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Positive assortative fertilization as a result of allopatry between Drosophila americana and D. novamexicana

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POSITIVE ASSORTATIVE FERTILIZATION AS A RESULT OF ALLOPATRY BETWEEN DROSOPHILA AMERICANA AND D. NOVAMEXICANA

by

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MASTER'S THESIS

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ABSTRACT

The level of reproductive isolation between the closely related *Drosophila americana* and *D. novamexicana* has been examined. Egg hatch rates in heterospecific crosses indicate that the reduction in hatch rate is due to prezygotic incompatibilities between heterospecific individuals. The two species exhibit an asymmetric level of postmating-prezygotic incompatibility that is caused by an incapacitation and/or loss of sperm from the heterospecific female's reproductive tract. Measurements of this phenomenon between iso-female lines within *D. americana* and a *D. americana* iso-female line suggests that the phenomenon is species wide and results from rapid divergence in male-female postmating-prezygotic reproductive compatibility between allopatric populations.

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CHAPTER I

PATTERNS OF REPRODUCTIVE ISOLATION

Introduction

The process of speciation, or the formation of distinct groups of interbreeding individuals, is a central feature of evolutionary change that has resulted in the diversity of species observed today. The processes that lead to speciation have concerned biologists even before the publication of Charles Darwin's *On the Origin of Species* (1859). Even though speciation has been actively investigated since Darwin's revolutionary theory, most of the knowledge about the process and its genetic basis has emerged over the past quarter century (Coyne & Orr, 2004). This resurgence of interest in the process of speciation is due in part to Ernst Mayr's contributions, namely the formulation of a criterion for defining species and the delineation of mechanisms that maintain species boundaries.

In his 1942 publication, *Systematics and the Origin of Species*, Mayr proposed the Biological Species Concept, which defines species as interbreeding groups of individuals that are reproductively isolated from other such groups (Mayr, 1942). This criterion for classifying species provides a framework for biologists to examine fundamental questions about the origin of reproductive isolation. These questions, as they relate to the time frame and mechanism of speciation, concern three primary problems. The first has to do with the initial splitting of a population into two separately interbreeding groups. Modes of speciation have been defined based on the spatial pattern of separation within a group that ultimately leads to genetic divergence between two groups. Generally stated, these speciation modes are divided into events in which the population becomes geographically

isolated (allopatry), and those in which the diverging populations occupy the same range (sympatry). Even though instances of sympatric speciation have been described, the evidence to suggest its commonality is lacking (Coyne & Orr, 2004). Furthermore, in cases where sympatry has been proposed as the mode of speciation it has been difficult to establish that the two species have not undergone a period of geographic isolation (Coyne & Orr, 2004). Allopatric speciation, on the other hand, presents a rather intuitive mechanism in which gene flow is prevented as a direct consequence of a limit on hybridization. This mode of speciation was proposed by Mayr to be the major cause of initial genetic divergence (Mayr, 1963).

The second major question about speciation concerns the evolutionary forces that cause the genetic divergence between two incipient species: whether natural selection or genetic drift plays a major role in species formation. Mayr proposed that genetic drift plays a more important role in the speciation process (Mayr, 1963). He described species formation in terms of founder effects and peripatric modes of divergence in which small population size of founder populations causes genetic drift to have a larger effect than natural selection (Mayr, 1963). Both forces may contribute to speciation, however, which plays a more important role is a matter of debate. Mayr's claim still remains an open question, even though the evidence thus far suggests that positive Darwinian selection has influenced the diversification of genes underlying mechanisms of reproductive isolation (Orr, 2005), although supporting evidence is available for only a handful of cases (Ting et al., 1998; Presgraves et al., 2003; Barbash et al., 2003). Thus it is clear that divergence in allopatry is a sufficient mechanism to account for a wide array of the existing biodiversity, and selection driven change in separated populations may be the mechanism underlying species formation.

The third question stems directly from the Biological Species Concept; i.e., what maintains separation of the two species? In other words, what are the isolating mechanisms that prevent gene flow between members of two incipient species that have

diverged in allopatry if, through secondary contact, they have the opportunity to mate? Mayr coined the term "reproductive isolation" to describe these mechanisms. Reproductive barriers are commonly classified into two categories: those acting prior to the formation of the zygote (prezygotic) and those acting after the zygote is formed (postzygotic). Even though this classification can be thought of as a convenience rather than a necessity, it represents a basic methodological distinction (with a few exceptions). Prezygotic barriers are a result of an interaction between the two parental genotypes, whereas postzygotic barriers are realized after the expression of hybrid genomes. In other words, prezygotic barriers result from incompatibilities between mating systems, whereas postzygotic barriers result from developmental and/or behavioral defects within hybrids. In allopatric modes of divergence, the accumulation of genetic differences between two populations generates these incompatibilities. Therefore, it is of interest to know the initial types of reproductive isolation that arise as a consequence of allopatry and how these differences contribute mechanistically and genetically to reproductive isolation.

In this chapter, patterns of reproductive isolation are reviewed. The purpose of this review is mainly to describe prevalent examples of both pre- and postzygotic isolation and present some of what is known about their mechanism and genetics. These examples are mainly derived from the fruit fly *Drosophila* because of its relevance to the thesis at hand and also because a large number of studies have been conducted using closely related species in this genus. Even though this is not an extensive review of all the known reproductive barriers across many species, it is nevertheless an informative overview that will set the second and third chapter in a general context.

Prezygotic isolation

Reproductive barriers that act prior to successful expression of the diploid zygotic genome are classified as prezygotic. A number of different types of prezygotic barriers

may arise. Those include: habitat isolation, temporal isolation, pollinator isolation, behavioral isolation, mechanical and gametic isolation. The first three types of prezygotic barriers represent different ecological factors, thereby discriminating them from behavioral, mechanical and gametic isolation, which are realized by direct interaction between two individuals, leading to reduced fertilization success. Ecological barriers are beyond the scope of the current topic: therefore, only the latter three examples of prezygotic isolation are reviewed.

Impediments to successful fertilization entail divergence within mating systems, which is likely driven by sexual selection that is causing rapid evolution of mating behaviors and mechanisms (West-Eberhard, 1983). Furthermore, theoretical predictions suggest that prezygotic isolation evolves more quickly than postzygotic isolation if the effects of sexual selection are strong (Lande & Kirkpatrick, 1988). Prezygotic barriers in major lineages such as frogs and birds, which are noted for sexual selection of mate-recognition, are thought to evolve very rapidly, suggesting the importance of this type of barrier in these lineages (Blair, 1964; Grant, 2001). Experimental evidence in *Drosophila* further indicated that the rate of evolution of prezygotic isolation in sympatric species is faster than postzygotic isolation; however, this may be due to reinforcement of species barriers (Coyne & Orr, 1997). In allopatric species of *Drosophila*, prezygotic and postzygotic isolation evolve at similar rates, indicating that the factors responsible for these incompatibilities evolve at similar rates (Coyne & Orr, 1997). Interestingly, the measurement of prezygotic isolation in all these examples is through behavioral isolation, thus creating a biased perspective on the evolution of prezygotic barriers in allopatry.

Behavioral and mechanical isolation

Behavioral isolation is characterized by a reduction in mating attraction between heterospecific individuals. This can be a byproduct of natural selection for local

adaptation or for species recognition (Coyne & Orr, 2004). Sexual selection may also play a major role in behavioral isolation. If one sex displays a mating signal, that signal stimulates a preference in the other sex. In many species, this type of sexual selection leads to elaborate sexually dimorphic traits which may cause sexual isolation from close relatives.

Extensive studies of mating preference have been performed in *Drosophila* (Coyne, 1996; Coyne & Charlesworth, 1997). Coyne and Orr (2004) conducted a survey of a number of genetic analyses of traits involved in behavioral or mechanical isolation between closely related species. The purpose of this survey was mainly to investigate patterns in the number of genes involved in behavioral isolation, thereby elucidating the genetic complexity of these traits. One conclusion drawn from this survey is that many genes are likely involved in behavioral isolation since the traits that are under selection are influenced by more than one gene. For example, nine different loci contribute to behavioral isolation between two races of *D. melanogaster* (Ting et al., 2001). Pheromone differences between another closely related allopatric trio, *D. mauritiana*, *D. sechellia* and *D. simulans*, are also based on polygenic differences (Coyne, 1996; Coyne & Charlesworth, 1997). Few examples of single gene effects have been described. For example, pheromone differences attributed to a single gene of large effect between the allopatric *Spodoptera latifascia* and *S. descoinsi* cause behavioral isolation (Monti et al., 1997). The role of adaptation to local habitats and/or sexual selection in the divergence of these genes is currently unknown.

Mechanical isolation results from incompatibility between the genitalia of heterospecific individuals which leads to failure in copulation or inability to transfer sperm or pollen. This form of prezygotic isolation likely evolves in a similar manner to behavioral isolation, i.e., through sexual selection driving the gradual coevolution of male traits and female preference for these traits. This could ultimately lead to differential divergence in morphological features of genitalia as demonstrated between *D. simulans*

and *D. mauritiana*, in which male genitalia are distinguishable by females in conspecific vs. heterospecific inseminations (Coyne, 1992). The isolating barrier in this case is reduced sperm transfer, as females in heterospecific matings terminate copulation prematurely, perhaps as a result of improper fit between genital organs. It has also been shown that the morphological difference in genitalia is due to at least 14 chromosomal regions, all of which have a similar effect (Zeng et al., 2000).

Mechanical and behavioral isolation represent two different types of prezygotic restrictions to gene flow, however, the same types of selective forces can result in both types of barriers. In essence, differential divergence in mating systems can generate incompatibilities relating to mating system interaction, specifically in cases of sexual dimorphism and strong selection for dimorphic traits. A study comparing the rate of evolution of pre- and postzygotic isolation in a diverse genus of sexually dimorphic fish (*Etheostoma*) reported the faster evolution of sexual isolation strictly as a byproduct of evolution in allopatric populations (Mendelson, 2003). Other comparisons from the literature support the prediction that sexual isolation evolves more rapidly than other forms of isolation (Mendelson, 2003). Even though the bias in ascertaining behavioral isolation and measuring its effect has shifted focus away from the study of other prezygotic barriers, other forms of isolation are also likely to result from sexual selection.

Gametic isolation

Gametic barriers are variable in the way they affect fertilization success across the species in which they have been studied. Any dysfunction in the sequence of events from insemination to gamete fusion can result in gametic isolation (also known as “assortative fertilization”). Gametic isolation can be expressed under both competitive and non competitive conditions (Coyne & Orr, 2004). Competitive isolation involves gametic incompatibilities that arise as a result of competition between sperm of multiple

ejaculates, each fully capable of successful fertilization in the absence of a second ejaculate within the female reproductive tract (Wade et al., 1994; Rieseberg et al., 1995; Carney et al., 1996; Price, 1997; Diaz & Macnair, 1999; Price et al., 2001). Noncompetitive isolation, on the other hand, involves a direct incompatibility between the heterospecific male's ejaculate and the female reproductive tract or egg such that the sequence of events leading to successful fertilization is disrupted (Patterson, 1946; Patterson & Stone 1952; Vick, 1973; Williams & Rouse, 1988; Lessios & Cunningham, 1990; Palumbi & Metz, 1991; Grimaldi et al., 1992; Gregory & Howard, 1993).

Competitive gametic isolation, otherwise known as conspecific sperm/pollen precedence (CSP/CPP), has been described in a number of invertebrate and plant species (see Coyne & Orr 2004, pp: 236-238). In *Drosophila*, CSP has been demonstrated in the allopatric *D. simulans* and *D. mauritiana* species pair (Price, 1997) and the partially sympatric *D. santomea* and *D. yakuba* species pair (Chang, 2004). In the first example, *D. simulans* females produce significantly less hybrid progeny when doubly mated to *D. simulans* and *D. mauritiana* males, regardless of the order of mating. Further analysis of this phenomenon revealed that the conspecific ejaculate displaces and incapacitates heterospecific sperm (Price et al., 2001).

Noncompetitive gametic isolation can affect fertilization success in various ways depending on the mating system. For example, external fertilization depends on a direct interaction between sperm and egg. Reproductive barriers between gametes of external fertilizers have been described in Abalones (*Halitois*) and Sea Urchins (*Echinometra*) (Williams & Rouse, 1988; Lessios & Cunningham, 1990; Palumbi & Metz, 1991). It has been shown in heterospecific fertilizations in abalones that the sperm acrosomal protein, *lysin*, fails to dissolve a major component of the egg's vitelline envelope, the VERL glycoprotein, thereby preventing sperm from penetrating the egg (Swanson & Vacquier, 1997; Kresge et al., 2001). Further analysis of the VERL protein revealed concerted evolution of repeated motifs with change accumulating through drift (Swanson &

Vacquier, 1998). The Lyisn sequence, on the other hand, is subject to positive natural selection (Vacquier et al., 1997), and it has been suggested that the selection pressure is a consequence of coevolution based on the ability to dissolve VERL protein (Swanson & Vacquier, 1998). In Sea Urchins, the recognition proteins (*bindins*) on the surface of egg and sperm from separate species fail to adhere to each other (Palumbi & Metz, 1991). Analysis of the *bindin* gene sequence reveal high levels of polymorphism in the protein sequences within species, characterized by an excess of replacement mutations relative to silent mutations (Palumbi, 1999). The process of competition for fertilization between sperm of different individuals results in rapid coevolution of these recognition elements, therefore leading to rapid divergence through natural selection (Palumbi, 1999).

Internal fertilization, which requires the transfer and storage of the entire male ejaculate, entails a series of events that may generate heterospecific gametic incompatibilities. In *Drosophila*, assortative fertilization may occur during copulation through a reduction in the amount of sperm transferred and/or the duration of copulation. There may also be problems during sperm storage such as sperm incapacitation or ejection. And finally, there may be incompatibilities that arise during sperm-egg interactions, such as the inability of sperm to permeate the egg (Markow, 1997). Reproductive barriers acting after insemination and before the fusion of gametes have been described in heterospecific crosses involving *Drosophila simulans* (Price et al., 2001), *D. pulchrella* (Fuyama, 1983), members of the *repleta* group (Patterson & Stone, 1952; Markow, 2001) and the *virilis* group (Patterson 1947). In these examples, an incompatibility between male and female reproductive structures leads to a reduction in fertilization success.

As in behavioral and mechanical isolation, gametic incompatibilities may result from concerted male-female coevolution (Markow, 1997; Rice & Holland, 1999; Miller & Pitnick, 2002;). Male-male behavioral competition and female preference are analogous to male-male sperm competition and female adaptation to the toxic effects of

sperm (Chapman et al., 1995; Pizzari & Snook, 2003; Moore & Pizzari, 2005). This arms race between males and females has been shown to produce a divergent set of sex-specific alleles which may in turn cause reproductive incompatibility with allopatrically diverged sister species (Miller & Pitnick, 2002; Jagadasheen & Singh, 2005). A mathematical model proposed by Gavrilets (2000) describes rapid evolution of reproductive barriers driven by male-female coevolution. This rapid evolution of reproductive barriers driven by sexual conflict can explain the increased rate of speciation after colonization of new habitats, which in essence is an allopatric model of speciation (Gavrilets, 2000). The implications of the rapid evolution of reproductive genes suggest that allopatry may result in rapid divergence of reproductive systems, as is the case in mating behaviors. It is of interest, then, to determine whether gametic incompatibilities are common among very closely related allopatric species, and whether this type of incompatibility is one of the first to arise in allopatry.

Postzygotic isolation

Barriers acting after the formation of the zygote can appear in two forms: those in which hybrids are unfit in either parental environment due to their intermediate phenotype (extrinsic postzygotic isolation), or those in which hybrids suffer developmental defects that disrupt their chances of survival and reproduction (intrinsic postzygotic isolation). Examples of the former have not yet been reported in *Drosophila*. This may be due in part to our poor understanding of differences in niche adaptation between closely related species of *Drosophila* that are still capable of producing hybrids with no apparent fitness disadvantage relative to either parental species. Intrinsic barriers have been, by far, the most extensively studied, especially in *Drosophila*, and those are mainly two kinds: hybrid sterility and inviability. A brief overview of these two barriers

is given below to highlight the approaches taken to understand some aspects of postzygotic isolation.

Hybrid inviability can be expressed at various stages in the life cycle of an organism. Any genomic incompatibility that arises within hybrids may cause a defect during development. One example in *Drosophila* was described early on by Sturtevant (1920): in the cross between *D. melanogaster* females and *D. simulans* males, only hybrid females appear because hybrid males experience lethality prior to eclosion, whereas in the reciprocal cross only hybrid males appear because females face embryonic defects (Sturtevant, 1920). Incompatibilities of this sort may be due to genetic interactions or endosymbionts such as *Wolbachia* that cause cytoplasmic incompatibilities. Of the former, the most prominent of these explanations is the "Dobzhansky-Muller" model of genetic incompatibilities. Broadly stated, this model proposes that a gene that has diverged in allopatry, coadapting with other genes in one particular genetic background, would be less efficient in another genetic background, i.e. the one which it has diverged from (Muller, 1942). Genetic analyses of hybrid inviability in which the number of genes involved was assessed reveal that a large number of genes play a role in hybrid inviability (e.g., True et al., 1996; Presgraves, 2003). This is consistent with what would be expected under the Dobzhansky-Muller model, given that at least two genes are required for this scenario. Presgraves (2003), for example, identified 20 chromosomal regions within the *D. simulans* genome that cause hybrid inviability due to interactions with the *D. melanogaster* genome. One of the genes that cause this inviability has been identified as a transcriptional regulator gene, *Hybrid male rescue*, and has been shown to have evolved rapidly through positive natural selection (Barbash et al., 2003; Barbash et al., 2004). Another gene causing hybrid inviability in this species pair encodes a component of the nuclear envelope, particularly the nuclear pore complex. This gene, *Nucleoporin96*, was also shown to be driven by positive Darwinian selection, even though genes encoding proteins within the nuclear pore

complex are usually highly conserved (Presgraves et al., 2003). These two genes represent only a fraction of the number of genes that are suspected to cause hybrid inviability, suggesting that genes causing hybrid inviability can evolve rapidly given that *D. simulans* and *D. melanogaster* have diverged only ~2.5 mya (Hey & Kliman, 1993). What can be concluded from this example is that allopatric divergence through positive Darwinian selection may bring about Dobzhansky-Muller incompatibilities at many loci.

The *D. melanogaster-D. simulans* system has contributed substantially to the understanding of the genetics of speciation by exploiting the genetic manipulability of *D. melanogaster*, but this system provides little input on the initial barriers arising in allopatry and the forces that cause them. On the one hand, *D. melanogaster* and *D. simulans* have evolved at least two types of isolating barriers, hybrid inviability and sterility. It is difficult to assess which of these barriers has arisen first and which was more important in maintaining species boundaries. Furthermore, measuring the degree of hybrid inviability has largely been done through counting the number of progeny produced in single and/or mass matings (Yamamoto & Sawamura, 1997; Chatterjee et al., 2008). Assessing the degree of hybrid inviability in this way does not allow for the detection of prezygotic effects that contribute to the reduction of progeny (Yamamoto & Sawamura, 1997; Chatterjee et al., 2008). Avoiding this bias is best accomplished by accounting for the frequency of oviposition that is a consequence of mating. Therefore, it is proposed that inviability of hybrids be distinguished from failures in gamete-gamete fusion using a detailed assessment method in which the defective stage is determined.

Conclusions

Theory predicts that sexual selection on behavioral and/or reproductive traits will result in rapid divergence of mating systems between allopatric species (West Eberhard, 1983; Gavrilets, 2000). This prediction was tested experimentally in *Etheostoma*, a

promiscuous spawner. These studies found that behavioral isolation within the genus evolves to completion much earlier than hybrid inviability (Mendelson, 2003). The mating lifestyle of this organism sets the stage for sperm competition, rapid evolution of fertilization proteins, and a potentially rapid evolution of barriers to fertilization (Rice, 1998; Howard, 1999). *Drosophila* species are, for the most part, also promiscuous in mating lifestyle; females mate with multiple males, which also leads to sperm-sperm competition, and females in turn adapt to the outcome of this competition. To investigate this phenomenon in Drosophila, two very closely related members of the *virilis* species group, *Drosophila americana* and *Drosophila novamexicana*, were examined to determine the stage at which the reproductive barrier between them is realized. The next chapters deals with this investigation.

CHAPTER II

REPRODUCTIVE INCOMPATIBILITY BETWEEN AN ALLOPATRIC SPECIES PAIR DROSOPHILA AMERICANA AND D. NOVAMEXICANA

Introduction

In determining patterns of speciation within a group of closely related species, it is of great interest to identify the common modes of speciation, the mechanisms of reproductive isolation and their underlying genetic basis, and the evolutionary forces responsible for speciation. The *virilis* group of *Drosophila* has been the focus of some of these broad range studies (Throckmorton, 1982; Orr & Coyne, 1989; Spicer & Bell, 2002; Orsini et al., 2004). This group of species is divided into two phylads: the *virilis* phylad and the *montana* phylad. The close evolutionary relationship between two members of the *virilis* phylad, *D. americana* (sometimes considered as two subspecies, *D. a. americana* and *D. a. texana*) and *D. novamexicana*, presents a great opportunity to address questions raised in the previous chapter, namely those concerned with the initial reproductive incompatibilities that arise in allopatry, because of the low level of genetic differentiation between them. A brief summary of the evolutionary history of this species pair is given below.

Biogeography

A rather extensive set of Phylogenetic analyses inform the evolutionary history of the *virilis* phylad. Figure 1 shows the phylogenetic relationships within this group. *D. virilis* separated from the common ancestor of *D. novamexicana*, *D. americana* and *D. lummei* approximately 4.5 mya (Nurminsky et al., 1996; Spicer & Bell, 2002; Caletka &

McAllister, 2004). The divergence of *D. lummei* from the common ancestor of *D. novamexicana/D. americana* was estimated to be 3 million years based on paleobotanical and climate data (Caletka & McAllister, 2004). This split occurred as a consequence of the dispersal of the *D. novamexicana/D. americana* common ancestor into North America and subsequent isolation around 3 mya with the onset of global cooling at the end of Pliocene period (Caletka & McAllister, 2004). An eastern and western split of the common ancestor within North America demarcated by the Rocky Mountains gave rise respectively to the contemporary forms *D. americana* and *D. novamexicana* (Figure 2). Divergence between these allopatric species is estimated at 0.4 mya (Caletka & McAllister, 2004).

Ecology & distribution

The general habitat conditions for members of the *virilis* group are riparian and they are known to maintain close associations with willows (Throckmorton, 1982). The habitat in which *D. novamexicana* is found is primarily that of old willow stands along river banks, which are rare in the southwestern deserts of North America (Throckmorton, 1982). *D. americana* inhabits similar, although denser, stands of willow and prefer high humidity close to river banks (Throckmorton, 1982). Larval feeding sites of *D. americana* on sandbar willow trees were identified by Blight & Romano (1953). It was also suggested that *D. americana* have low migratory ability and remain closely associated with rivers and other marshy regions (Carson, 1952). This habitat specificity, along with low migratory ability, may be a major factor in the rapid speciation within this group (Throckmorton, 1982).

Reproductive & genetic divergence

When considering reproductive barriers that have arisen between the two species, Patterson & Stone (1949) report results that indicate an asymmetric reduction in the number of progeny produced in crosses between *D. americana* and *D. novamexicana* (Patterson & Stone, 1949). Similar results were obtained in crosses between *D. novamexicana* and *D. virilis*, thereby not providing ascertainment of species relationships between the three species based on viability of progeny alone. Spieth (1951) conducted studies of mating preferences between the three species and concluded that "the degree of sexual isolation [between the three species] is almost randomly distributed among interstrain crosses" (Speith, 1951). This suggests that behavioral isolation has not evolved in allopatry between *D. novamexicana*, *D. americana*, and *D. virilis*.

Genetic divergence at the sequence level estimated for 8 genes between the two species is low (McAllister, unpublished data). This is not surprising given the recent split between the two species estimated to be ~0.38 mya (Caletka & McAllister, 2004). Nevertheless, the consequences of the geographic isolation are evident in morphological differences between the two species (Spicer, 1991; Witkopp et al., 2003) (Figure 2). Furthermore, crosses between the two species result in a reduction in the number of hybrids produced (Patterson & Stone, 1949). Given the very recent time of separation and low sequence divergence, the *D. novamexicana* and *D. americana* species pair provide a unique opportunity to study reproductive barriers that evolve in allopatry.

In addressing this problem, the approach for the study below has been to identify the stage in the reproductive/developmental cycle at which the inviability of hybrids is realized. An assessment of the variation of this phenomenon among multiple iso-female lines of each species was also conducted and is presented in Chapter III. In assessing the level of reproductive incompatibility, a hatch rate method is employed instead of counting the number of progeny in mass matings to produce an estimate of fertilization success that is not biased by the variation in the number of eggs laid by individual

females within and across species. Using the number of progeny as a measure of reproductive success can mask the potential effects of gametic incompatibilities because the number of eggs that do not hatch is not accounted for, therefore not allowing such barriers to be detected. Furthermore, using progeny counts from mass matings would mask the lethality cause by overcrowding within culture vials. There are also other advantages to using this method: namely, it allows the differentiation between interspecific and intraspecific reproductive incompatibilities, and furthermore, allows for the distinction between two types of inviability: the failure of embryonic development versus larval development.

Materials & methods

Progeny counts in conspecific vs. heterospecific crosses

All fly lines were maintained in culture on standard cornmeal medium at 22 ˚C and a 14:10, light:dark cycle. In order to asses the number of progeny produced in conspecific vs. heterospecific crosses, individuals from an *D. americana* iso-female line (NN97.4, genetically marked with a recessive red-eye mutation) and a *D. novamexicana* iso-female line (NM1031.4) were collected 1-2 days after eclosion and males and females were aged separately in groups of 20-25. For each cross a single virgin female was paired with a single virgin male in a new yeasted vial until copulation was observed. After copulation was complete, males were removed by aspiration and females were left in the vial maintained at 22 ˚C. Females were transferred to a new vial every 3 days to minimize effects of overcrowding of larvae. Progeny from each individual female eclosed 21-25 days later and the number of males and females was recorded. All possible crosses were conducted within and between the two species: N♀xN♂ (n=24), N♀xA♂ (n=34), A♀xA♂ (n=27), and A♀xN♂ (n=23). Data were analyzed using a Welch Two Sample t-test to test for equality in the number of offspring using R version 2.5.1.

Egg hatch rate in conspecific vs. heterospecific crosses

Hatch rate in conspecific vs. heterospecific crosses was assessed as follows: virgin flies from the same two lines used in the progeny count experiment were collected and aged as described above. Males and females for each cross were mated in groups of 10-20 in yeasted cornmeal vials. A total of ~200 females and ~200 males were used for each of the population cage crosses. Four population cages were established with the following crosses: N♀ x N♂, N♀ x A♂, A♀ x A♂, and A♀ x N♂. After introduction of the flies into the cage, mated groups were allowed to lay eggs in yeasted grape juice agar medium and were provided with a water source. A new egg collection plate was placed in the cage every 24 hours on 10 consecutive days. A random sample of ~100 eggs was collected each day and arrayed on a second grape-juice agar plate. The number of hatched eggs/total was recorded 48 hours after each collection. Data were input into SAS and a generalized linear mixed model to account for the random effect of blocking within days was fit using the PROC GLIMMIX procedure with a computation of the least squares means for each cross and contrasts between crosses (SAS V. 9.1).

Egg hatch rate in Backcrosses

Male progeny from both heterospecific crosses were collected and aged as described. The *D. americana* line used in these studies was ML97.5 (genetically marked with a recessive purple-eye mutation). Population cages were established as described for the following crosses (maternal type indicated in parentheses): N♀ x F1(N)♂, N♀ x F1(A)♂, A♀ x F1(A)♂, and A♀ x F1(N)♂. Data were collected and analyzed as described above.

Reproductive incompatibility assay

Three discrete stages in the reproductive cycle were examined to determine the type of reproductive isolation between *D. americana* and *D. novamexicana*: embryonic development, fertilization, and sperm storage.

Embryonic development: The pattern of nuclear syncitial divisions was used to determine the progression of embryos through early embryonic stages of development. This assay was conducted for two crosses, N♀ x N♂ and N♀ x A♂. A total of ~200 males and females for each cross were introduced into two cylindrical embryo collection cages (Genesee Scientific) and were allowed to mate for 2-3 days at 22 ˚C and were provided with grape juice agar medium supplemented with yeast paste. After females began laying large numbers of eggs, embryos were collected every 30 mins and were either dechorinated and fixed immediately or were allowed to develop at 22 ˚C in four discrete time intervals: 30-60 mins, 60-90 mins, 90-120 mins, and 120-150 mins. For example, embryos that were aged to the 60-90 mins interval were removed from the cage at 30 mins and were incubated for 60 mins before being dechorinated and fixed.

The procedure of Ashburner et al. (2000) was used to dechorinate and fix embryos prior to nuclear staining (Ashburner et al., 2000). Embryos were picked from the agar plates and washed into a mesh basket using distilled H2O. The mesh basket was placed in a glass petri dish containing 50% bleach for ~2 mins. The dish containing the egg basket was placed under a dissecting microscope to ensure the bleach only dissolved the vitelline layer without damaging the eggs. When the chorion had dissolved from approximately 80% of the embryos, the egg basket was removed from the bleach and placed immediately in another glass petri dish containing a PBS-based embryo-wash solution. Embryos were rinsed extensively with EWS and were allowed to settle to the center of the mesh basket. The mesh was removed and placed on the inside of a 5 ml glass tube and embryos were washed into the glass tube using 1 ml heptane. The mesh was removed and 1 ml of methanol was added. The tube was vigorously shaken for 20

seconds and embryos were allowed to sink to the bottom of the tube. Debris from the solution accumulated at the heptane-methanol border. The top heptane layer, along with most of the methanol was removed from the tube with a pipette and the tube was refilled with 3-4 ml of methanol. Fixed embryos were stored at 4 ˚C. Embryos were prepared for staining 1-2 days after fixation. Embryos were transferred in methanol to a 1.5 ml eppendorf tube and most of the methanol was removed. Another 0.5 ml of methanol was added, and 0.5 ml of PBTA was subsequently added two times to wash the embryos. The tube was gently inverted a few times and embryos were allowed to settle to the bottom of the tube. The embryos were then rinsed in PBTA 4-5 times and were incubated in DAPI diluted in PBTA for 5 minutes. Embryos were washed 8-10 times in PBTA and were prepared on a microscope slide and viewed under a fluorescent microscope.

Fertilization: Four embryo collection cages were established with four crosses, N♀ x N♂, N♀ x A♂, A♀ x A♂, and A♀ x N♂. Embryos from each cross were collected every 30 minutes after the introduction of yeasted grape juice agar plates until a sufficient number was obtained from each cross. The procedure for collection, dechorionation, fixation and rehydration are as described above. Fertilization was assayed using a sperm-tail specific rat polyclonal antibody, α-XT (Provided by Timothy Karr, University of Bath). Rehydrated embryos were incubated in 1:300 α-XT-PBTA dilutions on a rotator for 1 hour. Embryos were rinsed at least 10 times in PBTA and washed in PBTA overnight at 4 ˚C. The embryos were double labeled with green fluorescent Alexa anti-rat secondary antibody at 1:400 dilutions for 1 hour on a rotator. Embryos were extensively rinsed in PBTA (>20 times) and washed overnight at 4 ˚C. This rinse-wash cycle was repeated three times. PBTA was removed and embryos were rinsed 4 times in PBS-Azide. Glycerol mounting medium was prepared with 90% glycerol and 10% PBTA. Embryos were mounted on microscope slides and a cover slip was sealed over the sample. Using fluorescence microscopy, the number of embryos containing visible sperm tail, observed as a bundled coil in the anterior of the embryo, was recorded as the

proportion of fertilized embryos. Fertilization success was analyzed for each maternal type in conspecific vs. heterospecific crosses (e.g., N♀ x N♂ vs. N♀ x A♂) using a 2-sample test for equality of proportions using R version 2.5.1.

Sperm storage and motility: Sperm storage and motility was assessed under two regimes. For the first regime, *D. novamexicana* females were singly mated to a conspecific male (n=26) or a heterospecific male (n=24). A subset of females from each of the two crosses was subsequently dissected 1, 2 and 3 days after copulation. All dissections included only the vagina with attached spermathecae and seminal receptacles. These organs were place on a slide containing *Drosophila* Ringer's solution and a cover slip was placed over the sample. Sperm motility was qualitatively assessed under phase microscopy. Images were obtained using phase microscopy to visualize the motile sperm as colored lines within the storage organs.

In the second regime *D. novamexicana* females were kept in 2% agar. The number of eggs laid by each female before dissection (7 days after insemination) was recorded. Reproductive organs were dissected as described and viewed under a light compound microscope. Dissections in which any of the storage organs were severed or ruptured were discarded. The presence or absence of sperm in either the spermathecae or ventral receptacle was recorded for each individual female. Motility was assigned for each of the two storage organs separately on the basis of whether the sperm mass displayed steady oscillating motion. A 2-sample test for equality of proportions was performed on storage dynamics and motility separately using R version 2.5.1.

Progeny counts in double-matings

All flies used in double matings were collected and aged as described previously. Females from the *D. americana* line (NN97.4red) and *D. novamexicana* line (NM1031.4) were mated to a single male followed by a second male ~24 hours later in four different

orders: conspecific-conspecific (++), conspecific-heterospecific (+-), heterospecific-conspecific (-+), and heterospecific-heterospecific (--). Single conspecific and heterospecific matings were also simultaneously performed as described above. The progeny produced by each female were identified as conspecific or heterospecific progeny. In crosses involving *D. novamexicana* females, heterospecific progeny were darker than conspecific progeny. In crosses with *D. americana* females, heterospecific progeny lacked the mutant red eye color phenotype that is present in the *D. americana* line and were also lighter in pigmentation than their conspecific siblings. The number of conspecific progeny in double matings was analyzed statistically using the Welch Two Sample t-test in comparison with conspecific single matings (R version 2.5.1). The number of hybrid progeny in double matings was analyzed statistically using the Welch Two Sample t-test in comparison with heterospecific single matings (R version 2.5.1).

Results

Progeny counts in conspecific vs. heterospecific crosses and backcrosses

Crosses between *D. americana* and *D. novamexicana* produce a reduced number of progeny relative to crosses within species (Figure 3). A female *D. novamexicana* crossed to a male *D. americana* produces ~98% fewer progeny ($t = 20.0$, d.f. = 25, $p < 0.05$), while a female *D. americana* crossed to a male *D. novamexicana* produces ~70% fewer progeny ($t = 10.7$, d.f. = 26, $p<0.05$). The difference in the number of progeny produced conspecifically between *D. novamexicana* and *D. americana* is not significantly different ($t = -2.02$, d.f. = 37, $p> 0.05$) with both crosses producing 60-70 progeny. Heterospecific crosses with *D. novamexicana* females produce on average 1 individual, which is significantly less than the average produced by heterospecific crosses with *D.*

americana females ($t = -13.02$, $df = 25$, $p<0.05$). All crosses produced a 1:1 ratio of males to females ($\chi^2 = 0.021$, $p>0.05$).

Egg hatch rate in conspecific vs. heterospecific crosses and backcrosses

Conspecific vs. heterospecific crosses: The hatch rates in all four crosses as performed above are shown graphically in Figure 4. The conspecific hatch rate for *D. novamexicana* and *D. americana* is 95.2% and 94.3% respectively. The hatch rate between *D. novamexicana* females crossed to *D. americana* males is 2.4%, which is significantly lower than in the conspecific cross with *D. novamexicana* females ($t = 26.8$, $p<0.05$). In the reciprocal cross between *D. americana* females and *D. novamexicana* males, the hatch rate is 45.0%, which is also significantly lower than in the conspecific cross with *D. americana* females ($t = 13.4$, $p< 0.05$).

Backcrosses: The hatch rates in the four backcrosses are presented graphically in Figure 5. The results show that the two types of F1 males backcrossed to *D. novamexicana* females exhibit a considerable decrease in the frequency of hatched eggs relative to the parental conspecific cross involving *D. novamexicana* females ($t = 18.1$ for N♀ x F1(N) ♂ and 24.3 for N♀ x F1(A) ♂, $p<0.05$). F1 males from the cross with *D. novamexicana* as the maternal parent produce a significantly higher rate of egg hatch than F1 males with *D. americana* as the maternal parent when backcrossed to *D. novamexicana* females (19.8% and 8.8% respectively) ($t = 6.4$, $p <0.05$). In the backcross of the two types of F1 males to *D. americana* females, egg hatch is also significantly reduced relative to the parental conspecific ($t = 7.9$ for A♀ x F1(A) ♂ and $t = 11.5$ for A♀ x F1(N) ♂, $p< 0.05$). A similar pattern is observed in the backcross to *D. americana* females where F1 males from the parental cross of a *D. americana* female produce a significantly higher hatch rate than F1 males from the parental cross of a *D.*

novamexicana mother. This pattern suggests that there is a grand maternal effect in which, for example, F1 males carrying *D. novamexicana* alleles on the X-chromosome or mtDNA are more compatible with *D. novamexicana* females than are F1 males carrying the comparable alleles of *D. americana*.

Reproductive incompatibility assay

The similarity between hatch rate and average number of progeny indicates that the reduction in progeny number is primarily due to a failure prior to hatching of laid eggs which led to the design of three experiments to determine the mechanism of incompatibility in the heterospecific crosses. Only crosses with *D. novamexicana* females were used due to the higher level of incompatibility expressed in this cross.

Embryonic development: The pattern of syncitial development in embryos aged to 5 different time points described in the Methods section was assessed using fluorescence microscopy to determine the proportion of eggs corresponding to each stage of nuclear division. The data for the N♀ x N♂ cross (control) and N♀ x A♂ cross are shown in Figure 6 and Figure 7, respectively. In the control cross, the stages of syncitial nuclear division progress gradually with time (96% successful development). In the heterospecific cross (N♀ x A♂), the majority of embryos (95%) fail to initiate successful embryonic development. The difference in rate of successful development between the two crosses is highly significant ($t = 24.5$, $p<0.05$).

Fertilization: A sperm-tail specific anti-body, α-XT, was used to visualize sperm-tail in embryos laid in all four crosses between and within *D. americana* and *D. novamexicana*. Embryos were determined to have been successfully fertilized if they contained a coiled sperm tail in the anterior portion of the embryo (Figure 8). No case of

partial or incomplete fertilization was observed as has been previously noted in crosses between two races of *D. melanogaster* (Alipaz et al., 2001). The results, shown in figure 9 alongside hatch rate measurements from embryos produced from the same batch of crosses, indicate that the percentage of fertilized eggs corresponds to the percentage of hatched eggs in all four crosses. This result, along with the embryonic development assay, indicates that the incompatibility is occurring before or at the onset of fertilization.

Sperm transfer and storage: An assessment of motility and storage dynamics between both con- and heterospecific inseminations was conducted under two different regimes: one in which females were provided a medium for oviposition immediately after insemination, and another in which oviposition was inhibited. These data are summarized in Table 1. Example images are shown in Figure 10. Even though this method is not sensitive to differences in strength and/or number of motile sperm, it represents a qualitative measure of sperm motility during storage in the female reproductive tract. What can be concluded from this experiment is that under conditions conducive to oviposition, the pattern of motility within storage organs is indistinguishable between con- and heterospecific inseminations. However, this design does not allow the inference as to whether heterospecific sperm lose viability or are expelled from the female reproductive tract if stored for long periods of time, and furthermore, whether they are capable of successful fertilization.

In order to address long-term sperm viability, females were held in suboptimal conditions for oviposition (2% agar). To assess this method, the number of eggs laid by each female was counted (Table 2). Mated females from both crosses were also held in regular yeasted cornmeal medium to compare the number of eggs laid, but counting the exact number of eggs was difficult because of the large number of eggs and the difficulty in visually detecting embryos in the medium. Nevertheless, the number of eggs laid by *D. novamexicana* was conservatively estimated from both the average number of progeny

and the hatch rate data in the conspecific cross to be ~80 eggs/insemination, which is likely an underestimate. Table 2 shows that ~50-60% of females in both crosses fail to lay any eggs on 2% agar, while the maximum number of eggs laid by a female was 31. This suggests that holding females in 2% agar, though not ideal, may be useful in forcing long-term storage of sperm within the female storage organs. Only crosses involving *D. novamexicana* females were used to maximize detection of the effect since this is the cross in which reproductive success is reduced by ~98%.

In each dissected female, the intact ventral receptacle and two spermathecae were classified according to whether they contained sperm, and whether the sperm in that compartment was motile. Figure 11 shows the results for sperm storage within the two storage organs. In the conspecific cross, sperm was found in all cases to be stored in both storage organs, whereas 41% of heterospecifically inseminated females contained sperm only in the spermathecae ($\chi^2 = 11.2$, $p<0.05$). Figure 12 shows the results for sperm motility within the two storage organs. With conspecific insemination, motile sperm were detected in both storage organs in all inseminated females, whereas 18% of heterospecifically inseminated females contained motile sperm only in their spermathecae ($\chi^2 = 20.7$, $p<0.05$). These results indicate that there is substantial loss of sperm in the heterospecific cross particularly from the seminal receptacle. The low percentage of motile sperm found only in the spermathecae reveals the inviability of heterospecific sperm during long-term storage in these organs.

Progeny counts in double-matings

This experiment is designed primarily to test three hypotheses: (1) whether the ejaculate of heterospecific males incapacitates the female reproductive tract, which would be revealed by a reduction in fertilization success of the conspecific second male following a heterospecific insemination, (2) whether the heterospecific ejaculate directly

influences the reproductive success of conspecific sperm, and (3) whether the presence of a conspecific ejaculate influences the reproductive success of the heterospecific sperm. The summary of all double crosses is shown graphically in figure 13. In addressing the first hypothesis, a comparison between single conspecific crosses (e.g., N+) and double heterospecific-conspecific crosses (e.g., N-+) reveals that the number of conspecific progeny in the latter crosses (μ=57, stdv=2.9) is reduced relative to the former crosses (μ=70, stdv=2.2) when *D. novamexicana* is the female in the cross ($t = 3.4$, $df = 41$, $p < 0.05$) (Fig 13a). This result suggests that the heterospecific ejaculate reduces the reproductive success of conspecific sperm through incapacitation of the female since this reduction occurs with either order of mating. In the N+- cross the number of conspecific progeny is also significantly less (μ=56, stdv=5.4) than in single conspecific inseminations ($t = 3.3$, $p< 0.05$). This result is consistent with an overall reduction of the female's susceptibility to fertilization by conspecific sperm in the presence of the heterospecific ejaculate. In the cross in which *D. americana* is the female, the A-+ cross produced a mean of 77 (stdv= 8.1) conspecific progeny whereas the single conspecific cross produced a mean of 73 (stdv= 4.4), suggesting that sperm of conspecific males remains functional in the presence of heterospecific ejaculate ($t = 0.5$, $p>0.05$)(Fig 13b).

The second hypothesis suggests that the heterospecific ejaculate could directly interfere with the reproductive success of conspecific sperm. This would be observed in cases where the heterospecific sperm follows the conspecific sperm in order of insemination. In the cross in which *D. americana* is the female, the A-+ cross produced a mean of 77 (stdv= 8.1) conspecific progeny whereas the single conspecific cross produced a mean of 73 (stdv= 4.4), suggesting that sperm of conspecific males remains functional in the presence of heterospecific ejaculate ($t = 0.5$, $p>0.05$)(Fig 13b). However, the reciprocal order (A+-) produced a mean of 50.5 (stdv=4.84), which is significantly lower than progeny number in the single conspecific cross ($t = 2.9$, $df = 19$, $p< 0.01$). The difference in the number of conspecific progeny produced between the two

orders of mating can be explained by direct interference between sperm since it is observed only in one direction.

The third hypothesis addresses whether conspecific sperm precedence (CSP) is operating in double inseminations. One indication of CSP would be a reduction in the number of hybrid progeny produced in doubly inseminated females versus in females singly inseminated by a heterospecific male. *D. novamexicana* females produce a mean of 1 individual per insemination (stdv=0.4). In double inseminations, *D. novamexicana* females on average produce the same number of hybrid progeny regardless of the order of mating (N- compared to N+-: $t = 1.1$, $p > 0.05$; N- compared to N-+: $t = 1.3$, $p > 0.05$) (Fig 13a). In the reciprocal cross (Fig 13b), however, *D. americana* females on average produce fewer hybrids in double inseminations (A+- =8, stdv= 5.6 & A-+=9, stdv=2.2) than in both single and double heterospecific inseminations (A- =23, s.d.=1.6 & A--=26, stdv=1.3)(A- vs. A+-: $t = 4.2$, $p < 0.05$; A- vs. A-+: $t = 5.1$, $p < 0.05$). This is indicative of a reduction of fertilization success of heterospecific sperm in the presence of conspecific sperm. This may be an instance of CSP in one direction.

Discussion

Evidence for asymmetric assortative fertilization between the two closely related species, *D. americana* and *D. novamexicana*, has been provided. *D. novamexicana* females produce ~98% less progeny in heterospecific matings than in conspecific matings, while *D. americana* females produce ~70% less progeny in heterospecific matings than in conspecific matings. This reduction in reproductive success is primarily due to an incompatibility occurring prior to the hatching of embryos, as indicated by the correspondence between number of progeny produced and hatch rate in all four crosses.

The analysis of the backcross progeny was limited deliberately since the objective was to analyze the parental cross. These results, however, point to two observations: (1) F1 males sire more progeny when mated with *D. novamexicana* or *D. americana* than parental males in heterospecific crosses, indicating the involvement of multiple autosomal loci (Figure 2), and (2) F1 males sire more progeny when mated to females of their maternal type, indicating the occurrence of a grand maternal effect in the backcross progeny. Further genetic analysis is needed to identify the loci responsible for this incompatibility.

The reproductive incompatibility assay revealed that the incompatibility occurs after copulation and prior to fertilization. It was further determined that stored sperm is lost and/or incapacitated after prolonged storage. In order to improve the detection of differences in storage dynamics in conspecific vs. heterospecific inseminations, oviposition was minimized by keeping inseminated females in agar medium. Sperm was stored in both spermathecae and seminal receptacle and remained motile in all conspecific inseminations, whereas less than half of the dissected females from heterospecific inseminations contained sperm only in the spermathecae and only ~18% were classified as containing motile sperm. The assignment of motility in the latter case is likely an overestimate of the reproductive functionality of sperm in terms of reproductive success mainly because the observations lack quantitative measurements of the number of motile (i.e., functional) sperm, however, a qualitative assessment would suggest that the motile sperm in the heterospecific inseminations represent a small fraction compared to those in conspecific inseminations. In other words, it does not follow that heterospecifically inseminated females have a similar number of functional

sperm as conspecifically inseminated females just because the former females were classified as containing motile sperm. Rather, they contain less functional sperm based on qualitative assessment. Furthermore, these results do not rule out the possibility of an additional disruption such as sperm failing to penetrate the egg mycropyle. Nonetheless, the objective here is to determine the post-copulatory, pre-zygotic stage at which the incompatibility is realized.

The results discussed thus far provide a number of conclusions. First, it is clear that the dominant form of reproductive isolation between the two species acts prezygotically. Second, behavioral isolation was not contributing to the reduction in fertilization success in all crosses performed, and therefore is ruled out as a prezygotic barrier. Previous work has shown no significant sexual isolation between *D. americana* and *D. novamexicana* (Speith, 1951) and therefore can not be assumed to evolve faster than other forms of prezygotic reproductive incompatibilities between these two species. Third, gametic isolation between the two species is likely due to rapid divergence of biochemical components of the male ejaculate or sperm, which have been shown to diverge rapidly in allopatry (Swanson et al., 2001a; Swanson et al., 2001b; Swanson & Vacquier, 2002; Jagadasheen & Singh, 2005)

Results of double mating provided tests of three hypotheses: (1) whether the heterospecific ejaculate incapacitates the female thereby reducing the reproductive success of the second conspecific male, (2) whether the heterospecific ejaculate directly interferes with the reproductive success of conspecific males, and (2) whether conspecific males reduce the reproductive success of heterospecific males when both ejaculates are present in the female reproductive tract.

The first and second hypothesis predicts a decrease in reproductive success experienced by conspecific males in the presence of the heterospecific ejaculate. Two general explanations can account for this reduction. The first mechanism of fertilization reduction may be indirect, that is, through toxic effects to the female, which in turn result in lower success of conspecific sperm. This has been shown to be the case only in crosses with *D. novamexicana* females, regardless of the order of the double matings (N-+ or N+-). The second is direct interference between heterospecific and conspecific male ejaculates. As discussed, male-male competition and male-female coevolution resemble an arms race in which males evolve more effective toxic compounds that raises their likelihood of fertilization in the presence of other ejaculates (Chapman et al., 1995). Allopatrically diverged toxic compounds of *D. americana* males, for example, may present a unique set of toxic effects that are not normally encountered by *D. novamexicana* males, thereby reducing the fertilization success of the latter in the presence of the ejaculate of the former. A case of direct interference is observed in the double cross with *D. americana* females when mated first to *D. americana* male (A+-), but not in the reciprocal order.

The third hypothesis addresses the opposite effect: whether conspecific males reduce the reproductive success of heterospecific males as would be the case in conspecific sperm precedence. In crosses involving *D. novamexicana* females, the number of hybrid progeny produced on average is the same in single matings and the two double matings with conspecific and heterospecific males. Since the number of hybrid progeny in this cross is very low (~1), it would not be possible to detect instances of conspecific sperm precedence given the sample size.

In crosses involving *D. americana* females, however, the number of hybrid progeny produced is significantly less in both orders of double mating than in single inseminations with only heterospecific males. A possible explanation is that the conspecific ejaculate confers an advantage over the heterospecific ejaculate, thereby demonstrating a case of CSP, which leads to reduction in the number of hybrids. The mechanism of CSP would suggest that reproductive success of the heterospecific ejaculate is reduced in the presence of the conspecific male ejaculate, while reproductive success of the conspecific male remains the same; contrary to the increase in conspecific fertilization success as previously reported in *D. mauritiana* and *D. simulans* (Price et al., 1997).

Gametic isolation can be brought about by two types of evolutionary interactions: male-female co-evolution and male-male competition, which generally lead to faster evolution of genes involved in mating system interactions. It is of interest to understand how these mechanisms arise as a consequence of allopatry and whether they are more prevalent than postzygotic barriers. Theoretical predictions suggest that prezygotic barriers are likely to evolve faster than postzygotic barriers through sexual selection (Gavrilets, 2000). This has also been demonstrated experimentally through pairwise comparisons of the strength of pre- and postzygotic barriers among species within the same genus (Mendelson, 2003). One conclusion to be drawn from this and other studies on allopatric divergence and its initial consequences is that mating system evolution may contribute largely to prezygotic reproductive incompatibilities between recently diverged allopatric taxa.

Antagonistic sexual selection as a result of male-male competition would drive the divergence of the male reproductive machinery in allopatry (Rice, 1996; Rice & Holland, 1999). Rice (1996) performed an experiment in which *D. melanogaster* females were prevented from coevolving with males, who were in turn allowed to coevolve with a different strain of females (Rice, 1996). In essence, this experiment simulates allopatric divergence between males and females of the same species. This outcrossing design was continued for 30 generations after which it was found that experimental females had a higher mortality rate, due to reduced adaptation to foreign male's toxic ejaculate, when mated with experimental males than with control males. Furthermore, experimental males were more efficient at mating females and displacing the ejaculate of other males (Rice, 199). This result demonstrates the rapid coevolution between males and females in the same population. It further suggests that male-female coevolution is analogous to an "arms-race" dynamic in which males are adapting to more efficiently compete with other males' ejaculates while females are adapting to the increasing toxic effects of male ejaculates (Chapman et al., 1995).

Co-evolution between the sexes also results in rapid evolution of sex-specific phenotypes, such as sperm length, which has been shown to correlate with higher fertilization success (Miller & Pitnick, 2002). There have also been many studies on the seminal fluid components of sperm which demonstrate that accessory gland proteins (Acps) play a major role in the efficient storage of sperm, sperm competition, rate of oviposition, and inducing necessary morphological changes in the female reproductive tract which may be necessary for successful sperm storage (Tram & Wolfner, 1999; Neubaum & Wolfner, 1999; Qazi & Wolfner, 2003; Adams & Wolfner, 2007). This

super-family of proteins has been shown to have an elevated rate of evolution (dn/ds) (Swanson et al. 2001a, Swanson et al., 2001b; Swanson & Vacquier 2002). Furthermore, the elevated rate of evolution has been shown to be significantly higher in genes expressed in males (due to sexual selection) than in females, even though females do show an elevated rate of evolution in a small fraction of genes expressed in the female reproductive tract (Swanson et al. 2001a, Swanson et al., 2001b; Swanson & Vacquier 2002). Jagadeeshan & Singh (2005) report similar results when investigating genes expressed in testis, ovary, and head tissue. Rapidly evolving genes are differentially distributed with the majority being expressed in the testis. The ovaries contain the second largest group of these genes, and finally the head contains the remainder. They attribute this phenomenon to differential selection pressures driving the rapid evolution of genes expressed in sex-specific tissue, and furthermore, they suggest that this divergence is driven by positive selection (Jagadasheen & Singh, 2005).

The allopatric status of *D. novamexicana* and *D. americana*, the prezygotic barrier between them, and their recent divergence fulfill the requirement for independent reproductive system evolution within the two taxa. The findings presented in this chapter indicate that gametic incompatibility can arise rapidly in allopatry (~0.4 my), and may be a result of rapid male-female co-evolution. This would represent a clear example to support the prevalence of prezygotic isolation as a result of rapid mating system evolution. These findings reinforce the notion that most speciation events occur in allopatry. It further suggests that divergence in allopatry is likely driven by positive Darwinian selection and/or sexual selection.

CHAPTER III

INTRASPECIFIC VS. INTERSPECIFIC PATTERNS OF ISOLATION

Introduction

The previous chapter provided evidence of assortative fertilization between the closely related allopatric species pair *D. americana* and *D. novamexicana*. Understanding the genetic mechanisms involved in the realization of this gametic incompatibility is of significant interest. Furthermore, it is necessary to understand the changes in the genes involved in order to identify the evolutionary forces that caused their divergence. In multiple studies speciation genes have been described as genes undergoing positive Darwinian selection and do not fall under a particular functional class, but tend to be involved in "normal" physiological functions (Orr, 2005).

The gene(s) involved in the postmating-prezygotic barrier between *D. americana* and *D. novamexicana* are likely genes that are expressed (but not necessarily exclusively) during reproduction whereby the gene products from one sex are incompatible with their target in the other sex. Furthermore, the asymmetry of the incompatibility indicates a difference in the divergence pattern between the two species that could be caused by different genetic factors.

The genes involved in the incompatibility between *D. americana* and *D. novamexicana* are unknown, however, a number of studies have examined the pattern of genetic divergence at multiple loci within the virilis group. These studies include analyses of chromosomal divergence (Sturtevant, 1938; Sturtevant & Novitski, 1941; Patterson, 1941; Stone & Patterson, 1947; Hsu, 1952) and DNA sequence divergence (Hilton & Hey, 1996; Hilton & Hey, 1997; Spicer & Bell, 2002; Caletka & McAllister,

2004; Orsini et al., 2004; Morales-Hojas et al., 2008). These studies indicate two trends in sequence divergence between the two species. The first is that *D. novamexicana* variants at a number of loci constitute a subset of sequence variation within *D. americana* (Hilton & Hey, 1996; Hilton & Hey, 1997; Caletka & McAllister, 2004). Furthermore, phylogenetic incongruence is observed for genes associated with inversions, i.e., inverted karyotypes cluster together in a phylogeny irrespective of the species status (Morales-Hojas et al., 2008). An overview of these studies is given below.

Genetic divergence

Chromosomal rearrangements: Analyses of karyotypes within the virilis group have been performed in the early to mid 20th century through preparations of polytene chromosome squashes from salivary glands (Sturtevant, 1938; Sturtevant & Novitski, 1941; Patterson, 1941; Stone & Patterson, 1947; Hsu, 1952). These analyses have proven useful in determining genetic relationships among members of the group. The *D. virilis* karyotype, which is the primitive karyotype within the genus, consists of five pairs of rod chromosomes and a pair of dot chromosomes (Fig. 1). This karyotype has been preserved in the lineage leading to *D. lummei* and *D. novamexicana*; however, two chromosomal fusions have occurred in the *D. americana* lineage: one fusion consists of joining chromosomes 2 and 3 (Muller elements E and D respectively) and is evidently fixed in populations of *D. americana*. Another fusion has occurred between the X and 4th chromosomes (Muller elements A and B respectively) (Fig. 1). This X-4 fusion is polymorphic within populations of *D. americana* and exhibits a geographic frequency distribution that is highly correlated with latitude (McAllister, 2002; McAllister et al., 2008). Other than the differences between *D. americana* and *D. novamexicana* due to chromosomal fusion, several derived inversions are also distributed between these species (Hsu, 1952).

These chromosomal variations, particularly the X-4 fusion, have lead Patterson & Stone to propose a hybrid origin for *D. a. americana* (Patterson and Stone, 1952). Even though plausible, this explanation is not the most parsimonious (Throckmorton, 1982). Contemporary patterns of chromosomal variation are best explained by lineage sorting from the common ancestor, where particular rearrangements have been fixed in *D. novamexicana* but are still segregating in *D. americana* (Throckmorton, 1982).

Sequence divergence: Resolving the phylogenetic history of *D. americana* and *D. novamexicana* has been the focus of several molecular phylogenetic analyses (Hilton & Hey, 1996; Hilton & Hey, 1997; Spicer & Bell, 2002; Caletka & McAllister, 2004; Orsini et al., 2004; Morales-Hojas et al., 2008). Hilton & Hey (1996) and Hilton & Hey (1997) used sequences from the *period* and *oskar* loci to assess the evolutionary relationship between the two species. Sequences from both genes indicate that *D. novamexicana* comprises two monophyletic clusters embedded within paraphyletic alleles of *D. americana* (Hilton & Hey, 1996; Hilton & Hey, 1997). Orsini et al. (2004) used a multilocus microsatellite approach to resolve the phylogeny of the virilis group. Their results indicate that some of the microsatellite loci within the unfused X-4 variants of *D. americana* (*D. a. texana*) are more similar to those of *D. novamexicana* than they are to *D. americana* (Orsini et al., 2004). In another study (Caletka & McAllister, 2004) in which two mtDNA genes, Cytochrome b and Cytochrome-c oxidase subunit II, were examined, all *D. novamexicana* alleles were monophyletic, although weakly supported by all methods, and embedded among alleles of *D. americana*, while the remaining *D. americana* sequences were paraphyletic and randomly distributed along the clade with respect to the X-4 rearrangement status and geographic locale, further indicating that the variants found in *D. novamexicana* are a subset of the variation that is currently segregating in *D. americana* (Caletka & McAllister 2004).

These studies show that reciprocal monophyly is yet to be established between *D. americana* and *D. novamexicana* at a number of loci. One explanation is that low

sequence divergence between the two species is a result of shared ancestral variants due to recent divergence. This is also consistent with the idea that the initial split between the two species has been a result of peripatric divergence of a small subset of the *D. novamexicana- D. americana* ancestral population from the larger population that ultimately gave rise to the *D. novamexicana* lineage. This type of phylogenetic incongruence resulting from sorting of ancestral polymorphism is commonly observed between closely related species. It has been proposed that the phylogenetic incongruence is due in part to large differences in population size and higher nucleotide diversity in *D. americana*, which confounds efforts to establish genealogical independence for *D. novamexicana* (Caletka & McAllister, 2004). It has also been proposed that the incongruence may be largely due to associations of genes with inversions that have not yet completely sorted between the two lineages (Morales-Hojas et al., 2008; McAllister, unpublished data). Therefore, different phylogenetic results are obtained based on whether the genes analyzed fall within inverted or collinear regions.

Reproductive divergence within and between *D. americana* and *D. novamexicana*

Considering the overall pattern of divergence that is indicated by the above studies along with the level of reproductive divergence suggests that the reproductive incompatibility between *D. novamexicana* and *D. americana* has been the result of rapid divergence in allopatry. The differences in the genes involved in this process are expected to be fixed in the respective interbreeding groups if the pattern of gametic isolation is indeed a species wide phenomenon.

The hatch rate method is used here to measure the degree of reproductive incompatibility between and within species. Two main questions can be addressed by performing these measurements. The first has to do with the pattern of isolation within

and between species and the contrasts of these results with the level of genetic divergence. Measurements of sequence divergence in eight genes have indicated that the level of divergence within *D. americana* is indistinguishable from divergence measurements between *D. americana* and *D. novamexicana* (average divergence within *D. americana* = 0.028, between *D. americana* & *D. novamexicana* = 0.023) (McAllister, unpublished data). This would further allow for ascertaining whether there is a correlation between reproductive incompatibility and sequence divergence at the genes analyzed.

The second matter that can be addressed is whether there is any variation in the level of reproductive incompatibility within species. This may be due to two reasons. First, it may be the case that laboratory lines exhibit a certain degree of gametic incompatibility as a result of independent evolution within each line. Second, reproductive incompatibility between iso-female lines of the same species may be a result of population substructure that simulates micro-allopatric divisions within species. The prediction would be that the hatch rate within iso-female lines would be higher than between lines if a given species exhibits high levels of population subdivision.

Materials & methods

The level of reproductive incompatibility within and between species was measured for six lines of *D. americana* and two lines of *D. novamexicana*. The *D. americana* lines were: NN97.4red, HI99.14, PM99.32, LA99.46, FP99.4 and ML97.5 (Collected by Bryant F. McAllister). The *D. novamexicana* lines used were NM1031.4 and NM1031.0. Population cages were established and egg hatch rates were measured as described previously for 27 out of the 32 possible pairwise crosses between and within species. Data were input into SAS and a generalized linear mixed model was fit using the PROC GLIMMIX procedure with a computation of the least squares means for each

cross and contrasts between crosses (SAS V. 9.1). A multivariate analysis of variance was also performed using the PROC GLM procedure for a regressions analysis of average hatch rate measurements for each cross as a dependant variable with the divergence estimates and cross type (inter- vs. intraspecific) and an interaction between the latter two variables as independent variables using SAS (V. 9.1).

Mitochondrial DNA sequences for two genes, Cyt-b and COII, for five americana lines (HI 99.14, PM 99.32, FP 99.4, LA 99.46, and ML 97.5-pur) and NM1031.4 were obtained as described in Caletka & McAllister (2004). Pairwise distance between each line was computed using the Kimura 2-Parameter method on Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

Results

In measuring the fertilization success between multiple iso-female lines from the same species, population cages were established for six lines of *D. americana* and two lines of *D. novamexicana*. Interspecific crosses with both *D. novamexicana* lines gave similar results, therefore only crosses with NM 1031.4 are shown. The hatch rates for all pairwise crosses performed are shown in Table 3. In order to assess the level of reproductive success within species, a comparison between within-line hatch rate and between-line hatch rate can be made. These results show that there are a number of cases in which the hatch rate between lines is significantly reduced relative to the hatch rate within lines. For example, NN97.4 females are most compatible reproductively with males from their own line and lose on average 10% of their reproductive success when crossed to males from any of the other lines used. Another example is LA99.46, which shows a ~10-15% reduction in hatch rate in three interline crosses with an even stronger reduction (~27%) in interline crosses with ML97.5. Incidentally, the reciprocal cross between LA99.46 and ML97.5 shows a similar reduction (~27%) relative to the intraline

cross involving ML97.5. Other crosses, however, show no significant difference between inter- and intra-line crosses. For example, FP99.4 and PM99.32 females are equally compatible with all other lines of D. americana used.

Interspecific crosses between the two species indicate that the pattern of reproductive incompatibility is the same across all iso-female lines. Crosses with *D. novamexicana* females exhibit a hatch rate of~1% except when crossed with The LA99.46 line of *D. americana* which results in a hatch rate of 15%. The hatch rate in reciprocal crosses between the two species ranges from 24% to 57%.

Figure 14 shows a plot of hatch rate within and between lines on the y-axis and genetic distance as computed for the two sequenced genes, cyt-b and CO-II. The plot indicates that there is no correlation between genetic distance in these two genes and hatch rate. Furthermore, the range of intraspecific hatch rate measurements within species falls as a cloud directly above the range of interspecific hatch rate measurements between species. The regression analysis between hatch rate as a dependant variable and divergence and cross type as independent variables show that cross type predicts hatch rate ($F = 28.61$, $p<0.05$). Divergence alone and divergence coupled with cross type are not significant predictors of hatch rate ($F = 4.01$, $p=0.05$ & $F = 0.00$, $p>0.05$ respectively).

Discussion

The results for inter- and intraline hatch rate show a hatch rate reduction in 11 of the 20 intraline crosses with *D. americana* relative to intraline crosses, which would be the null expectation. It should be noted that the reduction in 9 of these cases is in the order of ~10%. This pattern of reduction may be due to iso-female lines diverging in the lab or as a result of populations that exhibit a certain degree of subdivision.

These data allow the conclusion that the gametic incompatibility described in the previous chapter is indeed a phenomenon expressed in heterospecific crosses and not simply an artifact of laboratory strains. These data also indicate that there might be differential levels of compatibility between different lines of the same species, although this variation falls within range of what would be expected within lines of the same species. Finally, the level of reproductive isolation as measured by the hatch rate shows no correlation to the sequenced mitochondrial genes, suggesting that this form of isolation has accumulated much more rapidly than these genes have differentiated between the two species.

Contrasts between the level of reproductive incompatibility within and between strains and genetic divergence are consistent with the proposition that a subset of loci is undergoing rapid adaptive evolution within each population. Hatch rate measurements within and between species fall into three distinct clusters: two clusters representing the interspecific crosses between species and one cluster representing intraspecific crosses. These clusters do not overlap with respect to hatch rate; however, they do fall within the same range of divergence values for the genes analyzed. The variation in hatch rate within D. americana may be accounted for by polymorphisms that are segregating within the loci responsible. Furthermore, fixed differences at these loci between the two species must account for the severity of the effect in interspecific hybridizations.

Reproductive incompatibility between *D. americana* and *D. novamexicana* represents an example of prezygotic isolation arising rapidly in allopatry. This species pair is one of the few *Drosophila* species that is known to have diverged recently and is allopatric. In the general picture of speciation, the plausibility of the dominance of

prezygotic barriers that result from natural and sexual selection in allopatry is exemplified clearly between this species pair.

Figure 1: Phylogeny of the virilis phylad: A phylogeny representing relationships among the virilis phylad. The estimated times of divergence are indicated at each node. The karyotype for *D. virilis*, *D. lummei*, and *D. novamexicana* are shown to the right, and that of *D. americana* is shown to the left.

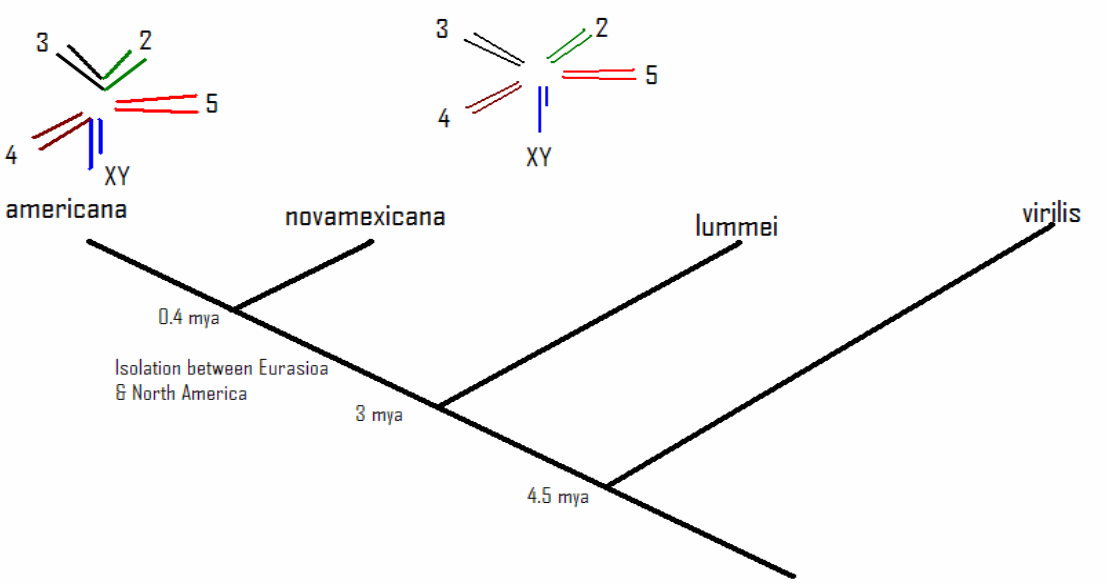


Figure 2: Geographic distribution of *D. americana* and *D. novamexicana*. Map of North America showing the geographic distribution and morphological features of of *D. americana* (blue) and *D. novamexicana* (yellow).

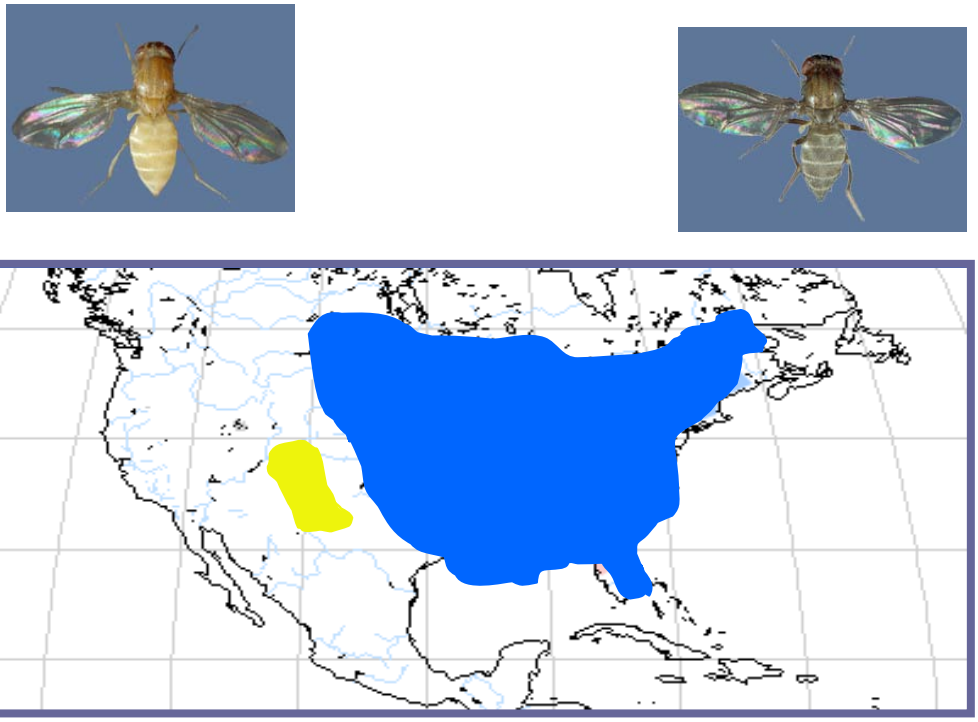


Figure 3: Progeny counts between *D. americana (NN97.4red)* and *D. novamexicana (1031.4)* from single copulations: The number of females analyzed for each cross is given in parentheses next to each cross type. Error bars represent 95% C.I.

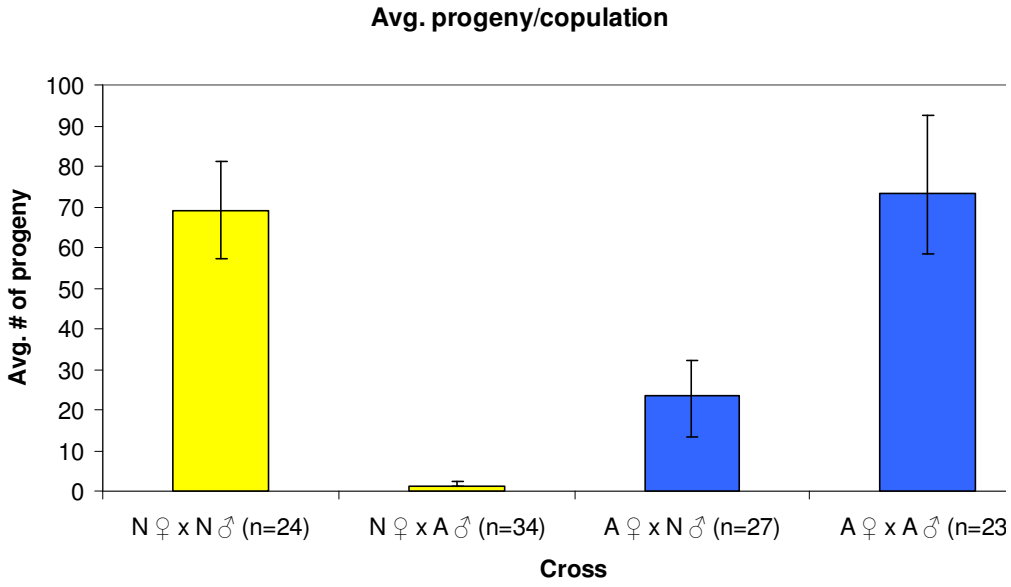


Figure 4: Egg hatch rate between *D. americana (NN97.4red)* and *D. novamexicana (1031.4)*: Cross type is indicated on the x-axis and proportion of hatched eggs on the y-axis. Error bars represent standard error.

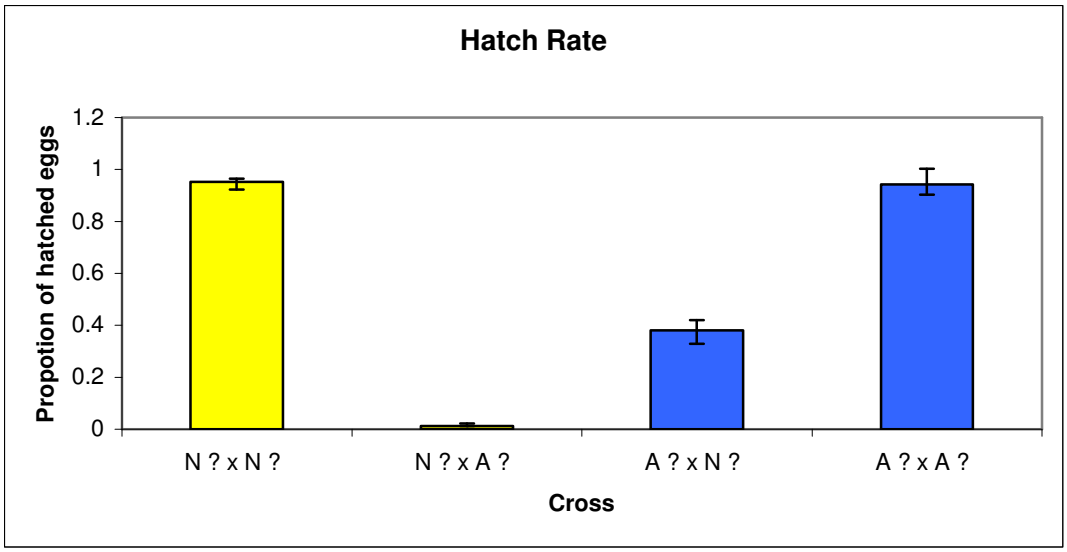


Figure 5: Backcross Hatch Rate: Cross type is indicated on the x-axis and the proportion of hatched eggs on the y-axis. For each backcross male, the maternal genotype of the F1 males is indicated in parentheses. Error bars represent standard error.

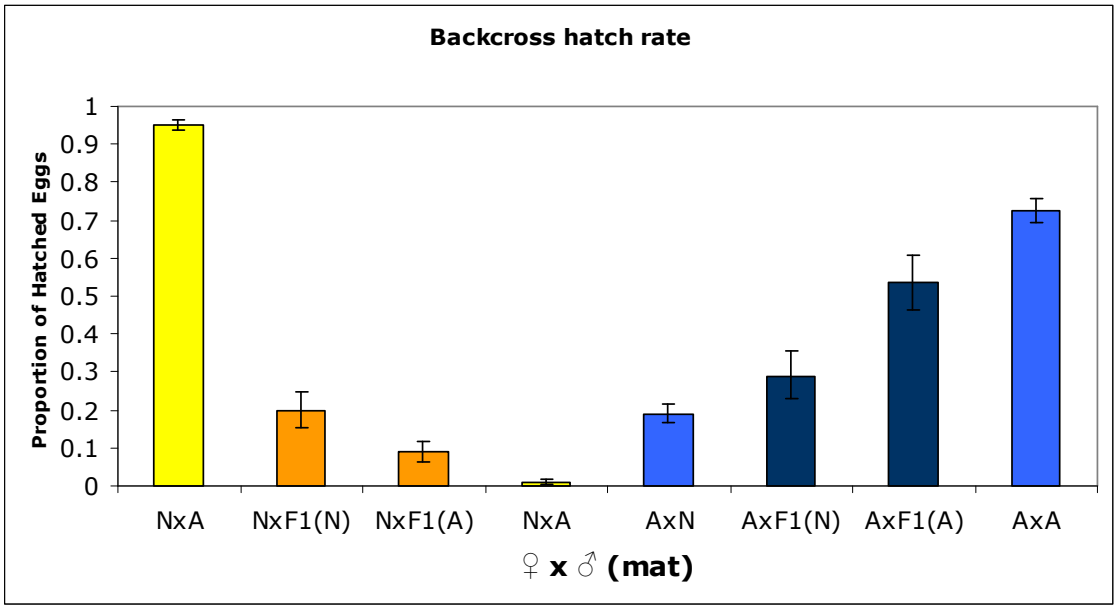
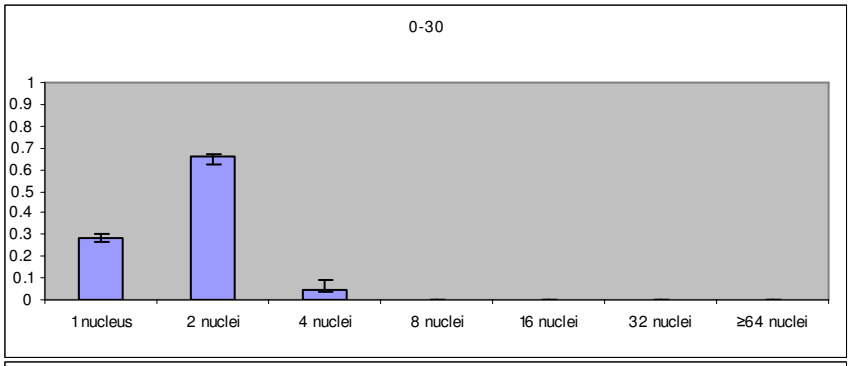
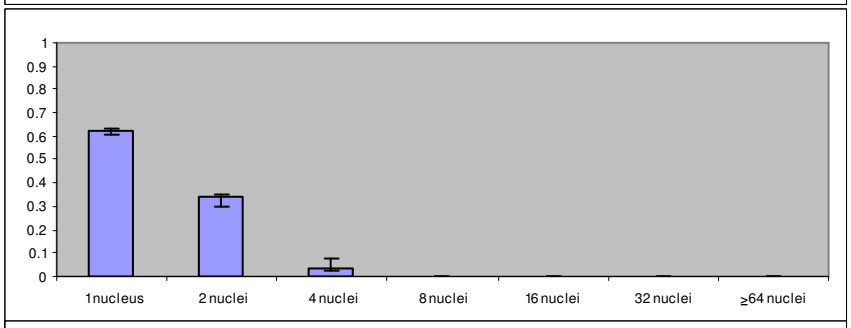


Figure 6: Embryonic Development Assay: N♀ x N♂: Graphs at each of the 5 time intervals represent the proportion of eggs displaying the 7 classifications of syncitial division. Error bars represent standard error.



0 - 30



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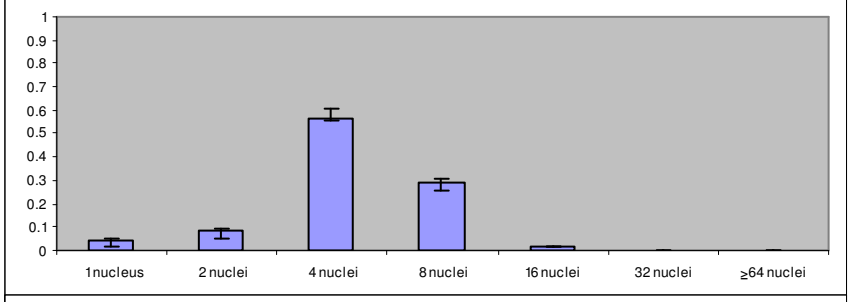

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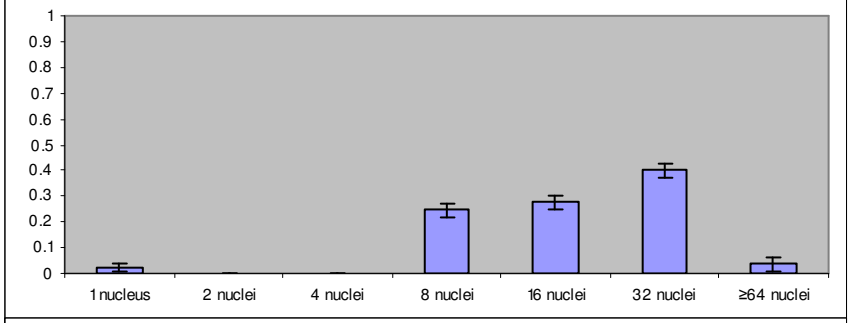
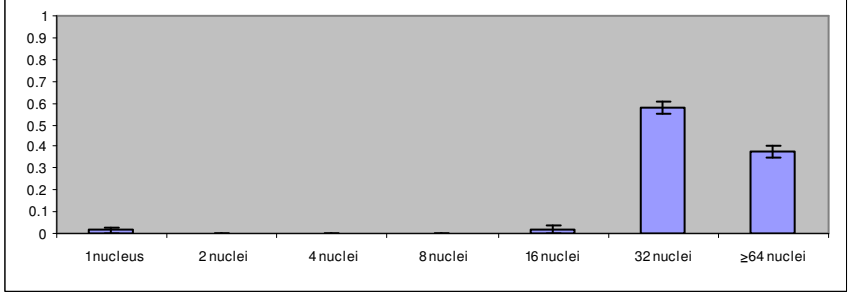
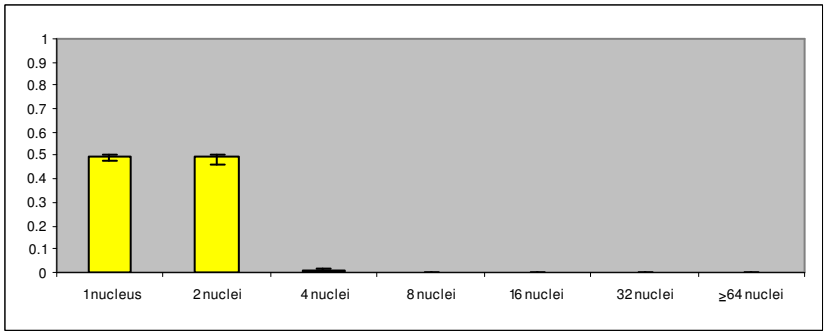


90 - 120

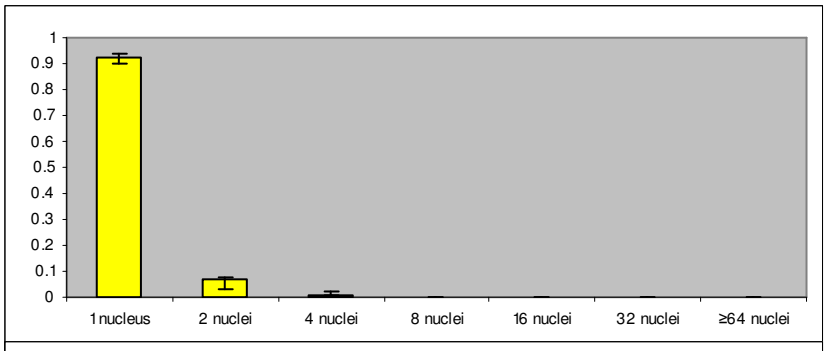


120 - 150

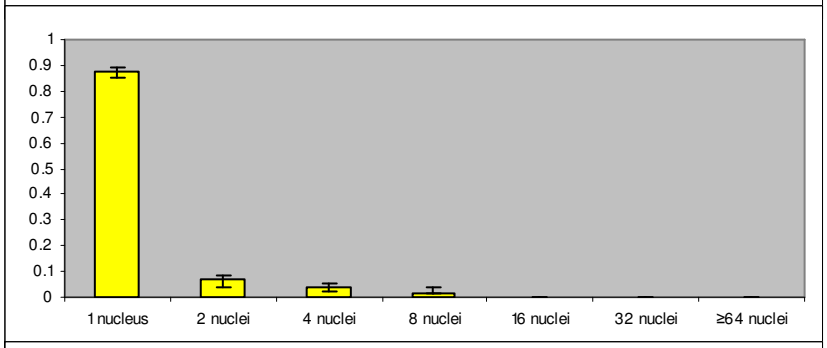
Figure 7: Embryonic Development Assay: N♀ x A♂: Graphs at each of the 5 time intervals represent the proportion of eggs displaying the 7 classifications of syncitial division. Error bars represent standard error.



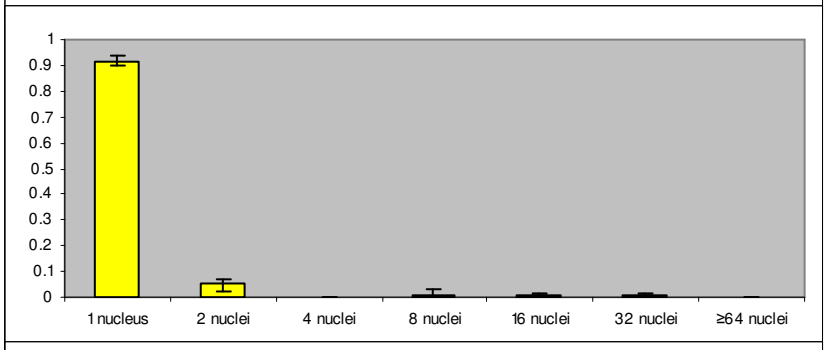
0 - 30



30 - 60



60 - 90



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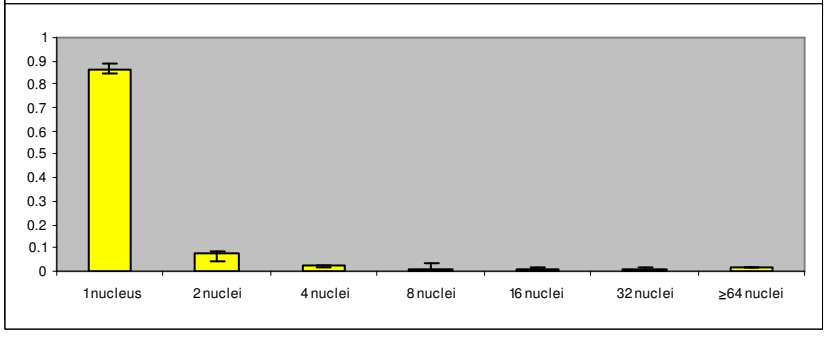
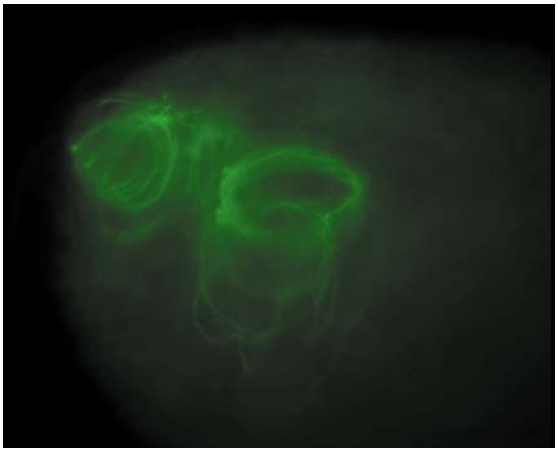


120 - 150

Figure 8: Visualizing Sperm Tail in Embryos of N♀ x N♂ vs. N♀ x A♂:

Sperm tail shown as coil in the anterior portion of embryos from the N♀ x N♂ cross.



Embryos from the N♀ x A♂ cross lacking sperm tail.

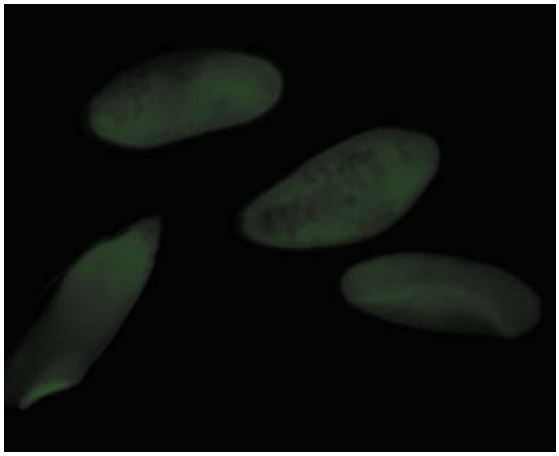


Figure 9: Proportion of Fertilized Eggs vs. Proportion of Hatched Eggs: The proportion of fertilized eggs is shown along side the proportion of hatched eggs. This demonstrates the close link between successful fertilization and hatchability. Lines used for the eggs collected for both fertilization and hatch rate assay were *D. americana NN97.4red* and *D. novamexicana 1031.4*. Error bars represent standard error

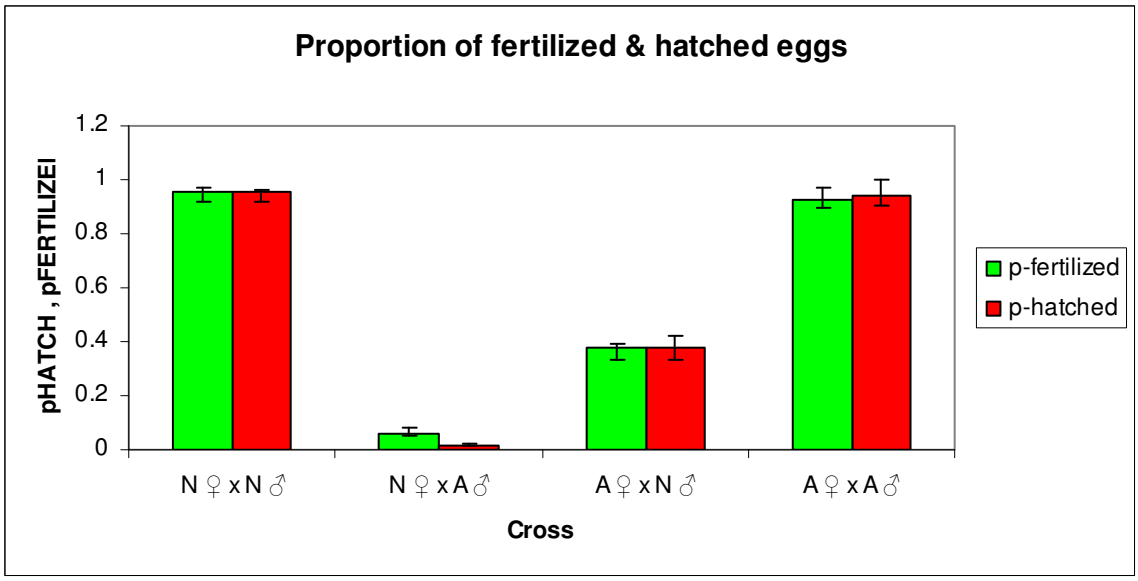
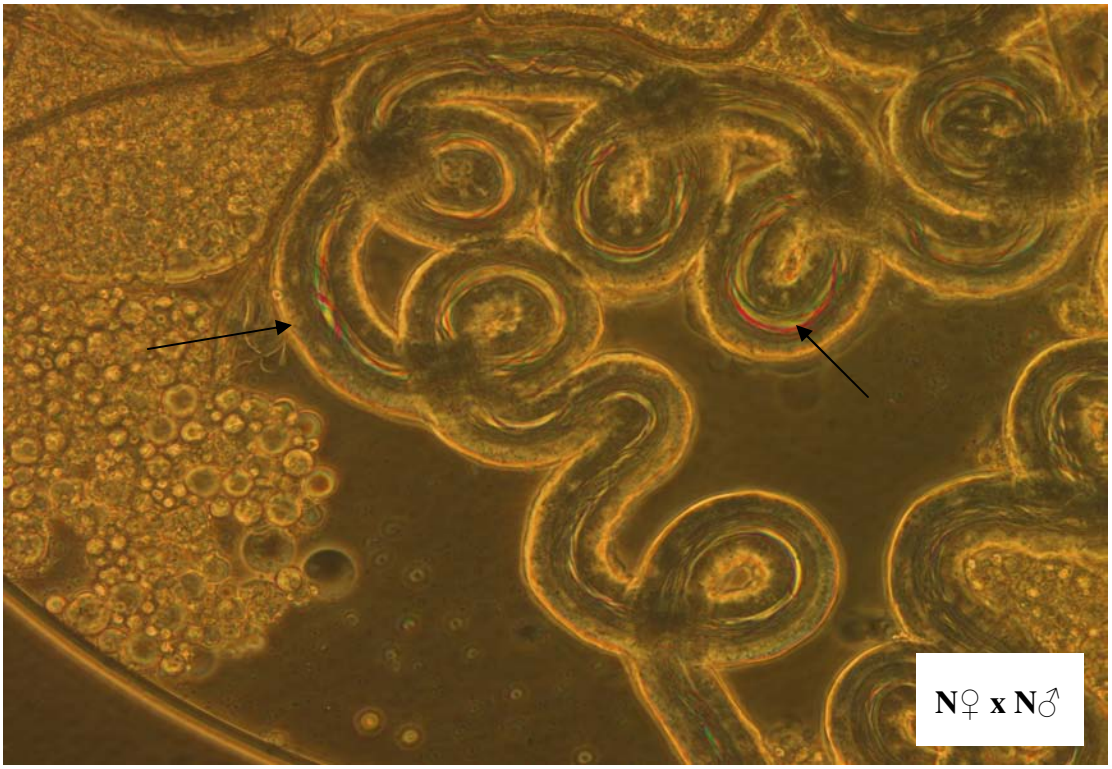


Figure 10: Motile sperm within storage organs: Representative images from four dissections across 4 consecutive days are shown for N♀ x N♂ and N♀ x A♂. Images are of seminal receptacles or spermathecae taken (a) ~6 hours after insemination, (b) ~24 hours after insemination, (c) ~48 hours after insemination, and (d) ~72 hours after insemination. Top image is from a conspecifically inseminated female, bottom image is from a heterospecifically inseminated female, as indicated to the right of each image. Motile sperm are represented by colored lines (red, green, yellow & blue) and are indicated by arrows.

10.a:



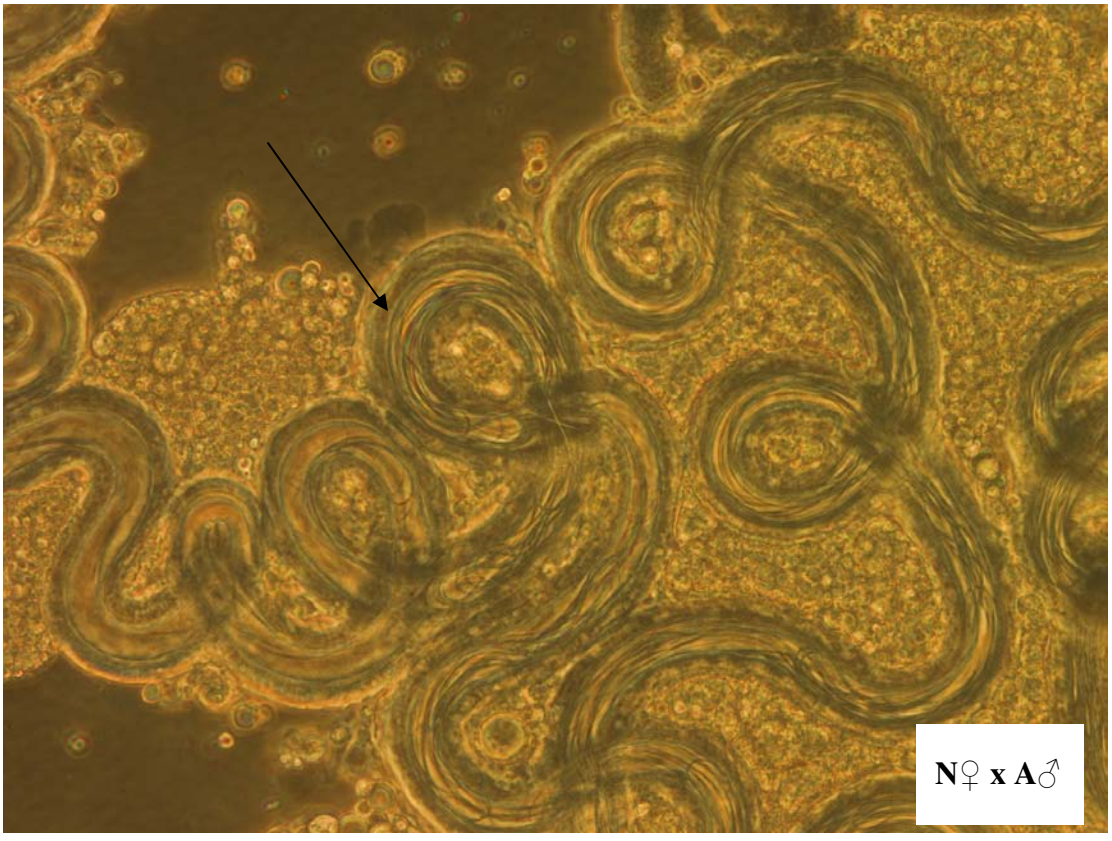
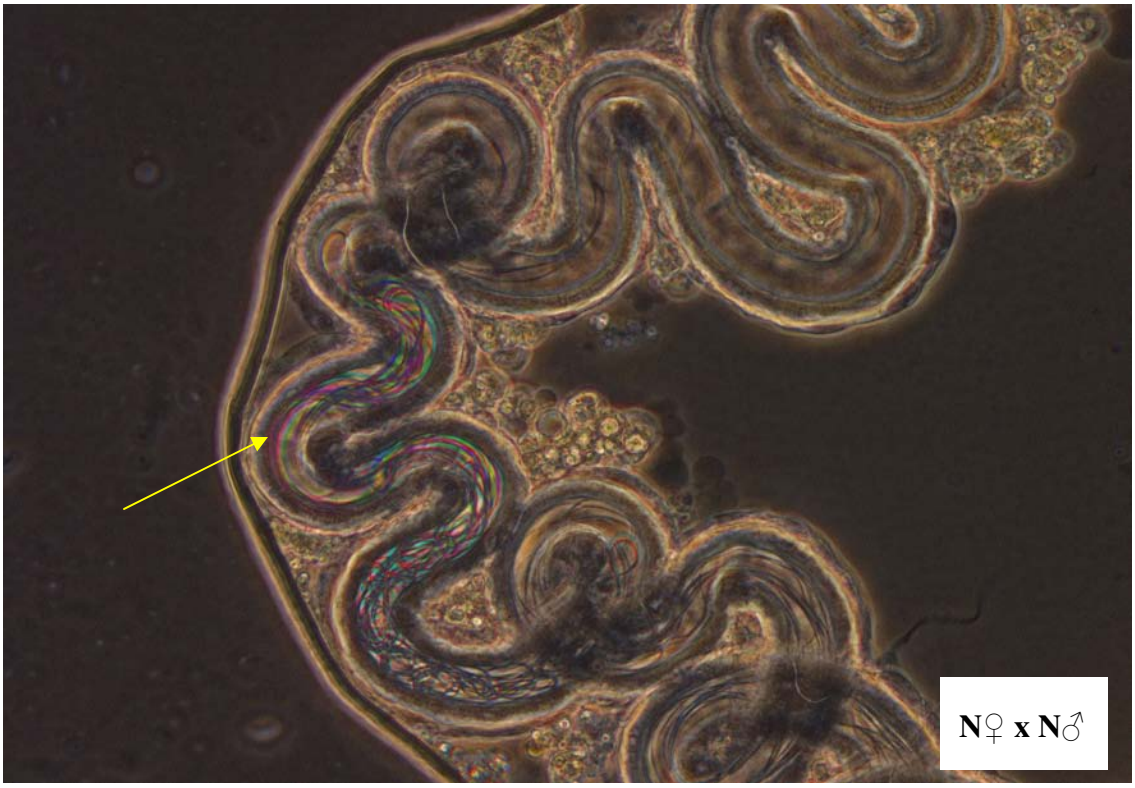


Figure 10. Continued:

10.b



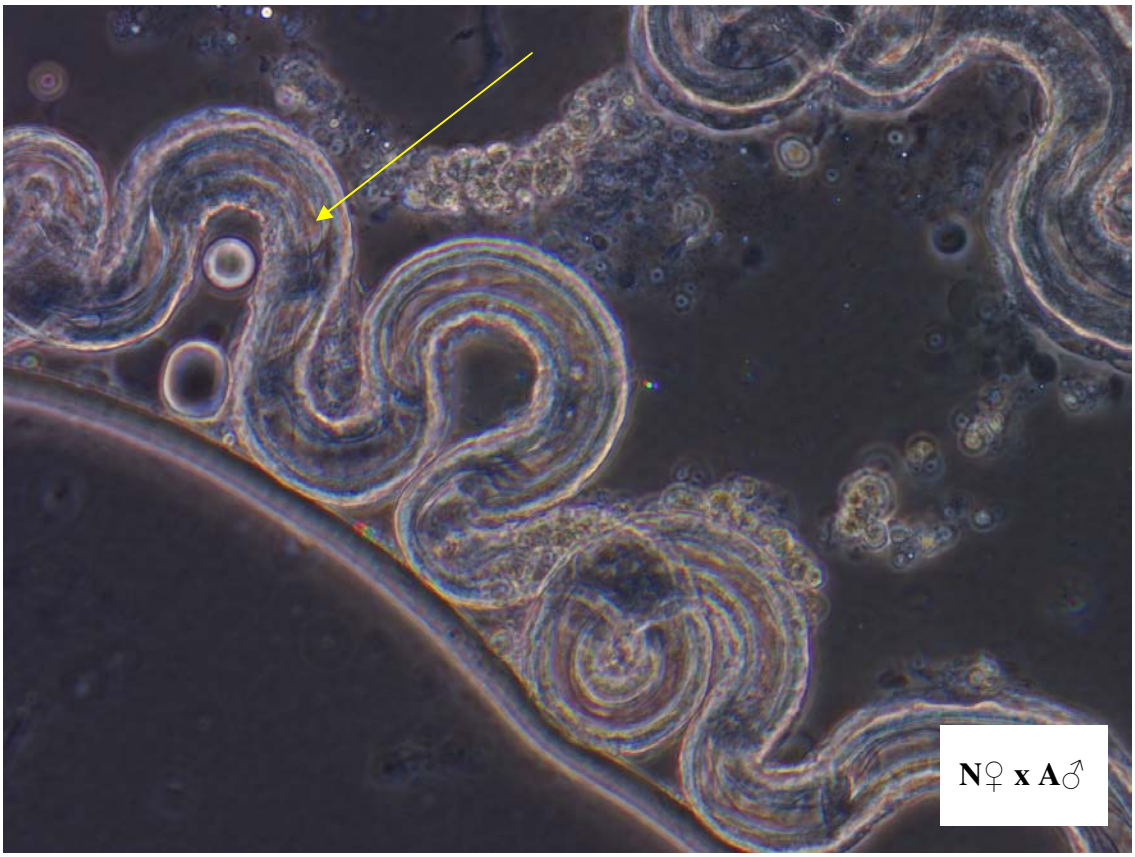
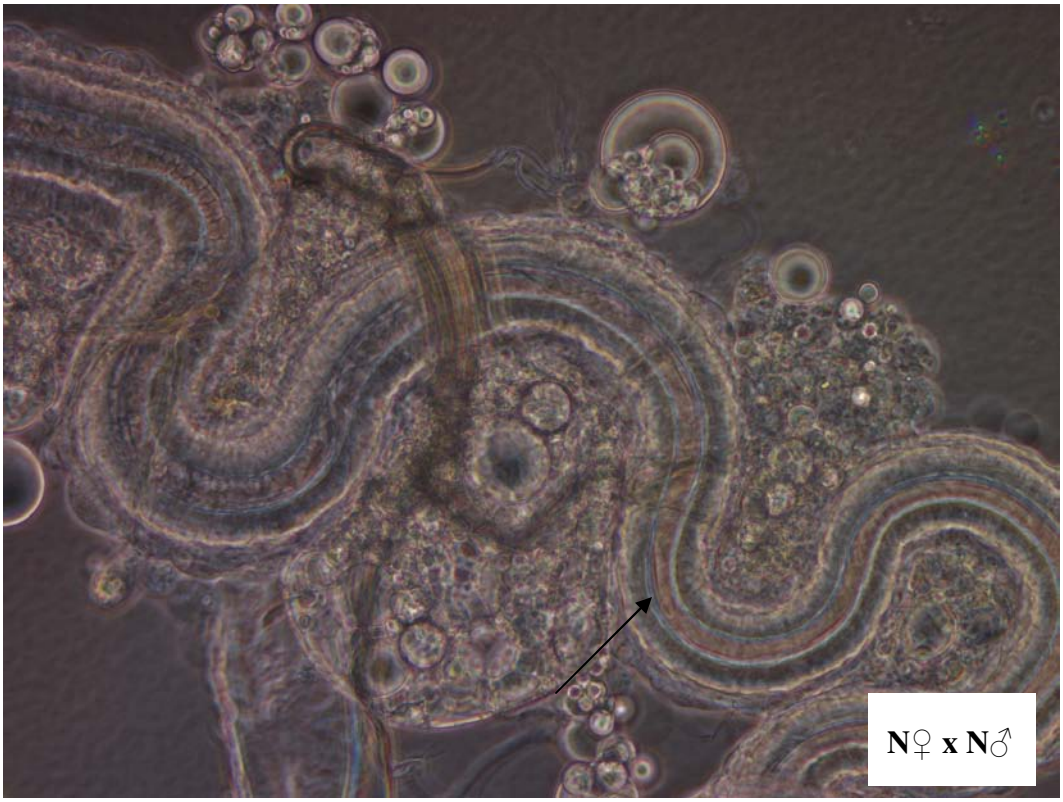


Figure 10 continued:

10.c



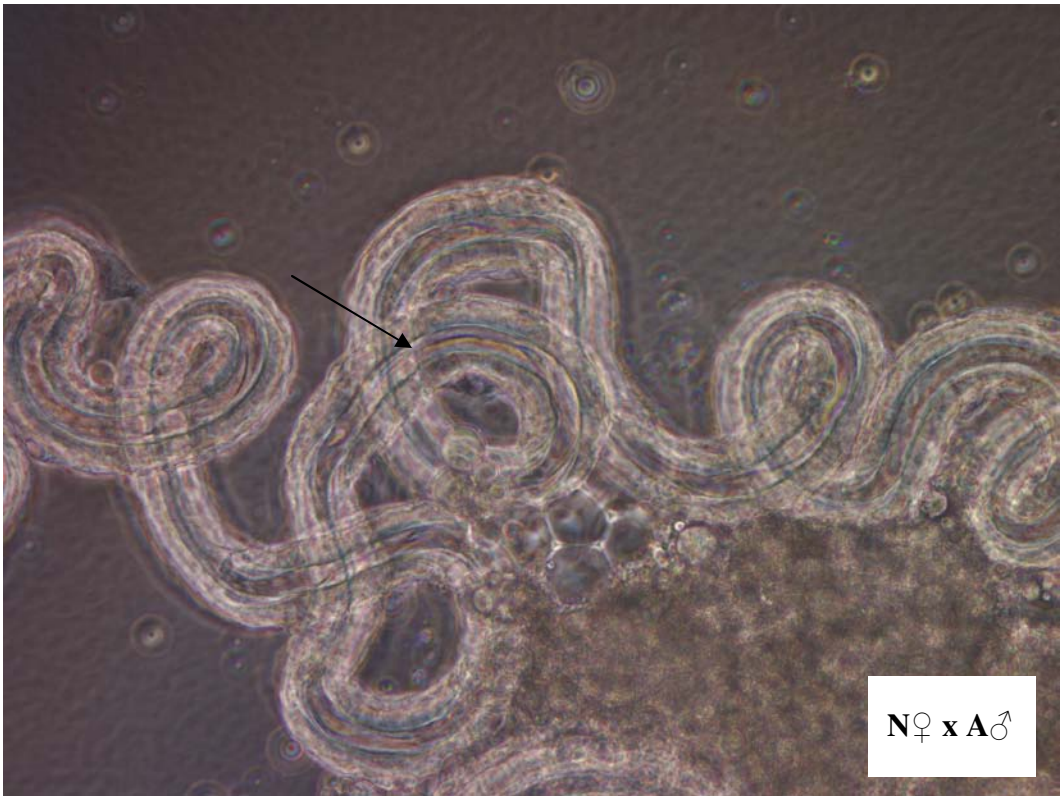
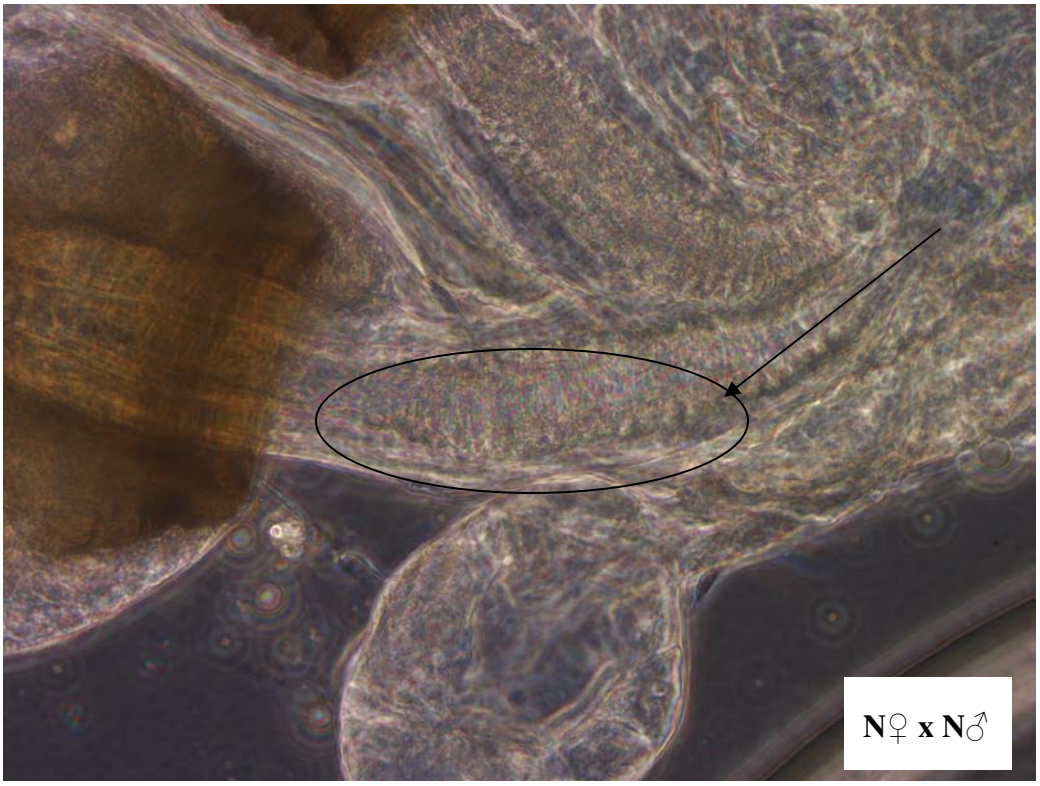


Figure 10 continued:

10.d



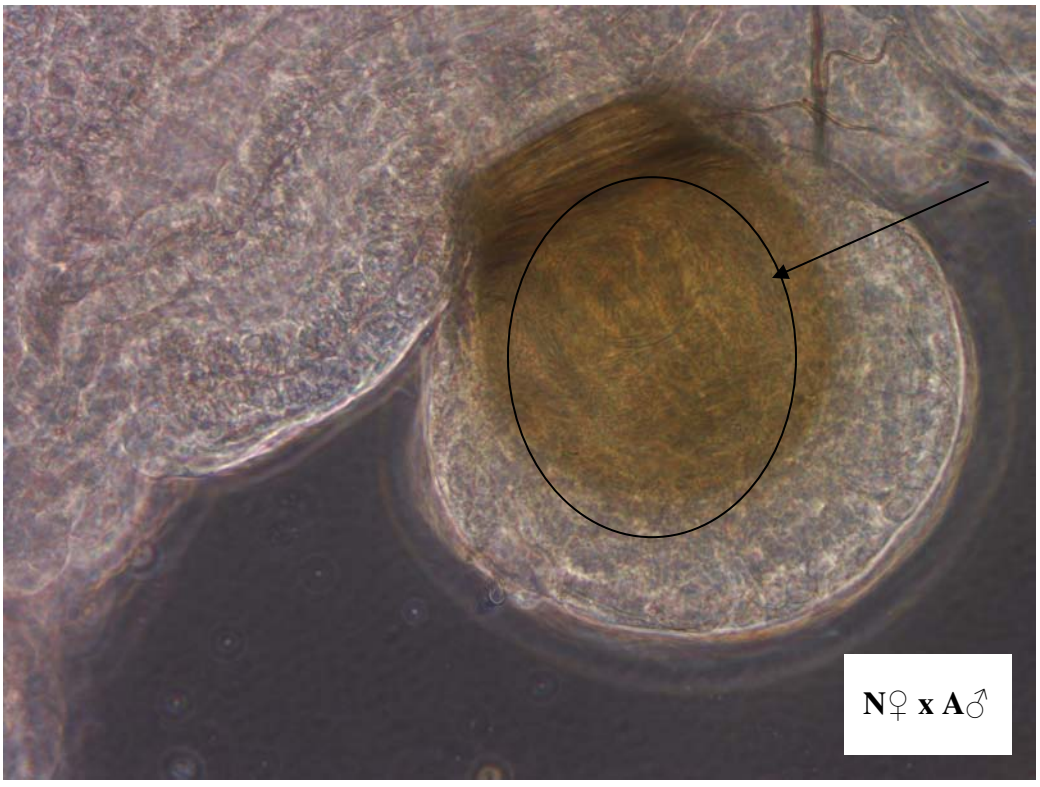


Figure 11: Sperm Storage after Seven days: The proportion of females with stored sperm in either the spermathecae, seminal receptacle, or both is shown graphically for both conspecific and heterospecific inseminations with *D. novamexicana* females. Proportion of females is given on the y-axis, and categories in the x-axis represent classifications for each individual female reproductive tract.

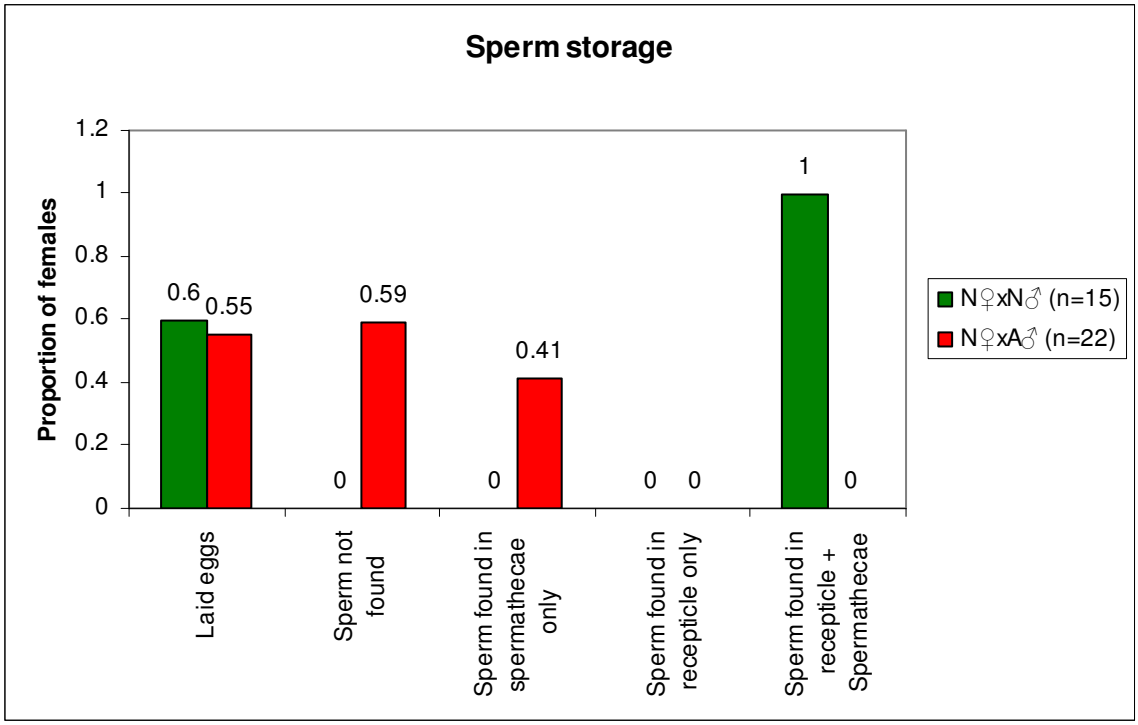


Figure 12: Sperm Motility after Seven Days: The proportion of females with motile sperm within either the spermathecae, seminal receptacle, or both is shown graphically for both conspecific and heterospecific inseminations with *D. novamexicana* females:

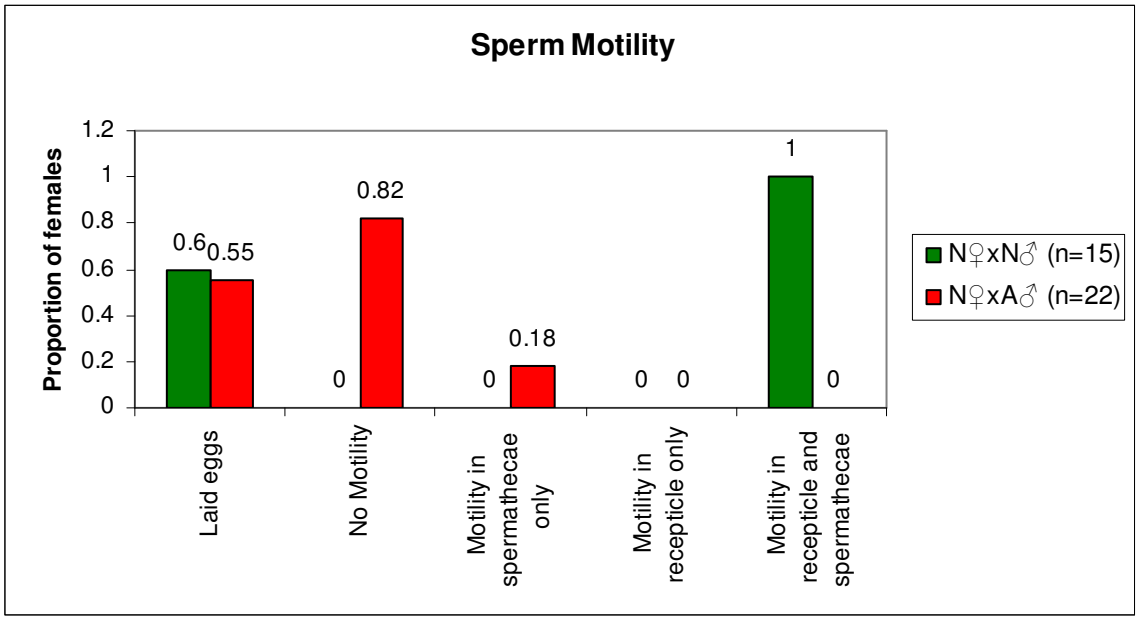


Figure 13: Progeny Counts from Double Matings: First + or – sign indicates first male (conspecific or heterospecific, respectively). Second sign indicates second male. Error bars indicate standard deviation. ($n \geq 50$)

a. Crosses with *D. novamexicana (1031.4)* females:

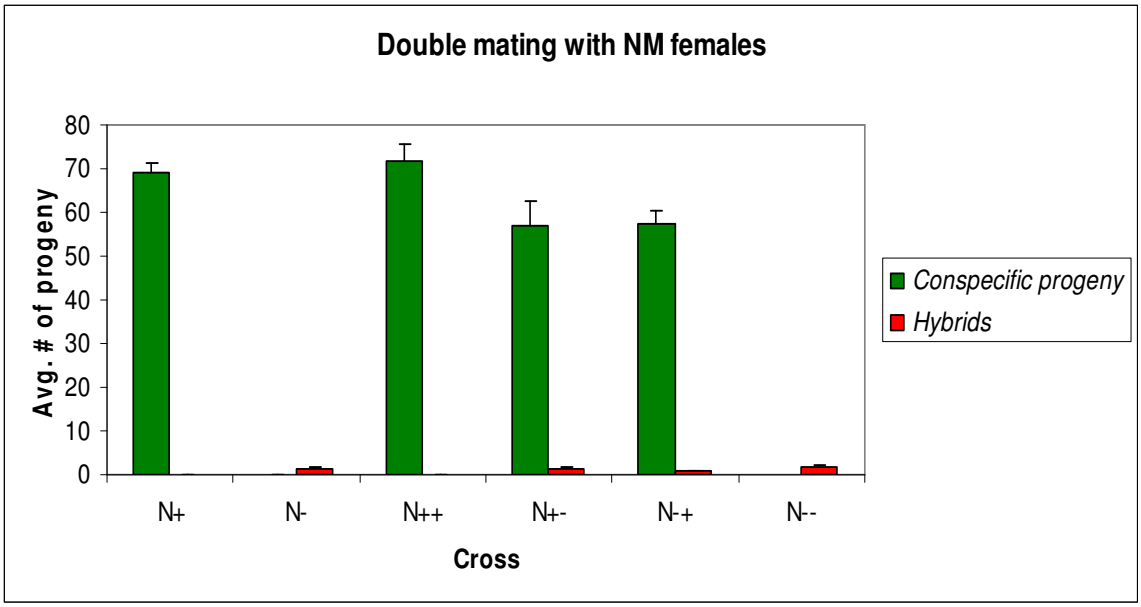


Figure 13 continued:

b. Crosses with *D. americana (NN97.4red)* females:

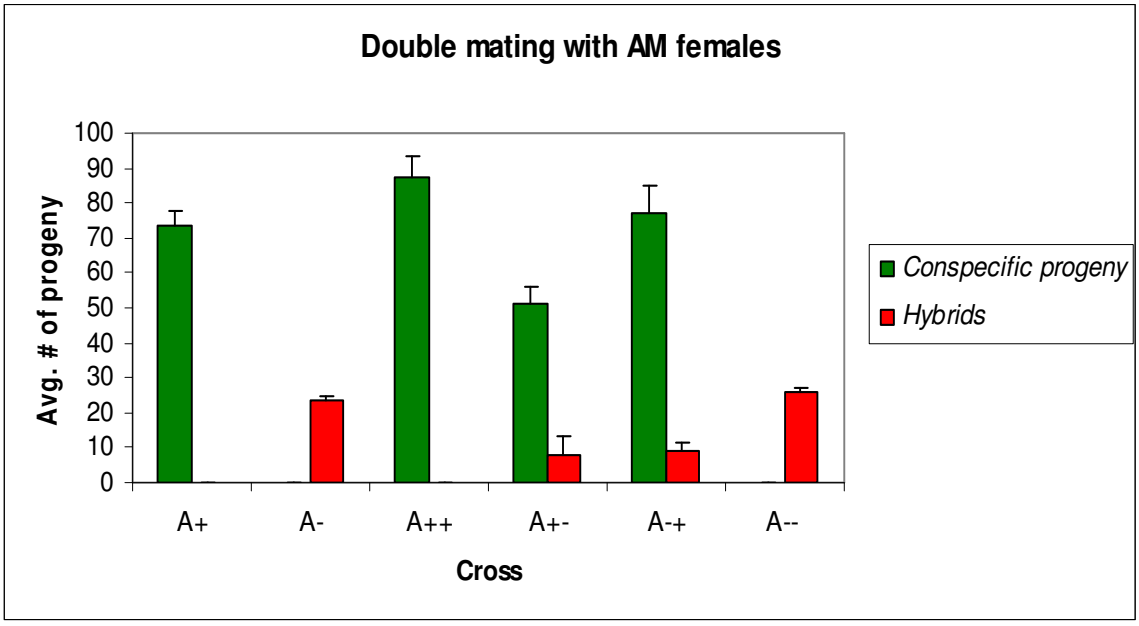


Figure 14: Intraspecific Hatch Rate vs. Genetic Divergence: plot of intraspecific (*D. novamexicana*: yellow, *D. americana*: blue) and interspecific (N♀ x A♂: red, A♀ x N♂: green) hatch rate against genetic divergence at the *Cyt-b* and *CO-II* genes. Each point represents the average hatch percentage of 10 replicates. Intraspecific hatch rates within lines are averaged across experiments and are represented by single points

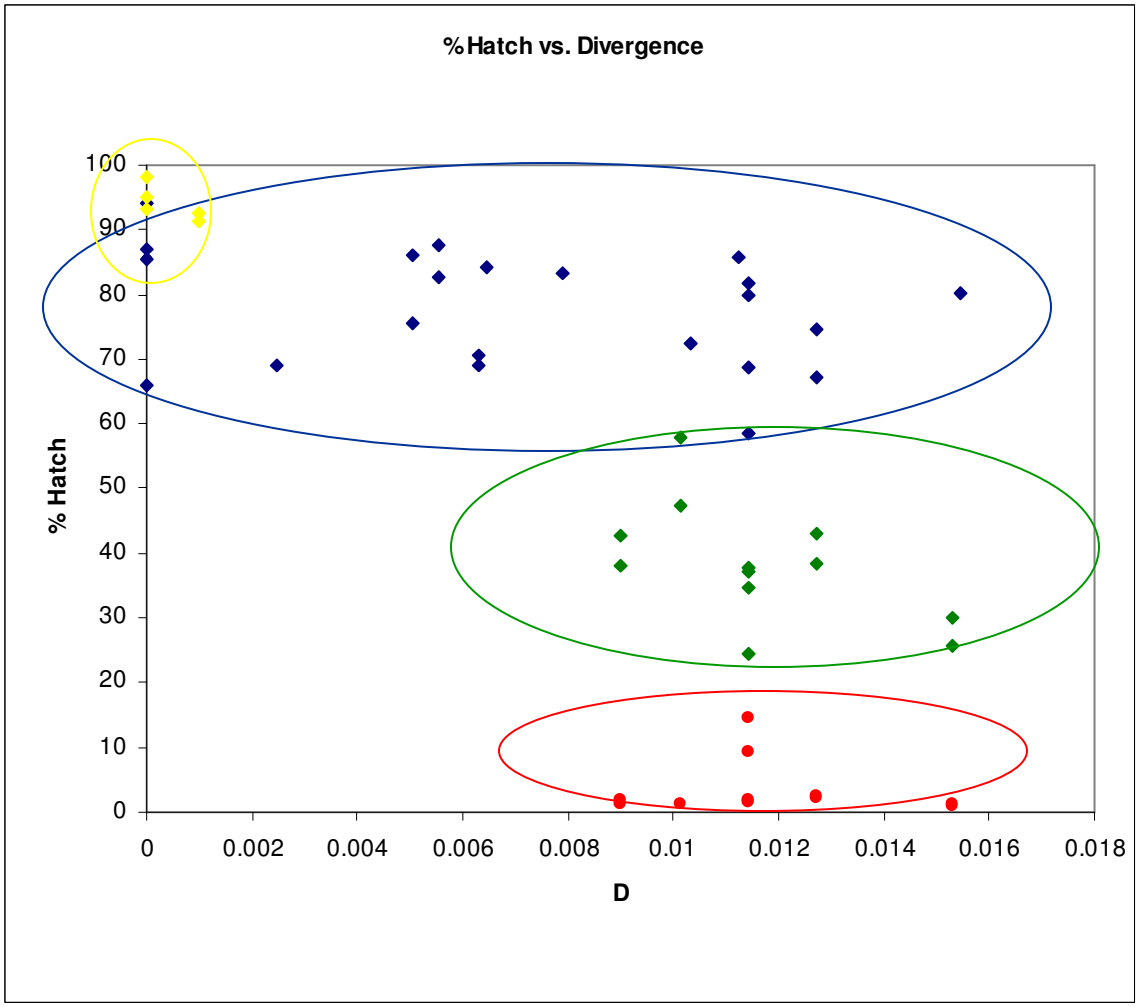


Table 1: Sperm Motility within Three Days in the Reproductive Tract:

Cross	6 hrs after insemination	24 hrs after insemination	48 hrs after insemination	72 hrs after insemination
N♀ x N♂ (n=26)	Dis.[a] = 4 Mot.[b] = 4	Dis. = 7 Mot. = 6	Dis. = 8 Mot. = 8	Dis. = 7 Mot. = 6
N♀ x A♂ (n=24)	Dis. = 5 Mot. = 5	Dis. = 6 Mot. = 6	Dis. = 7 Mot. = 6	Dis. = 6 Mot. = 5

a, The number of females dissected.

b, The number of females found to contain motile sperm.

Table 2: Number of Eggs Laid by Females Reared in 2% Agar:

N♀ x N♂		**N♀ x A♂**	
Female i.d.	**# of eggs laid**	**Female i.d.**	**# of eggs laid**
1	3	1	0
2	25	2	0
3	4	3	11
4	0	4	24
5	13	5	0
6	0	6	0
7	0	7	13
8	21	8	14
9	0	9	0
10	23	10	0
11	12	11	21
12	0	12	13
13	16	13	25
14	0	14	31
15	26	15	9
		16	0
		17	14
		18	0
		19	12
		20	0
		21	16
		22	0

Table 3: Intraspecific & Interspecific Hatch Rate:

Exp[a]	Female[b]	Male[b]	Type[c]	cross[d]	div[e]	hatch[f]
1	NN	NN	con	intra	0	0.93
1	PM	NN	het	intra	0.0056	0.83
1	NN	PM	het	intra	0.0056	0.88
1	PM	PM	con	intra	0	0.87
2	NN	NN	con	intra	0	0.92
2	LA	NN	het	intra	0.0079	0.72
2	NN	LA	het	intra	0.0079	0.83
2	LA	LA	con	intra	0	0.85
3	PM	PM	con	intra	0	0.86
3	PM	LA	het	intra	0.005	0.86
3	LA	PM	het	intra	0.005	0.76
3	LA	LA	con	intra	0	0.86
4	NN	NN	con	intra	0	0.91
4	NN	FP	het	intra	0.0065	0.84
4	FP	NN	het	intra	0.0065	0.69
4	FP	FP	con	intra	0	0.66
5	LA	LA	con	intra	0	0.84
5	LA	FP	het	intra	0.0063	0.71
5	FP	LA	het	intra	0.0063	0.69
5	FP	FP	con	intra	0	0.64
6	NN	NN	con	intra	0	0.93
6	NN	ML	het	intra	0.0113	0.86
6	ML	NN	het	intra	0.0113	0.8
6	ML	ML	con	intra	0	0.86
7	HI	HI	con	intra	0	0.86
7	HI	ML	het	intra	0.0114	0.8
7	ML	HI	het	intra	0.0114	0.82
7	ML	ML	con	intra	0	0.85
8	PM	PM	con	intra	0	0.85
8	PM	ML	het	intra	0.0114	0.8
8	ML	PM	het	intra	0.0114	0.69
8	ML	ML	con	intra	0	0.85
9	LA	LA	con	intra	0	0.86
9	LA	ML	het	intra	0.0114	0.58
9	ML	LA	het	intra	0.0114	0.59
9	ML	ML	con	intra	0	0.84

Table 3 continued:

10	FP	FP	con	intra	0	0.64
10	FP	ML	het	intra	0.0127	0.67
10	ML	FP	het	intra	0.0127	0.745
10	ML	ML	con	intra	0	0.84
11	N4	N4	con	inter	0	0.96
11	N4	ML	het	inter	0.0153	0.01
11	ML	N4	het	inter	0.0153	0.26
11	ML	ML	con	inter	0	0.86
12	N4	N4	con	inter	0	0.97
12	N4	FP	het	inter	0.0076	0.02
12	FP	N4	het	inter	0.0076	0.38
12	FP	FP	con	inter	0	0.66
13	N4	N4	con	inter	0	0.97
13	N4	LA	het	inter	0.0089	0.15
13	LA	N4	het	inter	0.0089	0.24
13	LA	LA	con	inter	0	0.88
14	N4	N4	con	inter	0	0.97
14	N4	PM	het	inter	0.0063	0.01
14	PM	N4	het	inter	0.0063	0.37
14	PM	PM	con	inter	0	0.85
15	N4	N4	con	inter	0	0.96
15	N4	HI	het	inter	0.0063	0.02
15	HI	N4	het	inter	0.0063	0.58
15	HI	HI	con	inter	0	0.83
16	NN	NN	con	inter	0	0.94
16	NN	N4	het	inter	0.009	0.34
16	N4	NN	het	inter	0.009	0.01
16	N4	N4	con	inter	0	0.97

a, Exp = experiment i.d.

b, Female & Male = female and male i.d. represented by the first two letters of the iso-female line i.d. (The *D. novamexicana* line used is N4).

c, Type = con- vs. heterospecific cross.

d, Cross = intra- vs. interspecific cross.

e, Div = divergence estimate at the two mtDNA genes sequenced.

f, Hatch = average hatch rate across 10 replicate trials.

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