numbers of females with or without stored sperm. I only found differences in sperm storage between intra and interspecific crosses for both the spermatheca and the seminal receptacle at 48 hours after mating, with a significantly higher number of females mated to heterospecific males having no stored sperm (spermatheca: $\chi^2= 11.31$, $P= 0.004$; seminal receptacle: $\chi^2= 25.23$, $P< 0.001$) (Table 4 ; Figure12, B and C). There were non-significant differences in the numbers of females with sperm in storage immediately after mating (0 hour), with all females having large amounts of sperm in the uterus (Table 4 and Figure12A). At 24 hours after mating, I observed a slight decline in storage for heterospecific crosses, but the most striking difference was the fact that only 1 out of 21 *D. virilis* females mated with *D. novamexicana* males had few sperm cells in the seminal receptacle at 48 hours after mating (Figure12C). Overall, the heterospecific cross shows a different pattern of either sperm movement and/or storage within the female reproductive tract than the conspecific crosses (Figure 12).

Table 4. 2×3 chi-square test and Fisher's exact test for presence of sperm in sperm storage organs

	Uterus	**Spermatheca**	**Seminal receptacle**
0 hour	χ^2 = 5.00 P = 0.082 Fisher value = 0.082	χ^2 = 0.92 P = 0.629 Fisher value = 0.999	χ^2 = 2.06 P = 0.355 Fisher value = 0.633
24 hours	χ^2 = 1.49 P = 0.473 Fisher value = 0.548	χ^2 = 6.22 P = 0.044 Fisher value = 0.055	χ^2 = 5.84 P = 0.050 Fisher value = 0.051
48 hours	— — Fisher value = 1.000	χ^2 = 11.31 P = 0.004 Fisher value < 0.001	χ^2 = 25.23 P< 0.001 Fisher value < 0.001

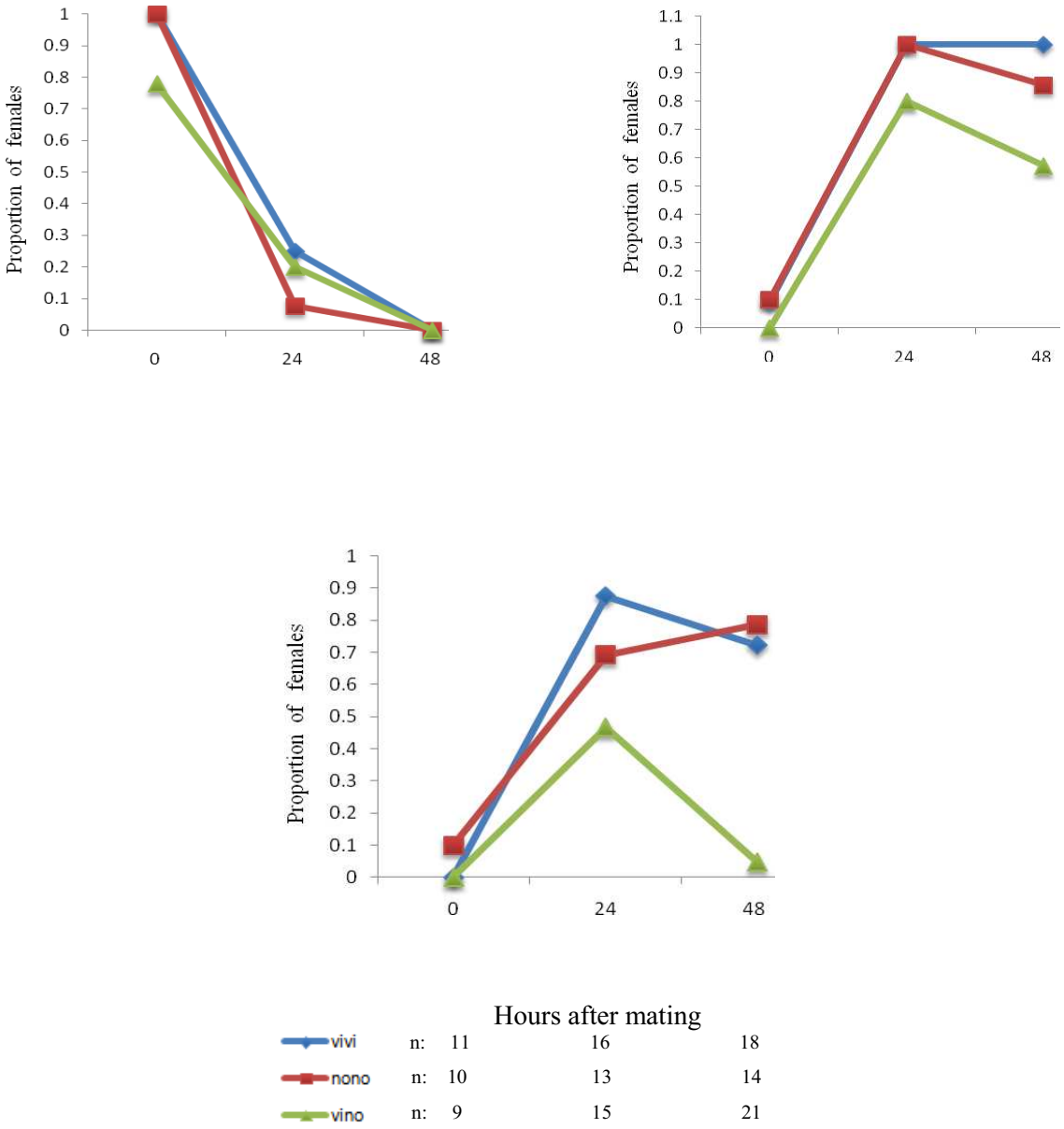

Figure 12. The proportion of females with sperm in storage organs (A, Uterus ; B, Spermathecae; C, Seminal receptacle) of conspecifically and heterospecifically mated females at different intervals after mating. Blue diamonds are *D. virilis* ♀ × *D. virilis* ♂, red squares are *D. novamexicana* ♀ × *D. novamexicana* ♂ and green triangles are *D. virilis* ♀ × *D. novamexicana* ♂. n: number of females tested.

3.2 Postmating, prezygotic isolation among other species of the *Drosophila virilis* subgroup

3.2.1 *D. novamexicana* × *D. a. texana*

The most closely related pair of species tested, *D. novamexicana* and *D. americana texana* showed significant differences in the numbers of eggs laid depending on the type of cross ($F_{3,73}$= 6.05; P< 0.001) but heterospecific crosses did not lay fewer eggs than conspecifics. Instead, the number of eggs laid seems to be determined by the species identity of the female, with *D. americana texana* laying fewer eggs than *D. novamexicana* (Figure 13).

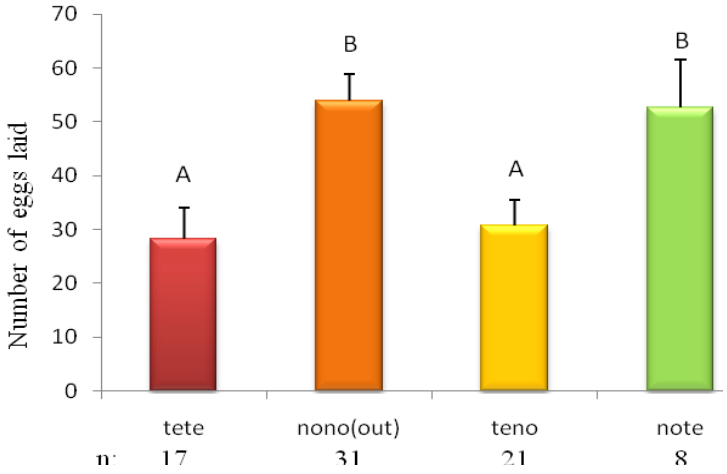

Figure 13. The average number and standard error of eggs laid by *D. a. texana* and *D. novamexicana* females mated to conspecific and heterospecific males. tete (*D. a. texana* ♀× *D. a. texana* ♂), nono (*D. novamexicana* ♀× *D. novamexicana* ♂), teno (*D. a. texana* ♀× *D. novamexicana* ♂), note (*D. novamexicana* ♀× *D. a. texana* ♂), n (number of crosses) and out (outbred). Crossing showing no significant differences in number of eggs laid are labeled with the same letter.

I found a significant effect on the proportion of eggs that hatched ($F_{3,73}$= 39.70; P< 0.001) with heterospecific crosses hatching a significantly lower proportion of eggs than conspecifics (Figure 14).

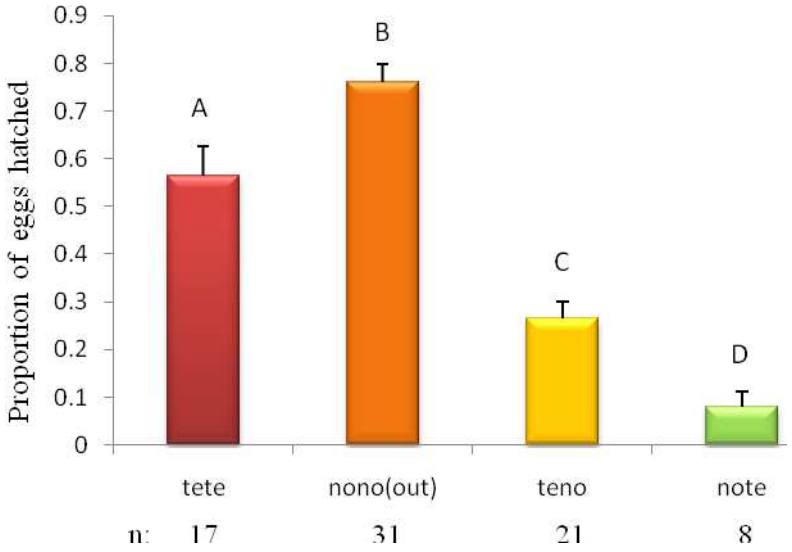

Figure 14. The average proportion and standard error of hatched eggs laid by *D. a. texana* and *D. novamexicana* females mated to conspecific and heterospecific males. The labels are as in Figure 13.

3.2.2 *D. lummei* × *D. novamexicana* and *D. lummei* × *D. a. texana*

The comparisons between more distantly related species such as *D. novamexicana* and *D. lummei*, and *D. americana texana* and *D. lummei* also show significant differences in the number of eggs laid by females ($F_{3,51}$= 9.17; P< 0.001; $F_{3,37}$= 4.59; P= 0.008 respectively), although once again females mated with heterospecifics did not lay fewer eggs. In fact, the *D. novamexicana* and *D. lummei* heterospecific crosses produced significantly larger numbers of eggs than conspecifics (Figure 15).

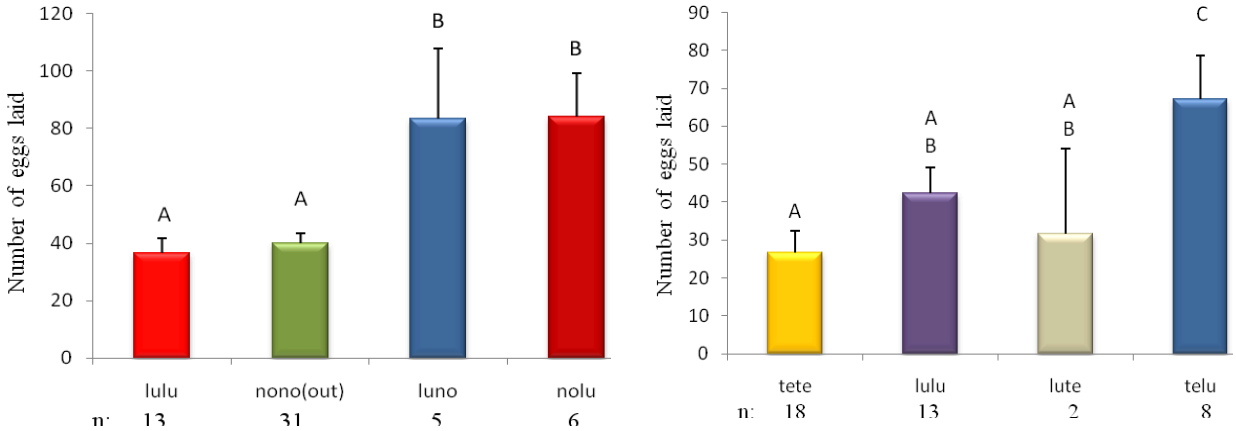

Figure 15. The average number and standard error of eggs laid by *D. lummei* , *D. novamexicana* and *D. a. texana* females mated to conspecific and heterospecific males. lulu (*D. lummei* ♀× *D. lummei* ♂), nono (*D. novamexicana* ♀× *D. novamexicana* ♂), luno (*D. lummei* ♀× *D. novamexicana* ♂), nolu (*D. novamexicana* ♀× *D. lummei* ♂), tete (*D. a. texana* ♀× *D. a. texana* ♂), lute (*D. lummei* ♀× *D. a. texana* ♂), telu (*D. a. texana* ♀× *D. lummei* ♂). Other labeles are as in Figure 13.

I also found a consistent result of significant differences in proportion of eggs hatched with the lowest proportions found for females mated to heterospecific males ($F_{3,51}$= 35.06; P< 0.001; $F_{3,37}$= 29.83; P< 0.001 respectively) (Figure 16).

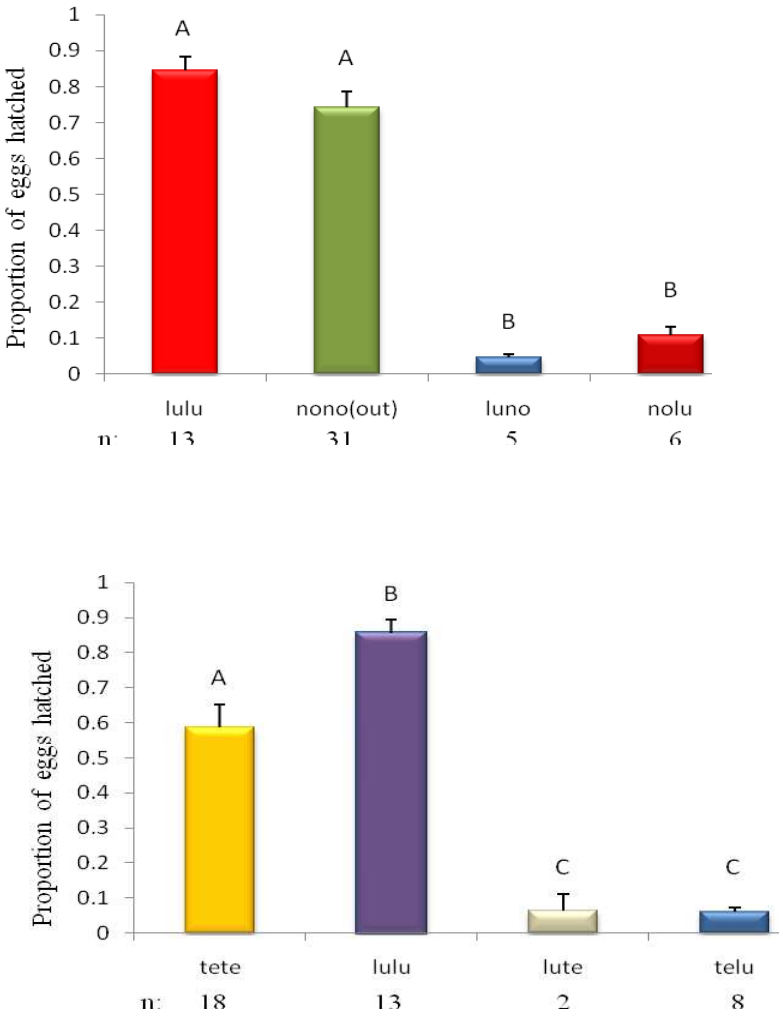

Figure 16. The average proportion and standard error of hatched eggs laid by *D. lummei* , *D. novamexicana* and *D. a. texana* females mated to conspecific and heterospecific males. The labels are as in Figure 15.

3.2.3 *D. virilis* ♀ × *D. a. texana* ♂

Finally, the most distantly related species pair of *D. virilis* and *D. americana texana* showed significant differences in numbers of eggs laid by females with the differences due to the lower number of eggs laid by *D. americana texana* females in conspecific matings ($F_{2,48}$= 10.77; P< 0.001, Figure 17).

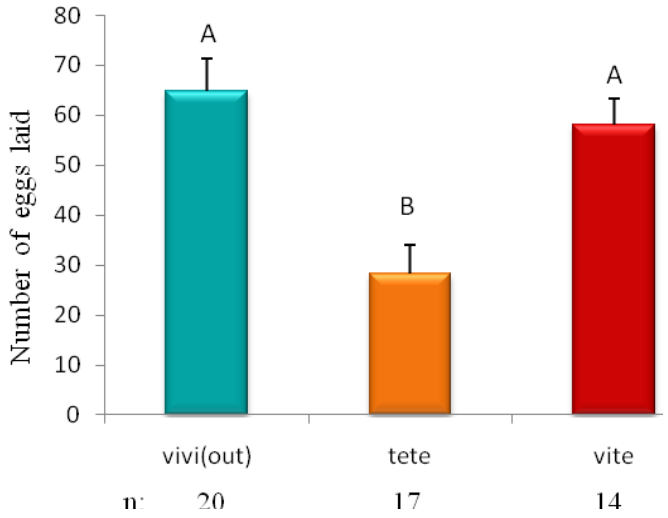

Figure 17. The average number and standard error of eggs laid by *D. virilis* and *D. a. texana* females mated to conspecific and heterospecific males. vivi (*D. virilis* ♀× *D. virilis* ♂), tete (*D. a. texana* ♀× *D. a. texana* ♂), vite (*D. virilis* ♀× *D. a. texana* ♂). Other labels are as in Figure 13.

Once again, *D. virilis* females mated with heterospecific *D. americana texana* males hatched a significantly lower proportion of eggs than both parental conspecific crosses (Figure 18).

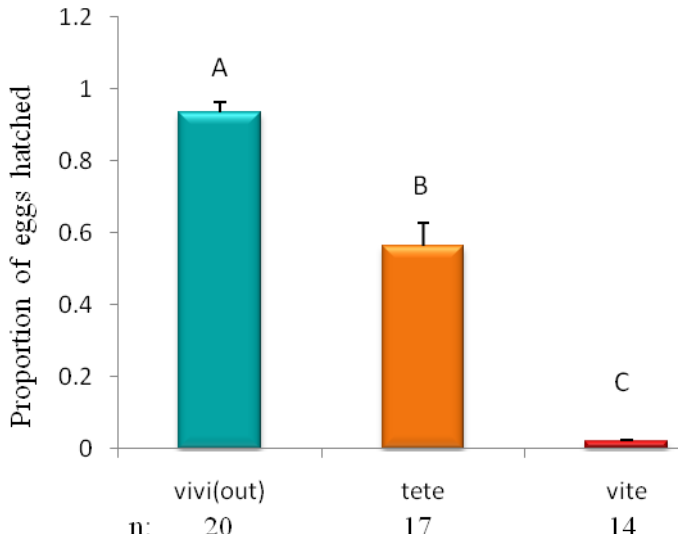

Figure 18. The average proportion and standard error of hatched eggs laid by *D. virilis* and *D. a. texana* females mated to conspecific and heterospecific males. The labels are as in Figure 17.

3.2.4 *D. virilis* ♀ × *D. lummei* ♂

The one cross producing different results than all others was the one between *D. virilis* females and *D. lummei* males. In this case a slightly lower number of eggs was laid by the conspecific *D. lummei* cross ($F_{2,49}$= 3.74; P< 0.031, Figure 19).

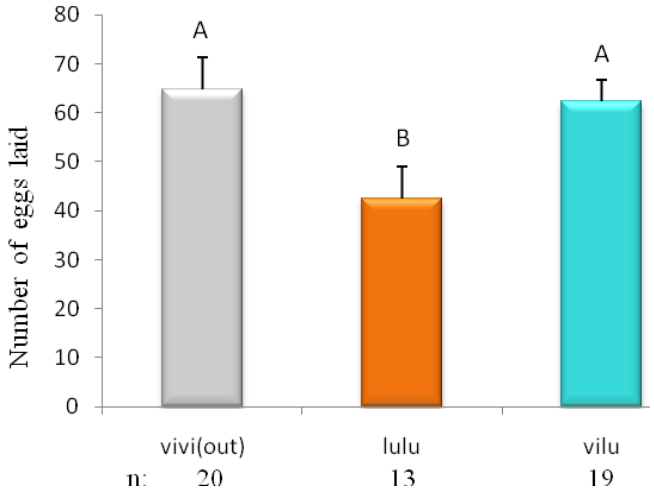

Figure 19. The average number and standard error of eggs laid by *D. virilis* and *D. lummei* females mated to conspecific and heterospecific males. vivi (*D. virilis* ♀× *D. virilis* ♂), lulu (*D. lummei* ♀× *D. lummei* ♂), vilu (*D. virilis* ♀× *D. lummei* ♂). Other labels are as in Figure 13.

There were no significant differences in the proportion of eggs hatched by *D. virilis* females mated with *D. lummei* males and *D. lummei* females mated with conspecifics, with only a significantly larger proportion of eggs hatched by *D. virilis* females mated with conspecific males relative to the heterospecific cross ($F_{2,49}$= 3.804; P< 0.029, Figure 20).

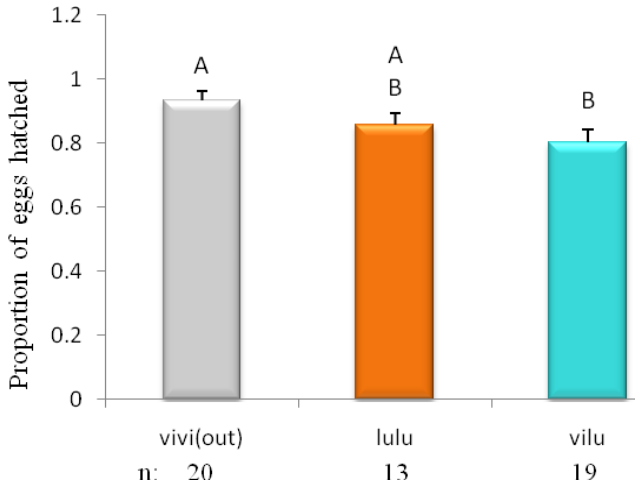

Figure 20. The average proportion and standard error of hatched eggs laid by *D. virilis* and *D. lummei* females mated to conspecific and heterospecific males. The labels are as in Figure 19.

3.3 Summary of results for crosses among species of the *Drosophila virilis* subgroup

Eggs laid in heterospecific crosses

♂ \ ♀	***D. virilis***	***D. lummei***	***D. a. texana***	***D. novamexicana***
D. virilis	—	—	—	—
D. lummei	N	—	Y	Y
D. a. texana	N	N	—	N
D. novamexicana	N	Y	N	—

N= Number of eggs laid are not different from those laid by the female in a conspecific cross.

Y= Eggs laid are higher than eggs laid by conspecific mated females.

Eggs hatched in heterospecific crosses

♂ \ ♀	***D. virilis***	***D. lummei***	***D. a. texana***	***D. novamexicana***
D. virilis	—	—	—	—
D. lummei	N	—	Y	Y
D. a. texana	Y	Y	—	Y
D. novamexicana	Y	Y	Y	—

Y= Significant differences in which hatched eggs are lower when compared to hatched eggs in conspecifics crosses.

N= No significant differences between eggs hatched in heterospecific and at least one conspecific cross.

4.0 DISCUSSION

This thesis investigates the postmating, prezygotic barriers that exist among species of the *Drosophila virilis* subgroup. My initial analyses on the average number of eggs laid by the conspecific and the heterospecific crosses did not show any significant differences for crosses involving *D. novamexicana* and *D. texana* females mated to *D. lummei* males as well as *D. lummei* females mated to *D. novamexicana* males. In these crosses females laid more eggs than in conspecific matings. However, all heterospecific crosses showed significantly lower egg hatchability than conspecific crosses. The only cross that showed no reduction in egg hatchability was that between *D. virilis* females and *D. lummei* males. It is possible that the lower offspring produced in the tested heterospecific crosses is a consequence of divergence between species of the *Drosophila virilis* subgroup in either male ejaculate proteins or female reproductive tract environmental conditions, as I discuss below.

4.1 The effect of the male ejaculate and female secretions on egg laying rates after mating

In Drosophila, the majority of changes in females' behaviour and physiology result during mating when females receive sperm and seminal fluid secretions from males, primarily the accessory glands proteins (*Acps*) (Harshman and Prout 1994; Chapman et al. 1995). Specific *Acps* target particular regions in the female reproductive tract and interact with females' ovulation and oviposition (Ravi-Ram et al. 2005). For instance, ovulin (*Acp26Aa*) and the sex peptide (*Acp70A*) are found to stimulate ovulation and oviposition (Soller et al. 1997; Heifetz et al. 2000). Both ovulin and the sex peptide stimulate egg production in different ways. When *Drosophila melanogaster* females were mated to *Acp26Aa* deficient males, the increase in egg laying by females was smaller than when females are mated to control males only on the first

day after mating (Herndon and Wolfner 1995). This suggests that *Acp26Aa* has a short term role in increasing egg production and that females need to receive other ejaculate component, such as the sex peptide, to maintain a high egg laying rate after mating (reviewed in Wolfner 1997). My results showed no differences in the egg-laying rate between females mated to conspecific and hetrospecific males, however, the increase in egg laying by *D. texana* females mated to *D. lummei* males, and between the cross of *D. lummei* and *D. novamexicana* in both directions was significant. The stimulation and elevation in egg-laying could be the result of the male ejaculate taking control over the female egg-laying, perhaps due to an inability of females to modulate the effects triggered by accessory glands proteins. This significant elevation of egg laying rates in heterospecific crosses compared to conspecifics was restricted to crosses between *D. lummei* and members of the *D. novamexicana – D. americana* clade. It did not occur among other species. In fact, in other heterospecific crosses the number of eggs laid after mating was similar to conspecific matings. It is unlikely that accessory glands proteins are evolutionarily conserved among species of the *D. virilis* subgroup. Proteins produced by the male accessory glands evolve rapidly even between closely related species of Drosophila (Coulthart and Singh 1988; Thomas and Singh 1992). Moreover, there appears to be a rapid turnover of *Acp* genes between species, with most genes being completely or partially lost (Begun et al. 2005; Haerty et al. 2007).

Alternatively, females might have retained the ability to recognize a wide variety of male-derived egg-laying triggering signals in the ejaculate. Egg-laying is stimulated by the male's ejaculate but is also mediated by the female's molecular counterparts (reviewed in Wolfner 2009). Therefore, it is possible that egg-laying in heterospecific crosses showing no differences with conspecifics is controlled by females retaining the molecular ability to recognize a wide variety of eggs-stimulating signals in the ejaculate. The current genome data available

from both Genome Browser (http://genome.ucsc.edu/) and FlyBase (http://flybase.org/) shows that *D. virilis* orthologs can be found for many of the female-derived molecules involved in the process of egg-laying (reviewed in Wolfner 2009) including the sex peptide receptor (SPR) recently characterized in *D. melanogaster* (Yapici et al. 2008).

4.2 The effect of the male ejaculate and female secretions on sperm fertilization success

Clearly, fertilization success was reduced in heterospecific crosses of *D. virilis* female mated to *D. novamexicana* males. An important observation in this cross is that stored sperms of *D. novamexicana* in seminal receptacle of *D. virilis* females were severely depleted within 48 hours after mating; whereas in conspecifically mated females sperm were found to be retained in the seminal receptacle and spermathecae for fertilization. Earlier studies suggested that sperm of *D. a. americana* and *D. a. texana* males mated to *D. virilis* females lost motility while in female storage (reviewed in Patterson and Stone 1952). Reproductive success is dependent on proper sperm storage in the reproductive tract of females and proper sperm utilization during fertilization. Under normal circumstances, sperm must transfer and enter the storage organs (spermathecae and seminal receptacle) after mating. Sperm must be nourished within the female storage organ until they can be utilized to fertilize eggs. Therefore, depending on the several components of the male ejaculate and the secretion of storage organs in females, differences in sperm storage between the conspecific and heterospecific crosses could be observed. Numerous studies have proven the distinctive role of Acps (*Acp62F* and *Acp29AB*) in sperm storage and retention in females (Neubaum and Wolfner 1999; Wong et al. 2008). Additionally, the secretions from the spermathecae and parovaria in females are required by sperm to fertilize the eggs (Anderson 1945; Allen and Spradling 2008; Prokupek et al. 2008). The enzyme glucose dehydrogenase (GLD), produced in both male ejaculatory bulb and the female spermathecae and

parovaria, has been shown to enhance the efficiency of sperm storage and release (Iida and Cavener 2004). In fact, GLD is found to be expressed in the spermathecae and the parovaria of *D. virilis* females but not in the parovaria of *D. a. americana* and *D. a. texana* (Schiff et al. 1992). Therefore, it is possible that variation in expression patterns of GLD within the females' organ might contribute to differences in sperm nourishment between species.

Insemination reactions, which are common in many taxa of Drosophila (Table 67 in Patterson and Stone 1952; Knowles and Markow 2001; Kelleher and Markow 2007), could cause incompatibilities that might affect the fertilization success by heterospecific sperm. An insemination reaction can block egg laying and re-mating. It can also result in sperm inactivation and improper sperm storage and movement (Patterson 1946). Insemination reactions occured only immediately after mating and not 24 hours after mating for species of the *D. virilis* subgroup (Grant 1983; Markow and Ankney 1988). I observed the occurrence of an insemination reaction immediately after maitng (0 hour) in the uterus of *D. virilis* females in both conspecific and heterospecific crosses. Thus, I found no clear evidence that the insemination reaction in this cross could cause different effects on the sperm of conspecific and heterospecific males.

Taken together, both the male ejaculate and the female reproductive tract secretions are likely under strong diversifying selection driven by species-specific female × male postmating interactions so that failure can occur in heterospecific matings. However, the most likely explanation of the impaired retention of sperm in storage is cryptic female control. Females tend to maximize their fitness by affecting the male sperm to be stored. Females might undernourish undesired sperm, thereby affecting fertilization success and egg hatchability.

4.3 The evolution of PPI among species of the *Drosophila virilis* subgroup

Postmating, prezygotic isolating barrier among the heterospecific crosses of the *Drosophila virilis* subgroup is proved to be strong. That the number of eggs laid remained constant in heterospecific crosses but hatching success fail, is likely costly to females. Females waste resources and energy by laying large numbers of unfertilized eggs. Males also suffer a fitness loss when they transfer sperm to a heterospecific female that subsequentially dumps or loses it from storage organs. However, it is interesting that at least in the *D. virilis* female × *D. novamexicana* male cross I examined, sperm was relatively rapidly lost from female storage after mating which might explain why there has not been strong selection against the high egg-laying phenotype. A female mating to a heterospecific male could actively dump or simply lose sperm from storage, and quickly became available and receptive to another mate. While I have not directly tested the possibility that *D. virilis* females dump sperm, it is clear that the sperm transferred by *D. novamexicana* males to *D. virilis* females do not remain in storage for long.

The fact that PPI was not restricted to strains of *D. virilis* collected from locations (i.e. southwestern USA) closer to *D. novamexicana*, with the caveat that given their different ecology they might not come into contact (Throckmorton 1982; Patterson and Stone 1952), suggests that PPI is a by-product of divergent evolution rather than reinforcement. In fact, my results show that the only cross for which no significant reduction in egg hatchability is observed is that between *D. virilis* females and *D. lummei* males. Therefore, my results and those that have reported reduced egg hatchability due to failure to fertilize eggs in crosses involving *D. virilis* females and *D. a. americana* males (Patterson and Stone 1952; Sweigart 2010) lend support for the evolution of PPI sometime during the diversification of the *D. novamexicana* - *D. americana* clade.

Finally, we know that *D. virilis* males show strong premating isolation from other species but *D. virilis* females readily mate with heterospecifics (Throckmorton 1982; Nickel and Civetta 2009). Coyne and Orr (1989, 1997) combined information on phylogenetic divergence and strength of premating and postzygotic isolation in the genus Drosophila to conclude that premating isolation barriers evolve earlier than other forms of isolation between diverging populations. It is therefore puzzling why *D. virilis* females do not show strong premating isolation with other species of the *Drosophila virilis* subgroup. One possibility is that ordering isolation barriers by time of divergence (as in Coyne and Orr 1989, 1997) is not fully informative of their actual contribution to isolation because one cannot assume total independence among isolation mechanisms. Therefore, premating isolation might not necessarily be the first barrier to hybridization. The other possibility might relate to the fact that males of the more ancestral species (*D. virilis*) are the ones showing premating behavioural isolation from derived female species. Asymmetric premating isolation might have evolved as a consequence of the evolution of polymorphism in receptors of derived male species to detect both short ancestral (*D. virilis*) and long derived species female cuticular hydrocarbons (Bartelt et al. 1986). Then, monomorphic male receptors in *D. virilis* males might not be able to recognize heterospecific females as suitable mates (Nickel and Civetta 2009).

5.0 CONCLUSIONS

- In some crosses among species of the *Drosophila virilis* subgroup, the number of eggs laid after mating does not differ between conspecific and heterospecific crosses and is determined by the female. This result suggests a cryptic female control over postcopulatory investment. However, in a few heterospecific crosses females laid a significantly large number of eggs suggesting a disruption of female × male postcopulatory interactions.

- For crosses in the *Drosophila virilis* subgroup the reduction in egg hatchability in heterospecific crosses is due to the production of unfertilized eggs thus lending support to the existence of postmating prezygotic isolation among species.

- The high production of unfertilized eggs by *D. virilis* females mated to *D. novamexicana* males was a consequence of the rapid depletion of sperm in storage organs of *D. virilis* females. My results suggest that the ineffectiveness of sperm in fertilization is due to cryptic control of *D. virilis* females, either by active dumping sperm or more subtle forms of undernourishment of heterospecific sperm.

- The fact that only the cross between *D. virilis* females mated to *D. lummei* males showed no evidence of PPI lends support to the evolution of PPI during the diversification of the *D. novaemxicana- D. americana* clade.

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7.0 APPENDICES

Appendix I - Standard cornmeal-yeast-agar-molasses medium (CYAM)**.**

Ingredient	Quantity
Cornmeal	65 g
Brewers yeast	13 g
Agar	6.5 g
Cold water	170 ml
Boiling water	760 ml
Refiners molasses	45.5 ml
10% Tegosept*	20 ml
99% Propionic acid	5 ml

***** 50 g methyl-hydroxybenzoate per 500 ml 95% ethanol

Protocol: Place steel pot with water on a hotplate and let it boil. Add cornmeal, yeast and agar into the cold water and whisk until stiff. Then, add this slurry to boiling water and stir constantly. When the mixture starts boiling, place the pot on a bench surface and add molasses. Let the mixture slightly cool to 65°C, add Tegosept and propionic acid. Pour prepared media in vials, bottles, or petri dishes.

Appendix Π - Preparation of 1X phosphate–buffered saline (PBS).

Component	Amount for making 1 liter
137 mM NaCL	8 g
2.68 nM KCL	0.2 g
10.14 mM Na_2HPO_4	1.44 g
1.76 nM KH_2PO_4	0.24 g

Protocol:

Dissolve all components in 800 ml of distilled water. Adjust pH to 7.2 using a pH meter. Bring up the volume to 1 liter by adding distilled water. Sterilize the solution by autoclaving and store at room temperature

Appendix III - Statistical analyses of eggs laid by conspecifically and heterospecifically mated females.

a) Analysis of variance test of between subject effects.

Test of Between-Subjects Effects

D. virilis and *D. novamexicana* (Japan Strain)

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	15092.801	2	7546.400	22.232	1.38E-08
Intercept	217158.678	1	217158.678	639.765	6.08E-43
Cross detail	15092.801	2	7546.400	22.232	1.38E-08
Error	30888.571	91	339.434		
Total	317607	94			
Corrected Total	45981.372	93			

$R^2 = 0.328$, $R^2_{adj} = 0.313$

Test of Between-Subjects Effects

D. virilis and *D. novamexicana* (Mexico Strain**)**

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2813.785	2	1406.890	1.517	0.226885
Intercept	166551.528	1	166551.52	179.604	1.67E-20
Cross detail	2813.781	2	1406.890	1.517	0.2268855
Error	61203.377	66	927.323		
Total	339645	69			
Corrected Total	64017.159	68			

R^2 =0.362, R^2_{adj}= 0.349

Test of Between-Subjects Effects

D. virilis and *D. novamexicana* (Argentina Strain)

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1145.241	2	572.620	1.021	0.364017
Intercept	368001.662	1	368001.662	656.329	1.05E-44
Cross detail	1145.240	2	572.620	1.021	0.3640172
Error	53826.839	96	560.696		
Total	456900	99			
Corrected Total	54972.080	98			

R^2 =0.021, R^2_{adj}= .000

Test of Between-Subjects Effects

D. virilis and *D. novamexicana* (California Strain)

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1163.615	2	581.807	1.006	0.371101
Intercept	216055.117	1	216055.11	373.453	1.49E-29
Cross detail	1163.615	2	581.807	1.006	0.3711013
Error	39918.829	69	578.533		
Total	347366	72			
Corrected Total	41082.444	71			

$R^2 = 0.044$, $R^2_{adj} = 0.015$

Test of Between-Subjects Effects

D. virilis and *D. novamexicana* (Russia Strain)

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	21042.306	2	10521.153	28.603	1.44E-10
Intercept	224759.769	1	224759.769	611.026	1.25E-44
Cross detail	21042.306	2	10521.153	28.603	1.44E-10
Error	37151.847	101	367.840		
Total	326662	104			
Corrected Total	58194.153	103			

$R^2 = 0.362$, $R^2_{adj} = 0.349$

Test of Between-Subjects Effects

D. virilis and *D. novamexicana* (Outbred Strain)

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3710.707	2	1855.353	2.8347	0.064882
Intercept	294348.606	1	294348.606	449.719	6.91E-34
Cross detail	3710.707	2	1855.353	2.835	0.0648816
Error	50397.779	77	654.516		
Total	354111	80			
Corrected Total	54108.487	79			

$R^2 = 0.069$, $R^2_{adj} = 0.044$

Test of Between-Subjects Effects

D. novamexicana and *D. a. texana*

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11287.025	3	3762.341	6.045	0.000981
Intercept	104105.312	1	104105.312	167.269	1.45E-20
crossout16	11287.025	3	3762.342	6.045	0.000981
Error	45433.780	73	622.381		
Total	191459	77			
Corrected Total	56720.805	76			

$R^2 = 0.199$, $R^2_{adj} = 0.166$

Test of Between-Subjects Effects

D. lummei and *D. novamexicana*

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17687.760	3	5895.920	9.166	5.93E-05
Intercept	125501.121	1	125501.121	195.104	4.61E-19
Cross detail	17687.760	3	5895.920	9.166	5.93E-05
Error	32805.948	51	643.253		
Total	177598	55			
Corrected Total	50493.709	54			

$R^2 = 0.350$, $R^2_{adj} = 0.312$

Test of Between-Subjects Effects

D. lummei and *D. a. texana*

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9357.3621	3	3119.121	4.588	0.007898
Intercept	37172.362	1	37172.362	54.680	8.5E-09
Cross detail	9357.362	3	3119.121	4.588	0.007898
Error	25153.077	37	679.813		
Total	99472	41			
Corrected Total	34510.439	40			

$R^2 = 0.271$, $R^2_{adj} = 0.212$

Test of Between-Subjects Effects

D. virilis and *D. a. texana*

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13402.91	2	6701.453	10.766	0.000137
Intercept	126623.2	1	126623.2	203.423	6.97E-19
Cross detail	13402.91	2	6701.453	10.766	0.000137
Error	29878.27	48	622.464		
Total	174711	51			
Corrected Total	43281.18	50			

$R^2 = 0.310$, $R^2_{adj} = 0.281$

Test of Between-Subjects Effects

D. virilis and *D. lummei*

Dependent Variable: eggtot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4478.471	2	2239.236	3.745	0.030658
Intercept	160297.4	1	160297.4	268.081	1.66E-21
Cross detail	4478.471	2	2239.236	3.745	0.030658
Error	29299.3	49	597.945		
Total	210800	52			
Corrected Total	33777.77	51			

$R^2 = 0.133$, $R^2_{adj} = 0.097$

b) Tukey post hoc test results.

eggtot (d)

Tukey HSD *D. virilis* (Japan) and *D. novamexicana* (Utah)

Cross detail	N	Subset	
		1	2
1	24	41.583	
2	25	41.64	
3	45		66.977
sig.		0.999	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = 339.435.
Uses Harmonic Mean Sample Size = 28.877
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi; 2=vino; 3=nono

eggtot (d)

Tukey HSD *D. virilis* (Russia) and *D. novamexicana* (Utah)

Cross detail	N	Subset	
		1	2
1	36	36.666	
2	23	41.304	
3	45		66.977
sig.		0.598	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = 367.840
Uses Harmonic Mean Sample Size = 32.093.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed
Alpha = .05.
1=vivi; 2=vino; 3=nono

eggtot (d)

Tukey HSD *D. novamexicana* and *D. a. texana*

Cross detail	N	Subset	
		1	2
1	17	28.176	
3	21	30.762	30.762
4	8		52.75
2	31		54
sig.		0.992	0.058

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = 622.381.
Uses Harmonic Mean Sample Size = 15.169.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=tete; 2=nono (out); 3=teno; 4=note

eggtot (d)

Tukey HSD luno

Cross detail	N	Subset	
		1	2
1	13	36.615	
2	31	40.193	
3	5		83.4
4	6		84.166
sig.		0.991	0.999

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = 643.254.
Uses Harmonic Mean Sample Size = 8.406.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed
Alpha = .05.
1=lulu ; 2=nono(outbred); 3=luno; 4=nolu

eggtot(d)

Tukey HSD *D.virilis* and *D. a. texana*

Cross detail	N	Subset	
		1	2
2	17	28.176	
3	14		58
1	20		64.9
sig.		1	0.706

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = 622.
Uses Harmonic Mean Sample Size = 16.643.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi (outbred); 2=tete ; 3=vite

eggtot (d)

Tukey HSD *D.virilis* and *D. lummei*

Cross detail	N	Subset	
		1	2
2	13	42.384	
3	19	62.368	62.368
1	20		64.9
sig.		0.056	0.952

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = 597.945.
Uses Harmonic Mean Sample Size = 16.708.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi (outbred); 2=lulu ; 3=vilu

Appendix IV - Statistical analyses of proportion of hatched eggs by conspecifically and heterospecifically mated females.

a) Analysis of variance test of between subject effects.

Test of Between- Subject Effects

D. virilis and *D. novamexicana* (Japan strain)

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.806	2	3.403	123.819	1.08E-26
Intercept	27.113	1	27.113	986.379	1.27E-50
Cross detail	6.807	2	3.403	123.819	1.08E-26
Error	2.501	91	0.027		
Total	42.486	94			
Corrected Total	9.308	93			

$R^2 = 0.731$, $R^2_{adj} = 0.725$

Test of Between- Subject Effects

D. virilis and *D. novamexicana* (Mexico strain)

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.249	2	2.124	60.768	1.08E-15
Intercept	11.606	1	11.606	331.953	1.9E-27
Cross detail	4.249	2	2.124	60.768	1.08E-15
Error	2.307	66	0.035		
Total	30.781	69			
Corrected Total	6.556	68			

$R^2 = 0.648$, $R^2_{adj} = 0.637$

Test of Between- Subject Effects

D. virilis and *D. novamexicana* (Argentina strain)

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11.079	2	5.539	257.326	7.44E-39
Intercept	29.398	1	29.398	1365.583	8.84E-58
Cross detail	11.079	2	5.539	257.326	7.44E-39
Error	2.024	94	0.021		
Total	51.771	97			
Corrected Total	13.103	96			

$R^2 = 0.846$, $R^2_{adj} = 0.842$

Test of Between- Subject Effects

D. virilis and *D. novamexicana* (California strain)

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.995	2	1.997	67.809	6.39E-17
Intercept	11.215	1	11.215	380.665	1.44E-29
Cross detail	3.995	2	1.997	67.809	6.39E-17
Error	2.003	68	0.029		
Total	30.563	71			
Corrected Total	5.998	70			

$R^2 = 0.666$, $R^2_{adj} = 0.656$

Test of Between- Subject Effects

D. virilis and *D. novamexicana* (Russia strain)

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7.651	2	3.825	155.962	2.78E-30
Intercept	23.196	1	23.196	945.640	3.43E-50
Cross detail	7.651	2	3.825	155.962	2.78E-30
Error	2.256	92	0.0245		
Total	55.966	95			
Corrected Total	9.908	94			

$R^2 = 0.846$, $R^2_{adj} = 0.842$

Test of Between- Subject Effects

D. virilis and *D. novamexicana* (Outbred strain)

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9.292	2	4.646	176.846	1.64E-29
Intercept	24.853	1	24.853	946.012	5.29E-45
Cross detail	9.292	2	4.646	176.846	1.64E-29
Error	2.022	77	0.026		
Total	41.974	80			
Corrected Total	11.314	79			

$R^2 = 0.821$, $R^2_{adj} = 0.817$

Test of Between- Subject Effects

D. novamexicana and *D. a. texana*

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.742	3	1.581	39.696	2.52E-15
Intercept	10.602	1	10.601	266.215	4.66E-26
Cross details	4.742	3	1.581	39.696	2.52E-15
Error	2.907	73	0.039		
Total	27.845	77			
Corrected Total	7.649	76			

$R^2 = 0.620$, $R^2_{adj} = 0.604$

Test of Between- Subject Effects

D. lummei and *D. novamexicana*

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.329	3	1.443	35.057	1.93E-12
Intercept	6.366	1	6.366	154.661	4.6E-17
Cross details	4.329	3	1.443	35.05695	1.93E-12
Error	2.099	51	0.041		
Total	28.522	55			
Corrected Total	6.428	54			

$R^2 = 0.673$, $R^2_{adj} = 0.654$

Test of Between- Subject Effects

D. lummei and *D. a. texana*

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.634	3	1.211	29.825	5.61E-10
Intercept	3.251	1	3.251	80.038	8.71E-11
Cross details	3.634	3	1.211	29.825	5.61E-10
Error	1.503	37	0.041		
Total	17.291	41			
Corrected Total	5.136	40			

$R^2 = 0.707$, $R^2_{adj} = 0.684$

Test of Between- Subject Effects

D. virilis and *D. a. texana*

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.872	2	3.436	121.058	1.77E-19
Intercept	12.784	1	12.785	450.438	4.87E-26
Cross details	6.872	2	3.436	121.058	1.77E-19
Error	1.362	48	0.028		
Total	24.214	51			
Corrected Total	8.234	50			

$R^2 = 0.835$, $R^2_{adj} = 0.828$

Test of Between- Subject Effects

D. virilis and *D. lummei*

Dependent Variable: proptot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	0.176	2	0.088	3.7997	0.029234
Intercept	37.381	1	37.381	1613.84	3.63E-39
Cross details	0.176	2	0.088	3.7997	0.029234
Error	1.135	49	0.023		
Total	40.287	52			
Corrected Total	1.311	51			

$R^2 = 0.134$, $R^2_{adj} = 0.099$

b)Tukey post hoc test results.

proptot (d)

Tukey HSD (Japan Strain)

Cross detail	N	Subset	
		1	2
2	25	0.148	
3	45		0.734
1	24		0.795
sig.		1	0.346

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .027.
Uses Harmonic Mean Sample Size = 28.877.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi; 2=vino; 3=nono

proptot (d)

Tukey HSD (Argentina strain)

Cross detail	N	Subset		
		1	2	3
2	23	0.045		
3	45		0.734	
1	29			0.936
sig.		1	1	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .022.
Uses Harmonic Mean Sample Size = 29.945.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05
1=vivi; 2=vino; 3=nono = 3

proptot (d)

Tukey HSD (Russia strain)

Cross detail	N	Subset		
		1	2	3
2	14	0.039		
3	45		0.734	
1	36			0.904
sig.		1	1	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .025.
Uses Harmonic Mean Sample Size = 24.706
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi; 2=vino; 3=nono

proptot (d)

Tukey HSD (Mexico strain)

Cross detail	N	Subset	
		1	2
2	15	0.123	
1	9		0.665
3	45		0.734
sig.		1	0.578

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .035.
Uses Harmonic Mean Sample Size = 15.000.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05
1=vivi; 2=vino; 3=nono

proptot (d)

Tukey HSD (California strain)

Cross detail	N	Subset		
		1	2	3
2	13	0.105		
1	13		0.565	
3	45			0.734
sig.		1	1	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .029.
Uses Harmonic Mean Sample Size = 17.039.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi; 2=vino; 3=nono

proptot (d)

Tukey HSD (outbred strain)

Cross detail	N	Subset		
		1	2	3
2	21	0.062		
3	29		0.726	
1	30		1	0.904
sig.		1		1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .0
Uses Harmonic Mean Sample Size = 25.989.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05
1=vivi; 2=vino; 3=nono

proptot (d)

Tukey HSD *D. novamexicana* and *D. a. texana*

Cross detail	N	Subset		
		1	2	3
4	8	0.080		
3	21	0.266		
1	17		0.563	
2	31			0.762
sig.		0.058	1	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .040.
Uses Harmonic Mean Sample Size = 15.169.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=tete; 2=nono (outbred); 3= teno; 4=note

proptot (d)

Tukey HSD *D. novamexicana* and *D. lummei*

Cross detail	N	Subset	
		1	2
3	5	0.047	
4	6	0.106	
2	31		0.741
1	13		0.845
sig.		1	0.727

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .041.
Uses Harmonic Mean Sample Size = 8.406
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed
Alpha = .05.
1=lulu ; 2=nono (out); 3=luno; 4=nolu

proptot (d)

Tukey HSD *D. lummei* and *D. a. texana*

Cross detail	N	Subset	
		1	2
4	8	0.060	
3	2	0.064	
1	18		0.587
2	13		0.856
sig.		0.999	0.151

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .041.
Uses Harmonic Mean Sample Size = 5.281.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=tete; 2=lulu; 3=lute; 4=telu

proptot (d)

Tukey HSD *D. vrilis* and *D. a. texana*

Cross detail	N	Subset		
		1	2	3
3	14	0.021		
2	17			
1	20		0.563	
sig.				0.934
		1	1	1

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .028.
Uses Harmonic Mean Sample Size = 16.643.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05
1=vivi (outbred); 2= tete; 3=vite

proptot (d)

Tukey HSD *D. virilis* and *D. lummei*

Cross detail	N	Subset	
		1	2
3	19	0.800	
2	13	0.856	0.856
1	20		0.934
sig.		0.539	0.311

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .023.
Uses Harmonic Mean Sample Size = 16.708.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05
1=vivi (outbred); 2=lulu ; 3=vilu

Appendix V - Statistical analyses of proportion of fertilized eggs by conspecifically and heterospecifically mated *D. virilis* and *D. novamexicana* females.

a) Analysis of variance test of between subject effects

Test of Between- Subject Effects (b) (Outbred strain)

Dependent Variable: tppropfert

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17.292	2	8.646	173.417	1.98E-27
Intercept	52.996	1	52.996	1062.931	3.06E-43
cross	17.292	2	8.646	173.417	1.98E-27
Error	3.390	68	0.049		
Total	75.195	71			
Corrected Total	20.683	70			

$R^2 = 0.836$, $R^2_{adj} = 0.831$

c) Tukey post hoc test results.

Tppropfert

TukeyHSD (outbred strain)

Cross detail	N	Subset	
		1	2
2	24	0.187	
3	19		1.170
1	28		1.266
sig.		1	0.315

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares the error term is Mean Square (Error) = .050.
Uses Harmonic Mean Sample Size = 23.075.
The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
Alpha = .05.
1=vivi(outbred) ;2=vino;3=nono(outbred)