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Evaluating the Rapid Divergence of Male Genitalia in Sibling Drosophila Species

Helene M. LeVasseur-Viens
The University of Western Ontario

Supervisor
Amanda Moehring
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

EVALUATING THE RAPID DIVERGENCE OF MALE GENITALIA IN SIBLING *DROSOPHILA* SPECIES

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by

Hélène LeVasseur-Viens

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The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

_____
Dr. Amanda Moehring

Supervisory Committee

_____
Dr. André Lachance

_____
Dr. Graham Thompson

Examiners

_____
Dr. Sashko Damjanovski

_____
Dr. Elizabeth MacDougall-Shackleton

_____
Dr. Graham Thompson

The thesis by

Hélène LeVasseur-Viens

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Abstract

The rapid divergence of male genitalia in a variety of animal groups is a well documented phenomenon for which there exists no universal explanation. The three prevalent hypotheses for the divergence of genitalia, the lock and key, pleiotropy and sexual selection, have all been tested in individual model organisms, but never have individual experiments been performed in one species pair to allow for direct comparison. *Drosophila simulans* and *D. mauritiana* have long been thought to be an example of the sensory lock and key model, but no concrete data has ever been presented to verify the validity of the model. This work looks to employ three different techniques to investigate each of the hypotheses, disproving the long held lock and key model and instead supporting the more generally accepted sexual selection hypothesis. These techniques could help to apply a more general explanation for the rapid divergence of male genitalia.

Keywords

Genitalia, Reproductive Isolation, Sexual Selection, Lock and Key, Pleiotropy, *Drosophila*

Dedication

This thesis is dedicated to the late Georges Levasseur: he wasn't sure if he believed in evolution but he certainly believed in me.

I would also like to thank my entire family who, no matter how stressed, or down on myself I can be, still thinks I'm big pimpin.

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In my seven years at the University of Western Ontario I have met a wide range of amazing scientists and academics that I would like to recognize for helping me to develop into the researcher that I am today.

Firstly, I would like to express my gratitude to my supervisor Amanda Moehring. Watching her speak about her research in a seminar steered me into a field I had never expected to become passionate about. I would like to thank her for helping me to realize my passion for the study of evolution, and that women in science can strive to have it all. Her intelligence and determination are an inspiration. I know that one day when I say I was a Masters candidate in her lab, that everyone will be thoroughly impressed I studied in such a prestigious learning environment.

This project would not have become what it is without the help of Dr Michal Polak at the University of Cincinnati, who graciously offered to assist me and led me in the right direction with his incredible intellect, and appreciation for my bad jokes.

My thesis could not have been completed without the help of my advisory committee. Getting to work with one of your idols from undergrad is rare but I was lucky enough to work with mine, Dr Andre Lachance. He was instrumental in developing my written thesis and ensuring my most glaring grammar mistakes didn't make it into these pages. His painstaking efforts made me look smarter and made this writing process easier. Dr Graham Thompson's assistance in my project's development and his humor throughout working with him has enabled me to both develop as a scientist and as a person, and for that I am truly grateful.

And lastly, the people who kept me sane. Your lab mates are really the only people who truly understand what a terrible failure and incredible success feels like in research. They're living it with you every day through the shit storm and the sunny moments. I would specifically like to thank two friends I hope to keep for the rest of my life, Meghan Laturney and Jessica Pardy who are to thank for me laughing every day, even when I felt like crying.

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Chapter 1

1 Literature Review

An overview of previous research, and the current state of the literature with regards to the rapid divergence of male genitalia.

Rapidly Diverging Genital Morphology

The divergent morphology of male genitalia across a variety of species is a well-studied phenomenon. Animal groups ranging from primates (Dixson 1989) to lizards (Böhme and Ziegler 2009) all show rapid evolution of male genitalia (Table 1.1) and yet biologists do not fully understand why this divergence occurs (Eberhard 1985). The literature (Eberhard 1985; Robertson 1988; Polak and Simmons 2009) includes in the definition of genitalia not only the inseminating, or primary, organs but also the secondary organs involved in copulation. Stimulation organs are thought to be important and also exhibit rapid divergence in a variety of animal groups (Eberhard 1985).

In insects, the rapid divergence male genitalia is so pronounced that even recently diverged sibling species show a high degree of variation in the male genitalia (Richards 1927; Liu *et al.* 1996; Song 2009). Although the phenomenon has long been known, an understanding of why it is so widespread is lacking (Arnqvist and Thornhill 1998; Simmons *et al.* 2009). Several different models have been developed to explain the evolution of genitalia in individual species, but none explains why it occurs across so many animal groups. The most prominent competing theories that attempt to explain why rapidly diverging male genitalia occur in such a variety of animal groups are the pleiotropy hypothesis, the lock and key hypothesis, and the sexual selection hypothesis

Table 1.1. Animal groups that have been documented as having rapidly evolving male genitalia (Adapted from Eberhard 1985)

Group	*Female genitalia soft and saclike or tubelike?*	*Sperm deposited directly onto eggs?*	*References*
Most insects	Some	No	**Liu *et al.* 1996; Arnqvist 1997, Arnqvist and Thornton 1998; Cordoba-Aguilar 1999 2002; Song 2009; Jensen *et al.* 2010; Simmons and Garcia-Gonzalez 2011**
Mammals:	Yes	No	
Artiodactyls	Yes	No	Gerhardt, in Walton 1960
Bats	Yes	No	Martin and Schmidly 1982; **Hosken *et al.* 2001**
Cavimorph and microtine rodents	Yes	No	**Matocq *et al.* 2007**
Primates	Yes	No	**Dixon 1989**
Many pulmonate molluscs	Yes	No	**Lace 1992**
Some opisthobrand molluscs	Yes	No	Edmunds 1970
Poeciliid fish	Yes	No	Rosen and Gordon 1953
Some cottid fish	Yes	No	**Morris 1956**
Sharks and rays	Yes	No	Applegate 1967
Many snakes	Yes	No	Saint-Girons 1975; **Nagy *et al.* 2007**
Some lizards	Yes	No	**Bohme and Ziegler 2009**
Many nematodes	Yes	No	Spratt 1979
Many turbellarian flatworms	Yes	No	Henley 1974
Some polychaete worms	Yes	No	**Merz and Woodin 2006**
Ostracod crustaceans			Pennak 1978
Some mites	Yes	No	Santana 1976; Griffiths and Boczek 1977
Aves	Yes	No	**Brennan *et al.* 2007**

(N.B. Citations in bold were added by H. LeVasseur-Viens)

Table 1.2. A summary of the predictions made by each hypothesis for the rapid divergence of male genitalia

Hypothesis	Reproductive Isolation	Selection Occurring	Type of Mates Rejected	Type of Isolation	Timing of Rejection
Pleiotropy	No	No	-	-	-
Lock and Key	Yes	Yes	Interspecific	Prezygotic	Precopulatory
Sexual Selection	Yes	Yes	Intraspecific	Prezygotic	Postcopulatory

(Eberhard 1985 1990 1993; Masly 2012; Hosken and Stockley 2004; Arnqvist 1997 1998; Shapiro and Porter 1989). All make different predictions as to why male genitalia are highly divergent and what the resulting effects are on speciation (Table 1.2). These hypotheses are used by researchers to explore the evolution of the morphology of genitalia and why it follows similar trends across such a broad range of model organisms.

The Pleiotropy Hypothesis

The pleiotropy hypothesis, first suggested by Ernst Mayr in 1963, describes the divergence of male genitalia as a by-product of their genetic link to general morphological genes. Selection acts on the overall morphology of diverging species, leading to the variation that is currently observed. The pleiotropy hypothesis predicts that changes in the morphology of the now differentiated species, in the absence of selection against the divergence of genitalia, cause an accumulation of neutral changes in the genitalia of sister species. It is difficult to reject the notion that genes leading to changes in body morphology also affect the morphology of genitalia. Consequently, the pleiotropy hypothesis has been looked upon favorably, but it is also controversial.

The pleiotropy hypothesis for the rapid evolution of male genitals differs from the other two hypotheses in that it does not predict that the male genitalia can be an initiating factor in reproductive isolation. According to the pleiotropy hypothesis, the variation observed, and therefore the subsequent mechanical isolation that can result, are by-products of the neutral evolution occurring on the organisms as a whole. By contrast, the lock and key and sexual selection hypotheses predict that the morphology of male genitalia is a key factor in initiating the isolation of species.

Evidence for the pleiotropy hypothesis for the neutral evolution of male genitalia has previously been observed in some model organisms. Work on sister species of Jamaican millipedes (*Anadenobolus*), for example, lends support to the pleiotropy hypothesis (Bond *et al.* 2003). In spite of the high degree of divergence between sister species of *Anadenobolus*, which is suggested by an analysis of their mitochondrial DNA, the male fertilizing genitalia in are remarkably similar. This is especially interesting because it contradicts what both the sexual selection and lock and key hypotheses would predict: the genitalia of the male millipedes do not play a role in isolating the sympatric species from each other. However, the similarity in the genitalia of these sister species is not what is commonly observed in nature.

Environmental stress has been shown to affect morphology in male water striders in accordance with what the pleiotropy hypothesis would predict; genital conformations were correlated with general morphology (Arnqvist and Thornhill 1998). The amount of variation in genital structures was comparable to that of body shape when water striders were food deprived, implying that similar genes were involved in regulating overall morphology. In experiments where copulations in water striders were allowed to occur and paternity was determined, it became apparent that selection also played a role in the divergence of genitalia. Arnqvist and his colleagues (1999) determined that sperm competition was important in the paternity of offspring. In the light of this discovery, it is difficult to determine the role of neutral evolution in the morphology of water strider genitalia, as sperm competition between males makes it impossible to eliminate sexual selection as a cause for morphological differences.

Although it has garnered support with some model organisms, the pleiotropy hypothesis has long been considered problematic as a universal explanation for the divergence of genitalia. Internal organs of many diverged species remain relatively unaltered even after millions of years of reproductive isolation and regardless of diversity in other morphological characteristics (Eberhard 1985). Adjustments have been made to the hypothesis to allow for the interaction between male and female genitalia to play at least some role in the morphological diversification of genitalia, but many questions still remain as to how neutral evolution can be invoked to account for the widespread occurrence of this phenomenon in such a large variety of groups.

When observing closely related sibling species it is often obvious that the divergence of morphology of male genitalia is not occurring only in conjunction with overall morphological changes (Song 2009; Liu *et al.* 1996). In many of these cases, most notably in insects, the male genitalia are very different in shape whereas the general body shape remains quite similar among species (Liu *et al.* 1996). This negates the notion that the evolution of genitalia is a consequence of neutral evolution at the level of overall morphology.

Evidence of co-evolution of the male and female genitalia would also contradict the pleiotropy hypothesis, as it would suggest that selection acts directly on the morphology of genitalia. Although very little research on the evolution of the genital tract has been done in birds, as most species do not have complex or external genitalia, waterfowl are a notable exception (Brennan *et al.* 2007). The length and complexity of the penis is highly variable in waterfowl (Brennan *et al.* 2007) making waterfowl an interesting group in which to study the co-evolution of genitalia. Brennan *et al.* (2007)

compared male and female genitals in a variety of waterfowl species and determined that the complexity of the vagina increased in females as the phallus became longer in males, suggesting co-evolution. The corkscrew pattern of genitalia also appeared to be going in opposite directions when comparing the sexes, suggesting antagonistic evolution. This phenomenon is not unique to the waterfowl, whose genitalia have received the most attention; other organisms have also been shown to have co-evolving genitals. Simmons and García-González (2011) described how they were able to simulate co-evolution in a laboratory setting. Co-evolution of male and female genitalia in the dung beetle, *Onthophagus taurus,* was observed using two treatments in which beetles were reared either in forced monogamy or under conditions of sexual selection. After a varying number of generations, the male and female genital morphology were observed using a principal component analysis to look for divergence from the initial population. Females with the choice to re-mate evolved deeper vaginas and as a consequence became harder to inseminate. In response to this, the male aedeagus (penis) increased in length over time, showing what appears to be antagonistic evolution as both sexes appear to be trying to control fertilization. No differences in general body morphology were noted between the populations, which therefore strongly opposes the predictions of the pleiotropy hypothesis, namely the occurrence of change in both treatments and a correlation between body morphology and evolution of the genitalia. The co-evolution of male and female genitalia does however support predictions made by the other hypotheses used to explain the rapid evolution of male genitalia.

The Lock and Key Hypothesis

The oldest hypothesis seeking to explain reproductive isolation based on morphology is the lock and key model. The male's genitals, or key, are thought to be rapidly evolving due to a selection pressure exerted by the female's genitals, the lock. First suggested by Dufour in 1844, this theory has long been supported because of its intuitive nature. It is logical to assume that if a male and a female have genitalia that are incapable of interlocking together, the pair could therefore not mate and the female cannot be fertilized by the male. The inability of a male to mate with, or inseminate a female would therefore lead to species isolation. To date, however, little evidence has been provided to support the predictions made by the lock and key model (Arnqvist 1997; Shapiro and Porter 1989).

Controversy surrounding the lock and key model has existed almost since its inception (Richards 1927) as many have argued that behavioural and postzygotic mechanisms are more likely to arise before mechanical isolation (Shapiro and Porter 1989). A long-standing argument against the lock and key hypothesis stems from the fact that female genitalia are often similar as compared to male genitalia in newly diverged species (Richards 1927). The question then became: if the female "lock" is essentially the same, how can there be recognition of the appropriate species "key" (Richards 1927; Shapiro and Porter 1989; Masly 2012)? As discussed previously, female genitalia can co-evolve with male genitalia (Brennan *et al.* 2007; Simmons and García-González 2011) although it is clear that female genitalia remain far less complex than those of males in almost all groups, putting the species-specific "lock" in question. Today some

explanations do exist as to why this might occur, making the lock and key model a viable explanation for species divergence.

Firstly, it is important to define the different modalities that exist within the lock and key hypothesis; the lock and key hypothesis can be described as being either structural or sensory. The structural lock and key model, classically known as mechanical isolation, occurs when differences in genital morphology make it impossible for a male to mate with, or inseminate a female of another species. This is what generally comes to mind when referring historically to the lock and key hypothesis. Variations in the female "lock" are crucial in validating this hypothesis, which is why so many scientists have contested it in the past.

The recently speciated carabid beetle species, *Carabus maiyasanus* and *C. iwawakianus*, are an excellent example of the structural lock and key model. They form a narrow hybrid zone where males will court females regardless of their species (Sota and Kubota 1998). Interspecific copulations do occur but they often lead to the female's death as the male genitalia of the opposite species causes fatal tears to the vaginal walls because of the difference in aedeagus size. *Carabus maiyasanus* males were also shown to break their own sexual organs due to mechanical incompatibilities during many of these matings, leading to an inability to fertilize subsequent females (Sota and Kubota 1998). These fitness costs have led to a reduction in the frequency of hybrids, making the lock and key model a successful reproductive isolation mechanism in these species.

Interestingly, reproductive isolation also occurs between *C. iwawakianus* and another closely related, sympatric species, *C. uenoi*, but it occurs differently. In this case,

although the aedeagi are divergent, with *C. uenoi* having a much longer phallus, death does not result in females who mate with the opposite species. Instead, interspecific matings are fewer and do not lead to fertilization (Usami *et al.* 2006). Insertion of the genitals of the inappropriate species rarely occurs due to mechanical isolation, possibly supporting the lock and key hypothesis, but the role of rejection behaviour in the form of sperm dumping by the female needs to be further investigated. If females are shown to recognize the appropriate genital morphology, as occurs in Macrodactylus beetles (Eberhard 1992), then these results would be an excellent example of reproductive isolation through the sensory mode of the lock and key hypothesis.

The sensory lock and key model involves female behaviour and recognition of the particular shape of the genitals of a species. It has brought new life to the lock and key model as it does not require female genitalia to be highly divergent. The shape of the male "key" serves as a cue that triggers rejection behaviour, leading to reproductive isolation between species. Few examples supporting strictly the sensory lock and key hypothesis and to date, no sensory pathways have ever been detected in females of recently diverged species. In fact, the example most commonly referred to: sibling species of *Drosophila* in the *D. melanogaster* subgroup (Masly 2012), has no empirical evidence to support it.

Further evidence of the lock and key hypothesis lies in that species with complex courtship behaviours have relatively simple male genitalia compared to those of males from related groups with less complex courtship behaviours (Eberhard 1985). This lends support to the lock and key hypothesis because it suggests that, in the absence of courtship cues, genital shape plays a stronger role in determining if copulation occurs. In

species with complex courtship, the species-specific morphology of male genitalia is potentially unnecessary for reproductive isolation of the sister species. This does not, however, disprove sexual selection as an explanation for rapidly evolving male genitalia.

The Sexual Selection Hypothesis

More recently developed by Eberhard in 1985, the sexual selection hypothesis suggests that copulation may be used for more than just transfer of gametes leading to insemination. Insertion of the male genitalia into a female is thought to contribute to selection at the fertilization stage either through sperm competition, cryptic female choice, or sexual conflict. This hypothesis sheds light on why not all copulations lead to insemination in some species. For example, initial copulation in the spider *Lepthyphantes leprosus* does not result in sperm transfer but rather acts as a cue for future sperm transfer in subsequent copulation events (van Helsdingen 1965). The abundance of new and ongoing literature on the sexual selection hypothesis suggests that it is currently the most widely accepted explanation for the divergence of male genitalia (Eberhard 1985 1990 2010; Córdoba-Aguilar 1999 2002; Birkhead and Pizzari 2002; Hosken and Stockley 2004).

Antagonistic evolution of the sexes is one possible mechanism of sexual selection acting on genital morphology. As in the Red Queen hypothesis, where evolution is necessary for a species to keep up with another competing species, sexual conflict describes the co-evolution of male and female genitalia to ensure fitness for each sex. Co-evolution of genitalia has already been exemplified by waterfowl (Brennan *et al.* 2007) and the dung beetle (Simmons and García-González 2011). In another case, male and female yellow dung flies (*Scathophaga stercoraria*) have been shown both to evolve

morphologically when polyandry is enforced (Hosken and Stockley 2001). Males showed increased size of the testes in response to polyandry and the ensuing sperm competition. Females evolved large accessory sex glands that exhibited spermicidal properties, thereby allowing female dung flies to have some control over fertilization. Although many examples of antagonistic evolution do exist with respect to genital evolution, other mechanisms have been invoked to explain why there is such high variability in male genital morphology. The various mechanisms are often difficult to differentiate from one another, as they are often thought to be acting together.

Cryptic female choice is now thought to be the most likely cause of rapidly evolving male genitalia (Eberhard 2010). A prezygotic and post-copulatory mechanism, cryptic female choice describes the isolation of species as being regulated by females. Often the female choice occurs only upon being presented with the opportunity to remate. The selection process occurs after insemination but before fertilization, making it cryptic and therefore more difficult to study. With regard to the divergence of male genitalia, females are thought to select the sperm that originates from the male with the most appropriate genital shape based on her sensory recognition. The reasons for this sexual selection are still considered complex, and both the sexy sons and the good genes hypotheses could explain female preference for species-specific morphology (Hosken and Stockley 2004).

It is important to distinguish between the cryptic female choice and sensory lock and key hypotheses for evolution of male genitalia. Both may lead to reproductive isolation, but in different ways and for different reasons. Although they both require recognition of the male's genital structure by the female after insertion, the lock and key

model describes that recognition as an important role for species isolation, whereas the sexual selection hypothesis suggests it is important for intraspecific selection of a trait. The lock and key model would often entail rejection behaviour before sperm transfer, whereas cryptic female choice is not an initially obvious form of reproductive isolation.

Females of animal species have long been known to be discriminatory when determining paternity of their offspring. Because of their increased commitment to parental care, females are often responsible for whether or not mating or insemination will occur. An example can be observed in *D. melanogaster* females, who extrude their ovipositor to prevent mating, consequently giving them control over which males can inseminate them (Fowler 1973). This is a prime example of female selection occurring prior to insertion. The sexual selection hypothesis seeks to determine if the role of the female in selecting paternity extends further into post-copulatory mechanisms. Since Fowler's work was published, more research has suggested that female preference arises before the male trait and that the two phenotypes may be genetically linked (Basolo 1995). In poeciliid fish, females were shown to prefer males that possessed a sword extending from the caudal fin regardless of whether or not it was present in the species (Basolo 1995). This is thought to occur because the ancestral species exhibit the sword phenotype and the female preference existed in the species before divergence. Determining how female preference can affect the evolution of male genitalia could explain why divergence is seen universally.

Dameselflies (*Calopteryx*) are also a well-studied group with regard to male genitalia. *Calopteryx macualta* males increase their chance of siring offspring with the use of highly specialized genitals that allow for the removal of the sperm of competitors

(Waage 1979). Males of their sibling species, *C. haemorrhoidalis asturica*, are unable to remove a competitor's sperm from the female sperm storage organ. Instead, the quantity of sperm stored within the female reproductive tract is correlated with the width of the aedeagus; the amount of sperm ejected by females increases with the width of the aedeagus (Córdoba-Aguilar 1999). This demonstrates the sensory recognition by females of a preferred genital trait. The same correlation was observed in other related species, including the ancestral *Hetaerina cruentata*, where females ultimately determined paternity cryptically, based on aedeagus width (Córdoba-Aguilar 2002).

Haplogyne spiders are another excellent example of a species with female cryptic choice. The females have complex internal genitalia that allow them to eject sperm and as a result directly control paternity (Burger *et al.* 2003), leading to sexual selection. More work needs to be done on this model organism, however, to determine if variability in male genitalia leads to differences in the amount of sperm ejected, or if other factors can lead to reproductive isolation.

The species-specific posterior lobe in the *D. melanogaster* subgroup

Ideally a model organism should lend itself to testing all three hypotheses presented above and conclusively evaluating their effect on rapidly evolving male genitalia. Many organisms have been studied extensively with the intent of assessing what affects male genital morphology (Arnqvist 1997 1998 1999; Córdoba-Aguilar *et al.* 1999 2002; Bond *et al.* 2003), but never have the three hypotheses been tested and evaluated all within the same organism. This is likely due to the fact that altering the male genitalia can lead to infertility or death, making many experiments impossible. The

advent of new technologies has allowed the design of experiments that were previously thought to be untenable (Manier 2010; Polak and Rashed 2010).

The *Drosophila melanogaster* subgroup is a well-known model organism in genetic research and has been well-studied with regard to male genital morphology (Coyne 1993; MacDonald and Goldstein 1999; Liu *et al.* 1996; Zeng *et al.* 2000). As well as the work done on genital morphology, work to elucidate differences in behaviour have also been done for this subgroup, with the use of quantitative trait loci (QTL) mapping (Moehring *et al.* 2004). The internal genitalia of the males in the subgroup are more or less invariant (Okada 1955). They all consist of the aedeagus used for sperm transfer (Figure 1.1, 1.2). What is of particular interest with regard to four of these species, namely, *D. melanogaster*, *D. simulans*, *D. mauritiana* and *D. sechellia*, is how similar they are morphologically, except for the posterior lobe of the male genital arch (Robertson 1983, 1988; Coyne 1993; Cobb *et al.* 1988; Jagadeeshan and Singh 2006).

Used by many scientists to identify the four closely-related species (Liu *et al.* 1996), the posterior lobes are species-specific in shape and highly divergent between species (Figure 1.3). Part of the external genitalia, the two bilaterally symmetrical lobes are located on either side of the male genitals and can be curled inward when at rest or everted for copulation (Figure 1.3 F-I). During copulation, when the aedeagus is everted and inserted into the female (Figure 1.2), the lobes are inserted between the 8^{th} and 9^{th} tergites of the female (Robertson 1988; Kamimura and Mitsumoto 2011). The posterior lobes are thought to be useful for enabling males to lock into place during copulation and therefore make it impossible for females to reject males (Jagadeeshan and Singh 2006).

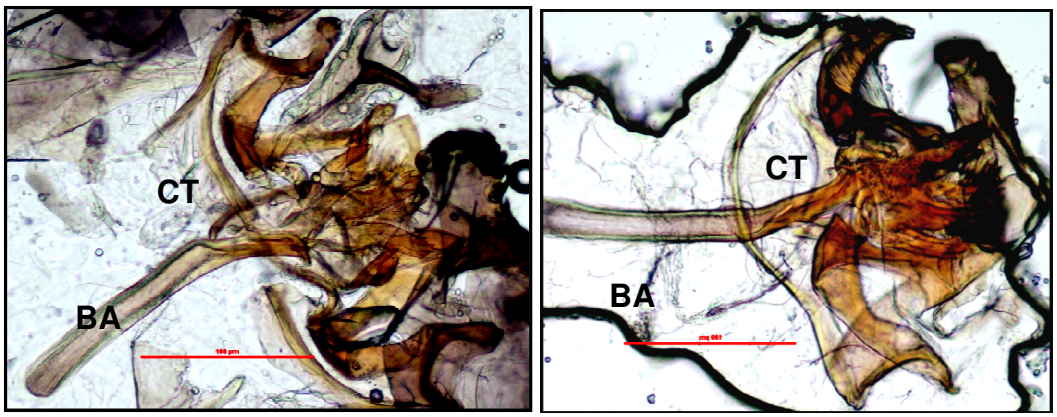

Figure 1.1. Comparing fertilizing male genitalia in the *Drosophila melanogaster* subgroup. A) Male primary internal genitalia in *D. mauritiana* (A) and *D. simulans* (B) that have been dissected and placed on a glass slide for imaging. The red scale bar represents 100 µm. The basal apodeme (BA), used to eject sperm, and connective tissue (CT) are labeled in both figures.

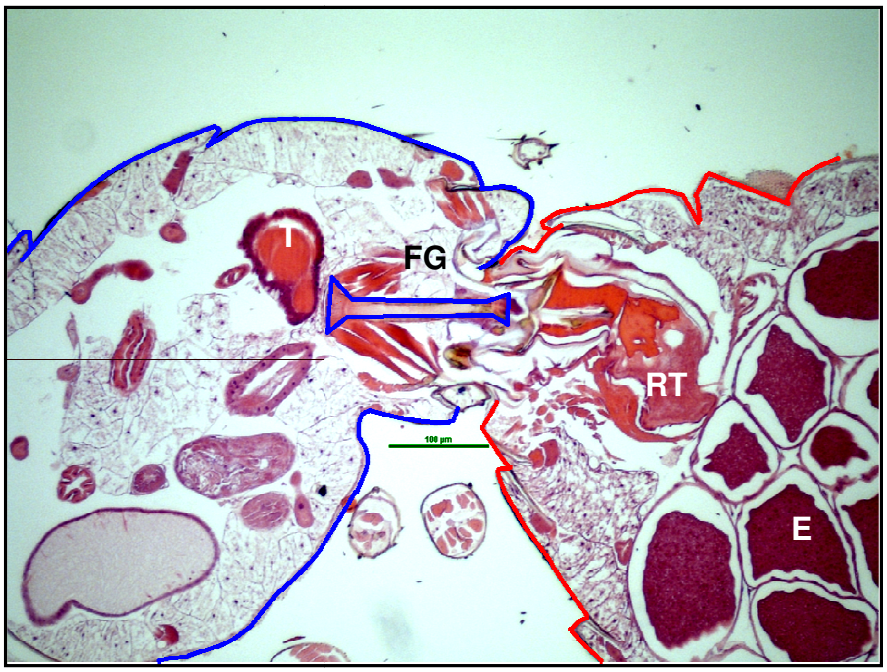

Figure 1.2. Interior view of a copulating *Drosophila* pair Histological section of a mated *Drosophila melanogaster* pair stained with haematoxylin. The male and the basal apodeme of the fertilizing genitalia (FG) are outlined in blue and the testes are depicted with the (T). The female is outlined in red, the eggs (E) and female reproductive tract (RT) are also labeled.

However, little is known as to why they would have evolved, as they are absent in *Drosophila* males of other species outside of the *D. melanogaster* subgroup. Their species-specific nature is also of interest as it has long been suspected that the difference in posterior lobe morphology may lead to species isolation (Robertson 1983).

Although many recent studies offer no support for the lock and key hypothesis (Shapiro and Porter 1989), two of the species in *D. melanogaster* subgroup, *Drosophila simulans* and *D. mauritiana*, have long been suspected to be an example of it (Robertson 1983 1988; Cobb *et al.* 1988; Masly 2012). Genetic studies have determined that these two species arose from a *D. simulans*-like ancestor within the last 250,000 years (Kliman *et al.* 2000). *Drosophila simulans* is a cosmopolitan species that is sympatric to the well-studied *D. melanogaster* (Lachaise *et al.* 1988). *Drosophila mauritiana*, on the other hand, is native to Mauritius, an island where *D. simulans* has yet to be found that is located 900 kilometres off of the east coast of Madagascar (Lachaise *et al.* 1988).

The two species do not come into contact in nature but exhibit an asymmetrical reproductive isolation when observed in the laboratory. *Drosophila mauritiana* females rarely allow for copulation with *D. simulans* males to begin, suggesting that there must be species-specific differences in males and their courtship behaviour which allows females to determine whether or not they are acceptable mates before insertion occurs (Coyne 1989). The same behaviour is not seen when the opposite cross is made with *D. simulans* females and *D. mauritiana* males: mating events readily occur for this pairing (Coyne 1989). Interestingly, although *D. simulans* females allow for interspecific mating events to begin, they exhibit a high

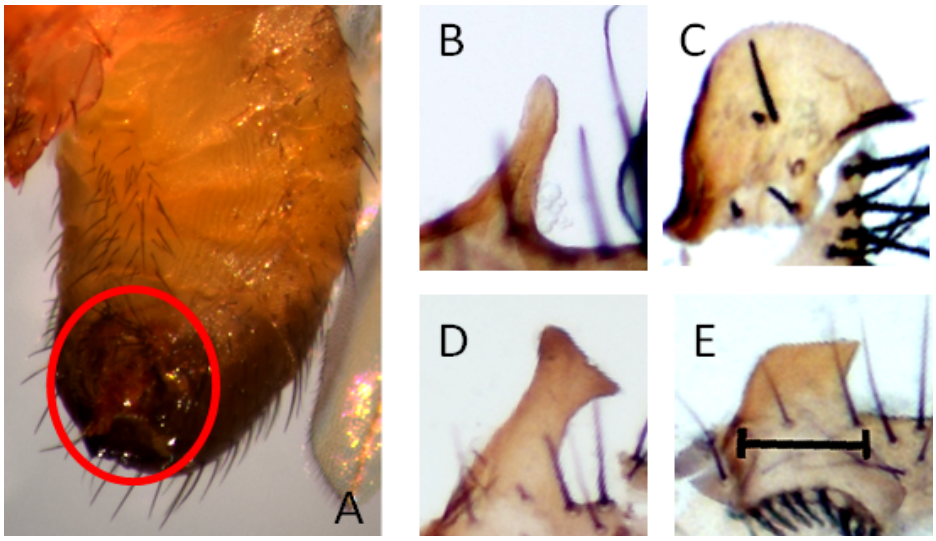

Figure 1.3. Male genital arch in the *Drosophila melanogaster* subgroup. A) The location of the genital arch in *Drosophila* males outlined on the male abdomen with a red circle. B-E species-specific posterior lobe shape in the *D. melanogaster* subgroup. A single lobe that has been dissected and laid flat is shown; the scale bar represents 50µm. B) *D. mauritiana* C) *D. simulans* D) *D. sechellia* E) *D. melanogaster*.

rate of rejection behaviour early in the copulation event, including wing displacement, kicking motions to dislodge the male, and diving off the side of the vial to end copulation (Robertson 1983; Coyne 1993). This behaviour is usually successful in reducing the duration of copulation, typically before sperm transfer can occur, showing that females play the leading role in the selection of an appropriate mating partner in this species pair.

The altered behaviour of *D. simulans* females suggests that the sensory lock and key model may be a factor in reproductive isolation but recent work suggests that a structural aspect of genital coupling may have been overlooked previously (Kamimura and Mitsumoto 2011). QTL mapping has also been performed to locate different regions in the genome that may account for the species-specific shape of the genital arch (Zeng *et al.* 2000) but no specific genomic regions were ever investigated to determine if the pleiotropy hypothesis may be responsible for genital variety.

Investigating all three of the hypotheses for rapid evolution of male genitalia is possible with this model organism: sibling species with divergent genitalia exist, the genetic regions responsible for species-specificity in shape are mapped, and observation of sexual selection is possible in a laboratory environment. The following three chapters describe the empirical evidence I collected with regard to each hypothesis, and therefore the likelihood of each hypothesis being crucial for the divergence of male genitalia in these sister species of *Drosophila*. Firstly, the lock and key hypothesis will be investigated with through microdissection of male genitalia using a laser. With this method, empirical data can be collected by comparing female behaviour when the posterior lobes are not species-specific in shape. Second, the pleiotropy hypothesis will be investigated by comparing posterior lobe morphology when one region of the genome

of one *Drosophila* species is introgressed into the other. Finally, comparison of sperm storage in *Drosophila* females based on alterations to the posterior lobe will begin the investigation of sexual selection as a factor in the divergence of male genitalia in these sister species.

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Chapter 2

2 Testing the Lock and Key Hypothesis

Evaluating the validity of the long suspected lock and key model in the species pair *D. simulans* and *D. mauritiana*.

2.1 Introduction

The idea that morphology plays an important role in species isolation is not novel. Dufour first suggested the lock and key model in 1844, describing the variations in genital morphology as being important to keeping different species apart. The lock and key model is based on the rapidly evolving male genitalia, the key, diverging and becoming unsuitable for the female genitalia, the lock, in another population. This divergence of genitalia can then give rise to reproductive isolation in an interspecific pair. For this model to hold true, females of the evolved species must be able to recognize variations in the species-specific male key.

The lock and key hypothesis has been a prominent explanation for the rapid divergence of male genitalia because of its intuitive nature. However, controversy does exist with regard to the hypothesis (Richards 1927; Shapiro and Porter 1989). The idea that morphological isolating factors would arise before other isolating mechanisms such as prezygotic (behavioural) or postzygotic (sterility, inviability) mechanisms, has been questioned. Although examples exist where the morphology of genitalia plays a part in the isolation of species (Mikkola 1992; Sota and Kubota 1998), in many of these instances irreparable damage is done to the female's reproductive tract and does not involve the female choice of rejecting males with an unsuitable genital morphology. The

main difficulty in resolving the controversy surrounding the lock and key hypothesis lies in that the model itself is difficult to test. Genital alterations are often fatal or lead to sterility in most organisms, therefore making most experiments impractical or even impossible. To further support or negate the lock and key hypothesis, a species pair in which alterations can be performed to the male genitalia has long been desirable (Arnqvist 1997, 1998).

The co-evolution of highly divergent male genitalia with female genitalia has been documented in a wide variety of organisms, ranging from waterfowl (Brennan *et al.* 2007) to dung beetles (Simmons and Garcia-Gonzalez 2011). Arguably the most widely studied group for identifying the genetic basis of genital morphology divergence in the light of the lock and key hypothesis is the *Drosophila melanogaster* subgroup. Females and males of this subgroup are very similar morphologically except for the shape of the posterior lobe of the male's external genital region, referred to as the "genital arch" (Coyne 1992, Figure 2.1), making this lobe a commonly-used tool to distinguish the species within the subgroup from each other (Coyne 1992, Liu *et al.* 1996). Within the group, the species pair *D. simulans* and *D. mauritiana*, which diverged allopatrically approximately 0.25MYA (Kliman *et al.* 2000), has been the most extensively studied as a genetic model for genital divergence (Coyne 1992, 1996; Liu *et al.* 1996; Zeng *et al.* 2000; Price *et al.* 2001). The flat, bilaterally-projecting and symmetrical posterior lobes surround the male genitalia (Figure 1.3A, 1.3B) and although they do not transfer sperm, they are inserted during copulation. The genital arch is thought to have arisen due to selection upon subtle differences within an ancestral species and differs considerably in shape between the two sibling species: *D. simulans* males have two large helmet-shaped

posterior lobes (Figure 1.3C) whereas *D. mauritiana* males have narrow, stick-like protrusions (Figure 1.3D). As the difference in the shape of the genital arch is species-specific and is not highly variable within a species, it has long been speculated that the difference may lead to mechanical isolation, allowing for reproductive isolation between species within this subgroup (Robertson 1983,1988; Cobb *et al.* 1988; Masly 2012).

Although *D. simulans* and *D. mauritiana* do no co-exist in the wild, previous research has described asymmetrical reproductive isolation between *D. simulans* and *D. mauritiana* in the laboratory. *Drosophila mauritiana* females are discriminatory in interspecific behavioural assays. When *D. mauritiana* females are paired with *D. simulans* males copulation rarely occurs. The lack of copulation suggests that there are species-specific differences in the male courtship behaviour that allow females to determine whether or not they are acceptable mates before genital insertion can occur (Coyne 1989). In contrast, mating readily occurs in the reciprocal interspecies cross (Coyne 1989). Interestingly, although *D. simulans* females allow for interspecific mating events to begin, they exhibit a high rate of rejection behaviour early in the course of copulation (Coyne 1992; Robertson 1983; Cobb *et al.* 1988), which often reduces its duration. Whereas in pure-species *D. simulans* the average duration of copulation duration is 25 minutes, compared to 15 minutes in *D. mauritiana*, the interspecies pairing has a reduced copulation duration of 5-8 minutes (Cobb *et al.* 1988). Due to the rejection behaviour exhibited by the female early after insertion, mating between the *D. simulans* female and *D. mauritiana* male often ends prior to complete external genital coupling (Jagadeeshan and Singh 2006), preventing adequate transfer of sperm for fertilization (Coyne 1992) and contributing to species isolation. Thus, courtship behaviours are

thought to determine whether copulation is initiated, whereas the genital arch shape is thought to affect the duration of copulation once it has begun. As the shortened copulation can contribute to behavioural isolation and is presumed to be affected by the different shapes of the genital arches of the two species, the *D. simulans* female and *D. mauritiana* male pairing is used as an example to support the sensory lock and key hypothesis (Masly 2012). Although the sensory lock and key hypothesis has long been formulated, no experimental evidence has ever been provided in support of it.

If the shape of the genital arch is an important factor used by *D. simulans* females in species recognition and the lock and key model is acting in this species pair, then females should reject the males of their own species if their genital arches deviate from the range normally found within the species. *Drosophila simulans* males do not have highly variable arches within the species (Liu *et al.* 1996). To test if deviation in the shape of the arch led to female rejection, I used a micro-dissecting laser to alter the genital arch shape of *D. simulans* males (Polak and Rashed 2010). Altered *D. simulans* males and controls were paired with females from their own species. As the experiment involves only conspecific partners, the only anomalous cue received by the female is the shape of the male genital arch. If *D. simulans* females use genital arch shape as a cue to prevent copulations with 'incorrect' males, rejection behaviour should be more frequent and the mean duration of copulation lower when males that have an altered arch shape. If, on the other hand, the genital arch shape does not contribute to mate selection or to a shortened copulation, then copulation should occur at the normal frequency and for the normal length of time. In this case, an alternative explanation must be found for the evolution of genital arch shape in this species. Interspecific pairs of *D. simulans* females

and *D.mauritiana* males were also observed when males had the posterior lobes altered to see if this would result in a change in female rejection. To address alternative hypotheses, I also measured copulation success when altered males were placed in competition for mating, and measured the occurrence and duration of copulation with altered males of all species of the *D. melanogaster* subgroup.

2.2 Methods

Stocks: Pure species stocks of *D. simulans* (obtained from the *Drosophila* Species Stock Center, stock #14021‑0251.199) and *D. mauritiana* (Synthetic; SYN, obtained from J. Coyne) were maintained on standard cornmeal-agar-molasses medium. All flies were housed on a 14:10 light:dark cycle, 21-23°C, 70% relative humidity.

Laser ablation: Males from the four species in the *D. melanogaster* subgroup were collected as virgins and left to age in food vials for 24 hours. Alterations were performed by anaesthetizing males on ice and altering the arch shape with a Zeiss Observer Z1 laser microscopy system using PalmRobo software. Four treatments were performed: 1) sham control males were placed in the same ice and laser environments as the altered males but no alteration was performed; 2) surgical control males were placed on the same ice and laser environments but only hairs from the genital region were removed using the laser; 3) single-arch altered males were anaesthetized on ice and one of the two posterior lobes was altered or removed; and 4) double-altered males were anaesthetized on ice, and both posterior lobes were altered or removed to produce a shape that was not species-specific.

Refined alterations were also performed on double-altered *D. simulans* males to test female responsiveness to different types of arch shapes. Males in this category were

altered in a variety of ways: removal of both tips, alteration to both crowns (minor and severe), and severe alterations, which consisted of alterations to both the tip and the crown of the helmet structure (Figure 2.1). After males were assigned to a treatment and the controls and altered males were generated, all were held with males of the same treatment for 2-4 days before behavioural assays were performed.

Behavioural assays: Males from all treatments that survived for at least two days after the alterations and retained full locomotion abilities (assessed qualitatively) were used in the behaviour assays; approximately 90% of the flies met these criteria. This was done to ensure that the males were not physically damaged from the alteration procedure and that they constituted viable mating options for virgin *Drosophila* females of the same age. Mating assays were performed in the first hour of "lights on" (Coye 1993)

For assays of copulation occurrence and duration, single males were paired in no-choice mating assays with single virgin females aged between 4-5 days old. The flies were observed for one hour in 3 dram (11 ml) vials that had been lightly misted with water to maintain the humidity. Courting behaviour exhibited by the male as well as the occurrence and duration of copulation were recorded. For pairs that were not used in sperm transfer assays, as described next, males with altered arches were then frozen at -20ºC for later arch visualization via dissection.

Sperm transfer assays were performed for *Drosophila* males who did not mate during the behavioural assays. These males were left with a virgin female for 7-10 days to determine if sperm transfer would eventually occur. Vials were scored for the presence

of larvae and the males' genital arches were dissected to ensure they were from the correct treatment.

Competition assays were performed to see if *D. simulans* males with altered arches were as successful as unaltered males in obtaining copulations when the two were placed in direct competition with one another. In competition assays, two *D. simulans* males of different treatments were placed with an individual *D. simulans* female and observed for one hour. Only assays where both males were seen courting the virgin female were scored. If copulation occurred during the assay, the unsuccessful male was removed to a separate vial, and both males were then dissected immediately after the copulation ended to score the arch alterations.

Genital arch visualization: Genital arches were dissected in TE buffer on a glass slide and observed using an E100 Nikon compound microscope equipped with a 5 megapixel digital camera. The computer software NIS-Elements 3.1 was then used to define the shape of the genital arch and the type of alteration (Figure 2.1) was scored.

2.3 Results

Duration of copulation is not affected by genital arch shape

The mean copulation durations were similar for all four treatments of *D. simulans* males in conspecific behavioural assays (Figure 2.2; P=0.921, F=0.124, N=101) comparing single-altered males (20.69 minutes), double-altered males (19.99 minutes), surgical controls (20.22 minutes), and sham controls (20.66 minutes). In other words, males with alterations to either or both posterior lobes did not differ significantly from the controls for mean duration of copulation. However, the standard deviation for the

altered males was higher than for the other treatments, suggesting a higher variability in the copulation durations, although no extreme outliers were noted. These results were replicated in a different laboratory environment using a different laser apparatus. Similar results were obtained in either setting when comparing altered males to surgical and sham controls (Supplementary Figure, Appendix A).

Only severe alterations affect male success in competition assays

When a control male and a double-altered male were placed together in the presence of a single *D. simulans* female, altered males had significantly fewer copulations (only 10% of the copulations compared to the expected 50%; Figure 2.3B; binomial test: N=20, $P<0.0001$). To determine if the difference in copulation rate was due to cuticular damage from laser surgery as opposed to differences in the arch shape itself, males with only one posterior lobe altered were also used in competition experiments with surgical control males. In these assays, the single-altered male copulated as frequently as expected if females did not discriminate against altered males (56%; binomial test: N=19, $P=0.648$), suggesting that the single intact posterior lobe can

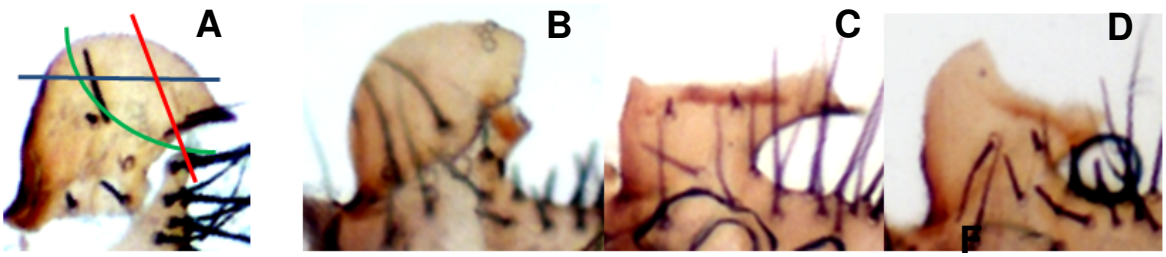

Figure 2.1: Types of alterations performed to the *Drosophila simulans* male posterior lobe. Alterations performed on both lobes of the arch of *D. simulans* males: A) Unaltered lobe, with three coloured indicating where the laser as used to cut and remove arch material, producing arches similar to (B) red, (C) blue and (D) green.

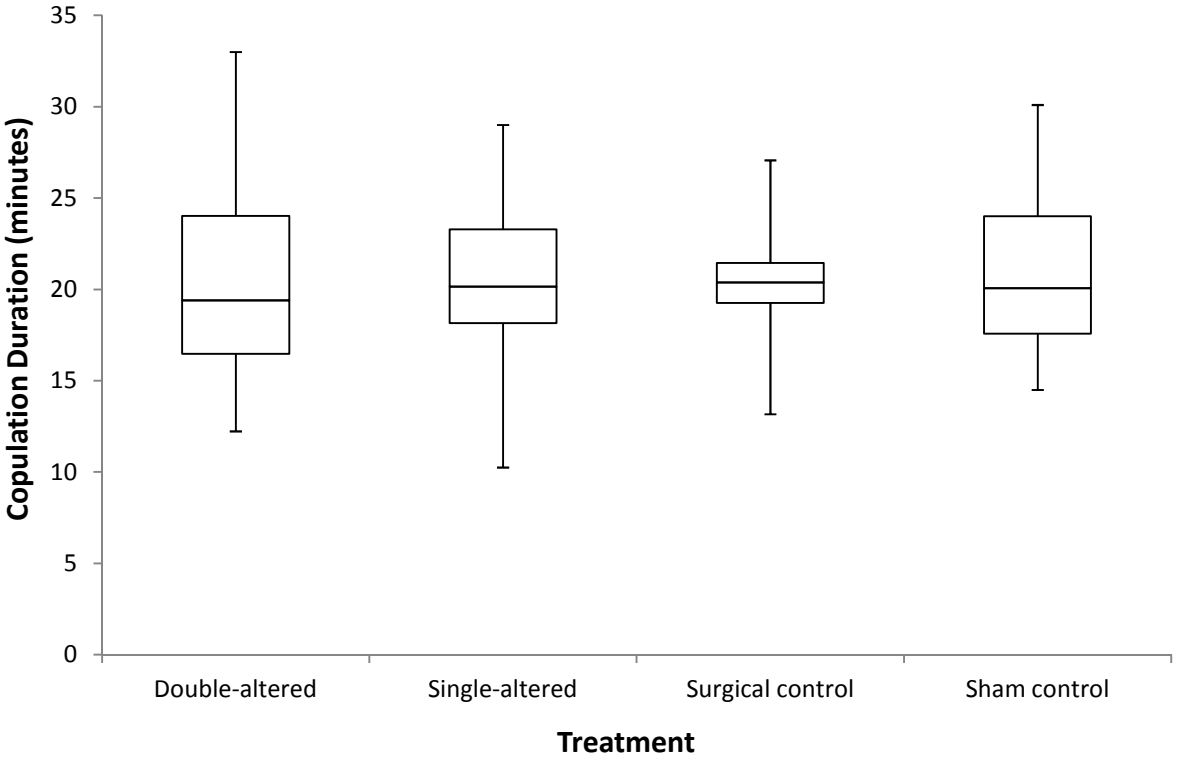

Figure 2.2. Mating of males with alterations to their genital arch. (A) Mean copulation duration for four treatments of *D. simulans* males using laser ablation. Copulation duration for conspecific pairs of *D. simulans* with males from four laser treatments: males with both posterior lobes altered, surgical controls with hairs removed, sham controls that have been in the laser microscope environment, but unaltered, and males with one posterior lobe altered using laser ablation (time in minutes, error bars minimum and maximum values). There was no significant difference in copulation duration between any of the three treatments (ANOVA $P = 0.921$, F= 0.124, N=101) and all durations were within the averages previously reported for the species.

sufficiently stimulate the female to allow for copulation to occur and that the laser surgery itself cannot be held accountable for the reduction in mating observed in double-altered males when another mate is available.

Frequency of copulation is affected by severity of alteration

Removing of part of the arch did not lead to a severe reduction in the duration of copulation in interspecies pairings but when comparing the alteration types (Figure 2.1G) it became apparent that differences in copulation rate could be attributed to the kind of alteration performed (Figure 2.3). *Drosophila simulans* males with severe alterations (Figure 2.1J) only mated in 2.9% of the behavioural assays performed (Figure 2.3A, N=35). They were also unsuccessful in transferring sufficient sperm when left with a female over a period of 5-7 days, as determined by the absence of larvae in the vials. Conversely, males with alterations consisting of tip removal (Figure 2.1H) were much more likely to mate successfully with the conspecific females as compared to males with crown alterations (Figure 2.1I). In the behavioural assays where courtship was observed, males with the tips removed mated 38% of the time whereas those with crown alterations only mated in 16% of cases (Figure 2.3A).

Arch alteration and intraspecific mating in the *D. melanogaster* subgroup

As removal of the arch in *D. simulans* led to a reduction in mating frequency, I wanted to see if this function of the arch was unique to *D. simulans* or whether it extended to the entire subgroup. I therefore removed the arch in males of the other three species in the *D. melanogaster* subgroup, namely *Drosophila mauritiana*, *D. melanogaster* and *D. sechellia*. No mating was observed in intraspecific pairs, in one

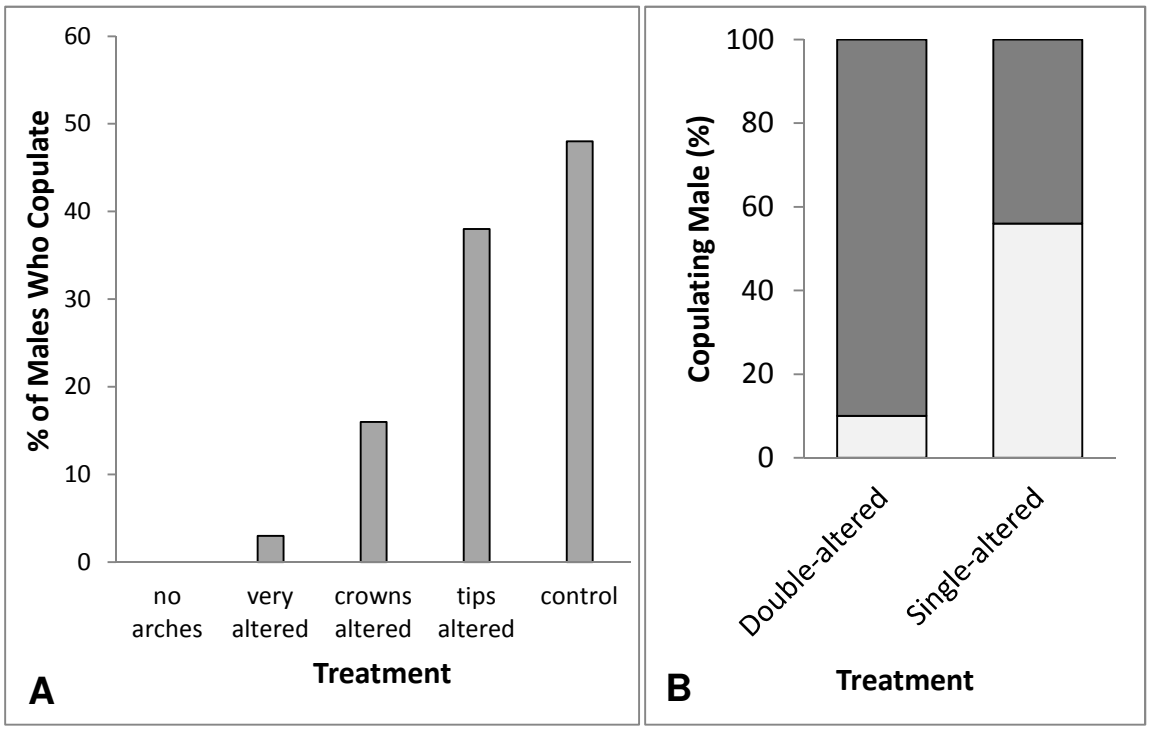

Figure 2.3. The Effect of Different Alterations to the Posterior Lobe on Copulation. (A) The percentage of *D. simulans* males who copulate in a one hour mating assay in four different laser treatments. (B) Percentage of copulation events in competition assays when comparing *D. simulans* males with both posterior lobes altered to control males and *D. simulans* males with one posterior lobe altered to control males. The shaded portions of the bars represent % copulation occurrence between control male and the intraspecific female whereas the white portions of the bars represent copulation occurrences of altered males with intraspecific females.

hour-long behavioural assays, after complete removal of the genital arch in any of the three species (N = 72).

In these experiments, vials were also scored for the presence of larvae after females were left with an intraspecific double-altered intraspecific male for a period of 7-10 days. *Drosophila sechellia* females proved to be the most selective based on arch shape and offered no evidence of sperm transfer when the males arches were fully removed and only limited success when minor alterations were performed (Figure 2.4A N=20, Binary test P<0.0001). Slight alterations to the posterior lobe of *D. sechellia* males also decreased the likelihood of sperm transfer to their conspecific females over the time span of a week with only 25% of vials having presence of larvae (N=12). Removal of the posterior lobe in *D. melanogaster* males resulted in the rare transfer of sperm; 3 vials out of 27 contained larvae (Figure 2.4A, Binary test, N=27, P<0.0001) which was significantly different when compared to amount of males who successfully fertilized conspecific females when slight alterations were performed (Figure 2.4A, Fisher's exact test, N=37, P=0.003). *Drosophila melanogaster* males with slight posterior lobe alterations did not show any signs of reduced fertilization success as 7 of 11 vials had larvae after 7-10 days and these males also often mated in a one hour-long behavioural assay. Altered *D. mauritiana* pairs also showed some larval presence when males had both genital arches removed. In 3 vials of 29 vials larvae was present, but as with *D. melanogaster*, there was a significant decrease in fertilization rate of severely altered males compared to subtly altered males (Figure 2.4A, Fisher's exact test, N=46, P<0.0001).

Arch alterations in *D. simulans*-*D. mauritiana* interspecific pairings

Copulation occurred in 5 of the 30 behavioural assays performed with *D. simulans* females and double-altered *D. mauritiana* males with no genital arches. As this mating frequency is similar to that observed with unaltered males (Robertson 1983), full removal of the posterior lobe does not appear to be a barrier to this interspecific mating. When mating did occur, post-copulation rejection behaviour was identical to what is seen in interspecific pairs with an unaltered male. Additionally, the average duration of copulation for the altered pairs was 5.85 minutes, which is not significantly different from what was seen in the unaltered males, which had an average of 5.48 minutes (N = 19, t-test, P=0.476, F=0.532; Cobb *et al.* 1988; Robertson 1988). Thus, the arch shape of *D. mauritiana* males does not seem to affect copulation frequency or duration when the males are paired with *D. simulans* females, counter to long-held beliefs for this species pair.

Vials were scored for the presence of larvae after females were left with a conspecific, double-altered male for a period of 7-10 days. When *D. mauritiana* males were paired with *D. simulans* females the fertilization rate after 7-10 days was not significantly different, regardless of their treatment (double-altered or slightly altered) (Figure 2.4B, G-test, N=33, P=0.137). Surprisingly, *D. simulans* females showed a significantly higher rate of successful mating with *D. mauritiana* males with both posterior lobes removed, as measured by presence of larvae (33%), than they did when paired with altered males of their own species (10%) (Figure 2.4B, G-test, N=51, P=0.04), suggesting that *D. mauritiana* males are more acceptable as mates when their arch is absent, or, more likely, that they are able to by-pass the female's ability to discriminate.

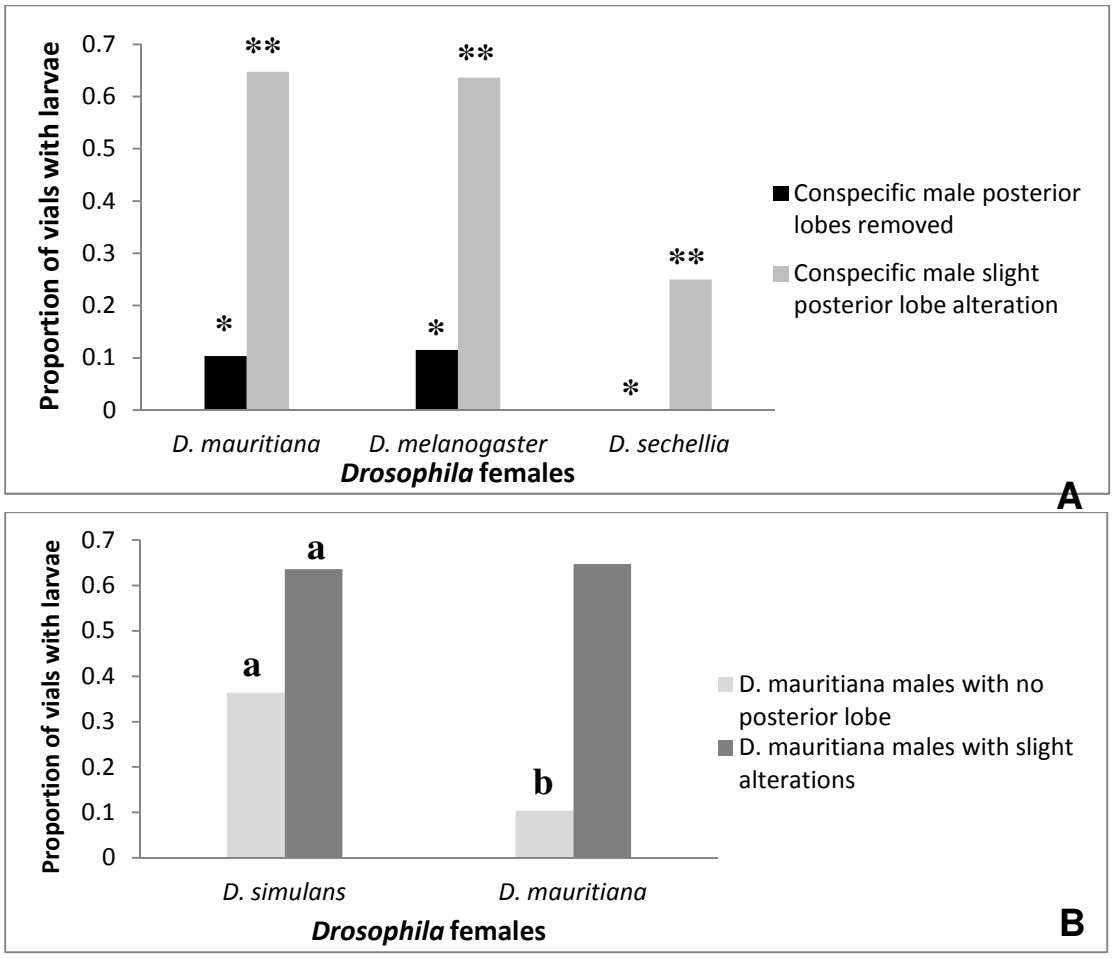

Figure 2.4. The Effect of Posterior Lobe Alterations on the Presence of Larvae. (A) *Drosophila* females from three species were left with males of their own species and the presence of larvae was evaluated at day 7. A significant difference was observed in all species when comparing presence of larvae, using a Fisher's exact test, when *Drosophila* males with their posterior lobes removed compared to those with slight alterations as depicted by the (*) and (**). (B) *Drosophila simulans* and *D. mauritiana* females were paired with *D. mauritiana* males, that either had no posterior lobes or slight alterations, and larval presence was evaluated after 7 days. The presence of larvae was determined as being not significantly different using G-test (N=33, *P*=0.137) when comparing the *D. simulans* females, although lower for males with no lobes, for the interspecific pairs. There was a significant difference when comparing the presence larvae in treatments where the *D. mauritiana* had no posterior lobes (the light grey bars): *D. simulans* females produced significantly more larvae than the conspecific pairs (G-test, N=51, *P*=0.04).

2.4 Discussion

The lock and key model, proposed over 150 years ago, is a prime example of a model that has been controversial in the scientific community due to a lack of direct empirical evidence. Highly divergent male genitalia in sister species is a well-documented phenomenon, but the reason for the rapid divergence remains an important question for biologists. A major difficulty with testing the lock and key model lies in the fact that, until recently, it was often impossible to alter male genitalia without eliminating the male's ability to mate. The sibling species *Drosophila simulans* and *D. mauritiana* have long been suspected to be an example of the lock and key isolation mechanism due to the species-specific shape of the male genital arch and the rejection of interspecific males after copulation, and thus have served as the primary genetic model for this hypothesis. I utilized laser microdissection to alter the shape of the posterior lobe, allowing for an empirical test of whether females would reject a mating partner based solely on a difference in genital arch shape; the use of intraspecific pairs allowed for all of the remaining cues (i.e. courtship song, courtship intensity, etc.) to be conspecific (Arnqvist 1997).

My study showed that *D. simulans* females did not show increased rejection behaviour of conspecific males after mating began in any of the treatments assayed. Both single-altered males and double-altered males had copulation durations within the average expected for the species, which is not in agreement with what would be predicted by the lock and key model. The insertion of arches that were not species-specific in shape into *D. simulans* did not lead to the same rejection behaviour seen when the same females are paired with *D. mauritiana* males. This suggests that if the arch shape is a cue for

species isolation, it does not serve as a cue after copulation has begun, as has previously been predicted.

What did vary between the four treatments was the rate of occurrence of copulation. In competition assays where *D. simulans* females had the option to mate with a double-altered or a surgical control *D. simulans* male, the females mated almost exclusively with the male that had the correct arch shape for their species. Interestingly, the effect was only present when both lobes of the arch were altered, as males with only one arch altered mated equally to controls. This implies that the laser treatment was not the cause for selection against the double-altered males, as the females were not choosy against males with a single arch altered, in other words a single arch of the correct shape is sufficient for females to accept a male, The selection of control males over those with two altered arches by the *D. simulans* females could imply the use of the stereotypical arch shape of both posterior lobes as a cue prior to insertion rather than after insertion, which is a previously unstudied aspect to the species isolation in these sibling species.

The rate of copulation of a double-altered *D. simulans* male was highly dependent on the degree of alteration made to the genital arch. The removal of the tips had little effect on successful mating (Figure 2.1H), whereas any *D. simulans* male with large alterations to the crown (Figure 2.1I) or with the majority of the posterior lobe removed (Figure 2.1J) failed to mate or inseminate a female of its own species, even after a five day period. This indicates that the arch does not act as a cue for copulation duration as previously thought, as rejection behaviour is not observed during a copulation event. The shape of the posterior lobe could instead act as a cue of an appropriate mating partner during courtship. Some *Drosophila* species such as *D. erectu* lack the posterior lobe

altogether, which has led previous researchers to assume that it is a quickly diverging male ornament that affects courtship but not the actual copulatory event. To determine if this is the function of the male genital arch in the *D. melanogaster* subgroup, I performed genital alterations on four species in the subgroup, all of which are commonly-studied and closely related to one another. Severe double-alterations where the genital arch was completely removed in the three species most closely related to *D. simulans* (*D. mauritiana*, *D. melanogaster* and *D. sechellia*) all led to a significant decrease in copulation and insemination in conspecific assays. In contrast, males that had minor alterations or only one arch altered were often successful in achieving copulations for all species except *D. sechellia*, which proved to be the most sensitive to arch shape. This suggests that, within the *D. melanogaster* subgroup, the posterior lobe's presence acts as a pre-copulatory cue used by females for rejection of an inappropriate mating partner. Surprisingly, as this effect is only present when severe alterations are made to the shape of the genital arch I examined, it appears that a female generally assesses the presence or absence of the arch but does not respond more subtle variations in arch shape. It is therefore unlikely that divergence in genital arch shape is in response to female selection upon the standing variation for this trait within a population.

This leaves the question: why would *D. simulans* females allow for copulation to occur with *D. mauritiana* males if they reject males of their own species whose arch shape is not true to the species? The answer lies in *D. mauritiana*'s aggressive courtship. Previous research has shown that *D. mauritiana* males are more likely to attempt copulation sooner and more aggressively than *D. simulans* males (Robertson 1983). Consistent with the idea that courtship plays a role in the success of *D. mauritiana* males,

double-altered males were not rejected prior to copulation with *D. simulans* females in behavioural assays, but rather after like with unaltered *D. mauritiana* males. The same rejection behaviour was observed for both altered and unaltered males after the initiation of copulation. Altered *D. mauritiana* males were also the most successful males in sperm transfer assays, successfully inseminating both their own females and *D. simulans* females after a seven-day period. *Drosophila mauritiana* females did show a lower rate of insemination than did *D. simulans* females when paired with altered *D. mauritiana* males, allowing for the possibility that in conspecific pairings, females have evolved more rapid rejection behaviours to reduce the frequency of unwanted copulations. It is therefore possible that the reduced size of the *D. mauritiana* arch (Figure 2.1C) arose because the more aggressive copulation makes it unnecessary for the males to produce a costly, larger structure for females to evaluate. In species other than *D. mauritiana*, the male arch is the key and the female's genitals are the lock, and females realize that the key is wrong even before it is inserted. In the pairing of a *D. simulans* female and a *D. mauritiana* male, however, the male is effectively kicking the door down.

Further investigations of post-copulatory mechanisms of species isolation may prove interesting as sperm storage in females has been shown to be different in interspecific pairs and conspecific pairs. Determining if *D. simulans* females have evolved a means of reducing interspecific paternity based on the genital arch shape would help to answer if both shape and sexual selection play a role in rapid male genital evolution.

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Chapter 3

3 Testing the Pleiotropy Hypothesis

Investigating the genetic basis for the morphological divergence of the posterior lobe in *D. simulans* and *D. mauritiana*.

3.1 Introduction

The pleiotropy hypothesis states that neutral evolution of overall morphology is responsible for the divergence of male genitalia (Mayr 1963; Arnqvist and Danielsson 1999). This hypothesis has long been questioned because several insect species pairs are recently evolved and have similar overall morphology, but have highly divergent male genitalia, which would suggest direct selection on genital morphology (Liu *et al.* 1996; Song 2009). A well-studied example of this phenomenon is the allopatric pair *D. simulans* and *D. mauritiana* (Robertson 1988; Coyne 1993; Liu 1996; Zeng 2000). These two species are morphologically indistinguishable from one another except for the shape of the male genitalia: the helmet shape of the posterior lobes in *D. simulans* males is highly divergent from the stick-like protrusions in *D. mauritiana* males (Robertson 1983; Liu *et al.* 1996; Masly *et al.* 2011).

Drosophila simulans females readily hybridize with *D. mauritiana* males in the laboratory. The interspecific pair produces offspring in a manner that is consistent with the predictions of Haldane's rule: female hybrids born from the *D. simulans* female and *D. mauritiana* male pair are fertile, whereas male offspring, who are the heterogametic sex, are sterile. Because female F1 offspring are fertile, the creation of lines containing known genomic information from these two species is possible. Lines of *Drosophila* can

be created that have the genetic background of one species and a known portion of another's genome; these are called introgression lines. Genetic markers can be used to define the borders of genetic insertions, and phenotypic variations in these lines can be ascribed to certain genetic regions from the other species.

Previous research describes the sterile F1 hybrid males as having an intermediate posterior lobe morphology when compared to the two parental species, while males resulting from a backcross to either parent species produce a continuous range of arch phenotypes (Liu *et al.* 1996; Zeng *et al.* 2000). If a single gene were responsible for the posterior lobe morphology, one would not expect to see such a large variety of phenotypes in the hybrids; this therefore suggests that several genes throughout the genome of *D. simulans* and *D. mauritiana* are responsible for the species-specific posterior lobe shape. Zeng *et al.* (2000) confirmed the polygenic nature of the posterior lobe of the genital arch with the use of quantitative trait locus (QTL) mapping. They showed that at least 19 different regions throughout the genomes of the two *Drosophila* species have an effect on posterior lobe morphology. Interestingly, the D. mauritiana morphology appeared to be somewhat dominant when comparing principal component analysis values in the backcrossed males (Liu *et al.* 1996; Zeng *et al.* 2000). Although these regions were located using QTL mapping, no specific genomic regions have ever been investigated to determine if they individually have an effect on male genital morphology. Indeed, since none of the individual regions had a large effect on the phenotype, it is possible that the effect of a single locus might be undetectable when it is measured individually.

Very little variation is observed in the arch morphology within a species, which facilitates comparisons of hybrids with the parent species. The posterior lobe of *D. simulans* laboratory lines containing small portions of the *D. mauritiana* genome can be compared in shape to the stereotypical posterior lobe of *D. simulans* males. The same can be done with *D. mauritiana* males that contain small portions of the *D. simulans* genome, making introgression lines useful in determining which genomic regions affect morphology, and whether individual genes alone can influence the trait. If it is observed that back-crossing of certain genomic regions of one species into the other does lead to posterior lobe variation, then it can be concluded that selection may have been acting directly on genes that affect lobe morphology, as body morphology has not been observed as being variable in these sister species.

3.2 Methods

Previous studies (Liu *et al.* 1996; Zeng *et al.* 2000) have located three large genomic regions on the third chromosome that are important to posterior lobe morphology in *D. simulans* and *D. mauritiana* (cytological regions 62, 82, and 98). Introgression lines for the third chromosome were previously created in the Moehring lab by repeated backcrossing of F1 hybrids to their parent species, then one generation of brother-sister mating to make the introgressions homozygous. Genetic markers were then used to determine the exact locations of the genomic region of the opposite species (McNiven and Moehring 2012 submitted). The resulting lines of *D. simulans* and *D. mauritiana* contain known inserted regions of the opposite species within their respective genomes (Figure 3.1, 3.2).

Genital arch visualization: Introgression lines containing each of the three cytological locations important for posterior lobe morphology (62, 82 and 98) were used for dissections of the posterior lobe. The three backcrossed *D. mauritiana* lines with known *D. simulans* genomic regions were line 52-2 (containing cytological region 62 from *D. simulans*), line 36 (cytological region 82) and line 79 (cytological region 98). The three backcrossed *D. simulans* lines with known *D. mauritiana* genomic regions dissected were line 45 and line 51 (containing cytological region 62), line 36-2 (cytological region 82) and line 100 and line 134 (cytological region 98). A microknife was used to remove the genital arch from the abdomen in TE buffer. A coverslip was then used to ensure that the posterior lobe was observed in a single focal plane. An E100 Nikon compound microscope equipped with a 5-megapixel camera was used to visualize the posterior lobes.

Comparing posterior lobe area, length, and width: The size and area of each posterior lobe was determined using the computer software NIS-Elements 3.1. A One-way ANOVA was used to determine if there was a significant difference in the area, length, or width of the posterior lobes when comparing the introgressed lines to their parental species as well as individual t-test's to compare each line individually to the parental line.

3.3 Results

Comparison of posterior lobe shape in backcrossed males

When comparing the overall morphology of the posterior lobes none of the backcrossed lines appeared to exhibit a hybrid phenotype, and in all cases but one, the morphology appeared to be species-specific and in accordance to the genetic background.

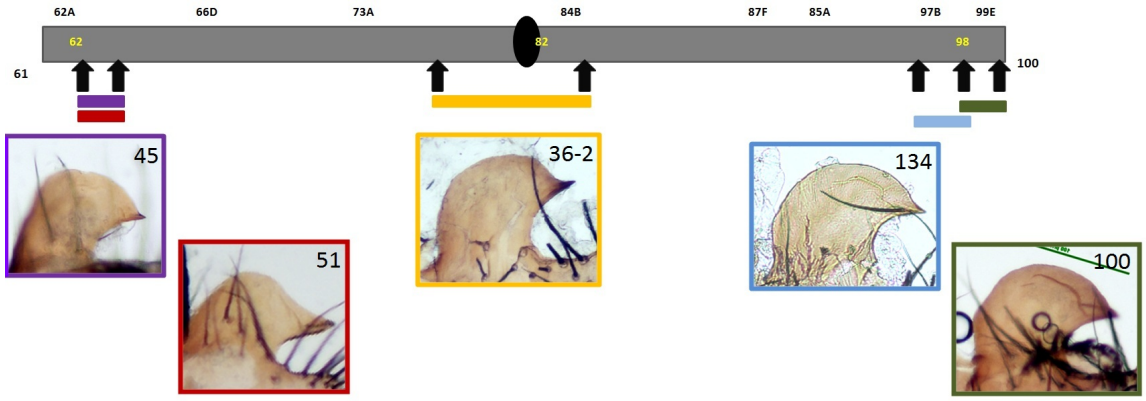

Figure 3.1. Cytological map of chromosome three in *D. simulans*. Chromosome three of *D. simulans* is represented by the grey bar, with the yellow numbers depicting three of the previously known regions important for posterior lobe morphology. The coloured bars below the chromosome represent the regions that were known to be from the *D. mauritiana* genome in the *D. simulans* background. Dissected posterior lobes from individuals from each introgression line are represented and outlined in matching colours along the chromosome.

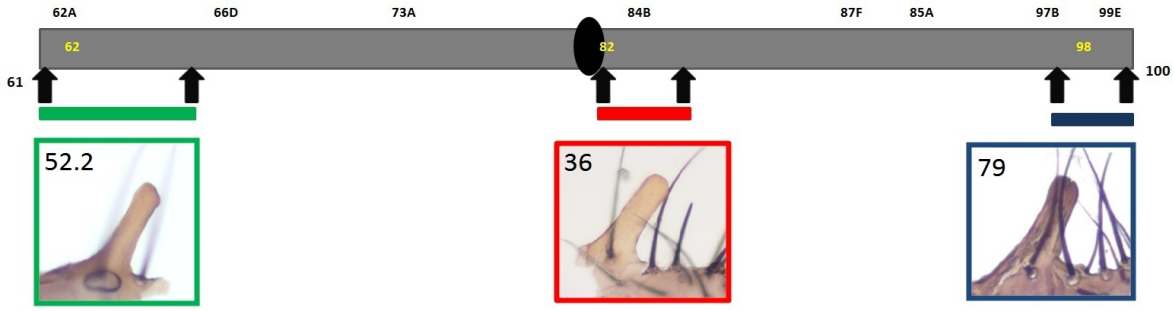

Figure 3.2. Cytological map of chromosome three in *D. mauritiana*. Chromosome three of *D. mauritiana* is represented by the grey bar, with the yellow numbers depicting three of the previously known regions important for posterior lobe morphology. The coloured bars below the chromosome represent the regions that were known to be from the *D. simulans* genome in the *D. mauritiana* background. Dissected posterior lobes from individuals from each introgression line are represented and outlined in matching colours along the chromosome.

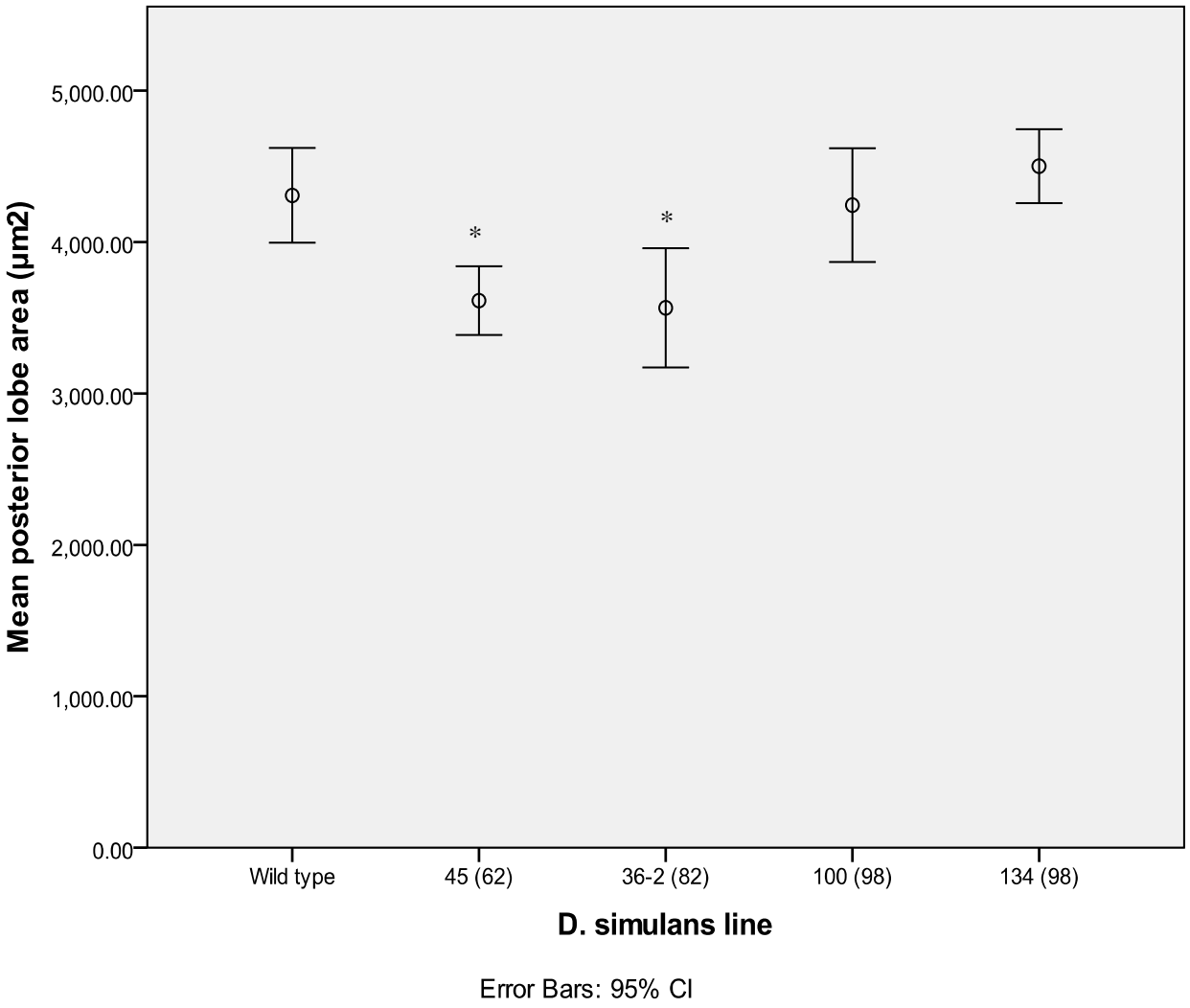

Figure 3.3. Comparison of mean area of the posterior lobe in *D. simulans* backcrossed lines and the parental line. Mean area of the posterior lobe from the 4 introgression lines were individually compared to mean area of the posterior lobe in the parental species. The mean area from four individual lines from the three regions known to affect posterior lobe morphology are shown along the X-axis. The lines that were significantly different compared to the wild type when using an independent t-test are marked with a (*) (N=40).

One *D. simulans* introgression line (51) was not species-specific in shape in some of the dissections performed (2/10; Figure 3.1). Another introgression line (45) whose backcrossed region from the *D. mauritiana* genome was identical to the one seen in line 51 was also dissected and no morphology differences were observed. Thus the sporadic differences observed in the one line are therefore unlikely to be due to the species-specific introgression.

Statistical analysis of posterior lobe area, width, and length

Posterior lobes in the parental *D.simulans* males were significantly wider and had a greater mean area when compared to the posterior lobes of males from introgression line 45, which has a genetic region from *D. mauritiana* at cytological location 61B to 66B (t-test, P=0.0001 and P=0.001, respectively). Significantly greater width and mean area were also observed for the introgression line 36-2, which has a known *D. mauritiana* genomic region from 76B to 93C on the third chromosome (t-test, P=0.0001 and P=0.005, respectively). The width of the posterior lobe was also significantly different when comparing the posterior lobes of parental *D. simulans* males to those from the introgression line 134, with an introgression at the cytological region 98 (t-test, P=0.023). The posterior lobes from the introgression line 100, also containing a *D. mauritiana* genomic region at cytological location 98, did not differ significantly in mean width when compared to the posterior lobes of parental *D. simulans*. Mean length of the posterior lobe was not significantly different in any of the four introgression lines or the wild type *D. simulans* males (ANOVA, F=0.554, P=0.697).

There was a significant difference in the mean length and area of the posterior lobe when comparing the parental *D. mauritiana* to the introgression line 36, which

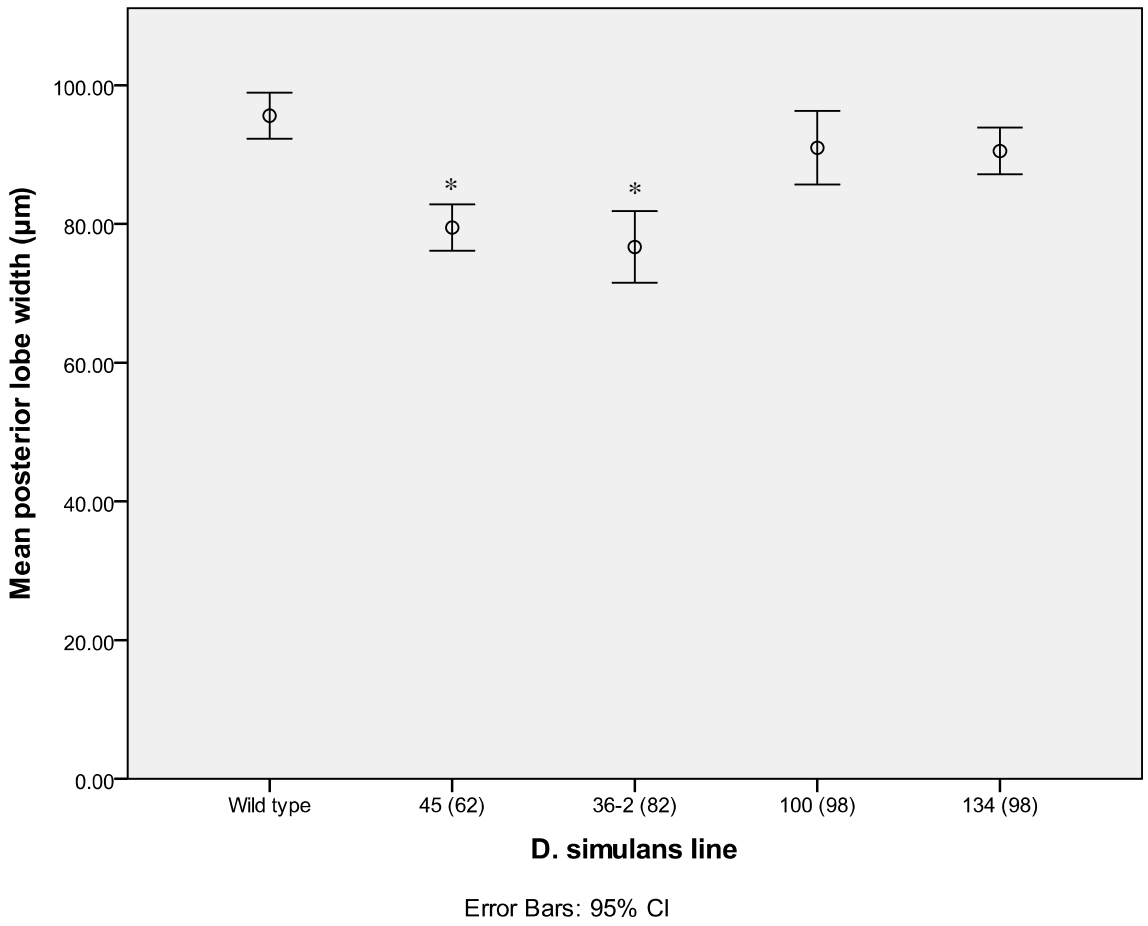

Figure 3.4. Comparison of mean width of the posterior lobe in *D. simulans* backcrossed lines and the parental line. The mean width of the posterior lobe from the four introgression lines were individually compared to the mean width of the posterior lobe in the parental species. The mean width from four individual lines from the three regions known to affect posterior lobe morphology are shown along the X-axis. The lines that were significantly different compared to the wild type when using an independent t-test are marked with a (*) (N=40).

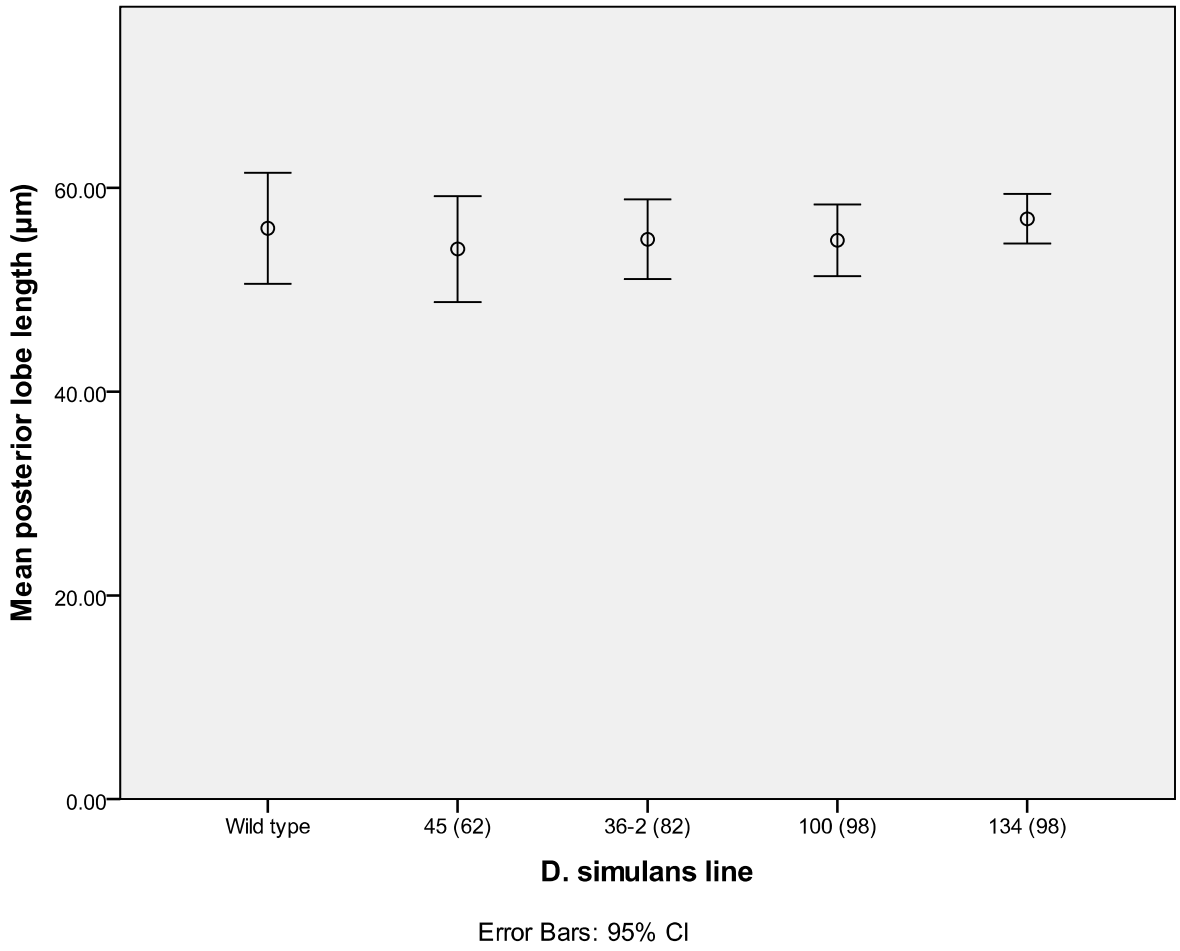

Figure 3.5. Comparison of mean length of the posterior lobe in D. *simulans* backcrossed lines and the parental line. An ANOVA was used to compare the mean length of the posterior lobe in the *D. simulans* backcrossed lines and the wild type parental. There was no significant difference when comparing any of the lines and length of the posterior lobe remained consistent regardless of what individual *D. mauritiana* genomic region was found in the *D. simulans* background. (P=0.697, F=0.554, N=40)

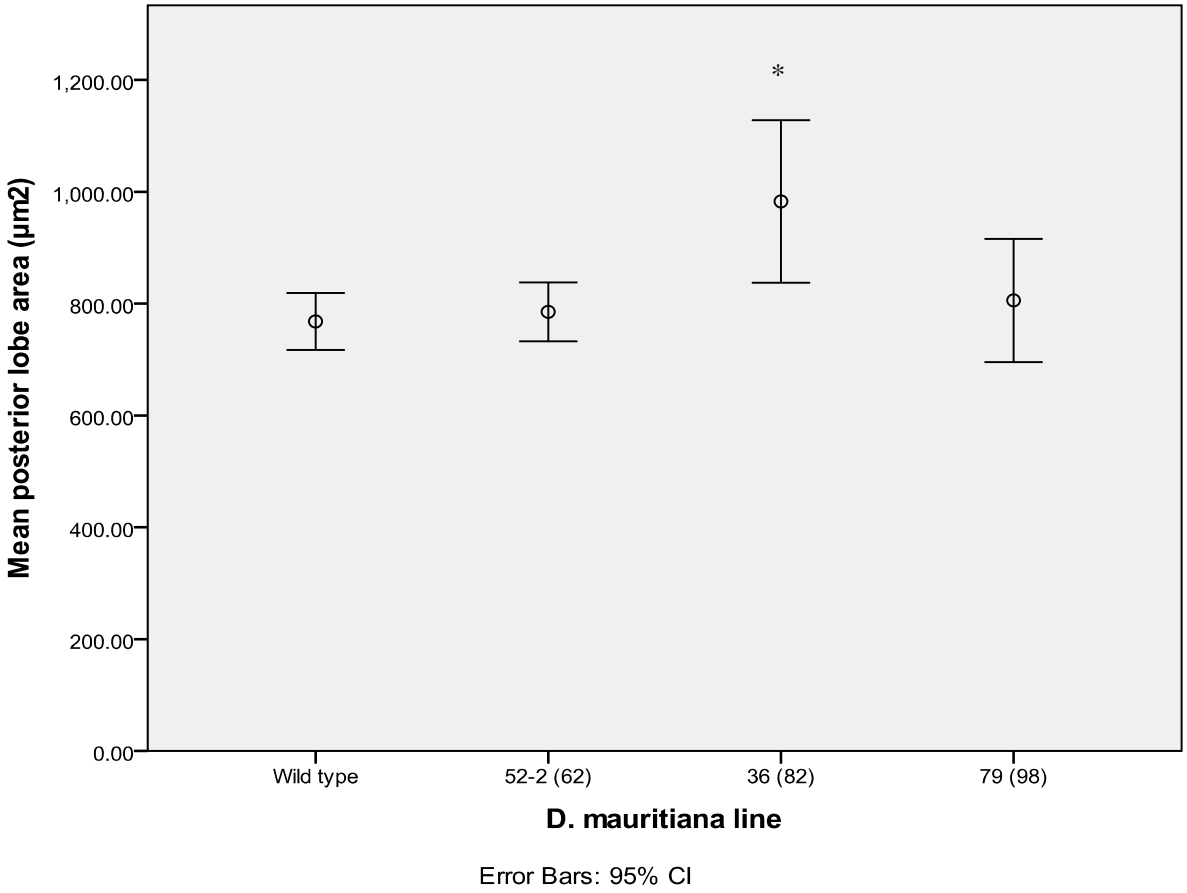

Figure 3.6. Comparison of the mean posterior lobe area in wild type *D. mauritiana* and the backcrossed lines. Mean area of the posterior lobe from the three introgression lines are individually compared to mean width of the posterior lobe in the parental species. The mean posterior lobe area from three individual lines from the three regions known to affect posterior lobe morphology are shown along the X-axis. The line that were significantly different compared to the wild type when using an independent t-test is marked with a (*) (N=33)

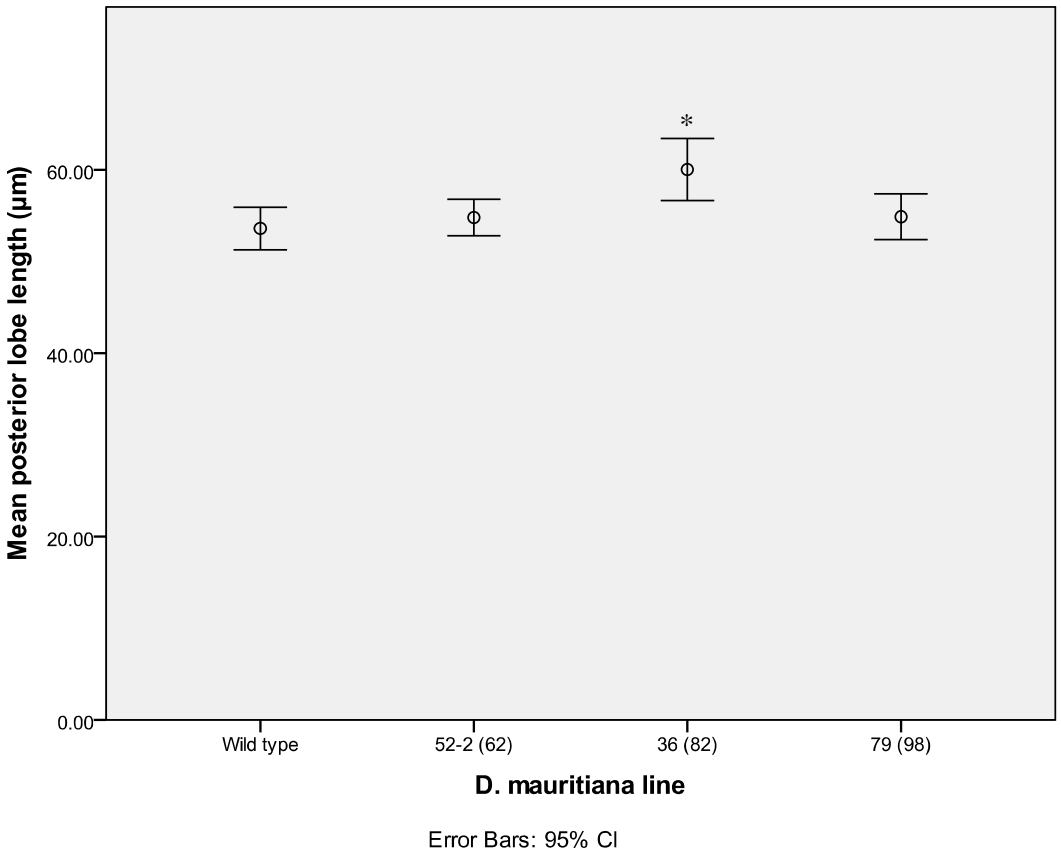

Figure 3.7. Comparison of the mean posterior lobe length in wild type *D. mauritiana* and the backcrossed lines. Mean length of the posterior lobe from the three introgression lines are individually compared to mean width of the posterior lobe in the parental species. The mean posterior lobe length from three individual lines from the three regions known to affect posterior lobe morphology are shown along the X-axis. The line that were significantly different compared to the wild type when using an independent t-test is marked with a (*) (N=33)

contains a *D. simulans* region at the cytological location 82 (t-test, P=0.003 and P=0.004, respectively). The introgression lines from the other two cytological locations, 62 and 98, did not show any significant difference in mean length (t-test P=0.079 and P=0.154, respectively) or area (t-test P=0.595 and P=0.488, respectively) of the posterior lobe when compared to those of the parental *D. mauritiana* males.

3.4 Discussion

The genetic basis for the high degree of divergence in male *Drosophila* genitalia is still largely unknown. The *D. melanogaster* subgroup is highly divergent with regards to the posterior lobe shape, but the specific genes responsible for these differences have yet to be located. Previous work has determined that all species with divergent posterior lobes in the *D. melanogaster* subgroup have several different regions that contribute to shape and size of the posterior lobe, suggesting a polygenic and additive effect (Liu 1996; MacDonald and Goldstein 1999; Zeng *et al.* 2000; Masly *et al.* 2011; McNeil *et al.* 2011). Sexual selection is considered to be one of the more likely causes for the divergence of male genitalia (Eberhard 1985, 1994, 2010) and could account for the high number of loci that affect male genital morphology.

The sister species *D. simulans* and *D. mauritiana* have been extensively studied with regard to the variation in posterior lobe morphology (Liu *et al.* 1996; Zeng *et al.* 2000; Masly *et al.* 2011; McNeil *et al.* 2011). *Drosophila mauritiana* genes have previously been shown to be dominant over those of *D. simulans* in backcrossed individuals (Zeng *et al.* 2000) in the 19 identified QTL regions of interest. To date though, no individual regions in these two species had ever been examined to determine if morphological variation could occur from a single genetic locus. Determining the

particular type of genitalia observed when only one area of the genome belongs to the other *Drosophila* species could help identify the genetic contribution of each locus, as well as assist in the future location of genes crucial in genital arch morphology.

My work has shown that when one individual QTL region, from the original 19 reported, is backcrossed into the other species' genome, an effect on posterior lobe morphology is observed. In this study, variation resembling F1 hybrid morphology of the posterior lobe was only noted in one backcrossed line. As the *D. mauritiana* genes are considered dominant over the *D. simulans* genes, it is not surprising that the males with unusual arches were from a *D. simulans* background containing one region of the *D. mauritiana* genome. Although the intermediate morphology is interesting, only a few individuals were observed as having the unusual genital morphology, and individuals from another line that contains the same genetic region of *D. mauritiana* in the *D. simulans* background did not display the non-species-specific posterior lobe phenotype. Further work can determine if inbreeding or selection was acting on the line that displayed an intermediate arch phenotype, and therefore may elucidate the cause for the variation in morphology in those individuals.

Liu *et al.* (1996) found that genetic regions that determined size and shape of the arch were indistinguishable, and therefore presumed to be genetically linked. It is surprising then that I observed differences in the size of the posterior lobe in lines from backcrosses to both species but that the intermediate morphology seen in F1 or backcrossed males from previous work (Liu *et al.* 1996; Zeng *et al.* 2000) was not observed. Two of the backcross lines with a *D. simulans* background, and one backcross line with a *D. mauritiana* background showed a significant difference in area (they had

smaller posterior lobes overall) compared to the parental line, suggesting that the genes within the tested genomic regions do contribute to size of the posterior lobe. These findings are supported by work done by Masly *et al.* (2011), who located regions in the *D. mauritiana* genome that are important for posterior lobe size when compared to their sibling species *D. sechellia*. One of those genomic regions was located on the right arm of the third chromosome, where I also located a region of interest, suggesting that there are potentially several genes of interest in this area. Previous work has determined that there are no significant differences in overall body morphology between the species and that there is very little correlation between body size and size or shape of the posterior lobe (Coyne *et al.* 1991; Liu *et al.* 1996; MacDonald and Goldstein 1999; Masly 2011). As no overall body morphology differences have ever been observed between the males from these parental species, it is likely that the genes affecting the size of the posterior lobe are not neutrally evolved general morphology genes as the pleiotropy hypothesis would suggest. To solidify these findings, future work would require statistical testing to evaluate if overall body morphology is significantly different in these backcrossed lines when compared to their parental lines.

One of the *D. mauritiana* genomic regions in a backcrossed *D. simulans* line that was significantly different for posterior lobe width is located near a previously documented candidate gene for posterior lobe morphology (Chatterjee *et al.* 2011; McNeil *et al.* 2011; Masly *et al.* 2011). The *D. melanogaster* gene known as *Drop* (*Dr*), located at cytological location 99B, has been identified as important in sex determination. *Dr* is repressed in females during development, and when mutated in *D. melanogaster* males, leads to misshapen posterior lobes (Chattejee *et al.*. 2011). A comparison of

published sequences (Flybase) confirmed that there is a homolog for *Dr* in both *D. simulans* and *D. mauritiana* in the same cytological region (Figure 3.8). Backcrossed *D. mauritiana* males did not have significantly altered posterior lobe shape or size when compared to the parental males and therefore the question remains as to what role a *Dr* homolog may have in other species, if any.

It is important to note that the genomic regions observed in this study were very large and that continued research into the backcrossed regions that showed significant variations in the mean area, width and/or length should be further investigated. By using introgression lines with overlapping genetic regions from the opposite sister species, it may be possible to narrow down the regions of interest, through fine mapping, and locate candidate genes important for posterior lobe size.

Understanding how male genital morphology diverges so rapidly cannot be examined fully without having knowledge of the genomic regions responsible for the divergence. The large quantity of regions important for posterior lobe morphology in *Drosophila* does not negate any of the three hypotheses currently used to explain rapidly evolving genitalia. However, determining if the genes that affect genital morphology are common to all species, and can independently act on morphology, may help to answer several of our remaining questions.

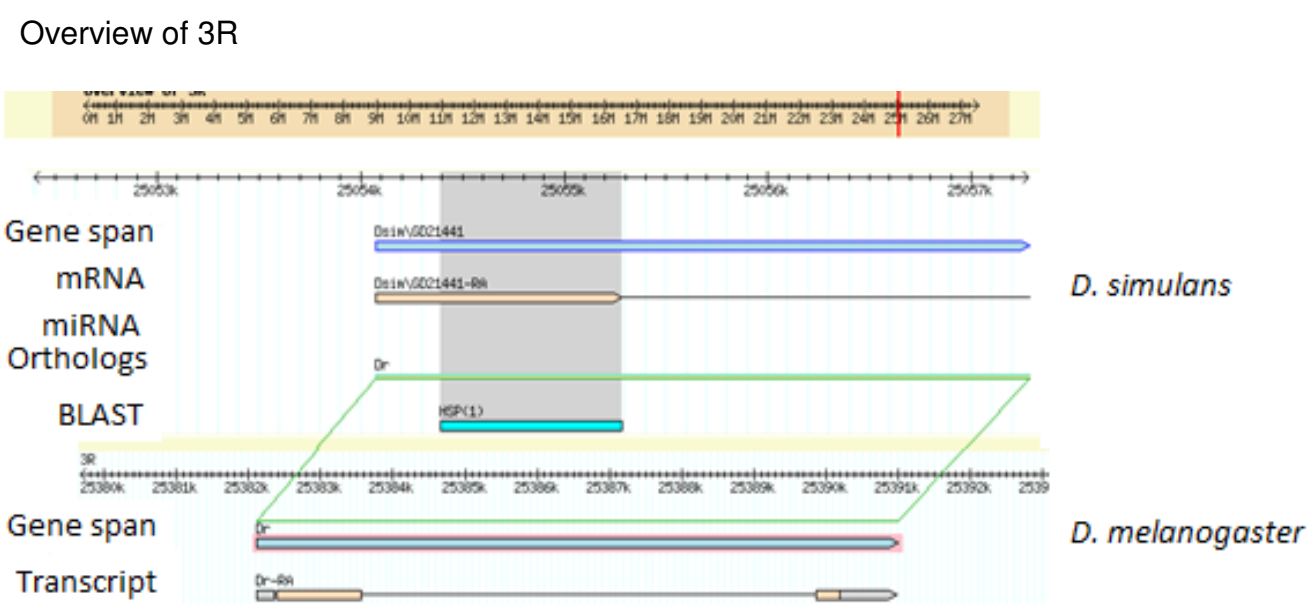

Figure 3.8. Cytological location of *Drop* gene in sister *Drosophila* species. The specific location of *Dr* on the right arm of the third chromosome in the *D.simulans* genome and the ortholog in its sister species *D. melanogaster. Drop* is located on the right arm of the third chromosome in both species. The mRNA and miRNA for *D.simulans* is shown, as well as the transcript in *D. melanogaster* which is known to be important for the development of the male posterior lobe. (Adapted from Flybase)

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Chapter 4

4 Testing the Sexual Selection Hypothesis

Preliminary research into the role of sexual selection on the divergence of male genitalia in *Drosophila* sister species.

4.1 Introduction

Recent studies on the rapid divergence of male genitalia largely focus on the role of sexual selection within a species and how it can lead to evolution (Eberhard 1985, 1990, 2011; Hosken and Stockley 2004). While the lock and key and pleiotropy hypotheses have been successful at explaining certain isolated occurrences of morphological divergence (Sota and Kubota 1998; Bond *et al.* 2003), neither can account for the wide variety of groups displaying rapid divergence of male genitalia. Different types of sexual selection, such as antagonistic evolution (Simmons *et al.* 2011), and sperm competition (Hosken and Stockley 2001), have been shown to lead to divergence of male genitalia in individual cases, but it is often difficult to determine the ultimate cause of morphological divergence in male genitalia. Eberhard (2011) describes cryptic female choice as being a likely mechanism of sexual selection that affects genital morphology in a wide range of animal groups.

Cryptic female choice is a postcopulatory and prezygotic isolation mechanism in which females of a species bias paternity based on a certain male phenotype (Eberhard 1985). In the case of rapidly diverging male genitalia, it is suspected that females use the correct sensory stimulation of the male genitalia as a cue to signal an appropriate mate. Detecting cryptic selection is more difficult when compared to other prezygotic isolation

mechanisms as it occurs during copulation. Often it is impossible to determine if the female has used sensory recognition as it is strictly internal. New molecular and genetic tools are now making it possible to analyze if females of a species can use cryptic choice to bias paternity.

Cryptic female choice has previously been observed in *Drosophila* (Price *et al.* 2001; Miller and Pitnick 2002; Polak and Simmons 2009). While it has been shown that it is possible for *Drosophila* females to bias paternity after copulation has occurred, the question remains as to whether or not they would do so based on the morphology of the male posterior lobe. Males from the four species in the *D. melanogaster* subgroup, *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*, are morphologically indistinguishable to a human observer except for the shape of the posterior lobe in the male genital arch (Figure 1.3). Previous research suspected the lock and key hypothesis for the divergence of the secondary male genitalia, specifically in the species pair *D. simulans* and *D. mauritiana* (Robertson 1983; Cobb *et al.* 1988; Masly 2012) but more recent work does not exclude the possibility that selection may play an active role in morphological evolution (Chapter 2, Jagadeeshan and Singh 2006).

By examining the contents of the reproductive tract of *D. simulans* females after copulation it is possible to observe sperm storage and determine if females actively bias paternity based on the morphology of the male's posterior lobe. Specifically, if *D. simulans* females are recognizing a species-specific phenotype, then a difference in sperm storage may be observed based on the posterior lobe shape. *Drosophila* females can dump sperm after males have transferred it to the bursa copulatrix (BC) or store them for later fertilization of their eggs in the spermatheca (ST) or seminal receptacle (SR).

Therefore, if mates are being distinguished by their ability to stimulate females during copulation, males who have alterations performed to the posterior lobe shape with the use of laser dissection may have a reduced amount of sperm in the female reproductive tract, and most notably in the long term storage organs.

4.2 Methods

Stocks: Stocks of *D. simulans* with green fluorescent protein (GFP) labeled sperm, originally acquired from the Scott Pitnick lab, were stored on standard cornmeal-agar media. The individual vials were stored in an incubator with a constant relative humidity of 70% and at approximately 23˚C. All flies were reared in a 14:10 light:dark cycle to facilitate virgin collection.

Laser ablation: The posterior lobes on *D. simulans* males were altered with the use of a Zeiss Observer Z1 laser microscopy system and the PalmRobo software. Males were left to age for one day on standard media prior to alterations. After 24 hours, they were put on ice for 20-30 minutes to anaesthetize them and then were randomly assigned to three laser treatments: 1) control *D. simulans* males had hairs from their genitalia removed, 2) single-altered *D. simulans* males who had one posterior lobe altered, and 3) double-altered *D. simulans* males who had both posterior lobes altered. Males from all treatments were then left to age over a period of 3-5 days to ensure survival after the laser ablations.

Behavioural assays: Mating assays were performed in the first 1.5 hours after "lights on" as that is when *Drosophila* are most active (Coyne 1993). A single *D. simulans* female and male, both between 4-6 days old, were place into a three dram (10.5 ml) glass vial that had been lightly misted with water to provide humidity and encourage mating. Each

pair was observed for 45 minutes to an hour and scored for courtship, copulation, and copulation duration.

Dissection and visualization of the female reproductive tract: Conspecific pairs who did mate in the behavioural assays were separated after copulation ended. The *D. simulans* females were then left in the three dram vial for 15 minutes to allow for sperm storage to occur. After the 15 minutes had elapsed, each female reproductive tract was removed on a glass slide using dissection forceps then covered with TE buffer and a coverslip for visualization. The GFP labeled sperm in the reproductive tract were visualized using a fluorescent Zeiss Z1 microscope in the University of Western Ontario Biotron.

4.3 Results

Sperm storage in the female reproductive tract: Reproductive tracts of *D. simulans* females showed variation in sperm storage based on the morphology of the posterior lobes of the male they had mated with. The assumption was made that males with posterior lobe alterations were still capable of sperm transfer, this is a rational assumption as there was no contact to the male fertilizing genitalia and there were some sperm present in females who were mated to altered males. When observing the bursa copulatrix and seminal receptacle there appeared to be fewer sperm when a female had mated with an altered male as compared to a control male *D. simulans* in all of the observed female reproductive tracts. The two images (Figure 4.1) show a large difference in the amount of sperm stored, and are representative of the females tested in these two treatment groups. A trend was observed in that there was a decrease in the sperm stored, specifically in the seminal receptacles and the spermatheca, when the degree of alteration to the posterior lobe of the mated male increased (N=30).

Fewer or no fluorescent sperm heads were detected in the spermatheca of females, a long term storage organ, after copulations with altered males (Figure 4.2). Comparatively there were sperm detected in the spermatheca when males had the species-specific posterior lobe shape, as would be expected.

4.4 Discussion

The sibling species *D. simulans*, and *D. mauritiana* are often used as an example for the sensory lock and key model because *D. simulans* females reject *D. mauritiana* males early after insertion of the species-specific posterior lobe, resulting in low rates of sperm transfer. What has yet to be fully investigated is the role of other postcopulatory mechanisms in the isolation of the species. Price *et al.* (2001) determined that when sperm transfer does occur in the interspecific pairing of *D. simulans* females and *D. mauritiana* males, *D. simulans* females do not store the majority of the sperm and very few eggs are fertilized to make hybrids. This suggests that *D. simulans* females are biasing the paternity of their offspring when mating with males of another species but the mechanism underlying this selective fertilization is unclear as it could be a result of other isolating mechanisms, such as behaviour or pheromone profiles.

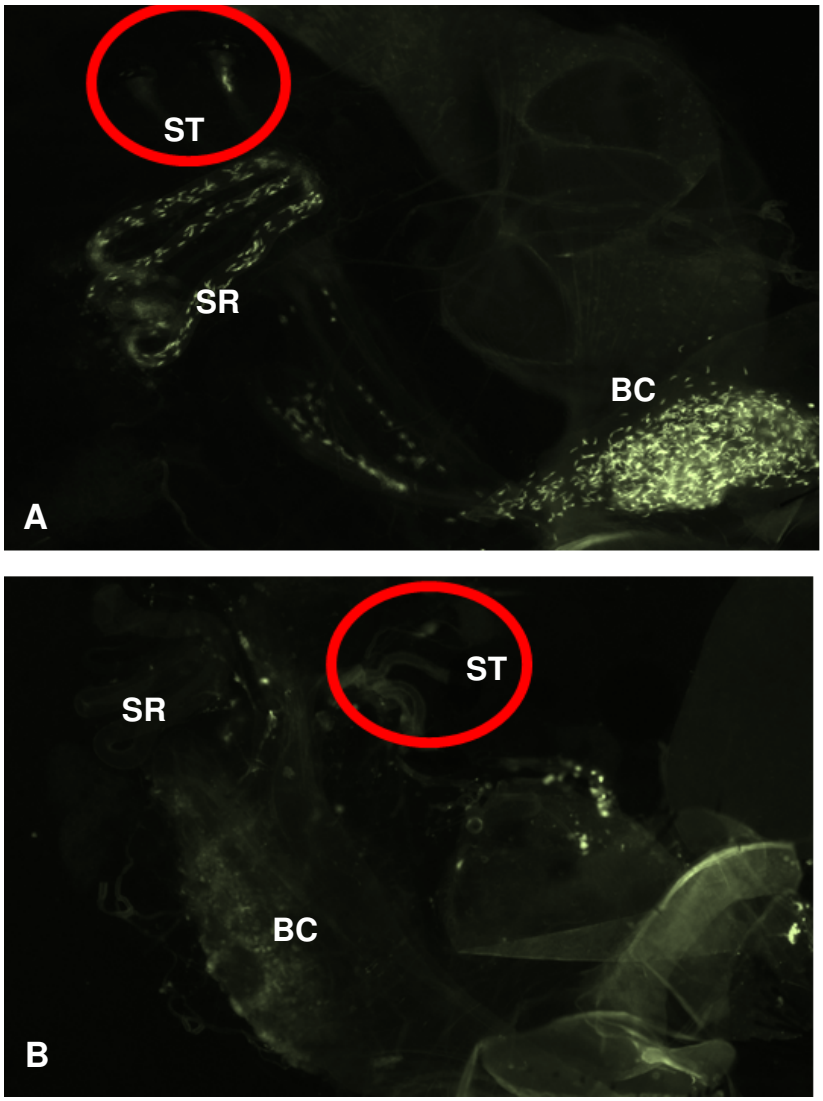

Figure 4.1. Sperm storage in the *D. simulans* female reproductive tract. The removed female reproductive tract of female *D. simulans* 15 minutes after the end of a copulation event with a *D. simulans* male who was either (A) a control, unaltered male or (B) a male with laser alterations on the posterior lobe. The fluorescence observed represent the stored GFP-tagged sperm heads. The red circle indicates the location of the long term sperm storage organ, the spermatheca (ST). The bursa copulatrix (BC), where the sperm is initially transferred, and the seminal receptacles (SR) are also labeled.

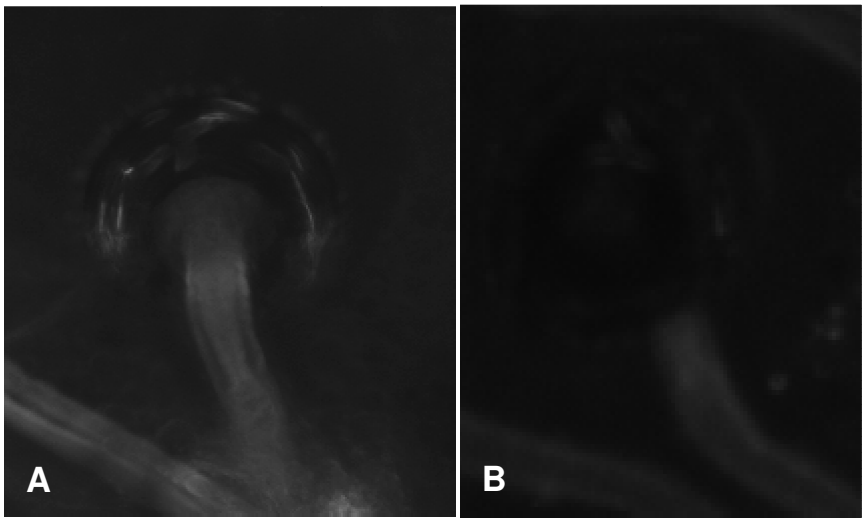

Figure 4.2. Dissected spermatheca in a mated *D. simulans* female. The fluorescence micrscope image of the spermatheca in a *D. simulans* female paired with a control male (A) and an altered male (B) of her own species. Fluorescence is observed in the sperm storage organ 15 minutes after copulation.

This study compared sperm storage in *D. simulans* females based on the morphology of the posterior lobe of *D. simulans* males. These males have the correct behaviour, courtship and pheromone profile for conspecific copulation with *D. simulans* females, and only differ in the shape of their genitalia. A few general trends were observed when comparing the storage of sperm by *D. simulans* females when they were mated with either control or altered males: there appeared to not only be more sperm within the female reproductive tract when males were unaltered, but there were also more sperm within the long term storage organ, the spermatheca. *Drosophila* females therefore utilize cryptic female choice as a prezygotic, and postcopulatory isolation mechanism. Evidence for differential storage of sperm based on the shape of the posterior lobe indicates that there is sensory recognition of the species-specific genital morphology.

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Chapter 5

5 Discussion

A summary of the findings of this thesis and future work that would continue to elucidate the rapid divergence of male genitalia.

Finding a Model Organism to Evaluate the Rapid Divergence of Genitalia

The rapid divergence of male genitalia is a well-documented phenomenon, and yet no concrete explanation has been provided as to why it occurs on such a wide scale. The three most studied hypotheses formulated to date are the lock and key hypothesis, the pleiotropy hypothesis, and the sexual selection hypothesis. Although specific examples of each model have been studied (Sota and Kubota 1998; Córdoba-Aguilar 1999; Bond *et al.* 2003), never have individual tests for all three hypotheses been performed in one model organism. Therein lies the novelty of my research.

Insects are often used to study the rapid divergence of male genitalia. Several examples of recently evolved insect species exist in which the male genitalia are highly divergent, often making male genitalia a useful tool for the assignment of different individuals to a species (Richards 1927; Córdoba-Aguilar 1999; Song 2009). An excellent model of recently evolved species that are distinguished by their male genital morphologies are four of the species in the *D. melanogaster* subgroup (Liu *et al.* 1996). The posterior lobes of the genital arch in *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana* are species-specific and have rapidly diverged within the past 2.5 million years (Kliman *et al.* 2000). That, in combination with the ease of rearing *Drosophila* in the laboratory, the possibility of mating them with each other and creating hybrids, and

the genetic tools available, make these sibling species ideal for further investigation of the three commonly studied hypotheses for the rapid divergence of male genitalia.

The Key Matters Less Than the Technique

The shape of the posterior lobe was previously thought to be an isolating mechanism between the species *D. simulans* and *D. mauritiana* because of the rejection behaviour exhibited by *D. simulans* females during interspecific copulations (Robertson 1983, 1988; Cobb *et al.* 1988; Coyne 1993). This rejection behaviour often results in shorter copulations and the absence of sperm transfer, which leads to species isolation (Coyne 1993). As the sensory lock and key hypothesis was long evoked as the explanation for this isolation, my research was intended to investigate the role of the species-specific morphology of the posterior lobe, if any. Few examples supporting the sensory lock and key model exist (Sota 1998), and although the *Drosophila* sister species have long been used as concrete evidence for the validity of the model, no empirical data had ever been documented. Recent research indicates that the posterior lobe caused tearing even in conspecific *D. simulans* mating events (Kamimura and Mitsumoto 2011), suggesting that there may be more than simple sensory recognition of the species-specific shape as previously suspected. My research determined that altering the posterior lobes of *D. simulans* males led to no increase in rejection behaviour by *D. simulans* females after copulation began, and therefore it was determined that the sensory recognition of stereotypical posterior lobe shape by females is not the only cue for an appropriate mate. This is not in accordance with the predictions of the sensory lock and key model: shorter copulations would be expected if the cue, being the species-specific posterior lobe or key, is removed or changed. These findings do not eliminate the possibility of sensory

recognition of the posterior lobe by *Drosophila* females, they only indicate that the rejection behaviour exhibited by *D. simulans* females may not be as easily categorized as previously predicted. If *D. simulans* females use the posterior lobe shape as a way to recognize appropriate mates after copulation begins, it is likely to happen cryptically, as suggested by the sexual selection hypothesis (Eberhard 1985).

A correlation between the lower frequency of copulation and the amount of posterior lobe removed by laser alteration was also reported, which suggests that *D. simulans* females recognize the posterior lobe shape prior to insertion. This kind of reproductive isolation mechanism has not been studied in these sibling species, and it presents an alternative reason for why selection would act on male genitalia. Females that recognize males of their own species prior to mating are less likely to produce sterile or unfit hybrids, therefore ensuring higher fitness for themselves. Indeed, this trend is not exclusive to *D. simulans*, as the posterior lobe appears to be a pre-copulatory cue for females in all four of the *Drosophila* species studied in the *D. melanogaster* subgroup, supporting this prediction. Behavioural differences in courtship had previously been documented, with *D. mauritiana* males being more aggressive and attempting copulation sooner, but this difference had been largely ignored by subsequent studies (Robertson 1983). These differences now appear to be the key reason for the interspecific mating events, and therefore other female rejection techniques may be used to bias paternity of offspring. Essentially, the *D. mauritiana* "key" does not matter, as males "kick the door down" with *D. simulans* females.

The discovery that posterior lobe shape may not be the interspecific cue for rejection behaviour in *D. simulans* females after copulation has begun puts into question

the validity of the sensory lock and key hypothesis for reproductive isolation from their sibling species *D. mauritiana*. Because my results did not provide support for such a mechanism, it becomes necessary also to evaluate other factors that may be responsible for the rapid divergence of male genitalia within the *D. melanogaster* subgroup.

The Pleiotropy Hypothesis Is Not a Likely Explanation for Genital Divergence

Finding the genes responsible for the species-specific nature of the posterior lobes in the *D. melanogaster* subgroup could also help clarify how rapid divergence of male genitalia has occurred. To date, there is no evidence that genes involved in the morphology of genitalia are correlated to genes involved in overall morphology in *Drosophila* males as the pleiotropy hypothesis would predict. Because no studies refute the correlation of genes regulating genital and overall morphology, until individual genes are located and evaluated separately, it is difficult to discredit the pleiotropy hypothesis for rapid divergence of male genitalia. Masly *et al.* (2011) recently contributed to the field by determining genomic regions important for size and shape of the posterior lobe in *D. mauritiana* and *D. sechellia*. The work presented here has done the same in *D. mauritiana* and *D. simulans* males, but further work is necessary to fine map and narrow down these regions to individual candidate genes. Quantifying length and weight of *Drosophila* males from backcrossed lines would allow me to determine if the differences among lines only occur in genital morphology or if they represent changes in overall body morphology, a logical next step to test the pleiotropy hypothesis. Neutral evolution has long been refuted as an explanation for the divergence of male genitalia (Eberhard

1985) and my research solidifies these previous findings, specifically in that selection does appear to be actively occurring in interspecific mating events.

Sexual Selection and the Future of Genital Divergence Work

Selection by females for an intraspecific male trait is the most commonly studied and well-supported hypothesis for the rapid divergence of male genitalia (Eberhard 1985, 1994, 2010; Hosken and Stockley 2004; Simmons *et al.* 2011). Cryptic female choice has been documented in a variety of organisms (Córdoba-Aguilar 2002, Burger *et al.* 2003) as a mechanism for the rapid divergence of male genitalia. In these instances, females of a species rely on sensory recognition of species-specific genital morphology to bias the paternity of their offspring after copulation, but prior to fertilization. The exact reasoning for this is still unclear, with most research focusing on the sexy sons hypothesis and the good genes hypothesis (Hosken and Stockley 2004). Cryptic female choice has been documented in some *Drosophila* species (Price 2001 *et al.*; Polak and Simmons 2009), but evidence that females can select sperm for fertilization of their eggs based on genital morphology (e.g. of the posterior lobe) in *Drosophila* is currently lacking.

Previous work on the species pair indicates that when a copulation event involving a *D. simulans* female and a *D. mauritiana* male does last long enough for sperm transfer to occur, females store a smaller percentage of sperm and fewer eggs are fertilized as compared to conspecific mating events (Price *et al.* 2001). Although this research does indicate that *D. simulans* females can bias paternity, the mechanism underlying this bias remains unclear. The goal of my research was to evaluate if the posterior lobe shape alone affected female sperm storage, therefore indicating sensory recognition of the posterior lobe shape during copulation by the *D. simulans* female. I

evaluated the presence of sperm in the reproductive tract of *D. simulans* females after they mated with males of their own species with posterior lobe alterations, allowing me to assess the role of male genital morphology in cryptic female choice.

My research determined that long term storage of sperm was less likely to occur when *D. simulans* females mated with *D. simulans* males who had non-species-specific posterior lobes. The differential storage of sperm based on the alteration of the posterior lobe implies that the morphology of the genitalia is a sensory cue for *Drosophila* females during copulation, but in a cryptic way, which is unlike the lock and key model. Further investigation into the topic could prove beneficial in understanding the evolution of male genitalia, as there did appear to be variation in sperm storage for long-term fertilization based on the alteration performed. These findings support the sexual selection hypothesis as a mechanism for divergence of male genitalia. To evaluate the validity of cryptic female choice based on posterior lobe morphology in *Drosophila*, future work should focus on how paternity of offspring is affected when the posterior lobe is not species-specific and females have the opportunity to re-mate. If the predictions about the sensory cues made here are accurate, one would expect *Drosophila* females to produce fewer offspring when insemination occurs with a male with posterior lobe alterations, specifically if other mates are available.

The rapid divergence of male genitalia is a well-studied field but many questions still remain as to why and how it occurs. Divergence of male genitalia is well studied and, as a result of my work, we now know that the long-suspected lock and key model is not responsible for the posterior lobe differences observed in the sibling species *D. simulans* and *D. mauritiana*. Although the revised definition of the sensory lock and key model has

given new life to the hypothesis (Masly 2012), empirical evidence, as seen in my research, has long fallen short when explaining how this model could lead to the rapid evolution of male genitalia (Shapiro 1989).

My research also shows that the pleiotropy hypothesis is not a likely explanation for the divergence of male genitalia as individual regions from the other species' genome can have an effect on posterior lobe size. Most new research into the divergence of male genitalia focuses on the sexual selection hypothesis, and this work suggests that this is warranted. Evidence for sexual selection for species-specific male genitalia exists in a wide variety of model organisms (Birkhead and Pizzari 2002; Córdoba-Aguilar 2002; Burger *et al.* 2003; Brennan *et al.* 2007; Simmons and García-González 2011), including this *Drosophila* pair, once thought to be one of the few remaining proponents for the lock and key hypothesis.

Continued research investigating the role of sexual selection in *D. simulans* and *D. mauritiana* may elucidate further what isolates this sister species and fully validate the sexual selection hypothesis. Further experiments that test all three suggested hypotheses in one model organism, such as what was completed here with two *Drosophila* species, can help to explain why the phenomenon occurs over such a large variety of groups, and further our knowledge on the basis of species divergence and isolation.

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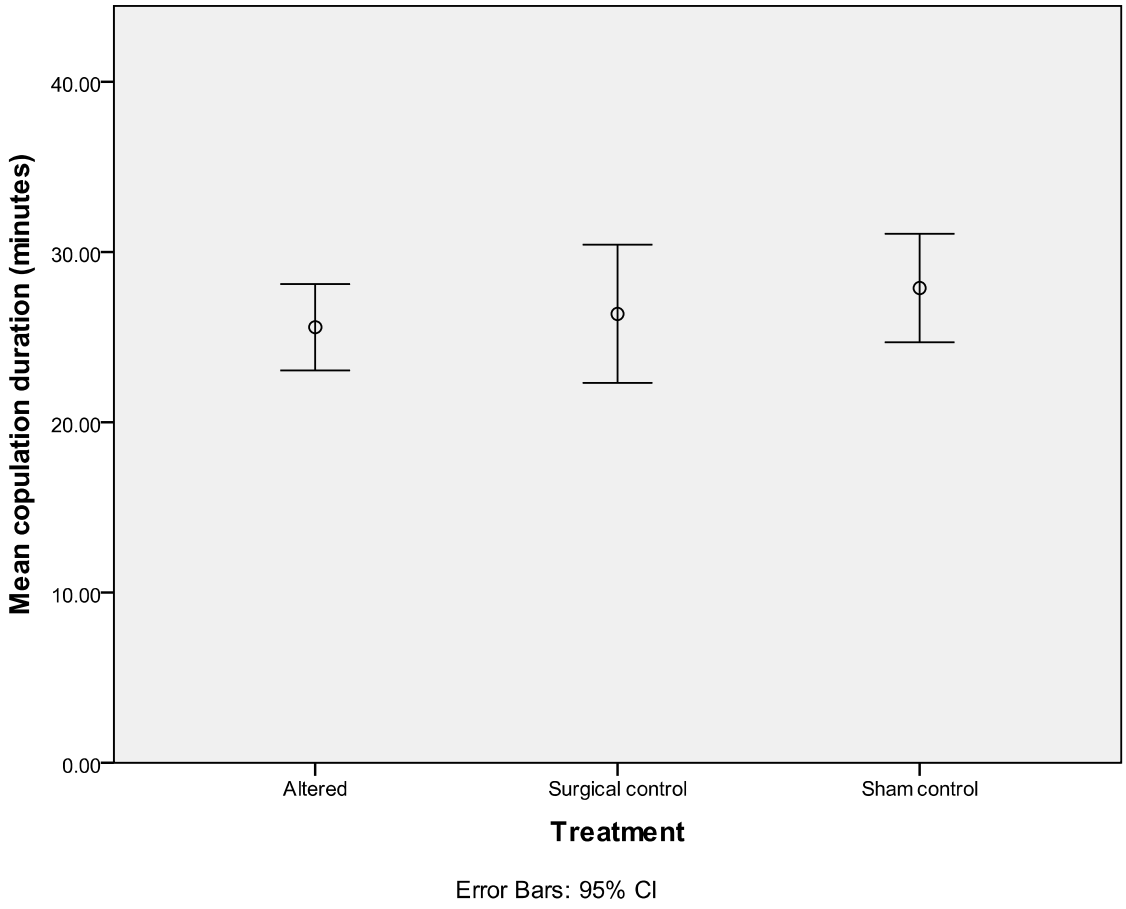
6 Appendices

Appendix A: Mean Copulation Duration for Altered *D. simulans* males.

Mean copulation duration for conspecific mating events of *D. simulans* females with *D. simulans* males from three different treatments 1) Males with both posterior lobes altered, 2) Surgical controls where hairs were removed from the abdomen and 3) sham controls who were placed in the laser treatment but no pieces were removed. As was seen in Chapter 2, there is no significant difference in mean copulation duration between any of the treatments (N=33, F=0.709, P=0.501). Performed with the help of Dr Polak at the University of Cincinnati.



Curriculum Vitae

Name: Hélène LeVasseur-Viens

Post-secondary Education and Degrees:
University of Western Ontario
London, Ontario, Canada
2005-2010 B.Sc.

The University of Western Ontario
London, Ontario, Canada
2010-Present M.Sc.

Honours and Awards:
Dean's List
2008-2009**,** 2009-2010

Nomination for a Teaching Award
2010-2011, 2010-2012

Presentations
Biology Graduate Research Forum 2010 - Poster presentation
Canadian Society for Ecology and Evolution 2011 - Symposium: Genetics of Speciation and Evolution
Evolution 2012 - Oral presentation, Sexual Selection and Evolution

Related Work Experience
Teaching Assistant
The University of Western Ontario
2010-2012

Publications:

McNiven V. T. K., H. LeVasseur-Viens, R. L. Kannipayoor, M. Laturney, and A. J. Moehring. 2011. The genetic basis of evolution, adaptation and speciation. Mol. Ecol.