Multivariate Post-copulatory Selection and Quantitative Genetics of *Drosophila simulans* Sex Comb

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as a thesis for the degree of
Master by Research in Bioscience
In April 2015

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

This thesis is concerned with investigations into the influence of *Drosophila simulans* sex comb characteristics on male fertilization success. Sex comb is a complex trait that has length, depth, and tooth number. Predicting the outcome of selection on a complex trait comprising of several traits requires an estimation of the impact of selection on both traits variances and covariances. The influence of multivariate post-copulatery selection on *Drosophila simulans* sex comb components has been investigated where the effect of offensive and defensive bouts of selection on males sex comb have been estimated by scoring both the proportion of offspring fathered by the focal male when he is the first to mate with the female (P1), and proportion of offspring fathered by the focal male when he is the second to mate with the female (P2). The heritablities for all sex comb components as well as their underpinning G matrix were calculated. Although *Drosophila simulans* sex comb components were all significantly heritable, constraints have been found to affect the comb and impede its evolution as a response to post-copulatory sexual selection. These constraints arise mainly due to: 1) the absence of directional post-copulatory selection. 2) The positive genetic correlation between *sex* comb components. 3) Physical constraints. All of the above constraints could also explain the high additive genetic variance underlying *D. simulans* sex comb components.

Contents

To my parents with love

Acknowledgments

“In the name of Allah, The Most Gracious and The Most Merciful”

I would like to express my deep gratitude to my supervisor Prof. David Hosken for the time that he devoted to me throughout this research work, and for his constant guidance and positive criticism that made this work interesting for me.

My deepest appreciation is to Dr. John Hunt and Dr. Clarissa House for their numerous moments of attention and for their support.

I also owe thanks to Dr. MD Sharma and Dr. Alastair Wilson for their help and kindness.

I am extremely grateful to the encouragement and moral support from my family at home, particularly my parents.

Most of all, I would like to thank my husband Mohammad for his wholehearted support throughout this work. I don’t think this would have been so comfortable without him.

General Introduction

Evolution can be thought of as across generational changes in allele frequencies within a population (Falconer & Mackay 1996). Darwin (1859) explained how this change is caused by selection, which is the process whereby those individuals better able to survive and reproduce leave more descendants, and because of this, the genes they carry increase in frequency from one generation to the next (Sheppard 1975; Ridley 1996).

Many characters possessed by organisms increase their chances of surviving to reproduce. However, there are other characters that would seem to reduce organism survival, and they are usually sexually dimorphic (Andersson 1994). Typically it is males that carry the extravagantly developed, secondary sexual characters, that in survival terms appear to be so costly. These characters are not primarily used in breeding like genitalia (primary sexual character), but they play important roles in reproduction. The peacock's tail is one iconic example. It reduces the male ability to fly and manoeuvre, and makes the bird visible to predators, and as noted above, such costly traits should be eliminated by natural selection (Ridley 2004; Snook *et al* 2013).

Darwin (1871) explained the evolution of these elaborated traits by sexual selection. Sexual selection is the differential reproductive success caused by reproductive competition. It occurs through male-male competition and female mate choice (Andersson 1994; Danielsson 2001), and these secondary sexual traits confer a reproductive advantage to their possessor relative to rivals, either by increasing their competitive power or by increasing their attractiveness (Andersson 1994; Ridley 2004). Consequently, males with conspicuous secondary sexual traits will compensate for their reduced survival by securing higher mating and siring success.

Darwin (1871) described sexual selection exclusively as a pre-copulatory process, and in keeping with this, many studies have used mating success as a measure of male reproductive success (e.g., Clutton-Brock 1988; Pemberton *et al* 1992; Abell 1997). However, it has recently been found that most females are polyandrous and mate with more than one male during the reproductive season

(Birkhead & Møller 1998). Polyandry means that sexual selection could continue after mating and this enables post-mating processes, which have the potential to affect male reproductive success (Smith 1984; Birkhead and Møller 1998; Hosken *et al* 2008). In other words, mating success alone may not be an accurate measure of male reproductive success. Therefore, in order to fully understand how male secondary sexual traits evolve, it is necessary to consider the effect of both, pre- and post-copulatory sexual selection episodes on these traits (Polak & Simmons 2009).

Post-copulatory sexual selection occurs through two mechanisms: sperm competition and cryptic female choice (Parker 1970; Eberhard 1996). Sperm competition, occurs when the sperm of different males compete to fertilize a female's ova, and it represents post-copulatory male-male competition (Parker 1970). Whereas, cryptic female choice, is the ability of the female to preferentially bias resources or paternity toward certain males, and it represents post-copulatory female choice (Smith 1984; Birkhead and Møller 1998; Hosken *et al* 2008). Either of these mechanisms has the potential to generate variation in paternity, and may reinforce or counteract pre-copulatory sexual selection (Birkhead & Pizzari 2002). Therefore, the sexual selection that males experience before mating and after insemination collectively affect the shape, strength, and direction of the net sexual selection acting on male sex traits (Hunt *et al* 2009; Polak and Simmon 2009). In spite of the importance of both pre- and post-copulatory sexual selection, only a few studies take into account the joint effect of these two selection episodes on male sex traits.

Trait evolution will not be driven by selection alone. Traits will not evolve, unless there is phenotypic variation associated with underlying heritable genetic variation among the members of the population (Falconer & Mackay 1996). Thus, genetic variation is necessary for selection to cause evolutionary change. For the vast majority of traits, many genes underlie their phenotypic expression as they are quantitative traits (Falconer & Mackay 1996), and the univariate breeder equation can be used to visualise how they should evolve in response to selection. This can be summarised as:

$$R = h^2 S$$

where R is the response to selection (evolution), $h^2$is the trait heritability (the proportion of the total phenotypic variance attributable to the additive genetic variance,V_A/V_P), and S is the selection differential (the mean phenotypic value of the selected individuals expressed as a deviation from the population mean before selection) (Hansen & Houle 2008). However, selection rarely acts on a single trait in isolation at any one time. In addition, selection may work on an integrated trait that looks like a single trait that in reality is a combination of traits (Brooks & Endler 2001).

Understanding how an integrated trait evolves, needs an estimation of the effect of multivariate selection on the traits, and on the additive genetic variance covariance between them (Lande 1979; Lande & Arnold 1983; Brodie *et al* 1995; Falconer & Mackay 1996), and this is summarized by the multivariate breeders equation:

$$\Delta\bar{z} = G\beta$$

where $\Delta\bar{z}$ is the column vector of changes in trait means, G is the additive genetic variance-covariance matrix, and β is the vector of linear selection (Lande 1979; Lande & Arnold 1983). The genetic variance-covariance matrix allows us to understand the extent to which covariances constrain or facilitate the evolution of correlated traits, and how the covariance structure among traits affect the rate of evolution of an integrated trait. This can be achieved by comparing the rate at which a set of traits evolve given the observed G matrix to the expected rate if all covariances in the G matrix are set to zero (Agrawal & Stinchcomb 2009).

According to the multivariate breeders equation, if we have the genetic variance-covariance matrix for a combination of traits, and we know the strength and direction of each linear selection gradient β_i operating on every single trait within the combined traits, then we can predict how they will evolve, and to what extent every single trait can constraint or facilitate the evolution of other traits.

A remarkable example of a trait that undergoes rapid evolution is the *Drosophila* sex comb. The sex comb looks like a single trait but in reality it has length, depth, and tooth number, which all together contribute to the secondary sex trait found on the forelegs of many *Drosophila* males (subgenus *Sophophora*). The

sex comb is an array of modified mechanosensory bristles that are used by males to grasp the female abdomen or to spread her wings during copulation (Ahuja & Singh 2008; Sharma *et al* 2011). In addition, sex combs are structurally variable and exhibit high intra- and interspecific variation in comb's tooth number, position, shape, and colour (Kopp and True 2002; Ahuja and Singh 2008). It has been suggested that sex comb evolution is driven by sexual selection. For instance, Hurtado-Gonzales *et al* (2014) found that surgical removal of the sex comb reduced male copulatory success in *D. melanogaster*. In *D. simulans,* males in copula from natural population had significantly fewer sex comb teeth than single males (Markow *et al* 1996). In contrast, sex comb's tooth number found to have a different effect on mating success in *D. bipectinata* where mating males showed significantly increased tooth number (Polak *et al* 2004).

These studies presented that the presence and the morphology of the sex comb could influence mating success in different species of *Drosophila*. However, little is known about the effect of sex comb morphology on post-copulatory sexual selection. An exception to this is a study by Polak and Simmon (2009), where they documented a positive correlation between fertilization success and sex comb bristle number in *D. bipectinata*. However, like other sex-comb studies they only focused on comb tooth number. The effect of sex combs as a trait comprising of length, depth and tooth number on male reproductive success and how episodes of sexual selection drive the evolution of such a complex trait remain unknown.

In this thesis I examined the effects of *Drosophila simulans* sex comb characteristics on male fertilization success, by examining how comb characteristics impact male sperm competitiveness, both defensively by scoring P1 (the proportion of offspring fathered by the focal male when he is the first to mate with the female), and offensively scoring P2 (the proportion of offspring fathered by the focal male when he is the second to mate with the female). I estimated the strength, shape, and direction of linear and nonlinear post-copulatory sexual selection imposed on sex comb characteristics by using multiple regression (multivariate approach) and canonical analysis (Land & Arnold 1983, Phillips & Arnold 1989). I also investigated the genetic architecture of the *D. simulans* sex comb by estimating its genetic variance-covariance

matrix. Finally, the effect of genetic covariances on the evolution of sex comb is assessed, and how genetic variances and covariances between sex comb traits may shape the male fitness surface.

CHAPTER 1

The Effect of Multivariate Post-copulatory Selection on D. Simulans Sex Comb Components

1.1 Introduction

Sexual selection acts mainly on males and is primarily responsible for the evolution of secondary sex traits (Darwin 1871; Andersson 1994). While, these sexual traits are costly, they also confer a reproductive advantage to their possessors relative to rivals, either by increasing their competitive power or by increasing their attractiveness (Andersson 1994; Ridley 1996). Because secondary sexual traits affect a male's ability to attract or otherwise secure a mate, many studies used mating success as an estimate of actual male reproductive success (e.g., Clutton-Brock 1988; Pemberton *et al* 1992; Abell 1997). However, most females are polyandrous (Birkhead & Møller 1998).

Polyandry means that sexual selection can continue after mating and this enables post-copulatory selection. Post-copulatory sexual selection is thought to be partly or completely under the control of females (Thornhill 1983; Eberhard 1985; Birkhead & Møller 1993; Birkhead & Møller 1998) and males compete at the ejaculate level in order to secure fertilization (Parker 1970). Consequently, mating success *per se* may not be an accurate measure of male reproductive success. Thus, in order to fully understand how male secondary sexual traits evolve, it is necessary to consider the effect of both, pre- and post-copulatory sexual selection episodes on these traits (Polak & Simmons 2009).

There is much evidence for the evolution of secondary sex traits through the combined effect of both pre- and post-copulatory sexual selection (Hosken *et al* 2008). It has been found that both episodes of sexual selection could complement each other. For example, in flour beetles (*Tribolium castaneum*) and fruit flies (*Drosophila simulans*), attractive males with high mating success achieved higher second-male precedence and thus higher fertilization success when allowed to mate with previously inseminated females (Lewis and Austad 1994; Hosken *et al* 2008). Furthermore, in guppies attractive males with more orange body colouration sire more offspring when females are artificially

inseminated with equal numbers of sperms from ornamented and less ornamented counterparts (Evans *et al* 2003). Alternatively, the two episodes of sexual selection may antagonise each other. For example, in water striders (*Gerris lacustris*) where larger males having a significantly higher mating success, but smaller males secure higher fertilization success from each mating, since they copulate for longer time which enable them to compensate for low mating success (Danielsson 2001). As a result, different episodes of sexual selection neutralise each other and reduce the variance in male reproductive success.

The vast majority of male secondary sex traits are quantitative traits and many genes underlay their phenotypic expression (Falconer & Mackay 1996). Quantitative traits are often a combination of characters that are genetically correlated and hence act as composites. For example, in guppies (*Poecilia reticulata*) the male colour looks like a single trait, but in fact it is a mixture of different colours (traits) that have different levels of integration. Some colours are correlated and evolve in concert (Brooks & Endler 2001; Blows *et al* 2003). This suggests that selection could acts on many characters simultaneously, and genetic correlations between traits are ubiquitous. For instance, both component of the male sex comb of *D. melanogaster*, tooth number and comb length are positively correlated, and both exhibited positive condition dependence, which explains why both traits have almost the same pattern of size changes when the flies are reared on poor and rich diets (Ahuja *et al* 2011). Thus, it is a tremendous oversimplification to study the effect of selection on characters in isolation (e.g., Haldane 1954; Van Valen 1965; O'Donald 1970), since selection rarely acts on a single traits at any one time (Brooks & Endler 2001). As a result, in order to understand how a composite of traits evolve, we need to measure the selection that acts on means, variances, and covariances of the composite components (Lande & Arnold 1983).

A remarkable example of a trait that has undergone rapid evolution is the *Drosophila* sex comb (Ahuja & Singh 2008; Sharma *et al* 2011). The sex comb is a secondary sexual character found on the forelegs of many *Drosophila* males (subgenus *Sophophora*). It is an array of modified mechanosensory bristles that are used by males to grasp the female abdomen or to spread her wings during copulation (Ahuja & Singh 2008; Sharma *et al* 2011). It has been

suggest that sex comb evolution is driven by sexual selection (Spieth 1952; Petrie 1988; Markow *et al* 1996; Polak *et al* 2004). Where, sex comb tooth numbers show a high intra- and interspecific variation and they affect male mating success (Coyne 1985; Kopp *et al* 2003; Tatsuta & Takano-Shimsu 2006). In *D. simulans* for example tooth number and comb size are negatively associated with mating success (Markow *et al* 1996), while in *D. bipectinata*, males with lager combs have greater mating success (Polak *et al* 2004). Most of the previous studies investigated the influence of sex comb's tooth number on mating success (e.g. Markow *et al* 1996; Ahuja & Singh 2008). However, little is known about whether the sex comb as a trait that has multiple characters, (i.e depth, length, and tooth number) could influence male fertilization success or not.

Here I examined the strength of linear and nonlinear, post-copulatory selection on *Drosophila simulans* sex comb characteristics when a male mates first (P1: the proportion of offspring fathered by the 1st male) or last (P2: the proportion of offspring fathered by the 2nd male). Using this approach, I estimate whether some variation in male fertilization success is explained by male sex comb morphology.

1.2 Methods

1.2.1 Fly stocks

The laboratory wild-type populations of *Drosophila simulans* were derived from twenty isolines that were supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. These isolines originally came from individuals that were caught in Tincurry, Eastern Australia, in March 2004. In the laboratory the isolines were mixed and maintained in population cages (ca. 800-1000 flies/cage) at 25^{o}C under a 12:12 H light: dark cycle, with overlapping generations for at least 4 years prior to the start of this investigation. The populations of ebony flies, which carry a homozygous recessive phenotypic marker, were established using a strain obtained from the Tucson stock centre and was maintained as above for over 50 generations. Ebony as well as wild-type flies were maintained under the same conditions. All cages had in excess of 600 flies with overlapping generations and free mate

choice. All flies were reared on *Drosophila* quick mix medium (supplied by Blades Biological, Edenbridge, Kent, U.K .).

For P1 and P2 estimation, ebony and wild-type flies were initially collected as virgins from stock populations. Egg laying vials were placed in the cages of each population daily and left for 24 hours. These vials were incubated until peak eclosion (ca. 8-9 days after egg laying). Offspring that eclosed overnight were killed and virgins were collected ca. 7hrs later. Virgin females were aspirated into individual vials containing culture medium and virgin males were pooled in standard vials with ca. 80 other virgin males. These virgin females and males were used for mating trials when the females were 3 days old and males were 3 - 4 days old, to ensure full sexual receptivity (Manning 1967).

Females were mated with a focal, wild type male and ebony male (P1) or an ebony male and a focal, wild type male (P2). Males mated once only and in a single role – defensive or offensive. Mating trials began at the beginning of the light stage of the light: dark cycle as this is when the flies are most reproductively active [Sakai & Ishida 2001]. In all trials, each male was aspirated into a female housing vial, and continuously observed for 2 hours (Spieth 1974). If copulation occurred, the male was removed from the chamber, aspirated into an Eppendorf and stored at -20^{o}C for dissection and measurement. Following the first mating, females were transferred daily into fresh food vials to oviposit for 4 days before their second exposure to virgin males. The mating procedure for mated females were identical to that was described above. Following their second mating, twice mated females were once again transferred daily into fresh food vials to oviposit for 4 days. On the 5th day the female was aspirated into an Eppendorf and stored at -20^{o}C.

Vials that had contained the mated females were stored at 25^{o}C and monitored daily until offspring emerged. Seven days after the first emergence, the vials were inverted and stored in the freezer and the ebony and wild type offspring from each of female's 8 vials was subsequently counted to determine the proportion of offspring that were sired by the focal, wild type male following a double mating.

1.2.2 Dissecting and Measurement on Flies

The variation in sex comb morphology of wild type males who successfully mated (P1= 307, P2= 378) was quantified to investigate possible correlations between sex comb characteristics and sperm defence/offence. The left and right fore-legs and wings were carefully pulled free from the body of each male, and then mounted on glass slides using Hoyers Medium. Digital images for wings (X30) and sex combs (X100) were captured by Leica dissecting microscope (M125) connected to a Leica camera (DFC295). Later wing length and sex comb characteristics were measured manually using Image J v1.46r.

As in Smith *et al* (2011) variation in wing length (WL) is obtained by estimating the distance between the intersection of the third longitudinal vein and the anterior cross vein to the distal tip of third longitudinal vein (Figure 1). The wing length is used as an index of body size (Sharma *et al* 2011), both left and right wings of each male were measured and an average value was calculated. The distance along the sex comb base was measured to indicate the comb length (CL), comb depth (CD) was also measured as the average length of the 1st, 3rd, and 5th pegs, and sex comb tooth (TN) number was counted as well (Figure 2). All sex comb characteristics, including CL, CD, and TN were estimated as the average of the measurements on the left and right body sides.

The precision of the measurements were assessed by blindly measuring all traits twice on a sub-sample (N=20) of wings and sex combs. Regression of measure one on measure two showed that measures were highly correlated and repeatable (CD: β= 0.959, r^2= 0.919, P< 0.05; CL: β= 0.991, r^2= 0.982, P< 0.001; TN: β= 1.00, r^2= 1.00, P< 0.001; WL: β= 0.996, r^2= 0.992, P< 0.001).

Figure 1: Wing length measured between A and B.

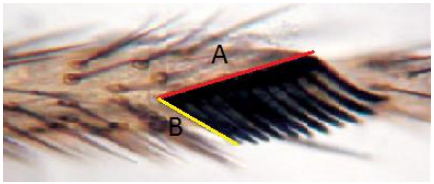


Figure 2: Foreleg of a *Drosophila simulans* male showing a sex comb with 11 teeth. Sex comb length (A). First tooth length (B).

1.3 Statistical analysis

The multiple-regression-based approach of Lande & Arnold (1983) was used to estimate the effects of linear and nonlinear selection on our variables (WL, CL, CD, and TN). Fitness scores were converted to relative fitness (ω) with a mean of 1 by dividing individual fitness success (W) by the mean mating success (i.e. fitness) of the population ($\overline{W}$).

$$\omega = \frac{W}{\overline{W}}$$

For statistical comparison, all traits were Z-standardized to have a mean of 0 and a standard deviation of 1. The Z-values of the traits is calculated as

$$Zi = \frac{(Xi - \mu i)}{\sigma i}$$

where Xi is the individual trait value, μi is the population trait's mean, and σi is the standard deviation of the trait (Hunt *et al* 2009). Then a first order multiple regression was fitted using only the standardized traits (WL, CL, CD, and TN) as the explanatory variables and relative fitness as the response variable, in order to estimate the standardized linear selection gradients βs (or the partial regression coefficients of relative fitness on the characters) and to evaluate the contribution of individual traits to fitness after removing the residual effects of other traits on fitness (Lande & Arnold 1983; Hunt *et al* 2009; Ower *et al* 2013):

$$\omega = \alpha + \beta_{WL} + \beta_{CL} + \beta_{CD} + \beta_{TN} + \varepsilon$$

where α is the regression intercept, and ε is the random error component. Then a second order multiple regression containing linear, quadratic and correlational terms was used to investigate the effect of selection on the variances and

covariances of the traits (Lande & Arnold 1983; Hunt *et al* 2009; Ower *et al* 2013):

$$\omega = \alpha + \beta_{WL} + \beta_{CL} + \beta_{CD} + \beta_{TN} + \frac{\gamma}{2}WL^2 + \frac{\gamma}{2}CL^2 + \frac{\gamma}{2}CD^2 + \frac{\gamma}{2}TN^2 + \gamma WLCL + \gamma WLCD + \gamma WLTN + \gamma CLCD + \gamma CLTN + \gamma CDTN + \varepsilon.$$

The above equation (second order multiple regression) cannot be used to determine the linear selection gradients, but it is used to drive the γ matrix which contains standardized γ coefficients (nonlinear selection gradients) associated with the quadratic and correlational terms, that describes the effect of selection on the variances and covariances of traits when the effects of linear selection are removed. The strength and shape of correlational selection acting on the covariance between two traits can be determined by the magnitude and sign of the γ coefficients associated with the correlational terms (Table 1) (Lande & Arnold 1983; Hunt *et al* 2009).

The curvature along the individual traits axes and the direct effect of nonlinear selection on the trait variances are indicated by the sign and value of the γ coefficients associated with the squared terms, as negative γ indicates a convex selection (stabilizing selection) affecting the trait, whereas positive γ indicates a concave selection (disruptive selection) affecting the trait (Table 1) (Lande & Arnold 1983; Phillips & Arnold 1989; Brodie *et al* 1995). Quadratic regression coefficients (γ coefficients associated with the squared terms) resulting from this model should be doubled as they are underestimated by a factor of 0.5 (Stinchcombe *et al* 2008).

Since the individual interpretation of the size and sign of γ coefficients may possibly underestimate the strength of nonlinear selection (Phillips & Arnold 1989; Blows & Brooks 2003), a canonical rotation of the γ matrix was used to locate the major axes of fitness surface (eigenvector) (Phillips & Arnold 1989). The result of this canonical analysis is the M matrix, that consists of four eigenvectors m_i (the subscript $_i$ depends on the number of traits measured), which define the major axes of the fitness surface (Phillips & Arnold 1989, Blows & Brooks 2003, Bentsen *et al* 2006). Each column in the M matrix represents a linear equation where the original values of the trait can be substituted to drive Y-score that places the original trait values into canonical

space for each individual included in the analysis (Hunt *et al* 2009). Next, the double regression method of Bisgaard and Ankenman (1996) was used, to estimate the linear θ_i and nonlinear λ_i selection gradients along each of the eigenvectors of the fitness surface, through using ω as the dependent variable and Y-scores and their quadratic terms Y_{ii} as the independent variables. Nonlinear λ_i selection gradients resulted from the above regression could have positive or negative values that represent respectively concave or convex (saddle shape fitness surface) fitness surface. The linear selection gradient θ represents the slope of the fitness surface along each of the major axes (Hunt *et al* 2009).

Since neither the explanatory variable (relative fitness) nor the predictor variables (WL, CL, CD, and TN) were normally distributed, a Monte-Carlo randomization test was used to evaluate the significance of all linear and nonlinear selection gradients resulted from the previously mentioned regressions, where the response variable (relative fitness) is randomised with 10,000 iterations (Mitchell-Olds & Shaw 1987).

1.3.1 Fitness surface visualization

The major axes of fitness surface recognized by canonical analysis were visualized by thin-plate splines (Phillips & Arnold 1989; Green & Silverman 1994). Thin-plate splines were estimated using the Tps function of the fields package (Furrer *et al* 2012) of R (version 2.13.5, available via http://www.r-project.org) using the value of the smoothing parameter (**λ**) that minimized the generalized cross-validation (GCV) score (Green and Silverman 1994). Finally, a perspective and a contour map views were plotted for the surface by using R.

1.3.2 Comparing the strength and form of sexual selection among P1 and P2

The strength and form of sexual selection acting on male wings and sex comb characteristics in P1 and P2 were compared using the sequential model building procedure (Drapper & John 1988; Chenoweth & Blows 2005). This procedure allowed the strength of standardised selection gradients between P1 and P2 to be statistically tested, but it cannot be used to compare fitness

surfaces across studies as canonical analysis places each study in its own unique canonical space (Ower *et al* 2013).

1.4 Results

1.4.1 Sex combs and sperm defence

Standardised linear, quadratic, and correlational selection gradients for the P1 experiment are presented in Table 1A. There was no significant linear or correlational selection operating on any of the traits. However, there was significant quadratic selection acting on CD.

Canonical rotation of γ matrix resulted in four eigenvectors - one with a positive eigenvalue (m_1) and three with negative eigenvalues (m_2, m_3, and m_4) (Table 2A). However, there was only significant selection on m_1 and m_4. Eigenvector m_1 showed disruptive (concave) selection and was heavily influenced by positive weighting from TN, and to a lesser extent, negative weightings from WL and CL (Table 2A). Eigenvector m_4 showed stabilising (convex) selection and was heavily loaded by a positive weighting from CD, and to a lesser extent, negative weighting from CL (Table 2A). The combination of disruptive and stabilizing sexual selection along eigenvectors m_1 and m_4 creates a saddle like fitness surface (Figure 3A). Figure 3B is a contour view that presents the same major axis with all data points and shows that many males measured are bunched in the valley between the two fitness peaks.

1.4.2 Sex combs and sperm offence

In the P2 experiment there was no significant linear selection working on any of the male traits (Table 1B). Nevertheless, there was a significant quadratic selection affecting TN, and a negative correlational selection between TN and CL (Table 1B).

Diagonalization of γ matrix gave rise to two positive eigenvectors (m_1 and m_2) and two negative eigenvectors (m_3 and m_4) although, only selection along eigenvectors m_1 and m_4 were significant (Table 2B). Eigenvector m_1 showed disruptive (concave) selection and was heavily influenced by positive weighting from CL, and negative weighting from TN. Whereas, eigenvector m_4 showed

stabilising (convex) selection and was heavily loaded by a positive weighting from WL, and to a lesser extent, negative weightings from TN and CL (Table 2B). The combination of disruptive and stabilizing sexual selection on the sex combs for P2 along eigenvectors m_1 and m_4 creates a saddle like fitness surface (Figure 4A). Figure 4B is a contour view visualization of the same major axis showing all data points, and again many of the males that were measured are gathered in the valley that goes between the two fitness peaks.

Finally, partial F tests from sequential modelling showed no significant difference in linear ($F_{4,653}$ = 0.261, P = 0.903), quadratic ($F_{4,645}$ = 1.487, P = 0.205), or correlational ($F_{6,633}$ = 0.700, P = 0.650) selection gradients among P1 and P2.

Table 1: The vector of standardised linear selection gradients (β) and the matrix of standardised quadratic and correlational selection gradients (γ) for the four male characters in P1 experiment (A) and P2 experiment (B).

		γ			
	β	TN	CD	CL	WL
A.	Standardized selection gradients for P1				
BN	-0.115	0.198			
CD	0.021	-0.027	-0.210*		
CL	0.101	-0.084	0.097	-0.076	
WL	-0.086	-0.100	0.040	0.080	-0.024
B.	Standardized selection gradients for P2				
BN	-0.017	0.416*			
CD	0.040	0.017	-0.030		
CL	0.031	-0.500*	0.024	0.464	
WL	-0.075	0.069	0.019	0.044	-0.134

BN, bristle number; CD, comb depth; CL, comb length; WL, wing length. Randomization tests: *P < 0.05, **P < 0.01, ***P < 0.001

Table 2: Linear (θ_i) and nonlinear (λ_i) selection gradients and the M matrix of eigenvectors from the canonical analysis of γ matrix, (A) P1 experiment and (B) P2 experiment.

			M			
	θ_i	λ_i	TN	CD	CL	WL
A.	Canonical analysis of P1					
m_1	-0.101	0.140*	0.851	-0.143	-0.326	-0.385
m_2	-0.047	-0.008	0.522	0.319	0.548	0.570
m_3	-0.136	-0.057	0.041	-0.323	-0.606	0.726
m_4	-0.025	-0.131**	-0.036	0.879	-0.475	-0.003
B.	Canonical analysis of P2					
m_1	0.035	0.472**	-0.691	0.005	0.723	-0.015
m_2	-0.002	0.009	0.486	0.596	0.469	0.435
m_3	0.051	-0.028	-0.358	0.803	-0.354	-0.319
m_4	-0.067	-0.092*	-0.399	-0.003	-0.364	0.842

Randomization tests: *P < 0.05, **P < 0.01, ***P < 0.001

(A) (B)

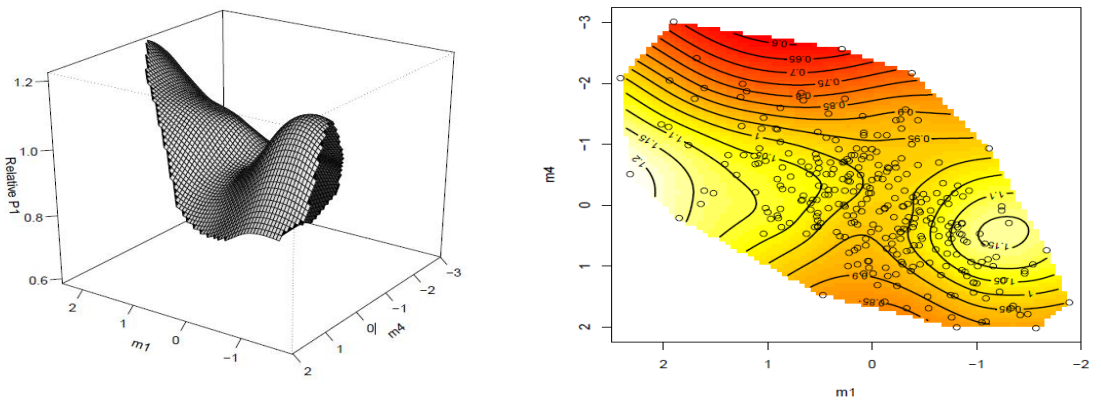


Figure 3: Thin-plate spline (A) perspective view and (B) contour map visualization of the two major axes of nonlinear selection (m_1 and m_4) operating on sex comb's traits of males mated in P1. Each point on the contour plot represents an individual male.

(A) (B)

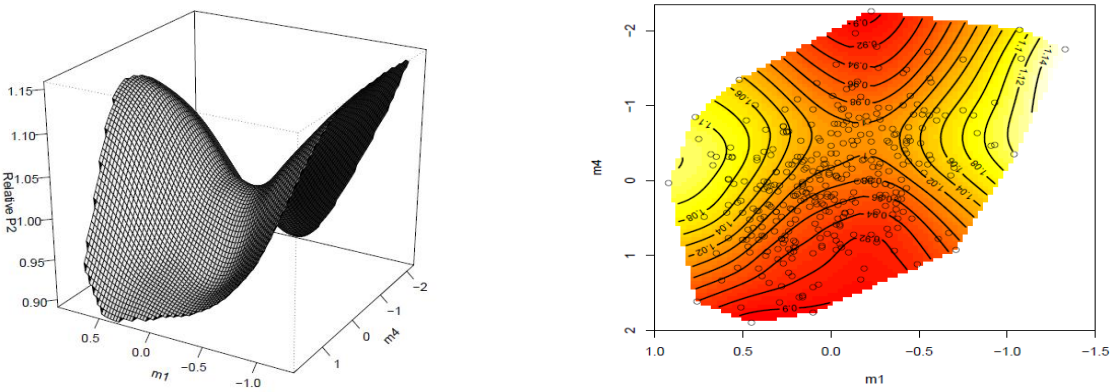


Figure 4: Thin-plate spline (A) perspective view and (B) contour map visualization of the two major axes of nonlinear selection (m_1 and m_4) operating on sex comb's traits of males mated in P2. Each point on the contour plot represents an individual male.

1.5 Discussion

Previous studies on *Drosophila* sex comb have emphasized the effect of sexual selection on tooth number only (Markow *et al* 1996; Tatsuta & Shimizu 2006; Ahuja & Singh 2008). However, the sex comb is comprised of a numbers of characters that are phenotypically correlated (Ahuja *et al* 2011), yet none of these studies showed how characters of the sex comb collectively respond to sexual selection. Particularly, little is known about the effects of post-copulatory sexual selection on *Drosophila* sex combs, with only a single study published to

date (Polak & Simmons 2009). Here, I estimate the effect of post-copulatory sexual selection on *D. simulans* sex comb length, depth and tooth number.

During sperm defence, I found significant non-linear post-copulatory sexual selection on sex comb traits of *D. simulans* males. Specifically, there was significant disruptive selection acting on m_1 and significant stabilizing selection acting on m_4. These two major axes of non-linear selection resulted in a saddle-shaped selection surface depicted in Figure 3A. Peaks in first male paternity occurred at extreme positive and negative values of m_1 and intermediate values of m_4. Positive values of m_1 corresponding with high tooth number and shorter comb length (CL) and smaller body size (WL) and intermediate comb depth. Negative values of m_1 correspond with low tooth number and longer comb length and larger body size and intermediate comb depth. However, the major feature of this rotated surface was a rising ridge as positive values of m_1 indicating that males bearing the shorter combs with higher tooth number will have the highest paternity share when they are the first to mate with a female.

During sperm offense, selection on male sex comb morphology was also significant and non-linear. Once more a saddle shaped fitness surface was seen as a result of disruptive and stabilizing selection acting on the major axes of non-linear selection m_1 and m_4 respectively. As before, peaks in second male paternity occurred at positive and negative values of m_1 and intermediate values of m_4. Positive values of m_1 corresponding with lower tooth number (TN) and longer comb length (CL) and intermediate body size (WL). Negative values of m_1 correspond with higher tooth number (TN) and shorter comb length (CL) and intermediate body size (WL). However, the dominant feature of this fitness surface was a rising ridge peaking at positive values of m_1, but in contrast to P1 results, these high values correspond to longer comb length and lower tooth number. Consequently, males with longer sex combs and lower tooth number appear to have better sperm offense. The above findings are further reinforced by the analysis of the standardized selection gradients for P2, where there was significant negative correlational selection acting on tooth number and comb length.

The sequential model building approach in the current research showed that there was no significant difference in the standardised selection gradients (linear, quadratic, and correlational selection) for sex comb characteristics across P1 and P2. Although, still these experiments cannot be combined because the canonical analysis places each study into its unique canonical space. Therefore, to combine P1 and P2 the angle between the dominant eigenvectors of selection in both experiments should be calculated (this will be considered in a future publication work). For example, in sagebrush crickets (*Cyphoderris strepitans*) the influence of multivariate sexual selection of female preference on male song parameters were estimated on songs of males captured in the wild and synthesised songs broadcasted to females in choice trials. The sequential model building approach showed little difference in the standardised selection gradients for call parameters across the field and playback experiment. However, there was a large angle (the dominant eigenvectors were almost orthogonal in the canonical space) between the dominant eigenvectors suggesting that the multivariate combination of call parameters under the strongest sexual selection in the field study differs significantly from that of the playback study (Ower *et al* 2013). Consequently, to combine P1 and P2, the angle between the main eigenvectors in these experiments should be significantly not different than zero.

As mentioned above the results indicate that male fertilization success is higher when they have a shorter comb with more teeth for sperm defence or a longer comb with fewer teeth for sperm offence. Despite selection on sex combs, the contour maps of the two major axes of non-linear selection in both P1 and P2 experiments (Figure 3B, and Figure 4B) show that most males sit in the fitness valley between the two fitness peaks, comb length and tooth number. This suggests that the response of sex combs to selection is constrained. These constraints could be physical and/or genetic.

P1 and P2 impose the same form of selection on the sex comb as shown by the sequential model building approach. Therefore, if P1 and P2 experiments can be combined together they will cancel each other's effect. This is because, at fitness peaks, the defensive bout favours shorter combs with higher tooth number, while the offensive bout favours longer combs with lower tooth number. Regardless of whether P1 and P2 can be formally combined or not, it is

physically impossible for the sex comb to be both longer and shorter and to have fewer and more teeth at the same time. However, this what is imposed by both bouts of post-copulatory selection on sex comb. Consequently, in order for males to be good in both bouts of selection as defenders and offenders, they must bear combs with intermediate values for length and tooth number, and thus they would achieve intermediate vales for fertilisation success. As a result, this would explain why many of the males are gathered in the fitness valley between the two fitness peaks as shown by the contour maps of the two major axes of non-linear selection in both P1 and P2 experiments (Figure 3B, and Figure 4B).

Genetic constrains could also explain the limited response of male sex comb to selection. Specifically, if comb length and tooth number are positively genetically correlated but correlational selection favours a negative correlation between them (Table 1, B). This would make it difficult for both, comb length and tooth number to evolve in the direction favoured by selection. However, negative correlational selection would have a very different effect on the response to selection. If comb length and tooth number are negatively genetically correlated, not only would the sex combs evolve in the direction favoured by selection but they should evolve rapidly (Agrawal & Stinchcombe 2009).

Markow *et al* (1996) reported that in natural populations of *D. simulans,* copulating males would have reduced mating success, if they are bearing sex combs with extremely low tooth number. In the current research findings, those males would also achieve an overall low fertilisation success, because they are only good, if they are the second to mate with a female. As a result, *D. simulans* males bearing sex combs with extremely low tooth number may achieve low reproductive success as reported by Markow *et a*l (1996) and the current chapter results (see Table 2B). In addition, it has been found that male body size is under stabilizing sexual selection in *D. simulans* (Markow & Ricker 1992) which is comparable to findings here which indicate that WL (an indicator of body size) is under stabilizing selection through post-copulatory sexual selection. Furthermore, thorax length, which is the most commonly used measure of body size in *Drosophila*, did not show any consistent relationship with male mating status in *D. simulans* (Markow *et al* 1996). This suggest that,

D. simlans male body size is under stabilising sexual selection in both pre- and post-copulatory episodes as shown by Markow *et al* (1996) and the results in Table 4 (B) respectively.

The observed association between sex comb's traits and both defensive and offensive fertilization success may be the results of either sperm competition and/or cryptic female choice (Thornhill 1983; Simmons 2001; Birkhead & Pizzari 2002; Polak & Simmons 2009). Sperm competition could increase *D. simulans* male's offensive or defensive competitive ability if the attractive traits of the males sex combs which are associate with the highest paternity share in P1 and P2 are positively correlated with the amounts and quality of the ejaculate's components, such as sperm numbers, sperm viability, or concentration of accessory gland proteins Acps (Simmons 2001).

Cryptic female choice may also be the post-copulatory mechanism responsible for the high fertilization success associated with the attractive sex combs. Under this scenario, a correlation between courtship performance and sex comb attractiveness may induce preferential utilization of male sperm by the female (Polak & Simmons 2009). During pre-copulatory courtship, males press their foretarsi and sex combs against the sides of the female's abdomen (Cooperman *et al* 2007; Polak & Simmons 2009). This contact stimulates the female peripheral nervous system and may confer cues regarding the shape and size of the sex comb. Depending on the quality of these cues about the attractiveness of the sex combs, females may be induced to keep the inseminated sperm and transfer those sperm from the spermatheca into the sperm storage organs (Polak & Simmons 2009). Alternatively, males with attractive sex combs could encourage the female to preferentially utilize sperm by inducing the female to dump previously stored sperm. Sperm dumping is a known copulatory behaviour in *Drosophila* that is used by females to increase current male fertilization success (Snook & Hosken 2004). In addition, in some *Drosophila* species, females eject sperm out of the spermatheca after mating, probably to bias paternity toward particular males (Alonso-Pimentel *et al* 1994; Polak and Simmons 2009). This suggests that female *Drosophila* have some control over fertilization and have the ability to bias paternity in favour of a particular male depending on whether the male is the first or the second to mate with her.

In conclusion, there are constraints affecting *D. simulans* sex comb and impede its evolution as a response to post-copulatory selection. These constraints could be physical and/or genetic. However, if each bout of post-copulatory selection is considered individually, males with the highest paternity share in P1 and P2 are those with specific sex comb characters. Specifically, at the peaks of fitness, shorter combs with many teeth confer high fitness in P1 and longer combs with few teeth confer high fitness in P2. The precise mechanism underlying this correlation is unknown and future research should aim to identify which of the above mechanisms is responsible for this correlation. In addition, further research is required to fully understand why the majority of males are gathered in the fitness valley and the potential constraints that underlie this.

CHAPTER 2

Quantitative Genetics of Drosophila simulans Sex Comb

2.1 Introduction

Evolution can be thought of as across generational changes in allele frequencies within a population (Falconer & Mackay 1996). For traits to evolve two factors should be present, selection and genetic variation. Regardless of the genetic variation underlying the trait, selection can acts on phenotypes and produce immediate phenotypic response (due to environmental source of variance) that can be measured. For example, the temperature in the nest influences the growth of the tails of young mice. There are differences in nest temperature between families, since mothers differ in the assiduity with which they nurse their young. Although, the nest temperature is not related to the mother's tail length, but this maternal effect produces an environmental component in the covariance of sibs in respect of tail length (Falconer & Mackay 1996). This example suggests that mice tails length is highly sensitive to environment particularly temperature and any selection on them could produce a response that does not depend on genetic basis. However evolutionary response to selection, which is the genetic change that occurs during a generation or from one generation to the next, does depend on genetic variation (Lande & Arnold 1983).That genetic variation is necessary for selection to cause evolutionary change can most easily be seen by considering the univariate breeders equation (Lande 1979). This equation, $R = h^2 S$, describes how a single quantitative trait evolves as a response to a particular selection. R describes the response to selection (evolution), h^2 the trait heritability (the proportion of the total phenotypic variance attributable to the additive genetic variance,V_A/V_P), and S the selection differential (the mean phenotypic value of the selected individuals expressed as a deviation from the population mean before selection) (Hansen & Houle 2008). It is clear from the equation that for any trait to evolve, genetic variation is needed (Hansen *et al* 2011).

The amount of additive genetic variation underlying traits has been used as a common estimate of traits evolutionary potential. Where the capability of a trait

to evolve is measured either by the variance scaled (h^2) or the mean-scaled (CV_A) additive genetic variance (Hansen *et al* 2011). Different trait types also vary in their heritabilities (Mousseau & Roff 1987; Pitchers *et al* 2014). For example, morphological traits have the highest heritabilities among all traits. Sexual traits also exhibit higher additive genetic variance compared with non-sexually selected traits (Pitchers *et al* 2014). In contrast, life-history traits have the lowest heritabilities, however, they tend to have high additive genetic variance but this is associated with even greater phenotypic variance (Houle 1992; Pitchers *et al* 2014). Physiological and behavioural traits have intermediate heritabilities (Mousseau & Roff 1987; Pitchers *et al* 2014). So different trait classes have different heritabilities but nonetheless for any trait to evolve there should be an additive genetic variance underlying it.

The near ubiquity of additive genetic variation in the vast majority of traits has been established through single-trait heritability and artificial selection experiments (Barton & Partridge 2000; Brakefield 2003). Furthermore, the univariate breeders equation is a gross simplification of true genetic architecture as traits are not isolated from each other but instead share genetic correlations with many other characters (Brooks & Endler 2001). For instance, it has been found that the numbers of traits which are pleiotropically independent are more probably to be in the order of 10s only (Walsh & Blows 2009).

In evolutionary quantitative genetics, the genetic covariances between traits capture the effect of pleiotropy, linkage disequilibrium, and the extent to which the evolution of one trait will be influenced by selection on another (Agrawal & Stinchcombe 2009). Genetic correlation between traits is one of the reasons for the observation that there are often limited responses to selection in natural and laboratory populations. The second reason is that, selection rarely acts on a single trait in isolation, instead selection usually affects a combination of traits simultaneously (Brooks & Endler 2001). Etterson and Show (2001) for example, used three heritable functionally related traits associated with drought stress and showed that the predicted univariate responses to selection were greater than the multivariate response to selection when the genetic covariance structure was included. Similarly in guppies (*Poecilia reticulata*), the effect of multivariate sexual selection was estimated for male colour pattern, which comprise of several genetically correlated traits, and it was suggested that

indirect selection on genetically correlated traits may impede the response of ornamental characters to directional selection (Brooks & Endler 2001). Thus traits are usually genetically correlated with each other, and sometimes, what looks like a single trait can be a composite of genetically correlated traits. Therefore, to fully understand how traits evolve, the multivariate selection on them and the additive genetic variation and covariation between them (G matrix) should be estimated (Lande 1979; Lande & Arnold 1983; Falconer & Mackay 1996).

Estimating the G matrix for a combination of traits show also if there is any negative genetic correlations (antagonistic pleiotropy) between traits, which could form one of the constraints on traits evolution due to indirect selection (Brooks & Endler 2001; Moor *et al* 2004; Agrawal & Stinchcombe 2009). For instance, Brooks and Endler (2001) estimated the both, G and the effect of multivariate sexual selection for male guppies colour pattern of *Poecilia reticulata*, where they found that indirect sexual selection which resulted from negative correlation between traits, generally either weakened or opposed the evolution of ornamental characters. This kind of constraints could particularly happen if there is a positive correlational selection but negative genetic correlations between these same traits. However, positive genetic correlations between traits could also weakened or oppose the evolution of characters if they are subjected to negative correlational selection (see Agrawal & Stinchcombe 2009). Not only do genetic correlations between traits could impede there response (evolution) to selection, but fluctuating selection may also constrain traits evolution (Cade 1981). However, these mechanisms could also maintain the additive genetic variance in traits that usually exposed to directional selection (e.g. sexually selected traits) by cancelling or weakened the effect of directional selection on traits genetic variance (Brooks & Endler 2001; Prokuda & Roff 2014).

Here I estimate the heritabilities and the genetic correlations (i.e. the G matrix) for the *Drosophila simulans* sex comb components (comb length, comb depth, and tooth number). It has previously been reported that there is substantial additive genetic variance underpinning male sex comb's tooth number in *D. melanogaster* (MarKow *et al* 1996; Ahuja and Singh 2008; Ahuja *et al* 2011). Although, still nothing is known about the amount of additive genetic variance

underlying comb's length and depth, and how *Drosophila* sex comb's components genetically covary. In the last chapter constraints have been found affecting *D.simulans* sex comb response to post-copulatory selection, where most of the males were gathered in the valley between the two fitness peaks. The counteracting effect of both episodes of post-copulatory selection might not be the only reason behind the constrained response of the sex comb. The genetic architecture underpinning the sex comb component could also be responsible. Thus, estimating the G matrix and the genetic correlations between sex comb's components of *D. simulans* would explain to us why not most of the males do have the perfect sex comb which associated with the highest fertilisation success as shown in the last chapter.

2.2 Methods

2.2.1 Fly stocks

The stock of wild-type populations of *D. simulans* used here were derived from twenty isolines that were supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. These isolines originally came from individuals that were caught in Tincurry, Eastern Australia, in March 2004. In the laboratory the isolines were mixed and maintained in population cages (ca. 800-1000 flies/cage) at 25^{o}C under a 12/12 light: dark cycle with overlapping generations for at least 4 years prior to the start of this investigation. All flies were reared on *Drosophila* quick mix medium (supplied by Blades Biological, Edenbridge, Kent, U.K).

For G matrix estimation flies were initially collected as virgins from stock populations. Egg laying vials were placed in the cages of each population daily and left for 24 hours. These vials were incubated until peak eclosion (ca. 8-9 days after egg laying). Offspring that eclosed overnight were killed and virgins were collected ca. 7hrs later. Virgin males were aspirated into individual vials containing culture medium and virgin females were pooled in standard vials with ca. 80 other virgin males. These virgin females and males were used for mating trials when the females were 3 days old and males were 3 - 4 days old, to ensure full sexual receptivity (Manning 1967).

2.2.2 The half-sib design

Sires and dams were randomly chosen from the above virgin males and females, where 130 virgin sires each mated with 5 different virgin dams. Sires and dams were paired randomly (i.e. one male was placed with one female) and were continuously observed until they copulated. When pairs had finished mating, the male was removed from the vial and placed with a new dam. The mated females then transferred daily onto fresh food for 4 days to lay. After that, vials containing eggs were stored at 25^{o}C for 12 days under a 12/12h light: dark cycle until all offspring emerged. Offspring of each dam were collected and housed in labelled vials as less than 10 flies per vial. These vials then kept in the freezer at -20 ^{o}C for subsequent dissecting and measuring, where 3 males from each female offspring were randomly chosen to provide the data (Figure 5).

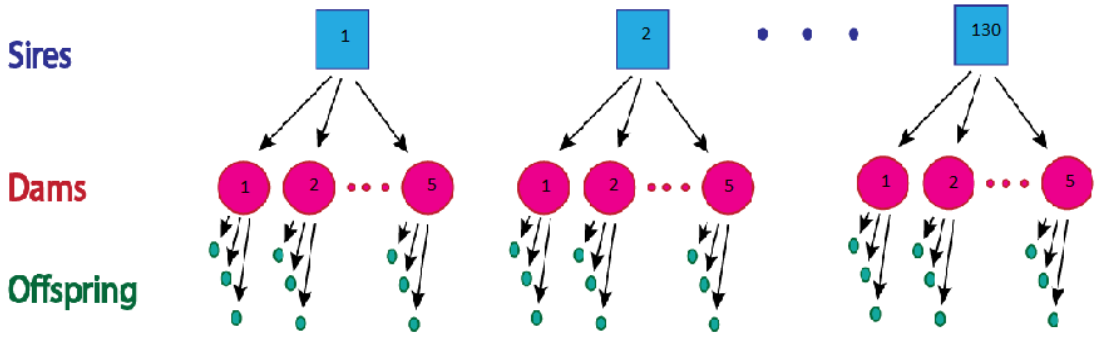


Figure 5: The basic experimental design, this generates the full sib/ half sib pedigree structure used in genetic assessments of *D. simulans* sex comb.

2.2.3 Dissecting and Measurement on Flies

20 sires out of 130 used in this experiment failed to produce offspring and thus they are excluded. 63 sires produced offspring from all the 5 dams they mated, and 24 sires produced offspring from 4 out of 5 dames they mated, while the remaining 18 sires produced offspring from only 3 out of 5 dams they mated. As mentioned above 3 males from each female offspring were randomly chosen to be dissected and measured to provide the data. Consequently, a total of 1395 randomly chosen sons were dissected, where the left and right fore-legs and wings were carefully pulled free from the body of each male, and then mounted

on glass slides using Hoyers Medium. Digital images for wings (X30) and sex combs (X100) were captured by Leica dissecting microscope (M125) connected to a Leica camera (DFC295). Later wing length and sex comb characteristics were measured manually using Image J v1.46r.

As in Sharma *et al* (2011) variation in wing length (WL) is obtained by estimating the distance between the intersection of the third longitudinal vein and the anterior cross vein to the distal tip of third longitudinal vein (Figure 1). The wing length is used as an index of body size (Sharma *et al* 2011), both left and right wings of each male were measured and an average value was calculated. The distance along the sex comb base was measured to indicate the comb length (CL), comb depth (CD) was also measured as the average length of the 1st, 3rd, and 5th pegs, and sex comb tooth (TN) number was counted as well (Figure 2). All sex comb characteristics, including CL, CD, and TN were estimated as the average of the measurements on the left and right body sides.

The precision of the measurements were assessed by blindly measuring all traits twice on a sub-sample (n = 20) of wings and sex combs. Regression of measure one on measure two showed that measures were highly correlated and repeatable (CD: β= 0.959, r^2= 0.919, P< 0.05; CL: β= 0.991, r^2= 0.982, P< 0.001; TN: β= 1.00, r^2= 1.00, P< 0.001; WL: β= 0.996, r^2= 0.992, P< 0.001).

2.3 Statistical analysis

The aim of this analysis is to estimate the additive genetic variance-covariance matrix (G-matrix), narrow-sense heritability (h^2), and the genetic correlation (r_G) of the *D. simulams* sex comb characteristics (comb length CL, comb depth CD, and tooth number TN) and wing length (WL). An animal model was fitted to the data in order to calculate the G-matrix from which the heritability of each trait and genetic correlations between each pair of traits were calculated (the animal model is explained by Wilson *et al* 2009).

Our genetic design was unbalanced, because as mentioned above some of the 130 males used in this experiment failed to mate with all the 5 dames, and several dams produced too few sons. To account for this unbalanced design,

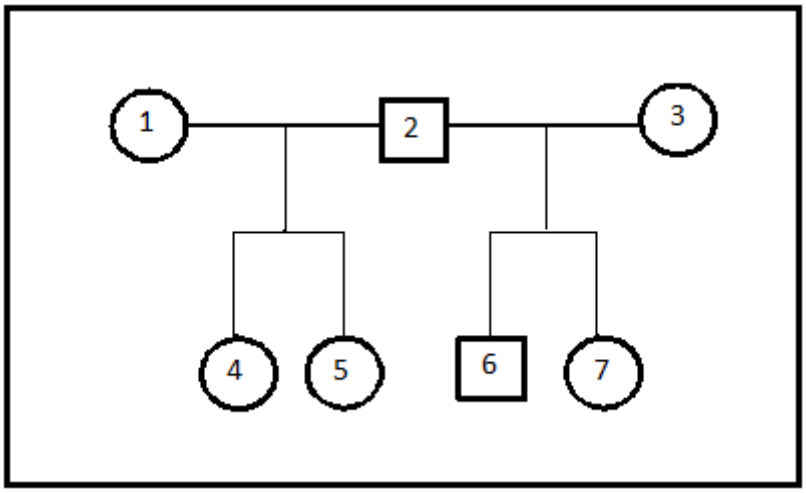
we fitted the current nested model using REML (animal model) implemented in ASReml (version 3.0; VSN International Ltd) which used to investigate the G-matrix for CL, CD, TN, and WL. The pedigree information was fed to it as shown in Table 3. The ASReml converts this pedigree information to an additive genetic relationship matrix known as the A matrix (e.g. see Figure 6, A and B). Since the A matrix is known (through the pedigree information) and the phenotypic covariance between individuals is known as well, the model can estimate the V_A as explained by the following equation,

$$COV_{ij}\ (Phenotypic) = 2\theta_{ij}\ (Relatedness)V_A.$$

Table 3: an example for the pedigree data structure as typically required by software packages.

ID	Sire	Dam	Offspring	CL	CD	TN	WL
1	1s	1d	1	54.683	41.184	8.5	1111.548
2	1s	1d	2	55.920	41.457	9.5	1117.941
3	1s	1d	3	56.782	38.286	9.5	1048.339
4	1s	2d	1	54.727	38.790	9.5	1171.983
5	1s	2d	2	61.910	40.493	10.5	1152.776
6	1s	2d	3	55.903	39.958	9.5	1122.229
7	1s	3d	1	58.957	39.504	10	1191.187
8	1s	3d	2	64.000	39.381	10.5	1184.957
9	1s	3d	3	62.847	36.459	11	1176.424
10	2s	1d	1	59.657	40.281	9.5	1198.681
11	2s	1d	2	55.425	40.854	10	1182.882
12	2s	1d	3	63.615	36.486	11	1184.891
13	2s	2d	1	55.444	38.560	9.5	1133.548
14	2s	2d	2	47.739	38.074	8	1123.654
15	2s	2d	3	58.454	39.251	9.5	1127.597
16	2s	3d	1	61.220	39.679	10	1154.040
17	2s	3d	2	48.845	38.117	8	1165.167
18	2s	3d	3	57.995	40.879	9.5	1179.350

(A)



(B)

$$A = \begin{bmatrix} 1 & 0 & 0 & 0.5 & 0.5 & 0 & 0 \\ 0 & 1 & 0 & 0.5 & 0.5 & 0.5 & 0.5 \\ 0 & 0 & 1 & 0 & 0 & 0.5 & 0.5 \\ 0.5 & 0.5 & 0 & 1 & 0.5 & 0.25 & 0.25 \\ 0.5 & 0.5 & 0 & 0.5 & 1 & 0.25 & 0.25 \\ 0 & 0.5 & 0.5 & 0.25 & 0.25 & 1 & 0.5 \\ 0 & 0.5 & 0.5 & 0.25 & 0.25 & 0.5 & 1 \end{bmatrix}$$

Figure 6: (A) An example pedigree with males as squares and females as circles, with illustrations of (B) a relatedness matrix, typically denoted A.

D. simulans females do nothing for their offspring except laying eggs and the maternal effect is minimal. Consequently, the simplest case of the model which contains only the fixed effect of trait mean and a random effect of breeding value ($y_i = \mu + a_i + e_i$) is suitable for the current experiment, this means that any covariance among observations must arise from sharing genes as determined by the pedigree structure. However, in the current experiment we have more than one trait, thus it is better to think about multivariate model and (co)variance matrices. So, for this four traits model, we would consider the phenotypic matrix P as comprising of phenotypic variances in CL (V_{P1}), CD (V_{P2}), TN (V_{P3}), and WL (V_{P4}) and phenotypic covariances between the four traits ($COV_{P1,P2}$), ($COV_{P1,P3}$), ($COV_{P1,P4}$), ($COV_{P2,P3}$), ($COV_{P2,P4}$), and ($COV_{P3,P4}$). P is then initially divided into the additive genetic matrix G and a residual matrix R, thus for four traits:

$$P = G + R$$

where,

$$P = \begin{bmatrix} V_{P1} & COV_{P2,P1} & COV_{P3,P1} & COV_{P4P1} \\ COV_{P1,P2} & V_{P2} & COV_{P3P2} & COV_{P4P2} \\ COV_{P1,P3} & COV_{P2,P3} & V_{P3} & COV_{P4P3} \\ COV_{P1,P4} & COV_{P2,P4} & COV_{P3,P4} & V_{P4} \end{bmatrix}$$

$$G = \begin{bmatrix} V_{A1} & COV_{A2,A1} & COV_{A3,A1} & COV_{A4,A1} \\ COV_{A1,A2} & V_{A2} & COV_{A3,A2} & COV_{A4,A2} \\ COV_{A1,A3} & COV_{A2,A3} & V_{A3} & COV_{A4,A3} \\ COV_{A1,A4} & COV_{A2,A4} & COV_{A3,A4} & V_{A4} \end{bmatrix}$$

$$R = \begin{bmatrix} V_{R1} & COV_{R2,R1} & COV_{R3,R1} & COV_{R4,R1} \\ COV_{R1,R2} & V_{R2} & COV_{R3,R2} & COV_{R4,R2} \\ COV_{R1,R3} & COV_{R2,R3} & V_{R3} & COV_{R4,R3} \\ COV_{R1,R4} & COV_{R2,R4} & COV_{R3,R4} & V_{R4} \end{bmatrix}.$$

The resulted additive genetic (co)variance matrix (G-matrix) can be used to calculate the heritability for each one of the four traits by dividing the V_A for each trait by the total phenotypic variance V_P of the trait. As it is known by definition that the heritability of a trait is the proportion of the total phenotypic variance explained by the additive effect of the genes underlying that trait, $h^2 = \frac{V_A}{V_P}$ and in common is expressed as narrow sense heritability h^2 (Wilson *et al* 2008). Also the genetic correlations r_G between traits could be calculated from the G-matrix through dividing the covariance between two traits by the square root of the cross product of both trait variances. For example, the r_G between CL and CD will be,

$$r_{GCLCD} = \frac{COV_{ACLCD}}{\sqrt{V_{ACL} \times V_{ACD}}}$$

The appropriate tools of statistical hypothesis testing are not universally agreed upon for mixed models (Wilson *et al* 2009). Thus, for statistical significance, an approximate test was used, where the estimated h^2 or r_G considered significant at $P = 0.05$, if it is more than twice its standard error. This is because 95% confidence interval is approximately estimates +/- 2 standard errors.

2.4 Results

Table 4 presents the estimated heritabilities h^2 and genetic correlations r_G for *D. simulans* male's sex comb characteristics and wing length as well as their associated standard errors. As mentioned above, the values in Table 4 are

considered significant at *P*=0.05, if each estimated h^2 or r_G is more than twice its standard error SE. Therefore all heritabilities in Table 4 are significant. CL has higher h^2 (0.6108 ± 0.0795) than CD (0.2581 ± 0.1068), while TN and WL have intermediate h^2 between CL and CD, where the V_A in each of TN and WL is almost responsible for about 50% of the total phenotypic variance ($h^2_{TN} = 0.5290 \pm 0.0627, h^2_{WL} = 0.4472 \pm 0.1301$). All traits are positively genetically correlated with each other (Table 4), and all of the correlations listed in Table 4 are significant except the r_G between CD and WL, which is not significant since it is less than twice of its associated SE ($r_{GCDWL} = 0.2716 \pm 0.2311$).

Table 5 shows the G matrix for the 4 traits, CL, CD, TN, and WL. This G matrix was also used to estimate the h^2s and the r_Gs listed in Table 4 by using the equations, $h^2_x = \frac{V_{Ax}}{V_{Px}}$ and $r_{Gxy} = \frac{COV_A xy}{\sqrt{V_{Ax} \times V_{Ay}}}$ respectively, (where x and y are two different traits). In Table 5 the diagonal values represent the $V_A$for the traits, underneath are the additive genetic covariances COV_A between traits, and genetic correlations are above the diagonal (which should be the same numbers listed in Table 4 for genetic correlations).

Table 4: Heritabilities and genetic correlations among CL, CD, TN, and WL.

	h^2	r_G			
		CL	CD	TN	WL
CL	0.6108 ± 0.0759*	1	0.3064 ± 0.1525*	0.8929 ± 0.0320*	0.6388 ± 0.0984*
CD	0.2581 ± 0.1068*	0.3064 ± 0.1525*	1	0.8929 ± 0.0320*	0.2716 ± 0.2311
TN	0.5290 ± 0.0627*	0.8929 ± 0.0320*	0.3589 ± 0.1441*	1	0.7039 ± 0.1074*
WL	0.4472 ± 0.0627*	0.6388 ± 0.0984*	0.2716 ± 0.2311	0.7039 ± 0.1074*	1

Values are considered significant at *P*=0.05, if each estimated h^2 or r_G is more than twice its standard error SE

Table 5: G matrix underlying sex comb components. Genetic covariances are listed on the lower part of the table. Genetic correlations are listed on the upper half of the table. Genetic variances are on the diagonal in bold.

	CL	CD	TN	WL
CL	**13.68**	0.3064	0.8929	0.6388
CD	1.037	**0.8363**	0.3589	0.2716
TN	2.059	0.2046	**0.3887**	12.56
WL	67.63	7.107	0.7039	**819.2**

2.5 Discussion

The major findings of this study are that *D. simulans* wing length and sex-comb length, width and tooth number are all significantly heritable. In addition, the estimated genetic correlations showed that these traits are positively genetically correlated except comb-depth and wing length, where the genetic correlation between them is not different than zero.

Sexually selected traits are genetically variable and tend to have higher heritability than non-sexually selected traits (Pomiankowski & Møller 1995; Bakker 1999). Our estimate of sex comb heritability (if we take the average of all sex comb components comb's length, depth, and tooth number: 0.47 ± 0.08) was not greatly different from previously published heritability for sexually selected traits (0.46 ± 0.03; Prokuda & Roff 2014). The wing length in this study is measured as a proxy for body size and the estimated heritability for wing length (0.45 ± 0.13) was also not greatly different from previously published heritability for *Drosophila* body size ($h^2 \sim 0.4$; Robertson 1957).

Trait heritability is a standard measure of the evolutionary potential of a trait (Falconer & Mackay 1996). This suggests that all our sex comb components have the potential to respond to selection and evolve as they are all significantly heritable. Consistent with this, sex combs of *D. melanogaster* males, where combs tooth number show rapid and robust phenotypic response to artificial selection (Ahuja & Singh 2008). Furthermore, It has been found in previous studies that there was dramatic variation in the morphology of sex combs between closely related species (Coyne 1985; Kopp & True 2002), this dramatic variation does not only indicate that sex combs have high heritability but also suggest that they exposed to directional selection (Kopp & True 2002). However, directional selection should deplete genetic variation (Walsh & Blows 2009), but as shown by the current chapter results (Table 4 and Table 5) there is still high additive genetic variance underlying sex comb.

Many mechanisms have been invoked to explain the high additive genetic variance in sexually selected traits (e.g. Promiankowski & Møller 1995; Row & Houle 1996; Jia *et al* 2000; Prokuda & Roff 2014). These include: 1) modifier loci, which act to increase the number of loci that directly affecting sexually

selected trait or increase the phenotypic variance by increasing the mean contribution of each locus to the total variance when sexually selected trait exaggerate under directional selection (Promiankowski & Møller 1995); 2) pleiotropic effects of linkage disequilibria among genes with major effects on fitness (Lande 1981). The positive genetic correlation underlying *D. simulans* sex comb traits is an example of pleiotropy; 3) newly arising mutation in polygenic traits (Lande 1976); 4) fluctuating selective pressure (Cade 1981). As a conclusion, from the above mechanisms and the results of the current research (see,r_G in Table 4), pleiotropy could be one of the mechanisms that explain the high additive genetic variance underlying *D. simulans* sex comb.

The genetic architecture underlying male sex comb was also examined by Ahuja *et al* (2011), where sex comb tooth numbers and length in *D. melanogaster* males, were found exhibiting heritable condition dependence. There is empirical evidence that costly male sex traits exhibit heightened condition dependence as compared to non-sex traits (Cotton *et al* 2004; Ahuja *et al* 2011). For example, in *D. melanogaster* male sex comb tooth number and its homologue, female transverse row bristles were confirmed to have heightened condition dependent expression as compared to non-sex sternopleural bristles (Ahuja *et al* 2011). Under the genic capture model which depends on two assumptions, condition dependence of sexually selected traits and high genetic variance in condition (since a larger proportion of the genome that is a complex summary of many processes affects condition). These two assumptions lead to the capture of genetic variance into sexually selected traits along with the evolution of condition dependence. Thus if male sex traits expression is condition dependent and if the additive genetic variance is highly harboured in condition, then it is expected for male sex traits to express and capture the high levels of variance in condition. (Rowe & Houle 1996). Condition dependent expression of costly sex trait increase their potential to avoid additive genetic variance depletion when they exposed to selection (particularly directional selection). Consequently, it is expected from sex combs to maintain high additive genetic variance since comb length and tooth numbers exhibiting heritable condition dependence as found by Ahja *et al* (2011) in *D. melanogaster*.

Sex comb traits are all positively genetically correlated and this is consistent with the findings of Ahuja *et al* (2011) where they presented a strong positive phenotypic correlation between both sex comb components of *D. melanogaster*, tooth number and comb length. These two traits showed the same response when they were exposed to poor and rich diet. This similar response may suggest a positive genetic correlation underlying them. The positive genetic correlation between sex comb traits found here (Table 4) could explain why not all *D. simulans* males have the optimal sex comb that is associated with the highest fertilisation success (Chapter 1). This is because there is a positive genetic correlation between tooth number and comb length, but negative correlational selection is favoured by selection (Chapter 1, Table 1B). Consequently, although there is additive genetic variance in both comb length and tooth number that allow them to respond to selection as shown by their heritabilities (Table 4), but the positive genetic correlation between them constrains there evolution in the direction favoured by post-copulatory selection. These genetic constrains protect the additive genetic variance against depletion (e.g. Brooks and Endler 2001). However, comb length and tooth number may evolve and respond to the post-copulatory selection imposed on them, if they are negatively genetically correlated.

Ideally, the breeder's equation, $\Delta\bar{z} = G\beta$, where evolutionary change ($\Delta\bar{z}$) is the product of the genetic variance-covariance matrix (G) and the vector of linear selection (β) (Lande & Arnold 1983) would be used to investigate whether the form of selection and genetic constrains limit trait evolution. For instance, Blows *et al* (2004) estimated both G and the fitness surface of *D. serrate* males CHCs. These hydrocarbons form the basis of the mate choice system in the species, and they are thought to be under strong directional selection (Blows *et al* 2004). It is found that G was not aligned with γ matrix that describes the curvature of the fitness surface. Which indicates lack of congruence between fitness landscape and G (Mcguigan 2006), and suggested substantial genetic constraints on the evolution of male sexually selected traits (Blows & Hoffmann 2005). Also, Homrigh *et al* (2007) used the multivariate quantitative genetics analysis on *D. bunnanda* sexually selected CHCs. Where they found that there was high genetic variance in all traits, but most of it was not in the direction of selection and therefore unavailable to sexual selection. As a result, for a

combination of traits to evolve there should be a substantial amount of genetic variation matching the direction of the selection β. Since even if there is genetic variation in all traits, but this variation is structured by covariances in a way such that there is no variation in certain directions of the multivariate space. And if β matches one of the direction with no genetic variation, then there will be no evolution (Agrawal & Stinchcombe 2009). A similar approach may be used to investigate the potential for the sex combs to evolve. This could be done by comparing the direction of the post-copulatory selection β acting on sex comb with the direction of the g_{max} (g_{max} is the main eigenvector in the G matrix where most of the genetic variation are present. g_{max} could be found by diagonalsing the G matrix). If β and g_{max} are in the same direction and there is no significant angle between them, then the reason that impede *D. simulans* sex comb evolution is not the genetic architecture underlying it (Pitchers *et al* 2014). The above approach is important, as it can show us whether a number of traits have the potential to evolve under certain selection scenario or not.

In conclusion, *D. simulans* male sex comb components, comb length, comb depth, and tooth number are all significantly heritable. Thus, they have the potential to evolve if they exposed to selection. In addition, they are all positively genetically covary, and this genetic correlation could be one of the mechanisms that explain the high additive genetic variance underpinning *D. simulans* sex comb component.

General Discussion and Conclusion

Drosophila sex comb is structurally variable and exhibits high intra- and interspecific variation in bristle number, position, shape, and colour (Kopp & True 2002; Ahuja & Singh 2008). Thus, it is a remarkable example of a secondary sexual trait that has undergone rapid evolution (Ahuja & Singh 2008; Sharma *et al* 2011). The sex comb is a character found on the forelegs of many *Drosophila* males (subgenus *Sophophora*) and it is important for grasping the female during copulation. While previous studies investigated the influence of the sex comb's tooth number on mating success (e.g. Markow *et al* 1996; Ahuja & Singh 2008) little is known about the effect of post-copulatory sexual selection on sex comb's length, depth, and tooth number and how they contribute to the male fertilisation success.

In this thesis *D. simulans* is used to test whether post-copulatory sexual selection affects sex combs and whether sex combs are heritable and what the nature of their genetic variation is. Therefore, the multivariate post-copulatory sexual selection on the comb and the underlying comb G matrix were estimated. The major finding of this research was that although there was no significant directional post-copulatory sexual selection acting on any of the *D. simulans* sex comb components, there was a significant non-linear post-copulatory sexual selection affecting them. Specifically, the two major axes of non-linear selection in both sperm defence and sperm offence experiments resulted in saddle-shaped selection surfaces. This indicated that one bout of selection (sperm defence) favoured males with shorter combs and higher tooth number while the other bout of selection favoured longer combs with lower tooth number (sperm offence). The quantitative genetic analysis showed that all sex comb components were positively genetically correlated and they were all significantly heritable.

The Lande equation (1979) $\Delta\bar{Z} = G\beta$, explains why both, the G matrix and the multivariate post-copulatory sexual selection for sex-comb components were estimated. This equation shows that in order to understand how sex comb as a

trait consisting of more than two traits evolve ($\Delta\bar{Z}$), the (co)variance matrix (G matrix) underlying the comb components as well as the linear selection (β) acting on them should be estimated. Previous studies suggested that *Drosophila* sex combs have been exposed to directional sexual selection, and that they have undergone rapid evolution (Kopp & True 2002; Ahuja & Singh 2008; Sharma *et al* 2011). However, our results showed that there was no linear (directional) post-copulatory sexual selection acting on any of the sex comb traits. The absence of directional post-copulatory sexual selection could be considered as one of the factors that explains the high additive genetic variance underpinning sex comb components. Especially, it is known that directional selection is one of the factors that could deplete genetic variation (Walsh & Blows 2009). This suggests that although sex comb is a secondary sex trait that evolves through sexual selection as an important organ for grasping the female during mating (Spieth 1952; Petrie 1988; Markow *et al* 1996; Polak *et al* 2004), it is not subjected to directional female preference as it used to be in the past. Consequently, this helps maintaining high levels of additive genetic variance (Cook 1977; Coyne 1985; Prokuda & Roff 2014).

Sexually selected traits are known as genetically variable traits and tend to have higher heritability than non-sexually selected traits (Pomiankowski & Møller 1995; Bakker 1999). Our estimate of sex comb heritability (0.47 ± 0.08) was not greatly different from previously published heritability (0.46 ± 0.03; Prokuda & Roff 2014), for sexually selected traits. This suggests that *D. simulans* sex comb is genetically variable. This variability is not only explained by the absence of post-copulatory directional selection, the genetic architecture underpinning the sex comb as well as the non-directional post-copulatory selection acting on it could also help maintaining high additive genetic variance. For example, the significant positive genetic correlation found between comb length and tooth number constrain their response to the negative correlational selection acting on them (Chapter 1, Table 1B). Therefore, this positive genetic correlation weakened or even cancelled the effect of the negative correlational selection acting on comb length and tooth number and helped maintaining the high additive genetic variance (Agrawal & Stinchocombe 2009).

Sperm defence bout of selection favoured males with shorter combs and higher tooth number, while the sperm offence bout of selection favoured males with

longer combs and lower tooth number. If it is possible to formally combine these two bouts of selection, they will cancel each other's effect and keep the additive genetic variance underlying the sex comb intact, especially because they affect the sex comb with the same amount of strength as was shown by the sequential model building approach in Chapter 1. Regardless of whether sperm defence and sperm offence bouts of selection can be formally combined or not, it is clear that males cannot both have fewer and more comb teeth and shorter and longer combs at the same time. This is physically impossible; however, this is what both bouts of post-selection have imposed on sex comb. This implies that it would be difficult for a male to achieve high fertilisation success as both defender and offender due to the above mentioned physical constraint. Therefore, it would be better for *D. simulans* males to achieve intermediate values for fertilisation success by bearing combs with intermediate values for length and tooth number in order to be good defenders and offenders at the same time. Consequently, what has been mentioned above does not only explain the high additive genetic variance underlying sex comb, but it also explains why many of the males are gathered in the valley between the two fitness peaks, comb length and tooth number (Chapter 1, Figures 3B and 4B).

All *D. simulans* sex com components are significantly heritable (Chapter 2, Table 4). This is an indication that *D. simulans* sex comb has the potential to evolve rapidly if it is exposed to strong selection. Similar findings were reported by Ahuja and Singh (2008) in *D. melanogaster*, where sex combs exhibited rapid and robust phenotypic response when they were exposed to artificial selection. Furthermore, previous studies found that *Drosophila* sex combs evolved rapidly and divergently (Kopp & True 2002; Ahuja & Singh 2008; Sharma *et al* 2011). However, the results of Chapter 1 showed that *D. simulans* sex comb did not evolve, where most of the males were gathered in the valley with intermediate values for sex comb characters. This unexpected evolutionary stasis could be again due to the factors mentioned above (absence of directional post-copulatory selection, the covariance structure underlying comb components and the physically constraints) that also help maintaining the high additive genetic variance in sex comb.

All of the above constraints could change since the form, strength, and direction of selection may vary over time (Rowe & Houle 1996) as well as the genetic

(co)variance matrix underpinning traits (Falconer & Mackay 1996). For example, when sexual traits which are under stabilising selection become costly in their production or maintenance they evolve condition dependence. As a result, only males in higher condition are better able to pay higher marginal costs of further exaggeration than those in lower condition. Males with higher condition would achieve higher mating success and thus stabilising selection can be changed to directional sexual selection (Rowe & houle 1996). This suggests that sometimes traits pass a period of evolutionary stasis but under certain circumstances this stasis may convert to rapid evolution (Kirkpatrick & Ryan 1991; Andersson 1994). There is ample of empirical evidences for the condition dependence of sexually selected traits (Andersson 1994; Johnstone 1995), including *Drosophila* sex comb (Ahuja *et al* 2011). For example, in *D. melanogaster* both, tooth number and length of the males sex combs, show positive condition dependence where both of them significantly increase under rich diet (Ahuja *et al* 2011).

In conclusion, although *Drosophila simulans* sex combs are expected to evolve rapidly as a response to sexual selection since they have high heritabilities (Kopp and True 2002; Ahuja and Singh 2008), constraints have been found to be affecting *D. simulans* males sex combs and impede their evolution as a response to post-copulatory sexual selection. These constraints arise mainly due to: 1) the absence of directional post-copulatory selection. 2) The positive genetic correlation between all sex comb components, particularly between comb length and tooth number, which counteracts the negative correlational selection acting on them and reduces or even cancel its effect. 3) Both bouts of post-copulatory selection asking the sex comb to be too short and too long and to have higher and lower tooth number at the same time. However, this is physically impossible. Therefore, it is better for males to bear sex combs with intermediate values for comb length and tooth number in order to be good as both defenders and offenders at the same time. As a result of the constraints mentioned above, many of the *D. simulans* males do not have the comb with the structures that maximise their fertilisation success. Furthermore, all of the above constraints could also explain the high additive genetic variance underlying *D. simulans* sex comb components.

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