You're not my kind of girl: The Genetics of Premating Isolation in the Drosophila virilis group

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Abstract

Sexual isolation between closely related species is expressed through uncoordinated courtship, mating or fertilization events. Behavioural components of mating often form an initial barrier to the production of unfit zygotes (prezygotic isolation). Species of the Drosophila virilis group display a wide range of interspecies prezygotic isolation. *Drosophila virilis* males do not mate with heterospecific females (different species) within their species clade, yet *D. virilis* females are not as discriminating. By observing and recording disruptions in courtship behaviour between species, I have found that *D. virilis* and *D. texana* males significantly fail to continue courtship after they contact heterospecific females through tapping. Tapping is the first stage of courtship, where a male samples female cuticular hydrocarbons, or contact pheromones, through gustatory receptors on the fore tarsi. To determine the genetic basis for behavioural isolation, progressive male courtship events indicating an interest in the female, including tapping, proboscing, singing, and attempting copulation, were quantified for hybrid males from reciprocal heterospecific crosses. Results indicate that recessive X-autosomal gene interactions are responsible for *D. virilis* male discrimination of *D. novamexicana* and *D. texana* females. Therefore, future studies will concentrate on locating the X and autosomal regions that mediate male reception and reaction to female cuticular hydrocarbons.

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Time flies...

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1.0 Introduction

There exists a continuum of events that not only affect the divergence and evolution of species, but also function to maintain genetic boundaries between species; from geographic, ecological, temporal, morphological or behavioural differences, to gametic incompatibility, sperm or zygote mortality, and hybrid inviability or sterility (Dobzhansky in Stalker, 1942; Spieth, 1951; Throckmorton, 1982; Orr & Coyne, 1989). Speciation is the ultimate result of genetic and phenotypic differentiation. Allopatric speciation occurs when population gene pools diverge as a result of geographic separation. Sympatric speciation is more complex because it often involves divergent selection of characters while subpopulations remain in a common geographic range (Stearns & Hoekstra, 2002). Though we may not be able to observe speciation directly, the type of isolating mechanisms that are most effective at reducing gene flow may illustrate primary components of incipient speciation (Coyne & Orr, 1989).

The wide range and degree of isolating factors between species of the genus *Drosophila* provide a valuable tool for the study of mechanisms causing evolution and speciation. Mate discrimination and the evolution of courtship repertoires represent two subtle components of sexual selection that maintain species boundaries (Campesan *et al.*, 2001). Mate discrimination also plays a role in sexual isolation. Behavioural, or ethological, isolation may serve to maintain genetic distance between species by preventing maladaptive hybridization (Coyne & Orr, 1989). The *Drosophila virilis* species group consists of 14 species that display a wide array of reproductive isolation ranging from premating behavioural isolation to postzygotic hybrid inviability (Spicer & Bell, 2002). Therefore, the

group provides an excellent opportunity to study several mechanisms that cause sexual isolation. There is a lack of evidence of gene flow between virilis group species in nature indicating that combinations of ecological and ethological isolation maintain effective barriers (Throckmorton, 1982).

1.1 The Drosophila virilis group

Other than a few amber preserved specimens, no fossil evidence exists to date the divergence of *Drosophila* species (Stone *et al.*, 1960; Spicer & Bell, 2002). Phylogenetic analyses have used comparisons of behaviour, gene sequence, karyotype, morphology, protein electrophoresis, and satellite DNA to define the evolutionary distance between species (Throckmorton, 1982; Nurminsky *et al.*, 1996; Caletka & McAllister, 2004).

D. melanogaster and *D. virilis* diverged from a common ancestor 40 to 60 million years ago (mya)(Nurminsky *et al.*, 1996). The virilis and montana clades, or phylads, diverged from a common ancestor between nine to fifteen mya (Figure 1) (Nurminsky *et al.*, 1996; Spicer & Bell, 2002). The *D. virilis* species originated in the temperate forest regions of eastern Asia but is now a cosmopolitan species found in both domestic and woodland areas of China, Korea, North America and Russia (Throckmorton, 1982; Saarikettu *et al.*, 2005). The current range of *D. virilis* overlaps that of the Nearctic (North American) species of its clade, which are in turn allopatric to each other. It is proposed that an intermediate ancestor to the virilis clade came to the New World late in the Eocene through Beringia and diversified along with the plant genera *Betula*, *Alnus*, *Populus* and *Salix* in the central Rockies and northward (Throckmorton,

1982). Once precipitation decreased, in the early Pliocene (5-1.8 mya), many of the willow trees that *D. virilis* associated with became restricted to mountains or lowland stream borders (Throckmorton, 1982). A decrease in winter temperatures at the end of the Pliocene caused a southern shift of biomes, which disrupted gene flow and established the *D. novamexicana/D. texana* ancestor on the American continent approximately 2.6 mya (Nurminsky *et al.*, 1996). The order and timing of more recent speciation events are still disputed. Some DNA sequence comparisons suggest that *D. texana* became the first distinct American species (Figure 1), approximately 2.6 mya, and then *D. novamexicana* and *D. americana* diverged 0.38 mya (Caletka & McAllister, 2004). Other studies comparing protein and chromosomal differences have suggested that *D. novamexicana* is ancestral to both *D. texana* and *D. americana* (Throckmorton, 1982; Orr & Coyne, 1989).

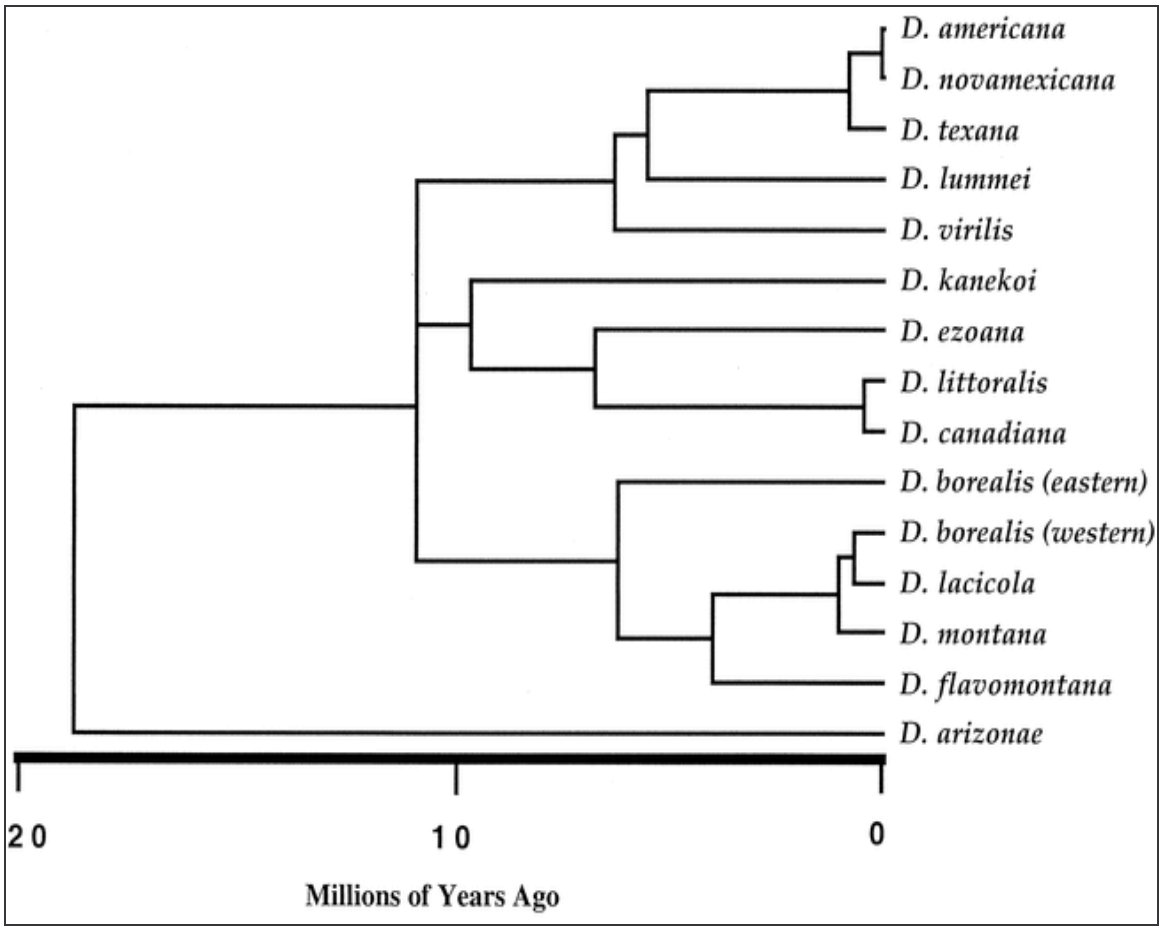

Figure 1. Divergence times for the Drosophila virilis group of species based on mitochondrial DNA and ribosomal RNA sequence comparisons (Spicer & Bell, 2002).

1.2 Reproductive isolation

Reproductive isolation is the product of uncoordinated behavioural or physical elements between males and females of different species (Stalker, 1942). Reproductive isolation between species can occur prezygotically (before gametes combine) through mate recognition, mate discrimination or sperm mortality, and postzygotically through zygote mortality, inviability or hybrid sterility (Coyne & Orr, 1989). Several studies have shown a direct correlation between reproductive isolation and genetic distance between species (Spieth, 1951; Coyne & Orr, 1989; Civetta & Singh, 1995). Among allopatric species, both prezygotic and postzygotic isolation evolve at a similar rate that is proportional to the accumulation of genetic differences (Coyne & Orr, 1989). Between sympatric populations mate discrimination evolves more rapidly, which is consistent with the need for several barriers to prevent gene flow (Coyne & Orr, 1989). It is proposed that premating isolation between sympatric species is caused by reinforcement against the production of unfit hybrids through direct selection for mate discrimination (Coyne & Orr, 1989). The genetic analysis of premating isolation could clarify whether speciation through behavioural isolation proceeds gradually, or if it involves fixation of genes with large effects (Paallysaho *et al.*, 2003).

Premating sexual isolation is manifest through the discrimination of a mate seeker, or the sought mate (Spieth, 1951). Species recognition cues and behavioural isolation are particularly pronounced among members of the virilis phylad. Though in most animals it is believed to be a function of female choice, mate discrimination within the virilis phylad is primarily expressed through male behaviour (Spieth, 1951). *D. virilis* males will rarely mate with heterospecific

females (different species), but *D. virilis* females will more often mate with heterospecific males when conspecific males (same species or strain) are not available (Spieth, 1951; Saarikettu *et al.*, 2005). Heterospecific fecundation of *D. virilis* females decreases fitness through reduced fecundity of both parents and their hybrid progeny (Orr & Coyne, 1989). Because *D. virilis* females do not strongly discriminate courting males, female choice is probably not the driving force behind the divergence of courtship rituals in this group of species (Saarikettu, 2005). The discriminatory behaviour of *D. virilis* males challenges the "choosy female, indiscriminate male" theory of sexual selection, indicating a broader or more flexible definition is necessary.

1.3 Courtship behaviour

The exchange of sensory information between organisms is essential to the species mate recognition system (SMRS) (McMahon, 2002). Individuals physically communicate through chemical, visual, acoustical and tactile signals in a particular chain of events where the timing of courtship signals may vary between species but is relatively stable within species (Liimatainen & Hoikkala, 1998; Saarikettu, 2005). Though courtship behaviour is sexually dimorphic, species-specific signal-response chains involved in the SMRS should coevolve between males and females to prevent sexual isolation within populations (Greenspan & Ferveur, 2000; Saarikettu *et al.*, 2005). Courtship repertoires, preserved by stabilizing selection, maintain effective reproduction within species (Saarikettu *et al.*, 2005).

In *Drosophila*, female locomotion creates a visual stimulus that prompts a male to move towards her. Visual mate recognition may not be of primary

importance to *D. virilis* – as species of this group are known to mate in the absence of light, and males do not normally display or orient in front of females as *D. melanogaster* males do (Markow & O'Grady, 2005). Courtship initiates when the male taps the female with his fore tarsi, the distal portion of the front legs containing gustatory receptors (Spieth, 1951). The female may also tap the male. Gustation is essential in recognizing conspecific male and female cuticular hydrocarbons that function as non-volatile pheromones (Oguma *et al.*, 1992). If the female is recognized as a conspecific, and concedes to courtship by remaining stationary, the male moves to her genitalia where he receives tactile and gustatory signals through his proboscis and maxillary palps respectively (Greenspan & Ferveur, 2000). At this stage the male vibrates one or both wings in a species-specific courtship song. Acoustic signals are communicated through several types of wing vibration "songs" in both males and females and received through antennal aristae (Greenspan & Ferveur, 2000). The female communicates her acceptance of a mate by lifting and spreading her wings, and by opening her genital plates, at which time the male immediately attempts to copulate (Spieth, 1951; Markow & O'Grady, 2005). There is variation in courtship traits among *D. virilis* strains from different geographic ranges, but rituals have not diverged to the point of causing assortative mating or sexual isolation (Saarikettu *et al.*, 2005).

1.4 The genetics of mating behaviour in *Drosophila*

The evolution of behavioural isolation may be initiated by single genes of large effect, but most likely requires changes at more than one gene (Coyne & Orr, 2004). Mate recognition is facilitated by several traits of the SMRS; genetic

changes at one locus may, or may not, affect discrimination at one stage of courtship. Between closely related species, isolation is commonly facilitated by weak multilocus interactions (Paallysaho *et al.*, 2003). Distinctly separate species most often differ in more than one aspect of the SMRS, implying that several gene loci have changed between them. It is difficult to separate genetic factors affecting sexual behaviour because like most complex traits, behaviour is coded for by more than one gene, modulated by numerous biochemical pathways, and subject to environmental influences. It has been suggested that several elements of non-sexual behaviour have been recruited by sexual selection on neuronal pathways to form elements of courtship behaviour common to numerous *Drosophila* species (Greenspan & Ferveur, 2000). The divergence of pleiotropic genes acting on the brain and nervous system produces a sequence of species-specific courtship actions and responses that promotes conspecific, rather than heterospecific, mating (Huttunen &Aspi, 2003). Two major groups of candidate genes responsible for premating reproductive isolation have been broadly studied in *Drosophila*: cuticular hydrocarbon genes and song genes.

1.5 Hydrocarbon genes

In most animals, pheromones provide several modes of communication. Pheromones consisting of volatile hydrocarbons provide a “homing” signal to potential mates. Contact pheromones, or non-volatile cuticular hydrocarbons (CHs), are common to most drosophilids. Non-volatile pheromones require close physical proximity or contact for their reception and recognition. Olfactory neurons recognize volatile compounds, whereas gustatory, or taste, neurons

require soluble compounds (Scott *et al.*, 2001). In *Drosophila* olfactory genes are expressed in the neurons associated with the antenna and maxillary palps, but gustatory receptors are more widespread. Taste is facilitated by chemosensory bristles on the legs, proboscis, wings and female genitalia (Scott *et al.*, 2001). Two different families of seven-transmembrane domain proteins mediate odorant and taste recognition; the proteins are coded for by *Drosophila* odorant receptor (DOR) and gustatory receptor (GRO) genes distributed throughout the genome (Scott *et al.*, 2001). In *D. melanogaster* the gustatory gene Gr32D1 is expressed in multiple neurons in the proboscis and the two to three neurons in the distal tarsal segments of the legs that transmit gustatory information to the subesophageal ganglion in the most anterior portion of the hindbrain (Scott *et al.*, 2001).

Drosophilid hydrocarbon profiles vary with sex, age, and ecology of a species; the genes responsible are therefore proposed to be under both natural and sexual selection (Markow & O'Grady, 2005). Cuticular hydrocarbons within the virilis group of species vary in chain length between 22 and 31 carbons (C), with *D. virlis* having a unique 21C male hydrocarbon (Markow & O'Grady, 2005). Analysis of heterospecific crosses between *D. virilis* and *D. novamexicana* have determined that additive genetic factors on the second and third chromosomes are responsible for the production of Z-11-pentacosene, the major sex pheromone of *D. virilis* females (Oguma *et al.*, 1992; Greenspan & Ferveur, 2000).

1.6 Song genes

Singing provides different cues according to changes in both wing vibration characteristics and context (Satokangas *et al.*, 1994; Liimatainen & Hoikkala,

1998). Numerous studies have shown that *Drosophila* males and females produce, recognize, and respond differentially to several types of songs (Huttunen & Aspi, 2003). *D. virilis* males produce primary courtship song, and secondary courtship song in prolonged courtships, with cyclic strings of wing vibrations called pulses (Satokangas *et al.*, 1994; Suvanto *et al.*, 1994). Males also produce inhibitory songs when courted by other males. *Drosophila* females may also produce several song types, though much less frequently than males. *D. virilis* female song, like that of males, is species-specific and includes courtship (and duet songs in some species) and rejection songs (Satokangas *et al.*, 1994). Females of the virilis group require male courtship song before they will display the acceptance signal of wing spreading (Saarikettu *et al.*, 2005).

The species-specific courtship songs of *Drosophila* males are controlled by multiple genes of minor effects (Paallysaho *et al.*, 2003). Male song characters subject to divergent or stabilizing selection include the length of a pulse train (PTL), number of pulses in a pulse train (PN), number of sound cycles in a pulse, interpulse interval (IPI), and carrier frequency (Huttunen & Aspi, 2003). In the virilis group IPI is relatively constant but PN and PTL vary between the species (Huttunen *et al.*, 2004). Except for PN, genes on the X chromosome are responsible for differences in song patterns between the virilis, montana, and littoralis clades (Huttunen & Aspi, 2003; Paallysaho *et al.*, 2003).

1.7 Objectives

1) Quantification of sexual isolation between species of the Drosophila virilis clade: A postzygotic isolation index was calculated from relative progeny counts yielded by single pair mating between and within the virilis group of species.

2) Identification of behavioural elements responsible for premating isolation between the species: Differences in mate discrimination were quantified through courtship event observations between *D. virilis* males or females offered conspecific or heterospecific mates.

3) The genetic basis for *D. virilis* male mate discrimination: Courtship behaviour of first filial (F1) hybrid males from reciprocal heterospecific crosses was used to test the role of dominant vs recessive autosomal, as well as the X or Y-linked chromosomal basis for premating reproductive isolation.

1.8 Hypothesis

Drosophila virilis males do not actively court heterospecific females of their clade. Are there specific elements that cause *D. virilis* males to refuse heterospecific females? If so, what is the chromosomal basis for behavioural isolation in this clade?

2.0 Materials and Methods

2.1 Fly stocks

Wild type and mutant laboratory stocks were obtained from the *Drosophila* species stock center in Tuscon, AZ. Wild type *Drosophila virilis* (stock # 15010-1051.00), *D. novamexicana* (stock # 15010-1031.04), *D. americana* (stock # 15010-0951.00), and *D. texana* (stock # 15010-1041.16) were used for progeny count and courtship behaviour observations. A mutant *D. virilis* (stock #15010-1051.46), homozygous for recessive mutations on the second through fifth chromosomes (b[1]; tb[1] gpL2[1]; cd[1]; pe[1]) conferring broken wing cross-veins; tiny bristles and wing vein two gap; cardinal eye; and peach eye respectively, was utilized for the production of reciprocal heterospecific hybrid males.

2.2 Stock maintenance

Parental fly stocks were maintained in round (237 ml) polypropylene bottles containing a cornmeal-molasses (CM) medium (Appendix I). Parental fly stocks were maintained by transferring adults to bottles containing fresh media. Bottles were cleared of parental flies after eleven to fourteen days, or when larvae were visible. At a temperature of 24°C, *D. virilis* species embryos take 18 days to develop into adult flies (*Drosophila* species stock center). Crosses were set up between a virgin male and female aged to sexual maturity. All crosses were set up in 28.5 x 95 mm cylindrical vials containing CM media. All parental stocks and crosses were raised in an incubator set to a twelve-hour light/dark cycle at 20°C.

2.3 Virgin fly collections

Virgin flies were collected between 10 AM and 3 PM from bottles that had been cleared 24 hours prior. Hybrid males were collected at one to three day intervals. Flies were lightly anaesthetized with CO_2 gas and separated by sex under light-dissecting microscope visualization. Virgin males and females were stored separately in fresh food vials containing no more than 55 flies, and aged to sexual maturity, for nine to fourteen days (Stalker, 1942; Spieth, 1951).

2.4 Progeny count crosses

Conspecific and heterospecific wild type crosses (Table 1) were set up with a single virgin male and female per vial. Pairs were left to breed and oviposit for one week and then transferred to a fresh vial for another week, after which time the pair was discarded. Fifty pairs were set up for each cross. Progeny counts were done using light CO_2 anaesthetic four weeks after initiation of the vials.

Table 1. Total number of single pair heterospecific and conspecific progeny count crosses between *Drosophila virilis* species.

Male \ **Female**	*virilis*	*novamexicana*	*americana*	*texana*
virilis	50	50	50	50
novamexicana	50	50	0	50
americana	50	0	50	0
texana	50	50	0	50

2.5 Generation of interspecific hybrids

Reciprocal crosses were set up between a mutant *D. virilis* female (or male) and a wild type mate from either *D. novamexicana* or *D. texana* species. The resulting first filial (F1) hybrid male progeny were tested for courtship behaviour toward a non-*virilis* female (Figure 2).

2.6 Courtship event descriptions

Courtship behaviour elements for the Drosophila virilis group include both male and female signals and responses (Spieth, 1951; Satokangas *et al.*, 1994; Liimatainen & Hoikkala, 1998; Saarikettu *et al.*, 2005). A behavioural event is defined as either an instantaneous action, or the onset of a behavioural state. A behavioural state is defined as an activity that is not instantaneous, but has a longer duration that can be accurately timed by the observer (Altmann, 1973).

Male events include: 1) No courtship, 2) Following an ambulatory female, 3) Orienting or facing the female, 4) Tapping or touching the female with fore tarsi, 5) Proboscing the female's genitalia, 6) Circling a standing female, 7) Wing vibrating, 8) Attempting copulation, and 9) Decamping – running or jumping away.

Female events include: 1) Kicking the male, 2) Standing while being courted, 3) Walking during courtship, 4) Decamping, 5) Wing vibrating, 6) Wing lifting – to accommodate attempted copulation, 7) Courting the male - orienting or touching the male, and 8) Copulation (a behavioural state).

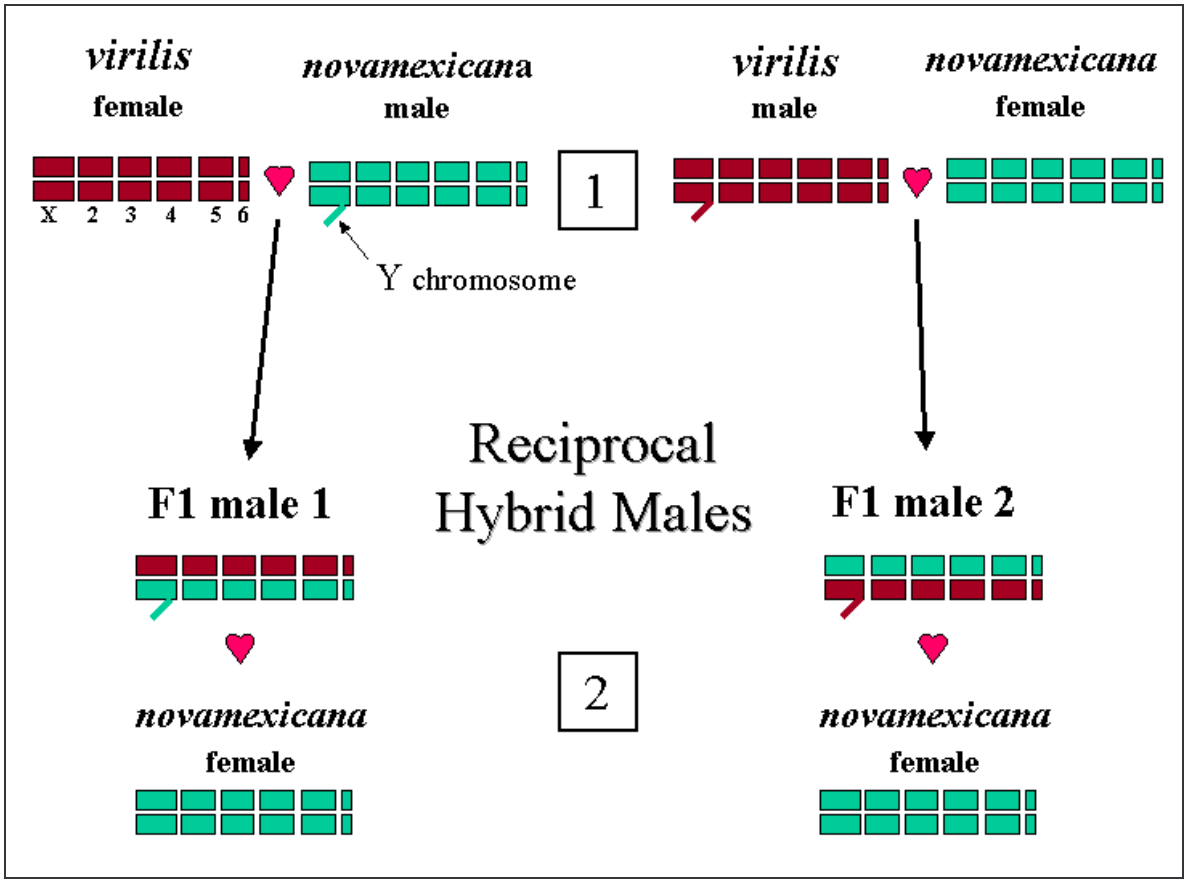

Figure 2. *Drosophila virilis* heterospecific crossing scheme to determine the genetic basis for behavioural isolation. [1] Reciprocal crosses were set up with *D. virilis* and either *D. novamexicana* or *D. texana* mates. [2] First filial (F1) males were tested for behavioural isolation toward a female of their non-*virilis* parental species. Please note – because only a small percentage of *D. virilis* males mate with heterospecific females, a larger number of crosses involving *D. virilis* males were set up to yield an adequate number of hybrid male progeny for testing.

2.7 Mating behaviour observations

Courtship observations were carried out following a no-choice experimental design where a male was paired with a female of one species. The no-choice method both provides statistical power to observations, and minimizes the effects of female choice, as females will mate more readily when courted by a single male (Ortiz-Barrientos *et al.*, 2004; Coyne *et al.*, 2005). Courtship elements, defined in the previous section, were quantified for each observed pair according to the focal animal sampling method outlined by Jeanne Altmann (1973).

Conspecific and heterospecific mating behaviour of wild type species were observed and scored to serve as controls. F1 hybrid males were tested for mating behaviour toward non-*virilis* wild type females. A sexually mature, ten to fourteen day old, male and female pair were placed together in a 28.5 x 95 mm cylindrical polyethylene observation vial containing CM media; courtship interactions were observed and recorded until copulation occurred or for 30 minutes (Spieth, 1951; Liimatainen & Hoikkala, 1998; Saarikettu *et al.*, 2005). Where copulation did occur, the duration was timed and recorded. Because active courtship events proceed in rapid succession, observed events were dictated to a recording device in real time, and later scribed to a data sheet in the order of occurrence for each pair.

3.0 Data analysis

3.1 Progeny Counts

Postzygotic isolation measures between species typically take into account the combined measures of hybrid progeny inviability and hybrid progeny sterility. Because hybrid inviability and hybrid sterility both evolve at a similar rate, an estimate of postzygotic isolation can be calculated from either measure (Coyne & Orr, 1989). To approximate to a postzygotic isolation index (**I**), hybrid inviability was calculated by subtracting relative progeny production from the potential inviability value of one.

$$\mathbf{I^{*} = 1 - relative\ progeny\ production^{\ddagger}}$$

*Where a value of 1 represents total isolation and a value of 0 represents no isolation.

‡Relative progeny production was calculated from the number of progeny produced by a heterospecific mating relative to the combined mean of both conspecific parental crosses (modified from Coyne & Orr, 1989).

Mean progeny values were calculated from pairs that produced viable offspring. Raw progeny count data was square root transformed to fit a normal distribution and analysed using analysis of variance (ANOVA) and a Tukey post hoc test.

3.2 Courtship Behaviour

Mean male courtship events indicating an active interest in the female, (tapping, proboscing, singing, and attempted copulation) were quantified according to occurrence (1) or absence (0) for each observed pair (Altmann, 1973). To prevent a type one error from comparing several courtship events for several crosses, each male was assigned a single courtship progression score (**C**). The courtship progression scores were assigned as follows: 1) males progressing

through all stages including attempting to copulate received a score of four, 2) males progressing only as far as singing received a score of three, 3) males progressing to proboscing received a score of two, 4) males that tapped females, but did not proceed to proboscing, received a score of one. Male courtship progression scores were analysed by univariate ANOVA and Tukey post hoc tests.

Crosses showing significant differences in post hoc groupings were further analysed by pair wise approximate randomization tests of male courtship events including tapping, proboscing, singing and attempting to copulate (software courtesy of Dr. S. Forbes). Approximate randomization tests the mean difference between two samples and provides the probability that they come from the same population. The software randomly resamples given data to generate a normal distribution of mean differences. The observed mean difference is compared to the randomly generated distribution of the mean differences.

An index of premating behavioural isolation (**Im**) was calculated, such that:

Im = 1 – relative mating frequency.

Relative mating frequencies were calculated from the observed mating frequency relative to the mean conspecific mating frequency of both species.

Mean copulation times were calculated, from the available data, for each cross. The copulation time of crosses with more than one observed copulating pair were analysed with ANOVA and Tukey post hoc tests.

4.0 Results

4.1 Progeny Counts

There were significant differences ($F_{11,588}$= 116.2; P< 0.001) in the proportion of progeny sired by females exposed to males of different species (Appendix II). Conspecific mating produced the greatest amount of progeny, with the exception of *D. americana*. Heterospecific crosses involving *D. virilis* males were much less productive than heterospecific crosses involving *D. virilis* females (Figure 3a). The failure of *D. virilis* males, exposed to heterospecific females for a period of two weeks, to produce progeny could be a consequence of premating and/or postmating isolation between species. Postmating incompatibility between *D. virilis* males and heterospecific females is evident from the observation of low progeny yield.

Although mating was not observed in the progeny count vials, an analysis of the proportion of vials yielding larvae, pupae, or viable progeny provides an approximate estimate to the proportion of mating (Figure 3b). Greater than 90% of conspecific pairs produced progeny (with the exception of *D. americana*, 78%). A high proportion (over 80%) of vials containing heterospecific pairs also produced some progeny. Once again, *D. americana* males were an exception, as only 44% of vials yielded larvae, pupae or progeny, and this species was therefore removed from further tests and analysis. Heterospecific pairs involving *D. virilis* males show the lowest proportion of vials with progeny, with stronger premating isolation between heterospecific pairs involving *D. novamexicana* and *D. americana* females (16% and 18% of vials show progeny, respectively) than *D. texana* females (42% of vials) (Figure 3b).

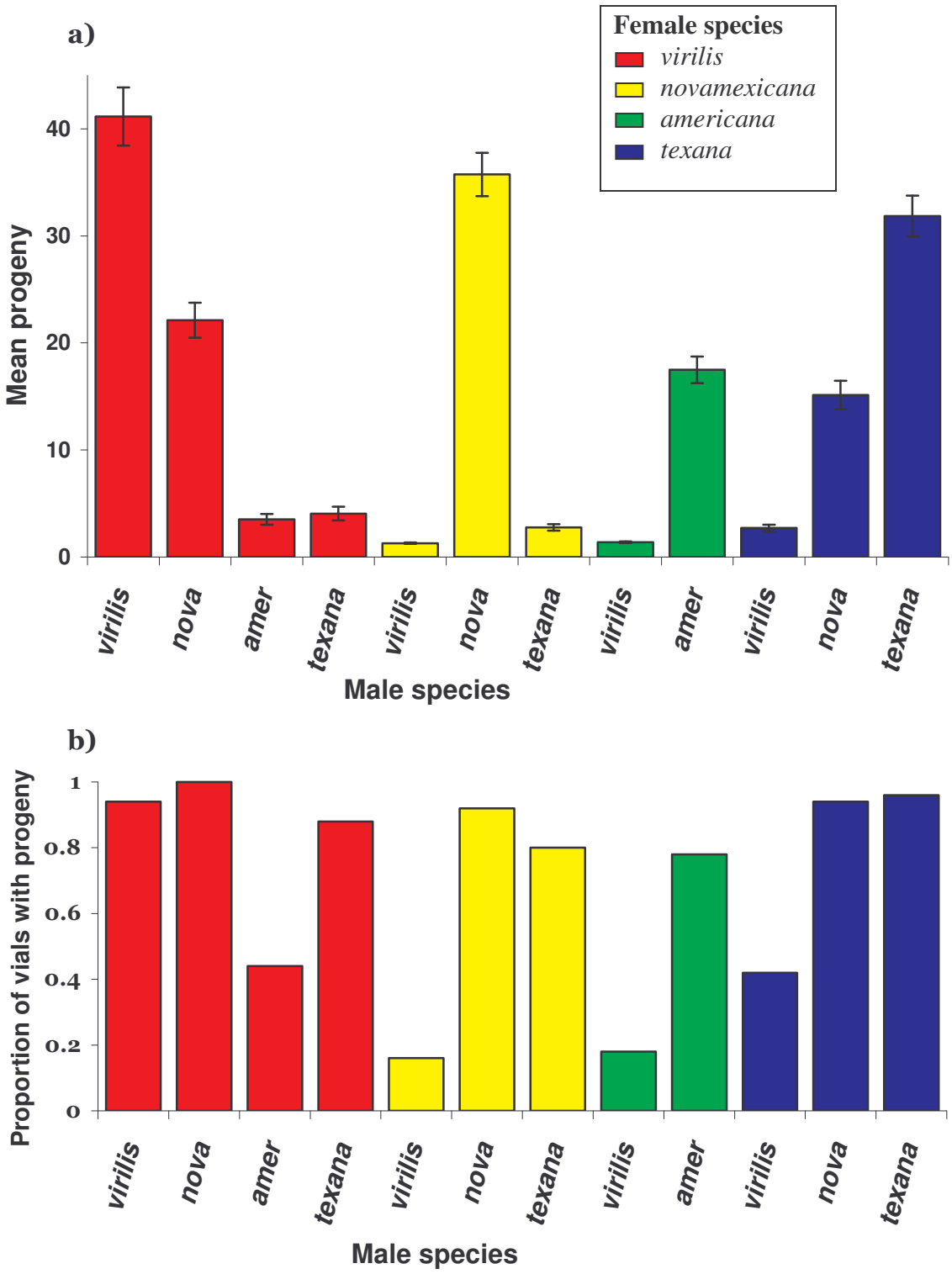

Figure 3. a) *Drosophila* single pair progeny production over an exposure time of two weeks (standard error bars included). X-axis labels indicate male species, and bar colours indicate female species (figure legend). **b)** Proportion of vials from progeny count crosses that contained larvae, pupae, or viable progeny indicating the occurrence of mating. Post hoc results in Appendix II and III.

Postmating incompatibility between *D. virilis* males and heterospecific females is evident from the observation of low progeny yield (Figure 3a). The failure of males to produce progeny does not provide an accurate measure of the proportion of males that failed to mate with heterospecific females (i.e. premating isolation). Therefore, observations of courtship and mating behaviour were used to quantify, and to identify, key behavioural elements causing premating isolation.

4.2 Courtship Events

Conspecific courtship progression scores (**C**) were not significantly different between *D. virilis* and *D. novamexicana* males, but *D. texana* males had lower scores than *D. virilis* males (Figure 4). *D. virilis*, and *D. texana*, males showed lower progression scores with heterospecific females than with conspecific females. *D. novamexicana* males displayed similar courtship progressions toward both conspecific and heterospecific females.

To determine the elements of behaviour that cause a decline in courtship towards heterospecific females, pair wise approximate randomization tests were run for each behavioural event (Appendix V). Heterospecific crosses involving either *D. virilis* females or males did not differ significantly from conspecific crosses in their frequencies of mate tapping ($P \geq 0.088$). Heterospecific crosses involving *D. virilis* males (Figure 5) showed a significantly lower proportion of proboscing, singing and attempting to copulate than *D. virilis* conspecific pairs ($P < 0.001$). Behavioural interactions involving *D. virilis* females and *D. texana* males did not differ from the reciprocal cross, and deviated from *D. texana*

conspecifics by less frequent proboscing, male singing and attempted copulation ($P \leq 0.007$). Observed courtship events between *D. virilis* females and *D. novamexicana* males (Figure 6) did not deviate from *D. novamexicana* conspecifics, and such observation is concordant with the analysis of the proportion of vials yielding progeny (Figure 3b).

The tendency of observed pairs to make the transition from one courtship event to a subsequent one was determined through event correlation for all pairs in the data set (Appendix IV). The correlation between male courtship song and female wing spreading ($r=0.776$) indicates that females do not strongly discriminate against heterospecific males in no-choice pairings, and that species-specific male courtship song may not be of utmost importance to female mate selection. Males differed in their tendency to proceed from tapping to proboscing ($r=0.558$). The males that continued to proboscing also proceeded to singing to a great degree ($r=0.885$) and carried through from singing to attempted copulation to the same degree ($r=0.792$) that females accepted them. Males that attempted to copulate were most often successful ($r=0.914$).

This study provides support to previous evidence of premating behavioural isolation between *D. virilis* males and heterospecific females. In addition, observations of courtship events show that male isolation is due to the inability of males to continue courting heterospecific females after tapping them (Figures 5 and 6).

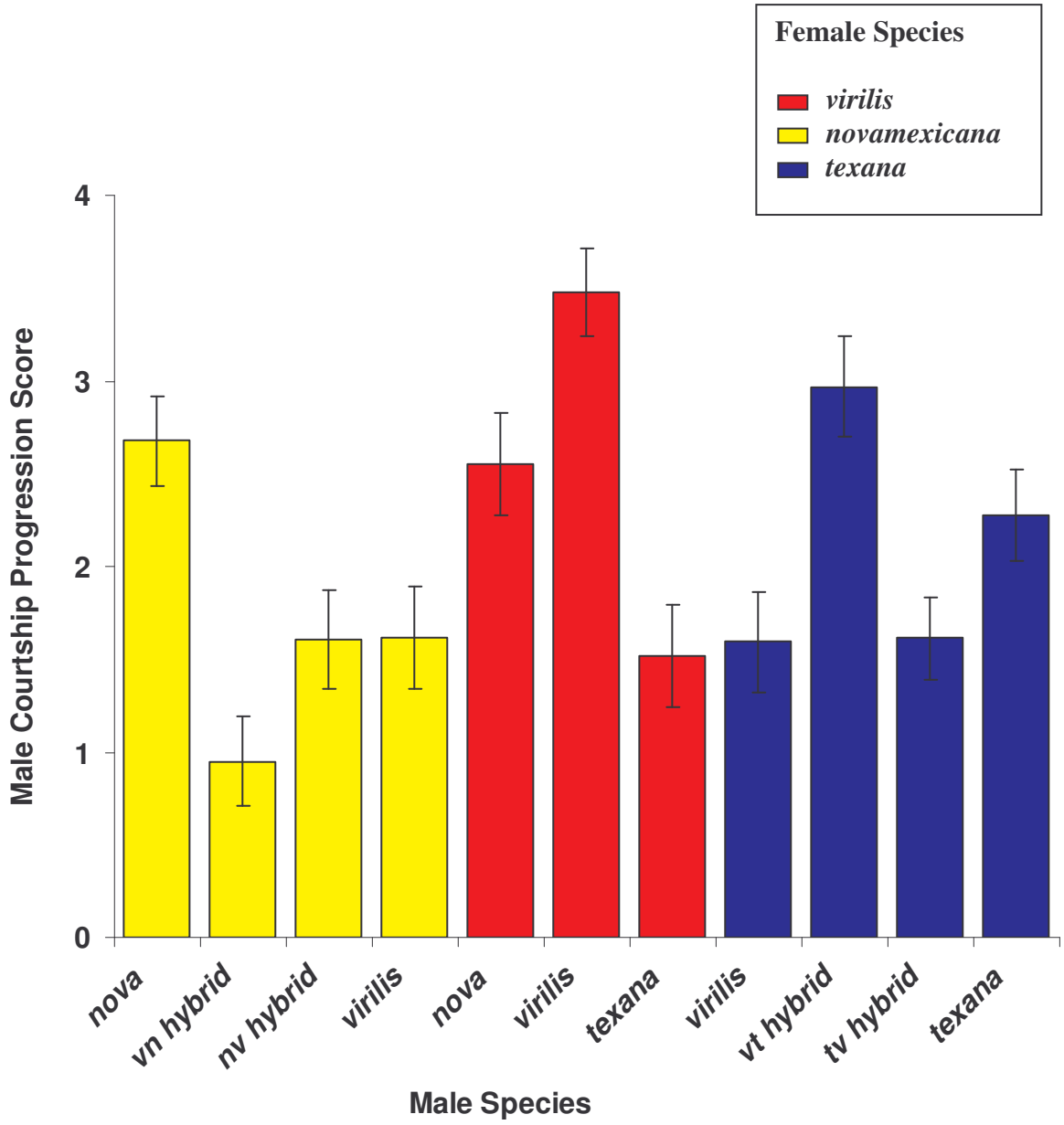

Figure 4. Mean *Drosophila* male courtship progression scores (including standard error bars). Male species are labelled on the X-axis and female species are indicated by bar colour. Hybrid male identification codes consist of two letters, the first identifies the species of the parental female and the second identifies the species of the parental male. Tukey post hoc results shown in Appendix VI.

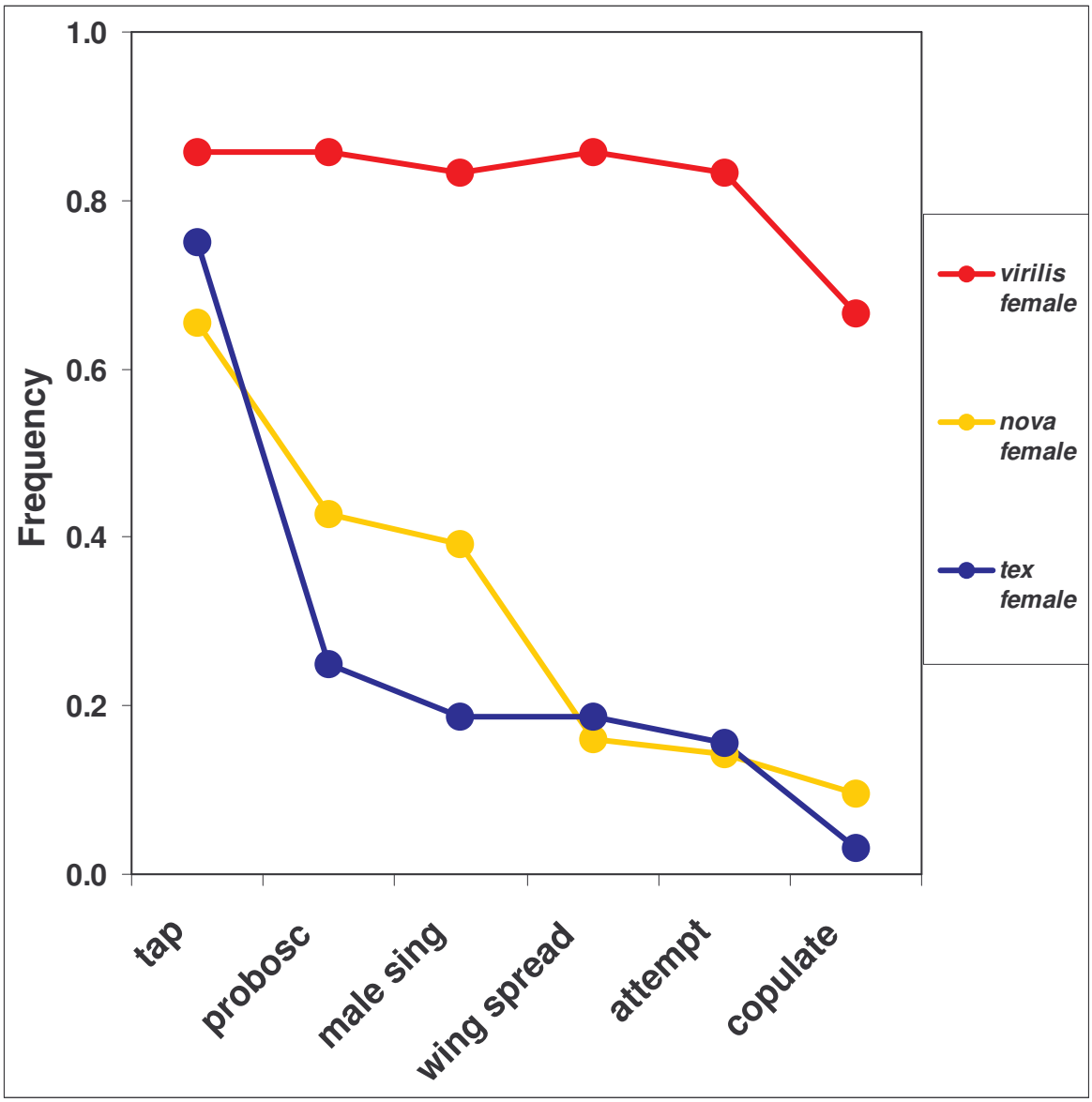

Figure 5. Observed male courtship event frequencies for *D. virilis* males paired with a conspecific or heterospecific female. Female species are identified in the figure legend. The female event of wing spreading is included in the graph to illustrate female interaction effects on courtship progression.

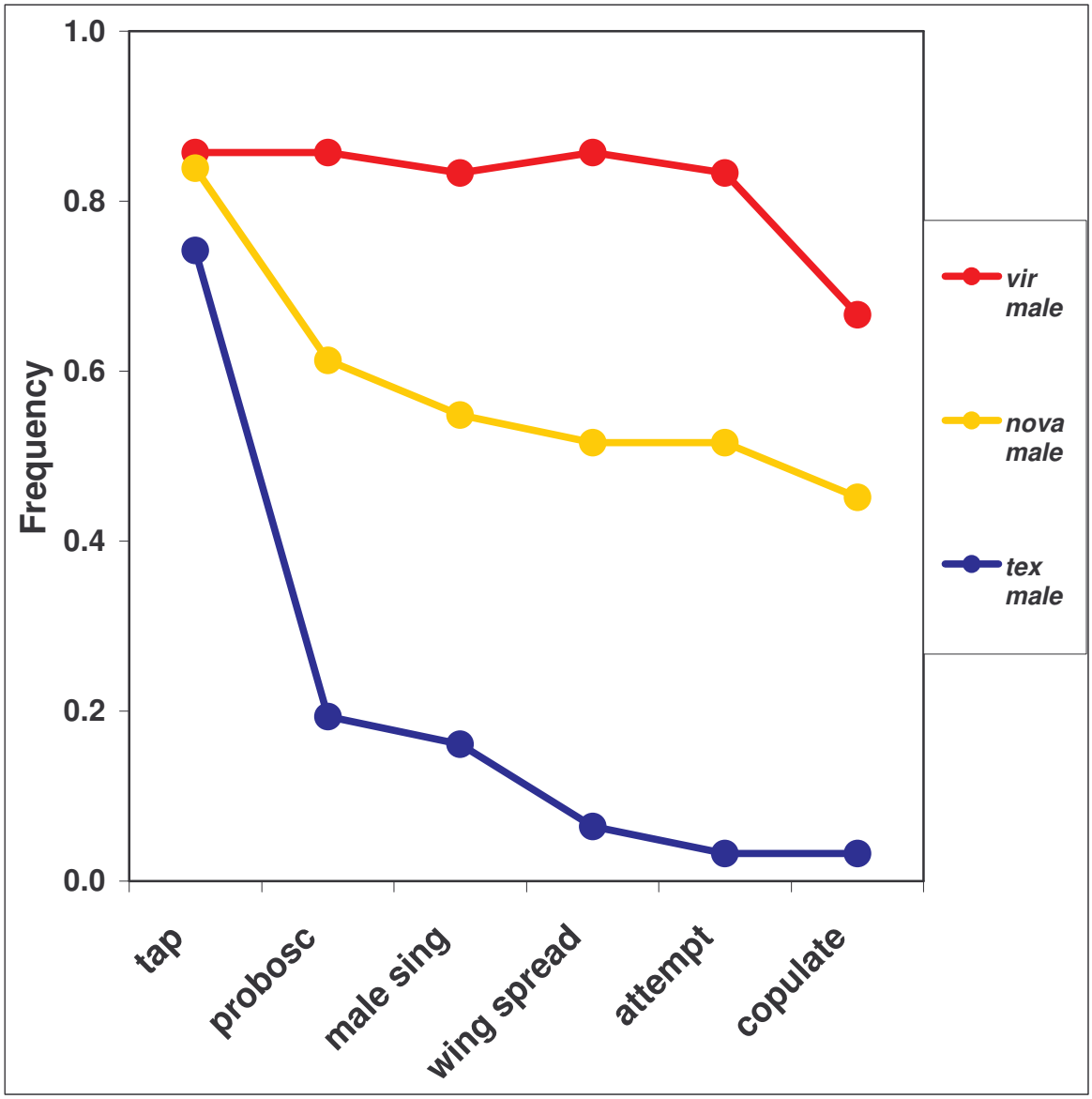

Figure 6. Observed male courtship event frequencies for *D. virilis* females paired with a conspecific or heterospecific male. Male species are identified in the figure legend. The female event of wing spreading is included to illustrate female interaction effects on courtship progression.

4.3 Hybrid male behaviour

Behavioural comparisons between reciprocal hybrid males were used to discern sex-linked effects. In comparison to *D. virilis* male heterospecific pairings, only the *vn* (from a *D. virilis* female and *D. novamexicana* male cross) and *vt* (from a *D. virilis* female and a *D. texana* male cross) hybrid males showed significantly different courtship progression scores (Appendix VI). Reciprocal hybrid males (ie. *vn* and *nv*) have the same autosomal constitution but the *vn* hybrid males, carrying the *D. virilis* X chromosome, showed stronger discrimination (C=0.95) than both the *nv* hybrid males and *D. virilis* males (C=1.61) toward *D. novamexicana* females (Appendix VI). This suggests that along with autosomal factors, the *D. virilis* X chromosome is a major contributor to isolation. Figure seven shows the behavioural breakdown of *D. novamexicana* hybrid males, relative to their parental species. Approximate randomization tests (Appendix V, line 7) show that *vn* hybrid males tap *D. novamexicana* females significantly less than *D. virilis* males do ($P \leq 0.017$). Hybrid males from *D. novamexicana* female and *D. virilis* male crosses (*nv* males) did not differ significantly in behaviour from *D. virilis* males offered *D. novamexicana* females.

Hybrid males from *D. virilis* female and *D. texana* male crosses (*vt* males) had a significantly higher courtship progression score (C=2.97) than *D. virilis* males (C=1.59) with *D. texana* females (Appendix VI). In fact, the *vt* hybrid male mean score was not significantly different from the *D. virilis* conspecific score (C=3.48). Hybrid *vt* males continued through proboscing, singing and attempting copulation significantly more ($P < 0.001$) than *D. virilis* males did when offered *D. texana* females (Appendix V, line 9). Hybrid males from *D. texana*

female and *D. virilis* male crosses (*tv* males) did not differ from *D. virilis* males in courtship toward *D. texana* females (P>0.05) (Appendix V, line 10). The *tv* hybrid male, carrying the *D. virilis* Y chromosome, had a progression score (C=1.61) quite similar to the *D. virilis* heterospecific male score (C=1.59). This suggests that the *D. virilis* Y chromosome or a form of *D. texana* X-autosomal interactions contribute to sexual isolation.

There is a consistent pattern throughout courtship event observations, and corresponding courtship progression scores, for hybrid male behaviour. The mate discrimination scores of *nv* and *tv* hybrid males, both carrying the *D. virilis* Y chromosome, are similar to *D. virilis* male discrimination scores toward heterospecific females. The *vn* and *vt* hybrid males, both carrying the *D. virilis* X chromosome, are more (*vn*) or less (*vt*) discriminate than *D. virilis* males.

4.4 Mean copulation duration

Reciprocal *D. texana* and *D. virilis* crosses were not included in copulation duration analysis due to the small sample size (N=1). The longest copulation time (N=3 samples) of four minutes and nine seconds was observed in the *tv* male and *D. texana* female cross. Of the crosses with statistically adequate copulation occurrence, the time ranged from two minutes and four seconds to two minutes and fifty-four seconds. Conspecific copulation durations were intermediate to the findings of Stalker (1942) and Spieth (1951), and clustered together in the second of three post hoc subsets (Appendix VII). The longer, and shorter, copulation durations observed in hybrid males and heterospecific crosses could indicate difficulties in sperm transfer.

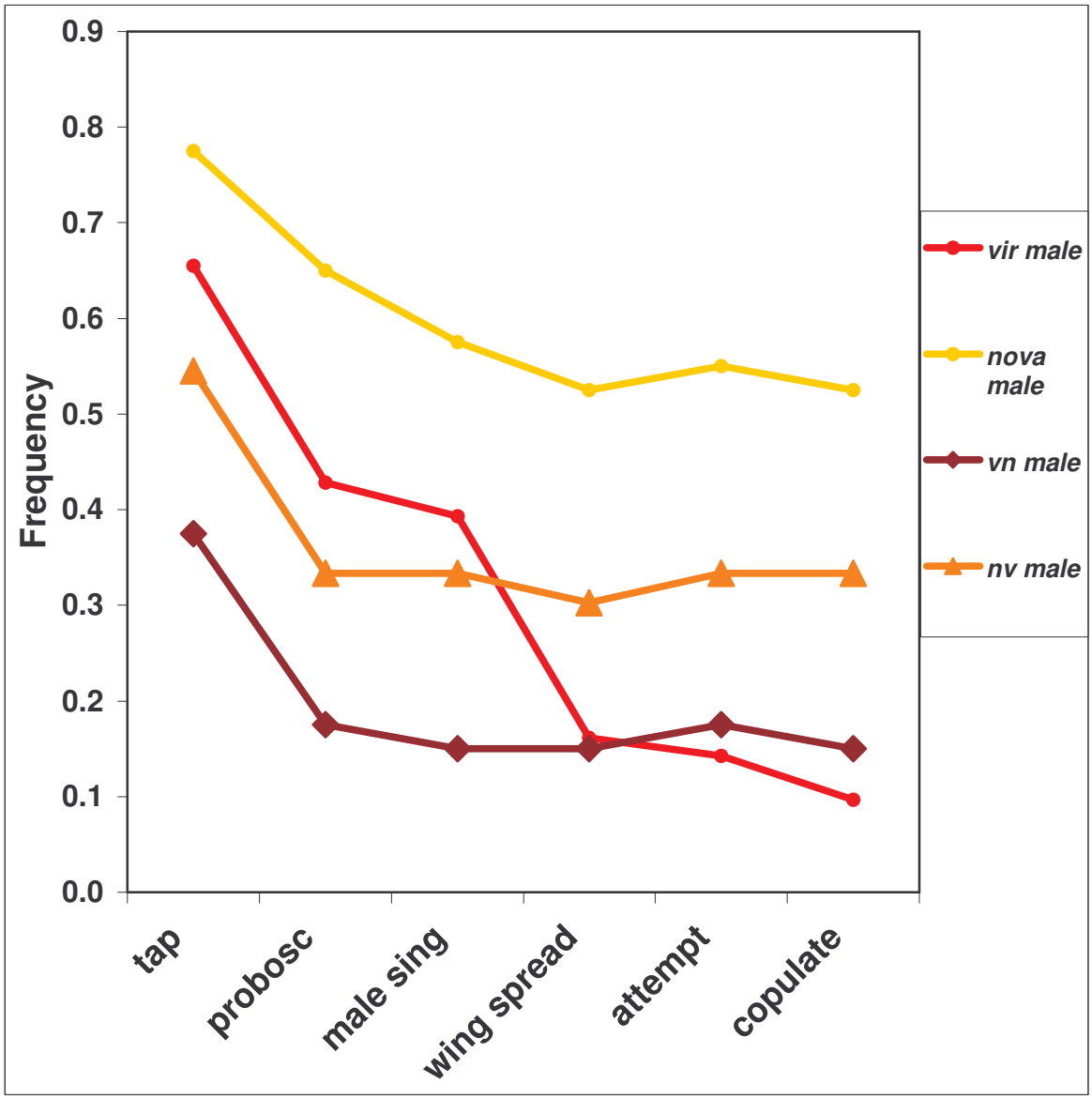

Figure 7. Courtship event frequencies, as directed toward *D. novamexicana* females, from conspecific, heterospecific and hybrid males. Male species are identified in the figure legend. The *vn* hybrid male was produced by a *D. virilis* female and *D. novamexicana* male cross, the *nv* hybrid male comes from the reciprocal cross. The female event of wing spreading is included in the graph to illustrate female interaction effects on courtship progression.

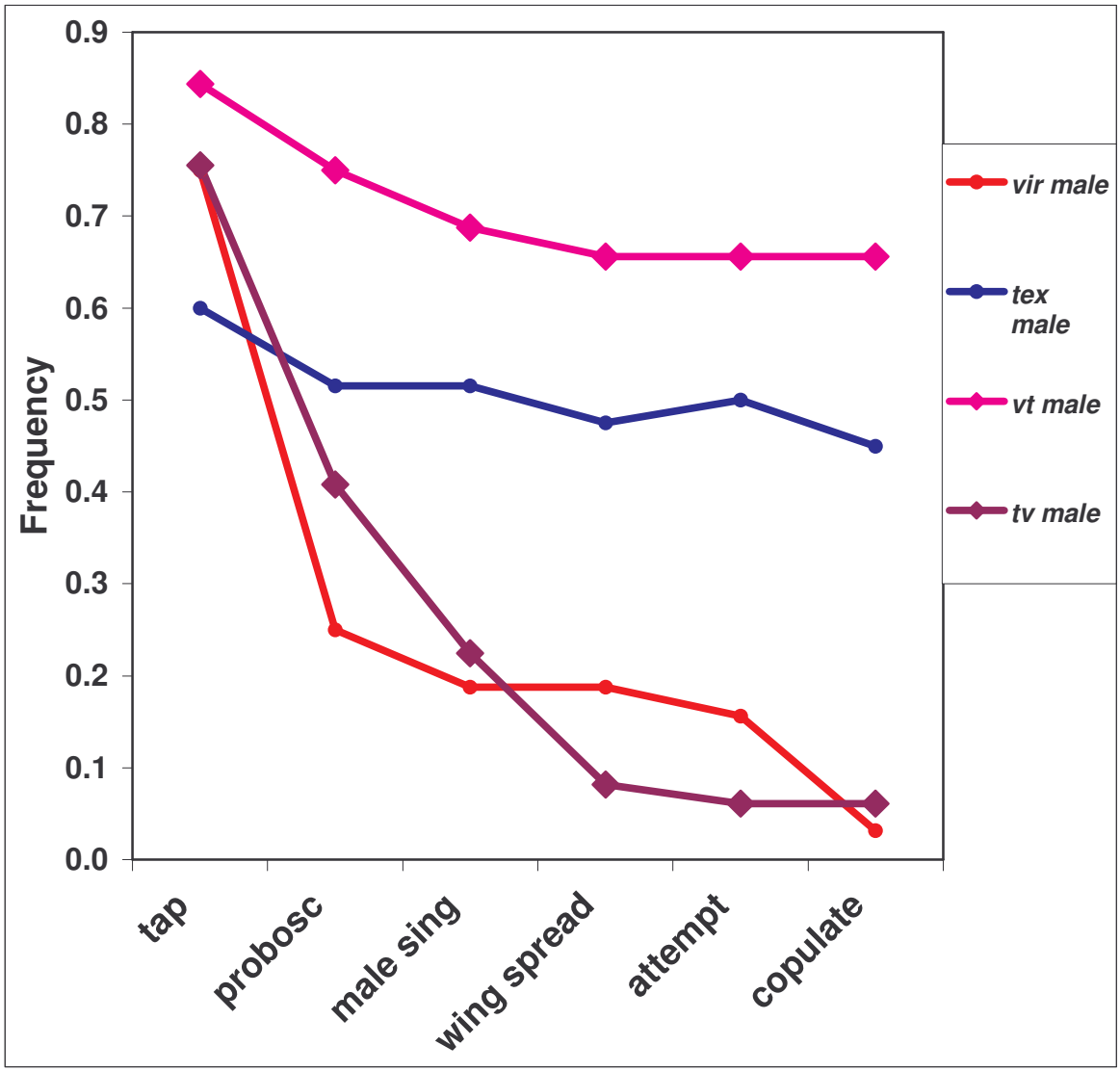

Figure 8. Courtship event frequencies, as directed toward *D. texana* females, from conspecific, heterospecific and hybrid males. Male species are identified in the figure legend. The *vt* hybrid male was produced by a *D. virilis* female and *D. texana* male cross, the t*v* hybrid male comes from the reciprocal cross. The female event of wing spreading is included in the graph to illustrate female interaction effects on courtship progression.

4.5 Isolation Indices

The postzygotic isolation index **I**, calculated using relative progeny production, was strongest (**I** ≥0.926) between *D. virilis* males and heterospecific females, yet also quite high for *D. texana* males with *D. virilis* females (**I**=0.889) (Table 2). Both the proportion of vials with progeny (Figure 3b) and **I** indicate isolation asymmetry, in that *D. virilis* males are isolated the most from *D. novamexicana* females and less isolated from *D. texana* females, yet *D. virilis* females are more isolated from *D. texana* than from *D. novamexicana* males.

The strongest premating index **Im**, calculated using relative mating frequency, was noted for reciprocal *D. virilis* and *D. texana* crosses (Table 2). *D. virilis* and *D. texana* males are the most isolated from heterospecifics, in terms of pre and post-zygotic isolation, though discrimination in *D. texana* males appears to break down over time (Figure 3b). Of the hybrid males, the *tv* and *vn* males showed the greatest behavioural isolation from non-*virilis* females (with **Im**=0.890 and 0.748 respectively). The relative strength of **Im** parallels that of **I**, except between *D. novamexicana* females and *D. virilis* males* where **I** is much stronger than **Im**.

Im › tex x vir, vir x tex, nova x vir*, vir x nova

I › nov x vir*, tex x vir, vir x tex, vir x nova

There is an asymmetry in each isolation index between males and females of the same species when they are paired with mates of another species (Table 2). *D. virilis* males are more isolated prezygotically and postzygotically from *D. texana* females (Im=0.944, I=0.926) and *D. novamexicana* females (Im=0.838, I=0.967) than *D. virilis* females are isolated from *D. texana* males (Im=0.942, I=0.889) and *D. novamexicana* males (Im=0.242, I=0.425). Sexual isolation asymmetries are greatest between *D. virilis* and *D. novamexicana* reciprocal crosses.

Table 2. Measures of sexual isolation between *Drosophila virilis* species. Hybrid male identification codes consist of two letters, the first identifies the species of the parental female and the second identifies the species of the parental male.

Female	Male	Mating frequency	**Im** *	Copulation time (min:sec)	Mean progeny	**I** ‡
tex	*tex*	0.4500	0	2:42	31.85	0
tex	*virilis*	0.0313	0.9440	¤2:28	2.71	0.9256
virilis	*tex*	0.0323	0.9422	¤0:40	4.05	0.8892
tex	*vt* hybrid	0.6563	-0.1754	2:54	NA	NA
tex	*tv* hybrid	0.0638	0.8903	4:09	NA	NA
virilis	*virilis*	0.6667	0	2:38	41.15	0
nova	*vn* hybrid	0.1500	0.7483	2:43	NA	NA
nova	*nv* hybrid	0.3333	0.4406	2:07	NA	NA
virilis	*nova*	0.4516	0.2420	2:04	22.12	0.4246
nova	*virilis*	0.0968	0.8376	2:55	1.29	0.9666
nova	*nova*	0.5250	0	2:15	35.74	0

NA - data not available.

¤ N=1.

* Prezygotic behavioural Isolation Index (**Im**) was calculated using relative mating frequencies such that: **Im** = 1 – relative mating frequency.

‡ Postzygotic Isolation Index: **I** = 1 – relative progeny production†
A value of 1 represents total isolation and a value of 0 represents no isolation.

† Relative progeny number for all crosses were calculated from the number of progeny produced relative to the combined mean of both species conspecific crosses.

Tukey post hoc results for mean copulation duration shown in Appendix VII.

5.0 Discussion

Drosophila virilis males are more strongly isolated, than *D. virilis* females, from heterospecific mates in both prezygotic and postzygotic measures. Premating isolation is affected through the courtship event of tapping, where gustatory receptors on the male's fore tarsi bind female cuticular hydrocarbons. I have not identified the specific genetic changes responsible for behavioural reproductive isolation, but the analysis of reciprocal hybrid male courtship behaviour suggests that interactions between recessive autosomal and X chromosome genes are responsible for the observed premating isolation.

5.1 Sexual selection and isolation studies

In this study I have shown that heterospecific mate discrimination is primarily the function of males. In most animals where anisogamy compels a greater maternal investment in offspring, we see strong selective pressure on females to exercise mate discrimination. The parental investment theory predicts that when females invest more than males do in offspring, females will be the discerning sex (Darwin in Gowaty *et al.*, 2003). If the assumptions of parental investment theory are met, it can be expected that female mate choice will drive sexual selection and isolation. However, several studies have indicated that males may also exercise mate discrimination regardless of parental investment (Spieth, 1951; Gowaty *et al.*, 2003). *D. virilis* (and *D. texana*) males provide an exceptional example where the selective tables, in terms of maintaining species barriers, are turned. In the virilis clade courtship discrimination is primarily the function of males, as females will copulate with males of other species – though

not to the same degree as with conspecifics (Spieth, 1951). The productivity of copulation for *D. virilis* males rests on their ability to discriminate against heterospecific females.

I have attempted to minimize the influences of mating propensity, or willingness to mate, and female mate choice (sexual selection) by utilizing a no-choice experimental design. Traditional studies of sexual selection and sexual isolation between *Drosophila* species have used one of four experimental crosses: 1) multiple choice, where several males and females of two (or more) species are placed together, 2) male choice, where the male of one species is placed with females of two species, 3) female choice, the reverse of 2, and 4) no-choice, where a single male and female are placed together (Spieth, 1951; Coyne & Orr, 1989). Mate discrimination is measured by either observing the number of copulations or quantifying the number of females inseminated (Coyne & Orr, 1989; Carracedo *et al.*, 2000).

Multiple-choice designs offer an actual choice of mates and therefore are more sensitive to subtle variations in mate preference that contribute to sexual selection (Saarikettu *et al.*, 2005). No-choice tests are less subject to the possible skewing effects of male and female mating propensity on measures of sexual isolation (Coyne *et al.*, 2005; Saarikettu *et al.*, 2005). No-choice tests provide true statistical replicates, with each pair observed, and present a more controlled (albeit artificial) environment as well as the opportunity for observers to identify the sex and species of each individual without the need to mark or alter the dipteran's phenotype (Coyne *et al.*, 2005).

Commonly used measures of sexual isolation do not allow a comparison of isolation asymmetry between males and females of a given species because a single index is computed from reciprocal (i.e. male1 × female2; male2 × female1) crosses (Rolan-Alvarez & Caballero, 2000). In this study I have estimated prezygotic (**Im**) and postzygotic (**I**) isolation indices for each direction of reciprocal crosses. Isolation asymmetry is apparent in premating and postmating indices, where *D. virilis* males are more strongly isolated from heterospecifics, than *D. virilis* females are, in both measures. Many *Drosophila* species show asymmetrical sexual isolation toward closely related species, one sex of a species is often more crossable than the other (Coyne *et al.*, 1994). Asymmetry may represent differences in male traits and female preferences, which may indicate that different genetic factors contribute to sex specific sexual isolation.

Coyne and Orr (1989) found that the degree of asymmetry between prezygotic and postzygotic isolation is negatively correlated with genetic distance. Among closely related species, it is commonly observed that only one sex of a species is strongly isolated from heterospecific mates causing asymmetry in isolation indices. Over time, reinforcement against the production of disadvantageous hybridizations will increase sexual isolation in the opposite sex (Coyne & Orr, 1989). Therefore, males and females of one species are expected to display less asymmetry, and stronger overall isolation, toward more distantly related species. Isolation indices (both **I** and **Im**) are near one, and symmetrical, between *D. virilis* and *D. texana*, whereas there is a greater asymmetry between *D. virilis* and *D. novamexicana* crosses. These findings lend support to the hypothesis that divergence from the founding *D. virilis* ancestor occurred in the

temporal order of *D. novamexicana*, and then *D. texana* and *D. americana*, which is consistent with classic phylogenetic analyses based on chromosomal differences in this group (Appendix VIII) (Caletka & McAllister, 2004).

5.2 Elements of the Species Mate Recognition System

This study shows that *D. virilis* males break off courtship with heterospecific females after tapping them. This initial stage of courtship, where female cuticular hydrocarbons (CH) are sampled, appears to be the critical point in mate evaluation for *D. virilis* males. Therefore, genetic factors contributing to CH reception are likely to play a large role in mate recognition and the activation of courtship behaviour in the virilis clade. The occurrence of sex and species specific hydrocarbons across numerous *Drosophila* groups suggests that their production and reception are an essential component of the species mate recognition system (SMRS) (Coyne, 1994; Scott *et al.*, 2001; Markow, 2005).

A few studies have utilized either decapitated or freeze-killed females to test male mate discrimination, suggesting that female chemical cues are more important than behavioural ones in eliciting male courtship (Marcillac *et al.*, 2005; Oguma *et al.*, 1992; Coyne *et al.*, 1994). Cuticular hydrocarbons, or contact pheromones, function in mate recognition and modulate male courtship intensity for several *Drosophila* species (McMahon *et al.*, 2002; Saarikettu, *et al.* 2005). Differences in cuticular hydrocarbon profiles affect sexual isolation between *D. simulans* and *D. sechillia*, two species of the melanogaster clade, and the genetics of these CH differences have been localized to the third chromosome (Coyne *et al.*, 1994).

In *D. melanogaster* two closely linked desaturase genes (*desat*) on the right arm of the third chromosome, *desat*1 and *desat*2, control the proportion of unsaturated CHs produced by females. The *desat*1 gene functions in a pleiotropic manner to affect not only the production of, but also the perception of CHs in both males and females (Marcillac *et al.*, 2005). Multiple *desat* products are proposed to interact with metabolic genes in the oenocytes, which participate in CH production, and with the accessory cells of chemosensory organs of the tarsi, antennal segments and proboscis (Marcillac *et al.*, 2005).

A large family of seven-transmembrane domain proteins mediates taste recognition in *D. melanogaster*; these putative G protein-coupled receptors are coded for by gustatory receptor (GRO) genes distributed throughout the genome (Scott *et al.*, 2001). Transgenic and in situ hybridization studies have localized expression of the Gr32D1 gene to chemosensory bristles of the tarsi, proboscis and cibarial organ (Scott *et al.*, 2001). Another gustatory gene, Gr68a, has been shown to code for pheromone reception. Disruption of the Gr68a gene causes a significant decrease in *D. melanogaster* male courtship progression (Markow & O'Grady, 2005). Locating candidate chromosomal regions that contribute to behavioural isolation in the virilis clade should begin with a focus on the *desat* and GRO gene regions.

5.3 The genetics of behavioural isolation

Recessive autosomal effects on premating isolation are apparent from hybrid male behaviour for each species pair crossed. All four hybrid male genotypes mated more frequently with non-*virilis* females than *D. virilis* males

did. Courtship behaviour was discrepant between reciprocal hybrid males of the same species, indicating sex-linked genes also contribute to sexual isolation in this group of species. The analysis of hybrid male mating frequency shows that *D. virilis – D. novamexicana* hybrids with a *D. virilis* X chromosome were as discriminative as *D. virilis* males towards *D. novamexicana* females, and *D. texana - D. virilis* hybrids with the *D. virilis* Y chromosome were as discriminative as *D. virilis* males towards *D. texana* females.

Presumably, because chromosomal recombination does not occur in males, gene loci on the Y chromosome should be relatively conserved between the three species studied, unless mutation or selection have facilitated genetic divergence. Only a few studies have suggested the *Drosophila* Y chromosome contributes to behaviour, yet several have shown a relationship between behaviour and the X chromosome, particularly in sexually selected traits (Huttunen & Aspi, 2003; Paallysaho *et al.*, 2003; Ortiz-Barrientos *et al.*, 2004).

One possible scenario is that mate discrimination occurs as a result of interactions between genes on the autosomes with sex chromosome genes modulating the effect of such interactions. My results show hybrid males (*nv* and *tv*) with a *D. virilis* Y chromosome did not differ significantly in courtship progression from *D. virilis* males encountering heterospecific females. However, hybrid males that carry the *D. virilis* X chromosome were either more (*vn*) or less (*vt*) discriminatory than *D. virilis* males. These results suggest that the *D. virilis* Y chromosome may not have an effect on recessive autosomal interactions, whereas the X chromosome increases or decreases recessive autosomal effects on premating isolation depending on the genetic background.

Another possibility is that a male's ability to be highly discriminatory in courtship behaviour towards heterospecific females is the consequence of interactions between loci on the autosomes and X chromosome. If so, breakdown of chromosomal interactions in interspecies hybrids, due to genetic divergence between species, could make hybrid males less discriminatory than either parental species. *D. novamexicana* is closely related to *D. virilis* and so its autosomal loci may be similar enough to properly interact with the *D. virilis* X chromosome, preserving the *D. virilis* male discriminatory behaviour. Autosomal loci in the more distantly related *D. texana* may not properly interact with the *D. virilis* X chromosome, making males non-discriminatory towards females. Courtship observations indicate that *D. texana* males are nearly as discriminate as *D. virilis* males, therefore undisrupted interactions between *D. texana* X chromosome and *D. texana* autosomes in the *tv* hybrid may preserve the *D. texana* male discriminatory behaviour with a possible enhancement of the effect due to *D. virilis* autosomal genes.

Overall the results indicate a complex genetic basis with recessive autosomal loci interactions together with X chromosome genes causing males to discriminate between females of different species based on their CH profiles. Further research is required to determine the gustatory genes responsible for the behavioural isolation of *D. virilis* males. Chromosomal homology and the completion of twelve *Drosophila* species genome sequences, make it possible to compare gene sequences among species, and allows the mapping of loci from one species to another. The postulated chromosomal homologies between *D. virilis* and *D. melanogaster*, respectively, are: X=X; 2=3R; 3=3L; 4=2L; 5=2R; 6=4 (Kress, 1993; Nurminsky *et*

al., 1996). Therefore, candidate gustatory genes should map to chromosome two (*desat*) and to chromosomes three and four (Gr32D1 and Gr68a) in *D. virilis*.

In several *Drosophila* clades sexual isolation is not absolute, providing the opportunity to manipulate genetic background in order to determine modes of inheritance responsible for behavioural divergence (Carracedo *et al*., 2000). There have been relatively few studies that have attempted to discern the genetics of prezygotic isolation (Coyne *et al*, 1994). It is particularly difficult to disentangle the many genetic influences on behaviour because even seemingly simple traits require interplay between several genetic, developmental and environmental influences. It is also possible that genes that maintain isolation at present have evolved after speciation was complete (Caracedo *et al*., 2000). Regardless of potential limitations, mapping genes that contribute to behavioural diversity and prezygotic isolation between species is a much needed first step towards the understanding of species isolation and would certainly provide a major contribution to unravelling the origin of species (Coyne *et al*., 1994).

6.0 Conclusions

1) *D. virilis* males produce a lower number of progeny than *D. virilis* females in heterospecific crosses.

2) *D. virilis* males are more discriminating than *D. virilis* females toward heterospecific mates.

3) *D. virilis* males are able to recognize heterospecific females by their hydrocarbon profiles early in courtship, thus conserving energy for more productive encounters.

4) Recessive autosomal and X chromosome interactions modulate the ability of *D. virilis* males to discriminate against heterospecific females.

5) There is evidence of asymmetry between males and females in both prezygotic and postzygotic isolation toward other species.

6) Traits involved in the emission and reception of signals relevant to courtship repertoires, and the genetic interactions that cause them, are not the same for males and females of the same species.

7.0 Future Directions

To determine the region(s) of *D. virilis* X chromosome and autosomes that interact to produce behavioural isolation through cuticular hydrocarbon reception, candidate gustatory receptor genes on chromosomes two, three, and four will be evaluated for their role in mate discrimination. Reciprocal crosses between *D. virilis* mutant (2) (stock #15010-1051.18) and wild type *D. novamexicana* will be used to produce F1 hybrid females, where recombination of heterospecific chromosomes will occur in the female gametes, to backcross to a *D. novamexicana* male (Figure 9).

The mutant (2) stock is homozygous for three recessive mutations on the X-chromosome (y [1] ch [2] mt [1]) conferring yellow body color, deep cherry red eyes, and miniature wings respectively. The fecundity of F1 hybrid males and females (postzygotic isolation) in backcrosses will be assessed following the previously described progeny count protocol. Backcross (B1) male progeny, hemizygous for different regions of the *D. virilis* X chromosome, and heterozygous for *D. virilis* autosomal introgressions, will be used to test X - autosomal effects on reproductive isolation. Molecular marker analysis will be used to determine B1 male chromosomal regions that are of *D. virilis* origin.

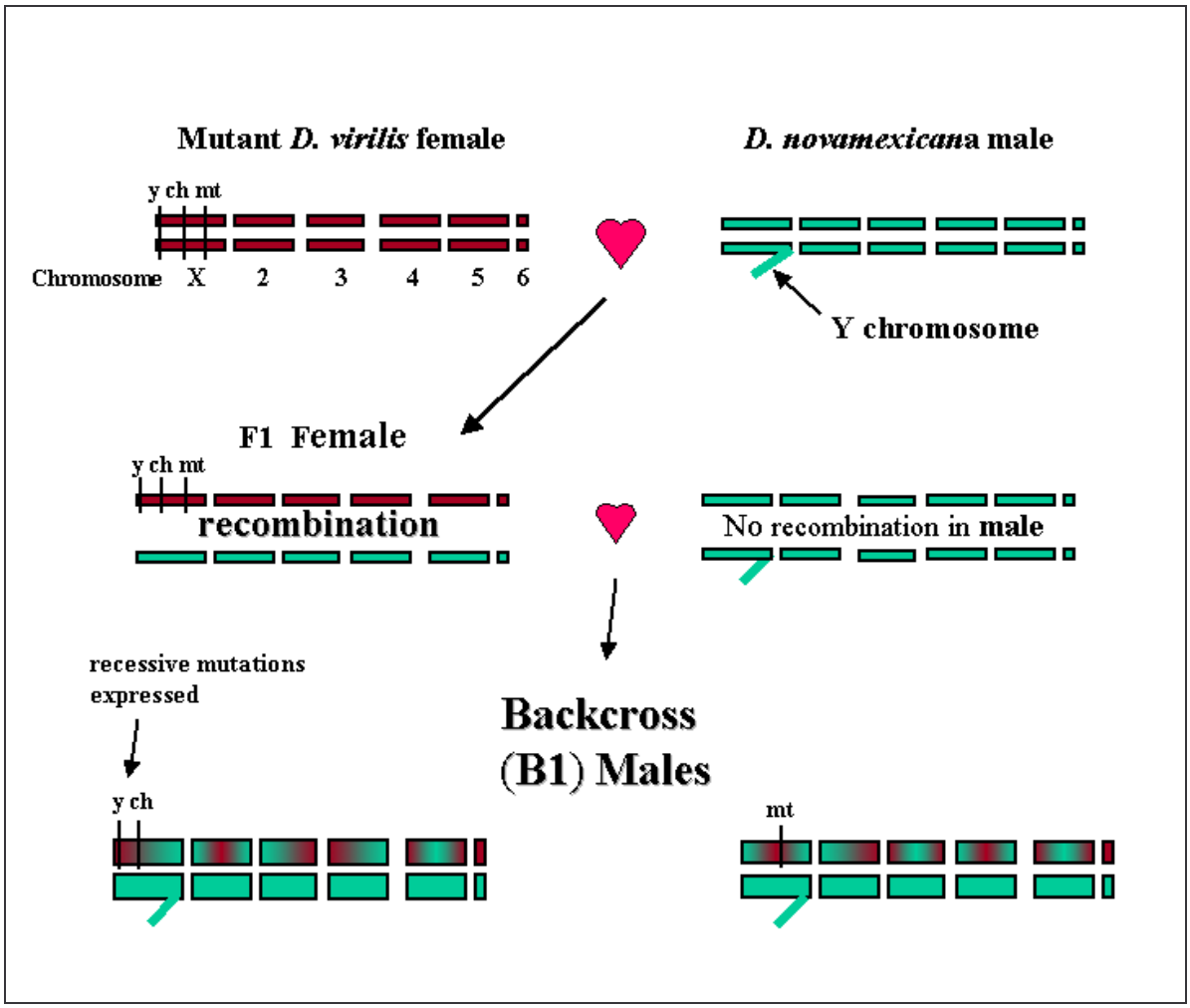

Figure 9. *Drosophila virilis* heterospecific crossing scheme for the determination of X chromosome and autosomal regions that contribute to behavioural isolation. Reciprocal crosses between *D. virilis* mutant (stock #15010-1051.18) and *D. novamexicana* will produce F1 hybrid females to backcross to *D. novamexicana* males. Backcross (B1) male progeny, hemizygous for different sections of the *D. virilis* X chromosome, and heterozygous for sections of *D. virilis* autosomes, will be tested for sexual isolation toward *D. novamexicana* females. Molecular marker analysis will be used to determine chromosomal regions of *D. virilis* origin in the backcross males (Huttunen & Schlotterer, 2002).

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9.0 Appendices

Appendix I. *Drosophila* standard cornmeal-molasses (**CM**) medium.
Pour water to boil into a steel pot on a hotplate at high heat. Combine cornmeal, yeast and agar with cold water; add this slurry to boiling water. Stir constantly until mixture comes to a second boil, then remove from heat and add molasses. Cool to 65°C in a water bath, add Tegosept and propionic acid and dispense into vials or bottles with a peristaltic pump. Yields approximately one hundred 28.5 x 95 mm vials or eighteen 237 ml bottles.

Ingredient	**Quantity**
Cornmeal	65 g
Brewers Yeast	13 g
Agar	6.5 g
Cold Water	170 ml
Boiling Water	760 ml
Refiners Molasses	45.5 ml
10% Tegosept*	20 ml
99% Propioninc Acid	5 ml

* 50 g methyl 4-hydroxybenzoate in 500 ml 95% ethanol.

Appendix II. Statistical analyses of conspecific and heterospecific progeny count data. Data was square root transformed to fit normality.

a) Analysis of variance test of between subject effects.

Tests of Between-Subjects Effects

Dependent Variable: SQRT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2581.501[a]	11	234.682	116.423	.000
Intercept	4276.230	1	4276.230	2121.394	.000
Species Cross	2581.501	11	234.682	116.423	.000
Error	1185.269	588	2.016		
Total	8043.000	600			
Corrected Total	3766.770	599			

a. R Squared = .685 (Adjusted R Squared = .679)

b) Tukey post hoc test results. The species cross column lists the female species first, followed by the male species.

Square Root of Progeny Count

Tukey HSD[a,b]

Species Cross	N	Subset 1	2	3	4	5	6
nov x vir	50	.1566					
amer x vir	50	.1849					
tex x vir	50	.6405	.6405				
vir x amer	50	.7132	.7132	.7132			
nov x tex	50		1.2142	1.2142			
vir x tex	50			1.6184			
amer x amer	50				3.1120		
tex x nov	50				3.4699		
vir x nov	50					4.4946	
tex x tex	50					5.2795	5.2795
nov x nov	50					5.3519	5.3519
vir x vir	50						5.8001
Sig.		.720	.679	.066	.983	.106	.799

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 2.016.

a. Uses Harmonic Mean Sample Size = 50.000.

b. Alpha = .05.

Appendix III. Statistical analyses of the proportion of progeny count vials indicating the occurrence of mating.

a) Analysis of variance test of between subject effects.

Tests of Between-Subjects Effects

Dependent Variable: indication of mating

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	53.898[a]	11	4.900	40.183	.000
Intercept	295.402	1	295.402	2422.541	.000
Species Cross	53.898	11	4.900	40.183	.000
Error	71.700	588	.122		
Total	421.000	600			
Corrected Total	125.598	599			

a. R Squared = .429 (Adjusted R Squared = .418)

b) Tukey post hoc test results.

indication of mating

Tukey HSD [a,b]

Species cross	N	Subset 1	Subset 2	Subset 3
nov x vir	50	.1600		
amer x vir	50	.1800		
tex x vir	50		.4200	
vir x amer	50		.4400	
amer x amer	50			.7800
nov x tex	50			.8000
vir x tex	50			.8800
nov x nov	50			.9200
vir x vir	50			.9400
tex x nov	50			.9400
tex x tex	50			.9600
vir x nov	50			1.0000
Sig.		1.000	1.000	.074

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .122.

a. Uses Harmonic Mean Sample Size = 50.000.

b. Alpha = .05.

Appendix IV. Pearson correlation co-efficient (r) between courtship events.

a) Pearson correlation between male tap and male probosc.

		male tap	male probosc
male tap	Pearson Correlation	1	.558**
	Sig. (1-tailed)	.	.000
	N	401	401
male probosc	Pearson Correlation	.558**	1
	Sig. (1-tailed)	.000	.
	N	401	401

**. Correlation is significant at the 0.01 level (1-tailed).

b) Pearson correlation between male probosc and male sing.

		male probosc	male sing in courtship
male probosc	Pearson Correlation	1	.885**
	Sig. (1-tailed)	.	.000
	N	401	401
male sing in courtship	Pearson Correlation	.885**	1
	Sig. (1-tailed)	.000	.
	N	401	401

**. Correlation is significant at the 0.01 level (1-tailed).

c) Pearson correlation between male sing and male attempt to copulate.

		male sing in courtship	male attempt to copulate
male sing in courtship	Pearson Correlation	1	.792**
	Sig. (1-tailed)	.	.000
	N	401	401
male attempt to copulate	Pearson Correlation	.792**	1
	Sig. (1-tailed)	.000	.
	N	401	401

**. Correlation is significant at the 0.01 level (1-tailed).

Appendix IV. Pearson correlation co-efficient (r) between courtship events.

d) Pearson correlation between male attempt to copulate and male/female copulate.

		male attempt to copulate	copulate
male attempt to copulate	Pearson Correlation	1	.914**
	Sig. (1-tailed)	.	.000
	N	401	401
copulate	Pearson Correlation	.914**	1
	Sig. (1-tailed)	.000	.
	N	401	401

**. Correlation is significant at the 0.01 level (1-tailed).

e) Pearson correlation between male sing and female wing lift.

		male sing in courtship	female wing lifting
male sing in courtship	Pearson Correlation	1	.776**
	Sig. (1-tailed)	.	.000
	N	401	401
female wing lifting	Pearson Correlation	.776**	1
	Sig. (1-tailed)	.000	.
	N	401	401

**. Correlation is significant at the 0.01 level (1-tailed).

f) Pearson correlation between female wing lift and male attempt to copulate.

		female wing lifting	male attempt to copulate
female wing lifting	Pearson Correlation	1	.941**
	Sig. (1-tailed)	.	.000
	N	401	401
male attempt to copulate	Pearson Correlation	.941**	1
	Sig. (1-tailed)	.000	.
	N	401	401

**. Correlation is significant at the 0.01 level (1-tailed).

Appendix V. Pair wise approximate randomization test results for male courtship events. Cross column entries list the female species first, then the male species.

Line	Cross 1	Cross 2	Two tailed Probability			
			Tap	Probosc	Sing	Attempt
1	nov x nov	vir x nov	0.566	0.804	1.0	0.810
2	vir x vir	nov x vir	0.088	0.000	0.000	0.000
3	vir x vir	vir x nov	1.0	0.028	0.010	0.005
4	tex x tex	vir x tex	0.325	0.007	0.003	0.000
5	vir x vir	tex x vir	0.368	0.000	0.000	0.000
6	vir x tex	tex x vir	1.0	0.763	1.0	0.194
7	nov x vir	nov x vn	0.0168	0.062	0.056	0.747
8	nov x vir	nov x nv	0.313	0.793	1.0	0.074
9	tex x vir	tex x vt	0.535	0.000	0.000	0.000
10	tex x vir	tex x tv	0.805	0.160	0.785	0.253

* Hybrid male identification codes consist of two letters, the first identifies the species of the parental female and the second identifies the species of the parental male.

Appendix VI. Statistical analyses of male courtship progression scores.

a) Analysis of variance of between subject effects.

Tests of Between-Subjects Effects

Dependent Variable: Male courtship progression score

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	223.018[a]	10	22.302	9.412	.000
Intercept	1688.533	1	1688.533	712.616	.000
Species Cross	223.018	10	22.302	9.412	.000
Error	924.099	390	2.369		
Total	2890.000	401			
Corrected Total	1147.117	400			

a. R Squared = .194 (Adjusted R Squared = .174)

b) Tukey post hoc test results.

Courtship Progression Score

Tukey HSD[a,b,c]

Species Cross	N	Subset 1	2	3	4
* **nov x vn**	40	.95			
vir x tex	31	1.52	1.52		
* **tex x vir**	32	1.59	1.59		
* **nov x nv**	33	1.61	1.61		
* **tex x tv**	49	1.61	1.61		
* **nov x vir**	31	1.61	1.61		
tex x tex	40		2.28	2.28	
vir x nov	31		2.55	2.55	2.55
nov x nov	40		2.67	2.67	2.67
* **tex x vt**	32			2.97	2.97
vir x vir	42				3.48
Sig.		.769	.060	.716	.283

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 2.369.

a. Uses Harmonic Mean Sample Size = 35.622.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

* Hybrid male identification codes consist of two letters, the first identifies the species of the parental female and the second identifies the species of the parental male.

Appendix VII. Statistical analyses of mean copulation duration (min : sec). Reciprocal *D. virilis* and *D. texana* heterospecific crosses were not included in the analysis due to copulation sample sizes of one.

a) Analysis of variance test of between subject effects.

Tests of Between-Subjects Effects

Dependent Variable: Copulation time

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	243697593[a]	8	30462199.12	10.812	.000
Intercept	6535819157	1	6535819157	2319.736	.000
Species Cross	243697593	8	30462199.12	10.812	.000
Error	307105732	109	2817483.784		
Total	1.063E+10	118			
Corrected Total	550803325	117			

a. R Squared = .442 (Adjusted R Squared = .402)

b) Tukey post hoc results.

Copulation time

Tukey HSD [a,b,c]

Species Cross	N	Subset 1	Subset 2	Subset 3
vir x nov	14	2:04		
nov x nv	11	2:07		
nov x nov	21	2:15	2:15	
vir x vir	21	2:38	2:38	
tex x tex	18	2:42	2:42	
nov x vn	6	2:43	2:43	
tex x vt	21		2:54	
nov x vir	3		2:55	
tex x tv	3			4:09
Sig.		.138	.139	1.000

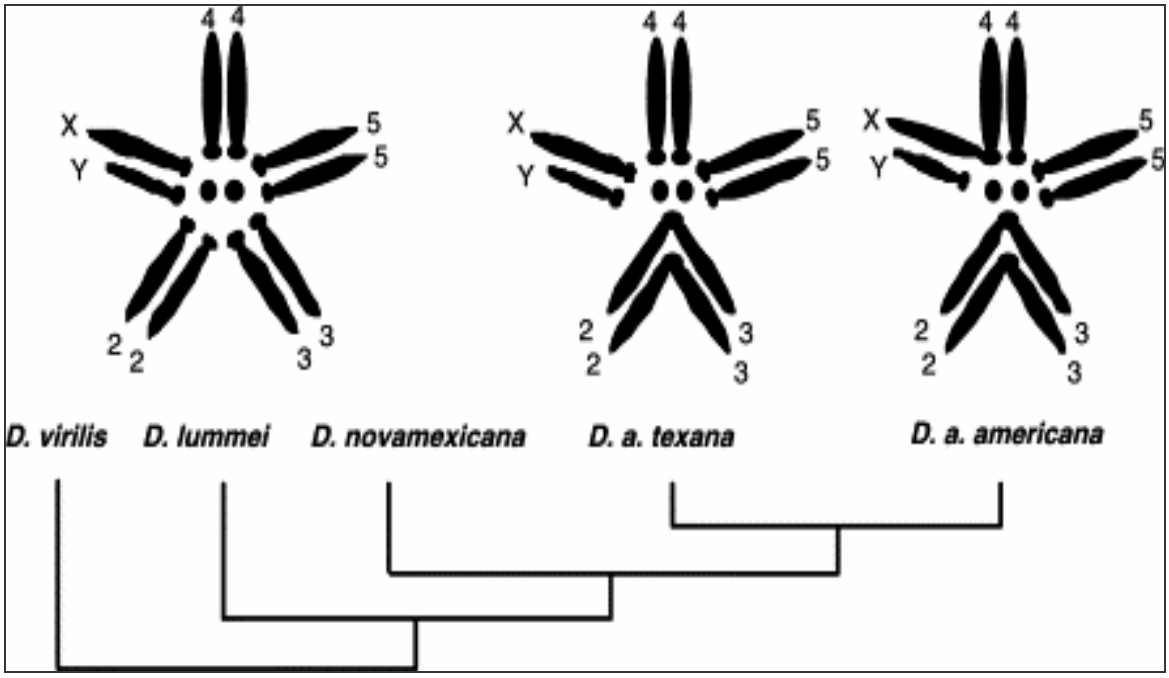
Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
Crosses vir x tex and tex x vir removed from analysis (N=1).

a. Uses Harmonic Mean Sample Size = 7.537.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

* Hybrid male identification codes consist of two letters, the first identifies the species of the parental female and the second identifies the species of the parental male.



Appendix VIII. Phylogenetic relationship among species of the Drosophila virilis group based on chromosomal rearrangements (Throckmorton, 1982; Caletka & McAllister, 2004).