Characterizing Sexual Selection in a Wild Population of *Protopiophila litigata* (Diptera: Piophilidae) and Analyzing the Combined Effects of Cuticular Hydrocarbons and Wing Interference Patterns on Male Mating Success in *Drosophila serrata*

Corey Godfrey

Master of Science in Biology

Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

University of Ottawa

In partial fulfillment of the requirement for the

M. Sc. degree in the

Ottawa-Carleton Institute of Biology

Abstract

One of the major research challenges is the ability to test selective forces in a wild population. A recent discovery of a new dipteran species, *Protopiophila litigata*, can enable researches to test selection in the wild. Most research has focused on mating behaviour, male mating success and senescence. In this study a small sample of wild mating and non-mating flies were collected, cuticular hydrocarbons were extracted and morphometric traits were obtained to assess the strength of sexual selection. There was significant linear sexual selection on cuticular hydrocarbons and, mid tibia length, hind tibia length and wing length. Overall, further establishes *P. litigata* as a model species for studying selection in the wild.

Earlier studies have demonstrated strong sexual selection on male cuticular hydrocarbons in *Drosophila serrata*. Recently wing interference patterns have been documented to be under sexual selection in *Drosophila melanogaster*. A sample of cuticular hydrocarbons and wing interference pattern values were analyzed to understand the combined effects on male mating success. Cuticular hydrocarbons were under sexual selection, however wing interference patterns were not. Overall, this study confirms selection on cuticular hydrocarbons, but highlights the difficulty in accurately capturing and measuring wing interference patterns.

Résumé

L'une des principales problématiques de la recherche en évolution est l'étude des forces de sélection en milieu naturel. La récente découverte d'une nouvelle espèce de Diptera, *Protopiophila litigata,* permet d'étudier la sélection dans le milieu naturel de cette espèce. La plupart des recherches dans ce domaine s'intéressent au comportement sexuel en lien avec le succès reproducteur des mâles et la sénescence. Dans mon étude, des petites populations de mouches sauvages (prélevées en état pré-copulatoire et post-copulatoire) ont été collectées. Les hydrocarbures cuticulaires ainsi que les traits morphométriques des individus ont été mesurés pour évaluer la force de la sélection sexuelle. Cette sélection sexuelle semble s'appliquer sur les hydrocarbures cuticulaires et la longueur du tibia des pattes du milieu et du tibia des pattes arrières, ainsi que la longueur des ailes. Cela confirme l'utilisation de *P.ligata* comme espèce modèle dans l'étude de la sélection en nature.

Des études récentes ont démontré l'importance de la sélection sexuelle des hydrocarbures cuticulaires chez les mâles D*rosophilia serrata.* En outre les patterns des inférences des ailes ont elles aussi été présentées comme étant un trait ciblé par la sélection sexuelle chez *Drosophila melanogaster*. Les hydrocarbures cuticulaires et les patterns des interférences des ailes ont été analysés pour examiner l'effet combiné des deux traits sur le succès reproducteur des mâles. Malheureusement les données obtenues n'ont pas montré que les patterns des inférences des ailes sont soumis à la sélection sexuelle dans notre population alors que les hydrocarbures cuticulaires le sont. Cette étude a donc confirmé la présence d'une sélection sexuelle

des hydrocarbures cuticulaires et révélé des difficultés de prise de mesures ainsi que de la pertinence de l'étude des patterns des inférences des ailes.

Table of Contents

Abstract ii

Résumé iii

Table of Contents v

List of Tables vii

List of Figures ix

Acknowledgements x

Chapter 1: Overview 1

Chapter 2: Characterizing Sexual Selection on Cuticular Hydrocarbons and Morphology in a Wild Population of Male *Protopiophila litigata* (Diptera: Piophilidae) 10

- Introduction 10
- Methods 17
- Results 23
- Discussion 24

Chapter 3: Analyzing Sexual Selection on Cuticular Hydrocarbons and Wing Interference Patterns in Male *Drosophila serrata* 35

- Introduction 35
- Methods 40
- Results 47
- Discussion 48

Appendix.. 58

List of Tables

Table 2. 1 Repeatability[a] estimates and loadings for the first principal component (PC1) for the 10 morphological traits in male *Protopiophila litigata*..... 30

Table 2. 2 Linear standardized selection gradients from the regression of relative mating success on the 17 standardized principal components of CHC variation in males. Gradients come from a standard least-squares model whereas P-values are from a logistic regression (see Methods). 31

Table 2. 3 Standardized linear selection gradients and their significance from the regression of relative mating success on thorax length thorax length-corrected variation in nine other morphological traits. All traits were standardized prior to analysis. The gradients come from a standard least-squares model whereas the P-values are from a logistic regression (see Methods). 32

Table 2. 4 Standardized linear selection gradients and their significance for the combined analysis of CHCs and morphology. Gradients come from a standard least-squares model whereas P-values are from a logistic regression (see Methods). 33

Table 3. 1. Repeatability estimates for whole-wing green and blue values for 20 male *D. serrata* wings..... 56

Table 3. 2. Standardized linear selection gradients for logcontrast cuticular hydrocarbons and total wing blue and green values in male *D. serrata*. 57

Table A. 1. Eigenvectors for the 17 principal components of CHC variation in male *P. litigata*. 58

Table A. 2. Eigenvalues and their total percent contribution for the 17 principal components of CHC variation in male *P. litigata.* 59

Table A. 3. Coefficient of variation for the 18 CHC peaks and the 10 morphometric traits investigated in *P. litigata.* 60

Table A. 4. Coefficient of variation for the nine CHC compounds and the two colour traits investigated in *D. serrata.* 61

List of Figures

Figure 2. 1. Cuticular hydrocarbon profile of a single male *P. litigata.* The 18 labeled peaks were integrated for subsequent analysis and were chosen because they were consistently observed in all 252 samples. 34

Figure 3. 1. Cuticular hydrocarbon profile of a single male *Drosophila serrata* with the nine labeled peaks used in sexual selection experiments. 53

Figure 3. 2. Wing Interference Patterns in *D. serrata.* Area and green and blue values were collected for the five different wing cells shown. 54

Figure 3. 3. The five different cell regions measured for blue and green colouring. Landmarks were chosen at locations where two veins intersected and at midway points between these vein intersections..... 55

Acknowledgements

First and foremost I would like to thank my supervisor, Howard Rundle. Howard has provided excellent guidance, encouragement and support. He was always able to provide me with constructive feedback.

Second, I would like to thank all current and past members of the Rundle lab, including Nicholas Rode, Brian Mautz, Kevin Kwok, Devin Arbuthnott, Mathieu Oudin, Julie Colpitts, Harmandeep Sekhon, Darla Williscroft and Kevin Xia. I would like to give particular thanks to Julie Colpitts for her invaluable help with running the GCMS machine and helping me organize my experiments; you truly kept myself and the Rundle lab together and moving forward. I would also like to give special thanks to Harmandeep Sekhon for his unwavering positive attitude and always making the lab a pleasure to be in. Lastly, I would like to thank Mathieu Odin for his help with my antler fly project. His knowledge of the species was crucial to troubleshooting many problems.

I would also like to thank my committee members Julie Morand-Ferron of the University of Ottawa and Alex Wong of Carleton University. They provided excellent feedback at my committee meetings and for bringing new insight to my research. My research as well as my conference was supported by a Natural Sciences and Engineering Research Council (NSERC) grant to Howard.

Chapter 1: Overview

An overview of sexual selection:

Sexual selection arises from variation among individual in their ability to acquire mates and sire offspring with them (Andersson 1994). Darwin (1859)first recognized the occurrence of sexual selection and described it as the "struggle between the individuals of one sex, generally the males, for possession of the other sex" (Darwin 1859). Darwin first suggested sexual selection to explain the evolution of traits that were clearly harmful to survival and other components of nonsexual fitness (Darwin 1859; Andersson 1994). Although sexual selection arises through the competition in acquiring a mate and producing offspring, rivals don't have to meet one another for this to occur. Two primary types of sexual selection are generally recognized: intrasexual selection and intersexual selection.

Intrasexual selection arises through competition in one sex, generally males, for access to the other, and was originally considered a precopulatory process. Precopulatory intrasexual selection can include males establishing and defending a territory to have access to females, male-male combat for access to females, and scramble competition (Andersson 1994). There are numerous examples of each of these. For instance, male lekking marine iguanas establish and defend territories in order to gain access to females (Partecke et al. 2002). Males establish and defend territories well before the mating season starts in order to secure the best location to attract and mate with females, and males that are better able to defend their territory have higher mating rates than unsuccessful males (Partecke et al. 2002).

Male kangaroos engage in combat with one another to establish a hierarchy in order to gain access to females. Fighting increases when females are oestrous and more receptive to mating (Warburton et al. 2013). The milkweed leaf beetle must compete with other males in order to find and mate with females. Males spend a majority of their time mate searching and will fight with mating males in order to gain access to females (Dickinson 1992).

More recently, intrasexual selection has been documented as also occurring post-copulation, leading to the development of traits like mate guarding, mating plugs, and larger ejaculates when other males are nearby (Gage 1991; Andersson 1994). There are also numerous examples of such traits. For instance, male whiptail lizards, *Aspidoscelis costata*, guard mated females to ensure they sire the most number of offspring in the clutch. Males will aggressively defend the female from other males and copulate with her multiple times to maximize their fertilization success (Ancona et al. 2010). In the scorpion, *Vaejovis punctatus*, males insert a mating plug to reduce sperm competition and reduce female receptivity to remating (Contreras-Garduño et al. 2006). Females are unable to remove the plug and remain unreceptive to mating until the plug degrades, thus allowing the first mated male to sire most of the offspring (Contreras-Garduño et al. 2006). Finally, male golden egg bugs, *Phyllomorpha laciniata*, copulate for a longer period of time when rival males are around in order to transfer more sperm to the female (García-González and Gomendio 2004). When males are subject to increasing sperm competition, the mean mating duration increases by as much as eight hours in order to maximize

sperm transfer and increase the male's chances of fertilizing the egg (García-González and Gomendio 2004).

Intersexual selection, in contrast, arises from non-random choice by individuals of one sex (or their gametes) among members of the other sex. Mate choice is any behaviour that limits the number of potential mates (Johansson and Jones 2007). Intersexual selection has been described to arise from female mate choice that can occur pre and/or post- copulation (Andersson 1994). Again, there are many examples of each. For instance, female house crickets, *Acheta domesticus,* demonstrate pre-copulation mate choice by preferring males with traits that make them more successful in fights such as body size, although attraction is not solely based on a male's ability to win a fight as females will sometimes chose the losing male (Savage et al. 2005). Female fowls, *Gallus gallus domesticus*, display postcopulatory mate choice by ejecting sperm of socially subordinate males (Dean et al. 2011). By selectively ejecting the sperm of certain males, females have a higher probability that a dominant male, which will increase the female's net fitness, will sire her offspring (Dean et al. 2011).

<u>Overview of thesis:</u>

While there are numerous studies addressing these different forms of sexual selection in a variety of species and for a range of traits, our understanding is nevertheless incomplete. In this thesis I address two understudied aspects of sexual selection via a study using wild-caught individuals of the antler fly, *Protopiophila*

litigata (Chapter 2) and a laboratory population of *Drosophila serrata* (Chapter 3). In chapter 2 I assess the effects male morphology and contact pheromones (cuticular hydrocarbons or CHCs) on mating success in antler flies. The majority of studies on CHCs as contact pheromones in insects have been lab based, so quantifying sexual selection in a field study is an important addition. In addition, relatively little is known about the traits important to mating success in antler flies so my research serves to further enhance our knowledge of the species. The unique biology of the antler fly allows males to be marked and tracked throughout their natural lifespan in the wild, making them an ideal system for studying various topics in evolutionary ecology. Identifying traits that are under sexual selection may aid future research on this species.

In Chapter 3 I assess the contribution of CHCs and structural wing colour patterns to male mating success in *D. serrata*. A large amount of work has been done on characterizing female mate preference for male CHCs in *D. serrata* (Chenoweth and Blows 2003; Chenoweth et al. 2005; Rundle et al. 2009; Gershman and Rundle 2016), but variation in CHCs generally only explains a small portion of the overall variation in male mating success in these studies. Little attention has been given to the potential contribution of other traits. A recent publication revealed that colour generated by interference patterns in transparent wings is under sexual selection in *Drosophila melanogaster* (Katayama et al. 2014), so in this chapter I test the combined effects CHCs and wing colour on male mating success in *D. serrata*.

In the sections below I provide a brief overview of sexual selection on chemical signals and colour, but to avoid repetition with the subsequent chapters I leave some of the specific details concerning antler flies and *D. serrata* for subsequent chapters.

Chemical signals:

Chemical signals can function at both long and short ranges (Costanzo and Monteiro 2007; Gershman and Rundle 2016) and can function as a means of species recognition, sex recognition, mating status recognition, and social dominance (Weddle et al. 2012). In insects in particular, chemical signals appear to be important in mate choice with pheromones having been identified in more than 3000 species (Johansson and Jones 2007). Moths and butterflies have been documented using long range pheromones in order to attract or locate a mate, and short range chemical signals in courtship behaviour (Costanzo and Monteiro 2007). Male butterflies have also been documented using anti-aphrodisiacs in order to reduce female attractiveness to other males post-mating (Gilbert 1976; Estrada et al. 2011).

Short-range chemical signals, specifically cuticular hydrocarbons in insects, have been documented as targets of sexual selection (Andersson 1994). Cuticular hydrocarbons are generally non-volatile, usually long-chain chemical compounds that form a waxy layer on the cuticle (Antony and Jallon 1982; Gibbs et al. 2003) that are thought to have evolved as a barrier to prevent water loss due to

evaporation (Thomas and Simmons 2008). However, in many species they have since been co-opted as a means of chemical communication during social interactions including mating, functioning as 'contact pheromones' (Montooth and Gibbs 2003; Hunt et al. 2012). Cuticular hydrocarbon expression is plastic within an individual and can change due to diet (Fedina et al. 2012), desiccation stress (Parkash et al. 2008), age (Kuo et al. 2012) and social environment (Gershman and Rundle 2016).

Cuticular hydrocarbons (CHCs) have been widely studied in *D. serrata*, a species of fruit fly native to Australia. Both sexes express the same suite of nine CHCs although their relative concentrations are sexually dimorphic (Chenoweth and Blows 2003). These traits have been shown to be important targets of mate choice in both sexes, although most attention has been given to female choice for male cuticular hydrocarbons (Hine et al. 2002; Skroblin and Blows 2006; Rundle et al. 2009). CHC expression is costly and females prefer a particular blend in males, generating persistent directional selection that is thought to be responsible for their condition-dependent expression (Delcourt et al. 2011).

In a review Kingsolver et al. (2012) addressed the strength and direction of selection in field studies. Their review paper looked into the approximately 140 field studies that have obtained measures of selection from natural populations (Kingsolver et al. 2012). Most field studies report that sexual selection (fecundity selection and mating success) is stronger in natural populations than survival

selection (Kingsolver et al. 2012). It appears that morphology is more commonly studied in wild populations (Kingsolver et al. 2012) than cuticular hydrocarbons. Only recently have CHCs started to be studied in wild populations of field crickets (Steiger et al. 2013). The results of this study showed directional selection on three principal components, disruptive selection on one principal component, and stabilizing selection on one principal component. Two of the standardized linear gradients exceededed the median absolute value of 0.18 in Kingsolver et al.'s (2001) review of the strength of selection in natural populations (Steiger et al. 2013).

Sexual Selection on colour:

Male colour patterns have been documented to be under sexual selection in a wide range of taxa such as birds, guppies, sticklebacks, butterflies and more recently hymenopterans and dipterans (Rowland et al. 1995; Senar et al. 2002; Shevtsova et al. 2011; Kemp et al. 2014; Auld et al. 2016). In birds, plumage and beak colour can both be signals of a male's condition and can be used in mate choice by females (Senar et al. 2002; Préault et al. 2005). Since both traits are dependent upon the quantity of carotenoids in a bird's diet, males with better foraging abilities will have brighter displays. These displays are hypothesized to help females choose high quality mates that provide better parental care (Senar et al. 2002; Préault et al. 2005).

Guppies are a well-documented case of male colouration being under sexual selection due to female choice. Although females have varying preferences for

males based on size, body shape and colour, most females prefer males with larger orange and black patches (Brooks and Endler 2001; Auld et al. 2016). Brighter male guppies are less likely to be parasitized and are better foragers, suggesting colour may act as a signal of overall male condition (Godin and McDonough 2003). Females in low predation environments prefer much more brightly coloured males than females in high predation environments since brightly coloured males are at a much greater risk of predation (Endler and Houde 1995; Godin and McDonough 2003; Auld et al. 2016), however when a guppy population is removed from a high predation environment females quickly express a preference for more orange males (Sandkam et al. 2015; Auld et al. 2016). In addition to guppies, female sticklebacks also show a preference for coloured males. Females sticklebacks are more willing to mate with males that have a red underside and have been documented showing a courting preference based on colour (Rowland et al. 1995).

Insect pigmentation is a variable trait under different selective pressures (Wittkopp and Beldade 2009). These visual signals can serve different functions such as predatory avoidance, either via camouflage or mimicry, and mating displays (Oliver et al. 2009b; Katayama et al. 2014; Thurman and Seymoure 2016). Butterflies, for example, have evolved various colour patterns in response to selection arising from mate choice and crypsis (Merrill et al. 2011; Kemp et al. 2014; Chouteau et al. 2016). In terms of sexual selection, females of various species prefer different colour patterns in their mates such as the blue markings on male *H. alimena* (Kemp et al. 2014). *Drosophila elegans* have a black melanin spot on their wings that they use in

courtship by waving their wings in front of a female in order to attract her (Yeh et al. 2006).

Recently, colour patterns have been described in the translucent wings of hymenopterans and dipterans (Shevtsova et al. 2011). This colour is generated by wing interference patterns and is claimed to be stable with respect to lighting conditions and to be species specific (Shevtsova et al. 2011). Variation in such wing colour was associated with variation in male mating success in a recent student in *D. melanogaster* (Katayama et al. 2014), suggesting they may be important in at least some *Drosophila* species.

Chapter 2: Characterizing Sexual Selection on Cuticular Hydrocarbons and Morphology in a Wild Population of Male *Protopiophila litigata* (Diptera: Piophilidae)

Introduction

Chemical signals are one of the major forms of communication between animals (Andersson 1994). While these often play a role in long distance mate location/attraction via highly volatile compounds (Costanzo and Monteiro 2007; Johansson and Jones 2007), the interest here is with contact pheromones found on the cuticles of many insects. Such compounds, often referred to as (epi)cuticular hydrocarbons (CHCs), are non-volatile long chained hydrocarbons and their derivatives. CHCs are thought to have evolved under natural selection to be the main barrier to prevent water loss due to evaporation, but have been secondarily co-opted as a means of chemical communication in social interactions (Montooth and Gibbs 2003; Hunt et al. 2012). Cuticular hydrocarbons have been documented to play a role in species recognition, sex recognition and mate choice(Chenoweth et al. 2005; Steiger et al. 2013; Gershman et al. 2014b). Given their role as a chemical signal they are likely a target of sexual selection and this has been documented in multiple insect species (McGuigan et al. 2008; Rundle et al. 2009; Hunt et al. 2012; Curtis et al. 2013; Steiger et al. 2013; Dyer et al. 2014).

The contribution of CHCs to mate choice and population/species discrimination has been particularly well studied in several *Drosophila* species. For example, the

mushroom-feeding *Drosophila subquinaria* has a partially overlapping range with its close relative, *Drosophila recens,* and a pattern of reproductive character displacement exists such that *D. subquinaria* females from sympatry are less likely to mate with a *D. recens* male than are those from allopatry (Jaenike et al. 2006). These sympatric *D. subquinaria* females also discriminate against their own allopatric males (Jaenike et al. 2006). Several lines of evidence including comparative studies of CHCs among populations, perfuming experiments, sensory modifications, and mating trials within and between species, all implicate CHCs as a key trait underlying these patterns of discrimination (Curtis et al. 2013; Giglio and Dyer 2013; Dyer et al. 2014; Rundle and Dyer 2015). CHCs have also been well documented as being under sexual selection in *Drosophila serrata* (see Chapter 3 for a detailed discussion). In brief, both males and females express a set of nine homologous pheromones although there is sexual dimorphism in their relative concentrations (Chenoweth and Blows 2003). CHCs appear costly to produce (Blows 2002) and their expression in males is condition-dependent (Delcourt et al. 2011). A series of experiments have shown that male CHCs are subject to persistent directional sexual selection due to female mate choice (Chenoweth and Blows 2003; Chenoweth et al. 2005; Rundle et al. 2008; Delcourt et al. 2011; Gershman et al. 2014b).

While numerous studies of sexual selection on insects have been conducted in both the laboratory and the field, estimates and strength of sexual selection on cuticular hydrocarbons stem almost exclusively from laboratory studies (but see Steiger et al.

2013). In order to provide insight into the effect cuticular hydrocarbons have on mating success in a wild insect, this study focuses on estimating sexual selection on CHCs using wild male *Protopiophila litigata* (the antler fly) collected from the Wildlife Research station in Algonquin Park. A sample of mating and non-mating males was collected and then phenotyped for cuticular hydrocarbons. In theory, both precopulatory mate choice and male-male (i.e. intrasexual) competition may contribute to the mating status of these males and previous research on the antler fly suggests that both of these occur in this species (i.e. male-male physical interactions are common; see below). This suggests that aspects of morphology are also likely to be important. Therefore, in addition to cuticular hydrocarbons I also measured a suite of morphological traits to provide insight into their contribution to mating success as well. A formal sexual selection analysis on this suite of morphological traits has not been previously performed in this species.

Due to the high site fidelity of males, *P. litigata* are an excellent species to address certain topics in evolutionary ecology in the field. To date, this work has focused on demonstrating and characterizing senescence in wild males. Given that males typically remain on a single antler for their entire adult life, they can be marked, introduced to an antler, and then observed to determine their lifetime mating success and longevity (Bonduriansky and Brassil 2002, 2005). Methods for maintaining ongoing laboratory populations and for manipulating larval diet have also been recently developed (Oudin et al. 2015), and such individuals can then be marked and introduced to the wild to observe the effects of such manipulations on

components of male fitness. The usefulness of *P. litigata* as a model system would be further enhanced by a more comprehensive understanding of sexual selection, including identifying the traits in males that are associated with increased reproductive success.

Given that most people are unfamiliar with the biology of the antler fly, in the following section I summarize what is known with a focus on their mating system given its relevance to sexual selection.

Biology of the Antler fly

The antler fly, *P. litigata*, has a life cycle that revolves around discarded cervid (moose or deer) antlers. Females oviposit into cracks or pores on the antler and the larvae develop by feeding in the porous interior bone matrix, after which they pupate in the dirt surrounding the antler. When ready to pupate, larvae come to the surface of the antler and use a "flicking" behaviour that catapults them off the antler into the leaf litter, covering a distance of up to 50 centimeters in a single leap (Bonduriansky 2002). This dispersal behaviour may be adaptive because it decreases dispersal time, thus reducing the risk of predation, or because it is metabolically cheaper than crawling slowly across the surface of the antler before falling off into the leaf litter (Bonduriansky 2002). Approximately 12 to 16 days later, adults emerge from the puparium (Bonduriansky 1995). Adult males most likely travel to the closest antler on which they appear to spend the rest of their life, competing with other males to acquire mates. One study identified that about half of

the marked males on a given antler remained on that antler despite the presence of another less than five meters away, and less than one percent moved to other antlers when these were located 100 meters away (Bonduriansky and Brassil 2005). In contrast, females spend most of their time away from the antler and appear to move among them, likely leaving to feed and possibly to avoid male harassment, and returning to mate and lay eggs (Bonduriansky and Brassil 2005).

P. litigata have been observed in Algonquin Park, Ontario, as well as in Nova Scotia (Bonduriansky 1995), but a detailed species distribution has not been determined. Male antler flies range from 1.6mm to 2.8mm and females range from 1.9mm to 3.1mm in body size (Bonduriansky 1995). Males are very aggressive and defend territories on the antler, frequently engaging in combat against other males and other species present on the antlers (Bonduriansky 1995). Male combat is often observed around oviposition sites favored by females (i.e. cracked, pores and damaged sites on the antler), with males attempting to establish and defend a territory presumably because of the enhanced access to females that come to lay eggs will increase a male's chance of acquiring a mate (Bonduriansky and Brooks 1999).

Males use an assault type of courtship (Bonduriansky and Brooks 1998b) in which they charge and attempt to mount females. A male will mount a female and remain on her back until oviposition is complete (Bonduriansky and Brooks 1998b), and guarding her from other males by push them away with his wings (Bonduriansky

1995). Such mate guarding may be a way for the male to reduce or prevent sperm competition or take-overs (Bonduriansky and Brooks 1998b). Males are often observed wrestling over females, thus preventing take-overs are important to male sexual fitness. In a small sample of 23 full mating pairs (from male-female encounter to separation) no take-overs were observed, however in an additional observation documenting mating behaviour approximately two-percent of mating attempts failed due to take-overs, which occurred exclusively when the male was attempting to mount the female (Bonduriansky and Brooks 1998b).

Mate preferences have been observed in both sexes in *P. litigata*. Male choosiness could arise from the direct fitness benefits gained by mating with more fecund females, or equivalently the cost of mating with a non-gravid or low fecundity female (Bonduriansky and Brooks 1998a). Females have been documented showing preference for males larger than themselves, possibly because larger males are better able to guard females (Bonduriansky and Brooks 1998a).

Larger males have been observed to have a higher lifetime mating success, presumably because they are better able to establish and defend a territory at an oviposition site (Bonduriansky 1995; Bonduriansky and Brooks 1999). Success at doing so has been linked to subsequent mating success. For example, males that were able to defend territories at oviposition sites were observed to have a mating frequency twice that of individuals that were unable to mate search in optimal areas (Bonduriansky and Brooks 1999).

Given the aggressive nature of males, it is highly likely that male body size in general, as well as the relative size of certain traits, are targets of sexual selection. For example, male limb length may be important because males appear to use their legs to assess how gravid a female is, they may affect his ability to remain on a female and prevent a take-over from another male, and they may contribute to a male's ability to win contests such a "boxing matches" in order to defend a territory on an antler. Wing length also has the potential to be under sexual selection since males use their wings to guard the female (i.e. fend off other males) while mating. Overall body size may contribute to the success of male-male interactions, both in defending a territory, preventing removal during mating, and in removing other males.

Methods

i) Sample collection

A sample of wild *P. litigata* was collected by my supervisor (Howard Rundle) from the Wildlife Research Station in Algonquin Park over several days between June 9 and July 7 of 2013. 126 mating males and 126 non-mating males were collected from two antlers located approximately 20m apart outside the Station's 'cookhouse' and 'bathhouse'. During each collection, all observed mating pairs on the antler or nearby ground/plants were aspirated into separate vials and an equal number of non-mating males were collected in the same way. Flies were then anaesthetized with carbon dioxide, mating pairs were separated, and CHCs were extracted from individual male flies by immersing them in 100 ul of hexane for approximately four minutes. After the four-minute period males were transferred to 99% ethanol to preserve them for subsequent dissection.

ii) CHC Measurements

All CHC samples were transported to the University of Ottawa where they were individually analyzed using a dual-channel Agilent 6890N fast gas chromatograph fitted with HP-5 phenylmethyl siloxane columns of 30 m length and 250 μm internal diameter (0.1 μm film thickness), pulsed splitless inlets (at 275 °C), and flame ionization detectors (at 310°C). The injection volume was 1 μL and the temperature began at 150°C and was held for 0.55 minutes, it then increased to 200°C at a rate of 120°C/minute, and then increased to 265°C at a rate of 7°C/minute, and finally increased to a temperature of 310°C by increasing at a rate of 120°C/minute. These

parameters were selected via an optimization procedure that sought to minimize the total run time while maintaining resolution (performed by my supervisor, Howard Rundle, using separate samples). The runtime of a single sample was just under 12 minutes.

From the resulting chromatograms, I integrated 18 peaks for each individual using the ChemStation software v. A.01.05 (AgilentTechnologies, Santa Clara, CA, USA). These 18 peaks represented all those that were consistently observed in all individuals, both mated and not. The chemical identity of these compounds has not yet been determined so I refer to them by their sequential number within the chromatographic profile of each fly (Figure 2. 1).

To correct for technical error associated with quantifying absolution concentrations via gas chromatograph, after integration the relative concentration of each CHC was calculated by dividing the area under each peak by the total area of all peaks for that individual. Proportions such as this are a form of compositional data to which standard statistical methods should not be applied (Aitchison 1986; Egozcue and Pawlowsky-Glahn 2011). To address this, the data were transformed to center-log-ratios (CLRs) using the following equation (Aitchison 1986):

$$CLR_n = \ln\left(\frac{p_n}{\left(\prod_{n=1}^{k} p_n\right)^{1/k}}\right),$$

where the numerator is the proportional area under peak n (k = 18 peaks) and the denominator is the geometric mean of the proportional area under all the peaks. Multicolinearity among the CLR-transformed traits was high (maximum variance inflation factor > 25) so these were converted to 17 principal components (the 18th principal component has an eigenvalue of zero due to the zero-sum constraint inherent in CLR-transformed data and was therefore discarded). Principal components analysis was performed across all males (mated and unmated) using the covariance matrix because CLR-transformation results in homogeneously-scaled trait values such that covariances among these traits play the role of correlations between non-scaled real variables (Aitchison 1986). Prior to this, three outliers were removed using the Mahalanobis distance-based technique in the multivariate platform of JMP v. 12.1.0 (SAS Institute Inc., Cary, NC), possibly representing contaminated samples or errors during integration.

iii) Morphometric Data

Prior to dissecting field-collected males, a repeatability analysis was conducted by measuring 20 male flies from a previously described laboratory stock population of this species (Oudin et al. 2015). Repeatability was estimated as the ANOVA-based intraclass correlation coefficient (Lessells and Boag 1987). Ten traits were measured as described in Oudin et al. (2015): head width, head height, thorax length, forelimb tibia and tarsus length, mid-limb tibia and tarsus length, hind-limb tibia and tarsus length, and wing length (from the inner r-m cross-vein to the distal end of the R_{4+5} vein).

Males used for the repeatability analysis were first preserved in ethanol prior to dissection to mimic the treatment of the field samples. Males were dissected with microcissors (Miltex/Integra LifeSciences, PA) under a Zeiss Discovery V.12 stereomicroscope with an ocular micrometer microscope and images were taken with the ZoomBrowser EX software using n A640 PowerShot Cannon camera. Images were measured using ImageJ v. 1.78 (National Institute of Health, Bathesda, Maryland). Dissected wings and limbs were mounted onto microscopes slides with double sided tape, while the head and thorax were placed in glycerol to prevent desiccation (Oudin et al. 2015). Males dissected for the repeatability analysis were photographed using the ZoomBrowser EX software twice. Each appendage, except for the wing, was photographed, removed from the slide using insect pins, and then repositioned before taking the second photo with both images being measured. The wing was photographed on two separate occasions and measured, but was not repositioned because it was impossible to remove the wing from the double sided tape without damaging it. The purpose of repositioning was to ensure that I captured as much of the measurement-induced (i.e. error) variability as possible. The repeatability values were above 0.8 for all traits (Table 2. 1).

Subsequent to the repeatability analysis, the same 10 morphological traits were measured on the field-collected males following the same protocol as above (but measuring each sample only once). As expected, these traits scaled allometrically with body size such that principal component 1 of the correlation matrix accounted

for 85.3% of the variance with all traits loading positively and with similar magnitude (Table 2. 1). Therefore, to allow sexual selection to be quantified separately on body size and shape, I used thorax length as an index of body size and calculated size-corrected values of the other nine traits by regressing each against thorax length and saving the residuals. Prior to this, eight outliers were removed using the Mahalanobis distance-based technique in the multivariate platform of JMP v. 12.1.0 (SAS Institute Inc., Cary, NC), possibly representing measurement or transcription errors during data collection. Multicolinearliy was not a concern in these data as the highest VIF value was 3.33.

iv) Statistical Analyses

Standardized linear (i.e. directional) sexual selection gradients on male CHCs were estimated via first-order multiple regression of relative mating success on the 17 standardized (mean = 0, standard deviation = 1) principal components of CLR-transformed trait variation, with the model fit via ordinary least squares (Lande and Arnold 1983). Collection location (fixed effect of cookhouse vs. bathhouse) was not significant and was therefore excluded from the model. The model was fit via ordinary least squares to estimate the selection gradients, but because mating success was binomial, significance tests of the individual gradients and the overall model employed logistic multiple regression fit via restricted maximum likelihood (Preziosi and Fairbairn 1996; Rundle et al. 2008). My analysis focused on linear selection alone because, given 17 traits, I lacked sufficient replication to estimate the

153 (i.e. 17 x (17+1) / 2) parameters of the gamma matrix of second-order quadratic and correlational selection gradients.

Linear standardized sexual selection gradients on male morphology were estimated in the same way by regressing relative mating success against body size (i.e. thorax length) and the body-size corrected variation in the other nine morphological traits. As above, these traits were all standardized (mean = 0, standard deviation = 1) prior to analysis and I again focused on linear sexual selection alone due to limited replication.

Sexual selection on CHCs and morphology were estimated separately to avoid issues of overfitting given the size of the data set (244 observations) in comparison to the complexity of a single model with 27 traits. Nevertheless, to provide some insight into partial effects of CHCs and morphology on mating success, I also fit the full model with all the traits and compared the results to that obtained from the separate analyses.

Results

Overall there was significant sexual selection on cuticular hydrocarbons (χ^2 = 52.723, df = 17, p < 0.0001) and the model explained 12.02 percent of the variation in mating success (R^2_{adjusted}) with coefficient of variation values for CHCs ranging between 0.1165 to 0.3663 and morphometric values ranging between 0.0871 and 0.1117 (Table A. 3). With respect to the individual traits, sexual selection gradients on principal components 3, 7, 9, 10 and 14 were significant (Table 2. 2), with selection on principal component 1 also approaching significance.

In a separate analysis, male morphometric traits were also under sexual selection overall (χ^2 = 23.805, df =10, p = 0.0081) and explained 5.5 percent of the variation in mating success (R^2_{adjusted}). With respect to the individual gradients, selection was significant on mid-tiba length (favouring smaller values), hind tibia length (favouring larger values), and wing length (favouring smaller values) (Table 2. 3).

When the two data sets were combined the results were fairly similar. The only differences between the previous analyses and the combined analysis are that principal component 3 and residual wing length were no longer significant, although the selection gradients on these traits (and all other significant traits) were still in the same direction (Table 2. 4).

Discussion

My results show that CHCs, mid tibia, hind tibia and wing length are under sexual selection and considered together, variation in CHCs and morphology explained a substantial portion of the variance in male mating success ($r^2_{adjusted}$ = 15.4%).

Individual gradients for several of these traits were also strong, exceeding the median strength of directional selection observed in a review of studies of wild populations (Kingsolver et al. 2001). In terms of CHCs, their importance to mating success (12.2% variance explained) is similar or higher than those seen in *Drosophila* in laboratory studies (Sztepanacz and Rundle 2012; Gosden et al. 2014; White and Rundle 2015).

The use of principal components was necessitated by strong correlations among CHCs, but it complicates the identification of the actual compounds targeted by sexual selection. From an examination of the gradients and loadings for the principal components (Table 2. 2 and Table A. 1.), it appears that high concentrations of peaks 5, 10, 11 and 15 and low concentrations of peaks 2, 3, 13, 16 and 18 were the most important to mating success. However, given the complexity of the gradients and loadings the exact peaks that are important to sexual selection would need to be confirmed via some sort of direct manipulation (see below).

Many studies in other insects have shown the importance of CHCs in mating success so it is not surprising that CHCs appear to be under sexual selection in *P. litigata*. A

majority of *D. serrata* studies have focused on the affects of female choice on male CHCs in the laboratory (Hine et al. 2002; Blows et al. 2004; Rundle et al. 2009), however one study in sagebrush crickets, *Cyphoderris strepitans*, looked at the affects of female choice on male mating success in the wild (Steiger et al. 2013). Previous antler fly studies have observed females rejecting males (Bonduriansky and Brooks 1998a; Bonduriansky and Brassil 2005), suggesting that male CHCs could be targets of female choice. However, much like *D. serrata* (White and Rundle 2015), male antler flies establish and defend territories (in their case on an antler) so CHCs could also play a role in male-male interactions involved in territorial defense and/or social dominance.

Interestingly, male body size does not appear to affect male mating success on its own. It is not entirely obvious what competitive advantage may be to males in having shorter mid-tibia and wings and a longer hind-tibia. Males flap their wings during mating in what is believed to be a signal to females to move to a different location on the antler (Bonduriansky and Brooks 1998b), although this behaviour may alternatively serve as a visual courtship signal so it is possible that females may visually prefer shorter wings. Another possibility is that males may use their wings as an acoustic signal like in *D. serrata* (Hoikkala and Crossley 2000). No attempt has been made to quantify vibrational wing song in the antler fly. Tibia length may play a role in male mounting ability (Bonduriansky and Brooks 1998b) and shorter mid-tibia and longer hind-tibia may give males a mounting advantage, better stabilizing them on top of the female while she moves to the underside of the antler and/or

helping males resist a takeover if the mating couple is attacked by another male. In terms of precopulatory male-male interactions, it is also possible that variation in mid- and hind-tibia could improve male fighting ability although it is unclear how this could be the case functionally.

A previous study in antler flies found that hind-tibia is a condition dependent trait with higher quality males (i.e. those raised on a better diet) having relatively larger values when compared to lower quality males (Oudin et al. 2015). This finding is in line with the current results demonstrating significant directional sexual selection for longer hind-tibias. This result is not surprising because traits that are targets of persistent directional selection are expected to evolve heightened condition dependence (Rowe and Houle 1996; Bonduriansky and Whitlock 2007). In contrast, neither mid-tibia nor wing length were sensitive to the diet manipulation. My results are partially consistent with patterns of sexual dimorphism described by Oudin et al. (2015). In particular, significant sexual dimorphism existed for all three of these traits and, consistent with the direction of sexual selection detected here, males had longer hind-tibia and shorter wings compared to females. However, sexual dimorphism in mid-tibia was such that males had relatively larger values than females whereas my results suggest sexual selection for shorter mid-tibia in males. This suggests that there are additional sources of sex-specific selection on mid-tibia (e.g., natural selection) and/or genetic correlations with other traits under selection that are responsible for the observed sexual dimorphism in relative mid-tibia length.

Both intra and intersexual selection may have contributed to the mating status of the males in this study and I am therefore unable to determine their relative importance in generating the sexual selection I observed. As the previous discussion highlights, both female choice and male-male competition are plausible and determining their relative importance would require further studies that isolated the opportunity for each. For example, with respect to intrasexual selection, assays could be performed in the laboratory to assess male territorial defense following White and Rundle (2015). In these assays pairs of males compete for control of a resource (e.g. food source and/or female oviposition site) and their success could be correlated with their phenotype. In nature, it might also be possible to collect males that do vs. do not hold territories on an antler and compare their phenotypes. With respect to intersexual selection, no-choice mating trials could be performed in the laboratory in which a single female is presented with a single male, thereby eliminating male-male interactions. During such trials it is possible to record mating success, time to mating, and mating duration, and variation in these can be correlated with male phenotype.

A selection analysis is an observational study that correlates mating success with certain traits and it is therefore possible that the true targets of selection were other unmeasured traits that were correlated with those that were measured. In order to better assess the exact traits under sexual in antler flies a manipulation would need to be done. With respect to CHCs, this could entail a classic perfuming experiment as

has been previously performed in *Drosophila* (Coyne et al. 1994; Blows and Allan 1998; Dyer et al. 2014). One of the main issues with perfuming experiments is that the preferred CHC blend is added overtop of a male's current CHC blend, which will combine the two profiles instead of replacing the male's own profile for the more attractive (or unattractive) profile. The perfumed male thus may or may not represent the attractive (or unattractive) blend that females chose and this could therefore skew the results. This is likely why perfuming studies have focused on species recognition instead of mate choice within a population (Blows and Allan 1998; Dyer et al. 2014) because between-species differences are likely much greater than among-male variation within a population. Another manipulation that can help identify the traits directly under sexual selection involves sensory ablation. The idea is that visual, olfactory or auditory receptors are removed or otherwise disable in individuals and the effect on mating success is quantified. For example, Giglio and Dyer (2013)preformed ablation experiments to better understand how *D. recens* and *D. subquinaria* males and females avoid mating with an individual from the other species. In their experiment files were either unmanipulated, blinded, or had their aristae, antennae or wings removed. Their study highlighted the importance of multiple modalities being important to mate acquisition with visual and chemosensory modalities being the most important to male mate acquisition (Giglio and Dyer 2013). It is more challenging to envision how morphological traits like leg and wing length might be manipulated, although assays that separate intra- from intersexual may shed light on how these trait may contribute to variation in mating success.

In conclusion, my results demonstrate sexual selection on CHCs and morphology among wild male antler flies, providing candidate traits for future studies. The power of this system lies in the ability to mark and track males throughout their life, including knowing their lifetime reproductive success. That populations can also be raised in the laboratory adds additional possibilities for future study. While postcopulatory components of sexual selection are more challenging to evaluate in this species, antler flies represent an excellent opportunity to study some important questions in sexual selection research including spatial and temporal variation in selection, the relative importance and potential interaction of intra- and intersexual selection, and the importance of multimodal male displays in explaining variation in mating success.

Table 2. 1 Repeatability[a] estimates and loadings for the first principal component (PC1) for the 10 morphological traits in male *Protopiophila litigata*.

Trait	Repeatability	PC1
Thorax	0.90	0.879
Head Height	0.92	0.944
Head Width	0.98	0.954
Fore Tibia	0.93	0.948
Fore Tarsus	0.83	0.921
Mid Tibia	0.93	0.936
Mid Tarsus	0.86	0.912
Hind Tibia	0.91	0.943
Hind Tarsus	0.84	0.900
Wing	0.99	0.895

[a]measured via the ANOVA-based intraclass correlation coefficient (n = 20 for each trait).

Table 2. 2 Linear standardized selection gradients from the regression of relative mating success on the 17 standardized principal components of CHC variation in males. Gradients come from a standard least-squares model whereas P-values are from a logistic regression (see Methods).

Trait	Selection Gradient	P-value
Prin1	-0.109	0.055
Prin2	-0.074	0.208
Prin3	**-0.139**	**0.0111***
Prin4	0.044	0.368
Prin5	0.006	0.972
Prin6	-0.066	0.179
Prin7	**0.226**	**<0.0001***
Prin8	0.098	0.085
Prin9	**-0.137**	**0.0072***
Prin10	**0.115**	**0.0312***
Prin11	-0.012	0.852
Prin12	-0.032	0.479
Prin13	-0.074	0.228
Prin14	**-0.152**	**0.0056***
Prin15	0.050	0.396
Prin16	-0.062	0.277
Prin17	0.081	0.173

Table 2. 3 Standardized linear selection gradients and their significance from the regression of relative mating success on thorax length thorax length-corrected variation in nine other morphological traits. All traits were standardized prior to analysis. The gradients come from a standard least-squares model whereas the P-values are from a logistic regression (see Methods).

Trait	Selection Gradient	P-value
Thorax	-0.079	0.202
Residuals Head Height	0.192	0.073
Residuals Head Width	-0.038	0.749
Residuals Fore Tarsus	-0.033	0.717
Residuals Fore Tibia	-0.079	0.426
Residuals Mid Tarus	0.065	0.505
Residuals Mid Tibia	**-0.269**	**0.009***
Residuals Hind Tarsus	0.078	0.358
Residuals Hind Tibia	**0.324**	**0.002***
Residuals Wing	**-0.193**	**0.032***

Table 2. 4 Standardized linear selection gradients and their significance for the combined analysis of CHCs and morphology. Gradients come from a standard least-squares model whereas P-values are from a logistic regression (see Methods).

Trait	Selection Gradient	P-value
Prin1	-0.087	0.242
Prin2	-0.087	0.101
Prin3	-0.081	0.136
Prin4	0.058	0.176
Prin5	0.021	0.897
Prin6	-0.074	0.159
Prin7	**0.247**	**<.0001***
Prin8	0.042	0.186
Prin9	**-0.144**	**0.0042***
Prin10	**0.103**	**0.0484***
Prin11	0.004	0.922
Prin12	-0.017	0.360
Prin13	-0.070	0.131
Prin14	**-0.175**	**0.0052***
Prin15	0.078	0.181
Prin16	-0.057	0.260
Prin17	0.084	0.127
Thorax	0.064	0.304
Residuals Head Height	0.080	0.312
Residuals Head Width	0.108	0.572
Residuals Fore Tarsus	-0.039	0.509
Residuals Fore Tibia	-0.096	0.556
Residuals Mid Tarus	0.098	0.379
Residuals Mid Tibia	**-0.218**	**0.0209***
Residuals Hind Tarsus	0.085	0.456
Residuals Hind Tibia	**0.242**	**0.0074***
Residuals Wing	-0.152	0.062

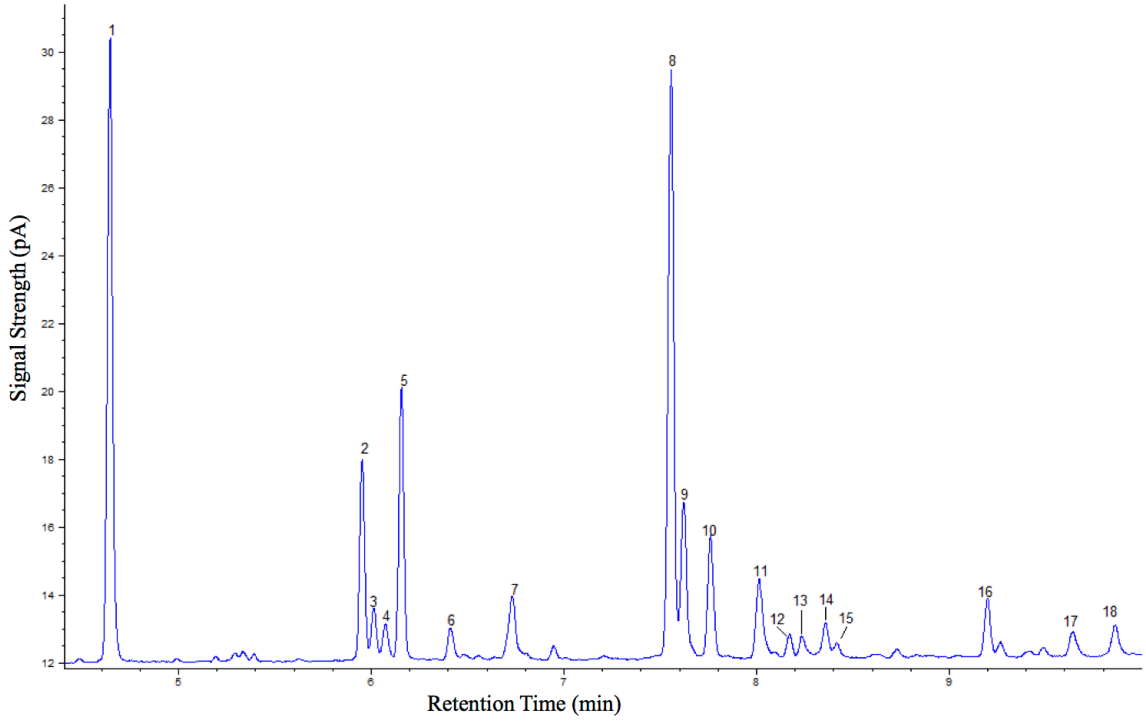

Figure 2. 1. Cuticular hydrocarbon profile of a single male *P. litigata.* The 18 labeled peaks were integrated for subsequent analysis and were chosen because they were consistently observed in all 252 samples.

Chapter 3: Analyzing Sexual Selection on Cuticular Hydrocarbons and Wing Interference Patterns in Male *Drosophila serrata*

Introduction

A lot of effort has been invested into identifying the various traits under sexual selection within species and quantifying the strength and form of selection on them. While increasing attention has been directed at quantifying the effects of multiple traits on reproductive success within a given species (Andersson 1994; Candolin 2003), significant gaps still exist in our understanding of the contribution of particular classes of traits in systems in which the role of others are well understood. In *Drosophila* in particular, the role of contact pheromones as targets of female choice has been extensively studied in several species (Higgie et al. 2007; McGuigan et al. 2008; Kwan et al. 2010; Sztepanacz and Rundle 2012; Curtis et al. 2013; Dyer et al. 2014; Gershman et al. 2014a). Wing vibrations (i.e. 'songs') have also been studied in other *Drosophila* species (Hoikkala and Crossley 2000; Hoikkala et al. 2000). There are other phenotypes, however, which have not been investigated. Recently, a new class of traits – stable colour patterns generated by thin film interference in transparent dipteran wings (Shevtsova et al. 2011) – have been identified. Little is known about their role, if any, in sexual selection. The goal of this chapter is to test the potential contribution to mating success of variation of structural colouration in the wings of male *Drosophila serrata,* a model species for the study of sexual selection studies on contact pheromones composed of cuticular hydrocarbons.

In insects, chemical signals can act both at long and short ranges in locating and/or attracting a mate (Costanzo and Monteiro 2007), and signals can change based on social environments (Weddle et al. 2012; Gershman and Rundle 2016). Cuticular hydrocarbons (CHCs) in particular are non-volatile compounds found on the cuticles of insects that improve desiccation resistance by reducing cuticular water loss (Antony and Jallon 1982; Gibbs et al. 2003; Montooth and Gibbs 2003). In many species, CHCs have been secondarily co-opted as a means of short range chemical communication and have been studied in *Drosophila* for their role in both mate choice (Chenoweth et al. 2005; Higgie et al. 2007; Rundle et al. 2009) and species recognition (Jaenike et al. 2006; Dyer et al. 2014).

D. serrata is commonly used in sexual selection studies focusing on CHCs. CHCs in this species have been shown to be sexually dimorphic (Chenoweth and Blows 2003) and male CHCs have been shown to be under persistent directional sexual selection as a result of female mate choice (Chenoweth et al. 2005; Rundle et al. 2008). Much attention has been given to the evolutionary consequences of this selection (Hine et al. 2002; Blows et al. 2004; Skroblin and Blows 2006; Rundle et al. 2009). Other studies have also looked at the importance of female CHCs to male mate choice (Chenoweth et al. 2005; Rundle et al. 2011). CHCs have been shown to be costly to produce for both males and females (Blows 2002), and have been shown to be condition dependent in their expression, at least in males (Delcourt et al. 2011). Further studies have looked into temporal variation in their expression

within and across days, as well as plasticity with respect to social environment (Gershman et al. 2014b; Gershman and Rundle 2016). Their role in male-male competition over territories has also been investigated, although in this case results suggested they were not important (White and Rundle 2015).

In addition to chemical signals, visual signals can play an important role in mate choice in insects. *Heliconius timareta* butterflies use wing colour as a cue for mate searching and mating (Mérot et al. 2015), *Bicyclus anynana* females select males based on the size and reflectance of male dorsal eyespots (Robertson and Monteiro 2005), and *Colias eurytheme* females prefer brighter UV reflecting males since UV reflectance is positively correlated with spermatophores size, which increases female egg production (Papke et al. 2007). In the damselfy, *Calopteryx haemorrhoidalis,* females select males based on their wing pigmentation as it is an indication of male condition (Córdoba-Aguilar et al. 2003). Within *Drosophila, D. elegans* males wave their wings in front of females in order to court them (Yeh et al. 2006), suggesting a visual signal of some sort.

Recently, structural colour patterns knows as wing interference patterns (WIPs) have been identified in the translucent wings of hymenopterans and dipterans (Shevtsova et al. 2011). WIPs are generated through the effect of the thin film interference where approximately 20 percent of the light is reflected and the colour patterns are visible when individuals are on darker substrates such that the signal is not over-powered by background reflectance (Shevtsova et al. 2011). WIPs arise

because wing membranes are composed of two chitin layers fused into one membrane and the different colours are a result of different membrane thicknesses (Shevtsova et al. 2011). Originally these colour patterns were largely ignored in behavioural and taxonomic studies because they were thought to be a form of iridescence, like the variable colours produced on a soap bubble, in which the colours are strongly influenced by the angle of incidence of the light and would therefore lack consist differences among individuals. In contrast, thin-film interference patterns are more stable to variation in lighting conditions (Shevtsova et al. 2011).

With this new discovery has come increased interest in the potential function of WIPs along with factors affecting their evolution. WIPs have been shown to be heritable in *Drosophila melanogaster* and may therefore evolve under natural and/or sexual selection (Katayama et al. 2014). WIPs are visible when contrasting on a non-white background (such as the green background of a leaf) and therefore may play an important role in mate choice in the wild (Shevtsova et al. 2011; Katayama et al. 2014). Thus far, only a single study has looked into the possibility of WIPs being a sexually selected trait. Using a set of isogenic lines of *D. melanogaster* (the Drosophila Genetic Reference Panel;Mackay et al. 2012), Katayama et al. (2014) provided evidence of sexual selection on male wing colours via female choice. In particular, after controlling for variation in several other traits (i.e. overall male attractiveness by calibrating attractiveness as a score of time-to-mating; with scores ranging from one – first male to mate – to zero – males that did not mate) two

different wing colour traits – hue and saturation – were correlated with male mating success (Katayama et al. 2014). Specifically, males with wings with more magenta colouration had higher mating success than did males with wings that were predominately yellow or blue, and males with more vivid colouration outperformed dull males (Katayama et al. 2014). Both hue and saturation are affected by wing thickness with thinner segments appearing more yellow and thicker segments appearing more blue (Katayama et al. 2014).

In previous studies using binomial choice mating trials, CHCs typically only explained a portion of the overall variation in male mating success in *D. serrata* (ranging from 5.1% – 28% (Delcourt et al. 2011; Sztepanacz and Rundle 2012; Gershman et al. 2014b; Gosden et al. 2014; White and Rundle 2015)), suggesting there are other as yet unidentified traits that matter. While male *D. serrata* use their wings to produce an acoustic signal, this may also serve as a visual cue to females. My goal is to test whether variation in male wing colour is associated with variation in male mating success and to do this I analyzed the effect of WIPs alongside CHCs given the known importance of pheromones in this species.

Methods

i) Stock population

In 2003 a stock population of *D. serrata* was created by mixing six laboratory populations collected from different sites along the Australian east coast. The stock was maintained at the University of Queensland under constant conditions (25° C; 12L: 12D cycle) at a large population size (16 half-pint stock bottles) on a standard yeast-agar media (see Rundle et al. 2005 for ingredients) with non-overlapping generations. In 2006, a large (>1000 individuals) copy of this stock was transferred to the University of Ottawa where it has been held since under the same conditions. The mating trials below were conducted in June of 2016 using flies from this population.

ii) Mating Assay

Flies were collected as virgins using light CO_2 anaesthesia within 24-hours of emergence and were separated into vials by sex and held at a density of five flies per vial. Holding vials contained 5 mL of food with live yeast sprinkled on top. Individuals were aged for five days before the mating trials. To conduct a trial, two males from the same holding vials were first introduced by aspiration (i.e. without anaesthesia) into a 35 mm diameter by 10 mm deep clear plastic petri dish with a disk of black paper placed upon a layer of prepared instant *Drosophila* media (Carolina Biological Supply Company, Burlington, NC) covering the entire bottom (to maintain humidity during the experiment). The lid of the petri dish had a hole in it to allow flies to be aspirated into it (after which it was plugged with a small foam

stopper). After the addition of the two males, a single female was then added. The black paper was placed in each petri dish to make WIPs visible to females. Given known time of day effects on mating (Gershman et al. 2014a), trials were performed within the first four hours of lights turning on in the morning, with approximately 90 replicate trials performed on each of the three consecutive days. Virgin collection was staggered across three days to ensure all flies were five days post-eclosion at the time they were tested (Sztepanacz and Rundle 2012).

During a trial the flies were continuously monitored until a mating was observed, at which point the flies were anesthetised with light CO_2 and then the chosen and rejected males were placed in separate paired vials for wing removal and the female was discarded. Usually within thirty minutes, but up to two hours near the end of the experiment due to a backlog of flies, the right wing was removed from each male using microcissors (Miltex/Integra LifeSciences, PA). Hydrocarbons were then extracted by submerging the fly in hexane for two minutes and then vortexting for an additional minute, after which it was removed and discarded. Wing removal was done before CHC extraction in order to preserve the wing for WIP analysis. Preliminary trials indicated no contamination of CHC samples due to wing removal. 271 mating trials were performed in total across the three days.

iii) CHC Measurements

CHC samples were analyzed using a dual-channel Agilent 6890N fast gas chromatograph fitted with HP-5 phenylmethyl siloxane columns of 30 m length and

250 μm internal diameter (0.1 μm film thickness), pulsed splitless inlets (at 275 °C), and flame ionization detectors (at 310°C). The injection volume was 1 μL and the temperature began by holding 140°C for 0.55 minutes, then increased to 190°C by increasing at a rate of 100°C per minute, then increased to 320°C at a rate of 45°C per minute and then holding for a minute (Sztepanacz and Rundle 2012). The runtime of a single sample was just under five minutes. Following numerous past studies in this species (e.g. (Delcourt et al. 2011; Sztepanacz and Rundle 2012; Gershman et al. 2014a), nine peaks (Figure 3. 1) were integrated using the ChemStation software v. A.01.05 (Agilent Technologies, Santa Clara, CA, USA). These peaks have been previously identified based on their retention times as: (Z,Z)-5,9-$C_{24:2}$, (Z,Z)-5,9-$C_{25:2}$, (Z)-9-$C_{25:1}$, (Z)-9-$C_{26:1}$, 2-Me-C_{26}, (Z,Z)-5-9-$C_{27:2}$, 2-Me-C_{28}, (Z,Z)-5,9-$C_{29:2}$, and 2-Me-C_{30} (Howard et al. 2003).

To correct for technical error associated with quantifying absolute concentrations via gas chromatography, after integration the relative concentration of each CHC was calculated by dividing the area under each peak by the total area of all peaks for that individual. Proportions such as this are a form of compositional data to which standard statistical methods should not be applied (Aitchison 1986; Egozcue and Pawlowsky-Glahn 2011). To address this, following previous studies (Rundle et al. 2008; Sztepanacz and Rundle 2012; Gershman et al. 2014a) the data were transformed into eight log contrasts using (Z,Z)-5,9-$C_{24:2}$ (peak 1) as the common divisor. Prior to the analysis, two outliers were removed using the Mahalanobis distance-based technique in the multivariate platform of JMP v. 12.1.0 (SAS Institute

Inc., Cary, NC), possibly representing contaminated samples or errors during integration.

iv) Wing Measurements

Although vision has not been investigated in *D. serrata*, the compound eye of *D. melanogaster* has been well studied. In this species, five types of rhodopsins are expressed in the eye with peak absorptions that range from 345–508 nm (i.e. from the UV to green; Zhu 2013). This differs from the spectrum of visible light in human (400–700 nm) in that *Drosophila* have increased sensitivity to UV, but are relatively insensitive to red. WIPs in *Drosophila* show distinct variation among the 'cells' of the wing that are defined by the wing veins and margins (Figure 3. 2). However, whether *Drosophila* have the visual acuity to discriminate these cells is unclear (Nathan Morehouse, personal communication). Therefore, for each wing I measured the amount of green and blue within each of the five major wing 'cells', as well as the area of each cell, allowing me to calculate a total wing colour score for an individual by summing values for each cell and weighting them by their area. UV was not measured because I lacked the necessary equipment to photograph under UV light.

Males were dissected under a Zeiss Discovery V.8 stereomicroscope with an ocular micrometer. Wings were then mounted on black 'murillo' 360 gsm card stock paper (Legion Paper, NY) by placing the wing on the paper with the dorsal side facing up, adding a cover slip, and then taping the cover slip down with black electrical tape. Images were taken under a Zeiss Discovery V.12 stereomicroscope with a Zeiss CL

1500 ECO cold light halogen light source (15V, 150W) on low lighting (level 2 of 5 levels) with the ZoomBrowser EX software using A640 PowerShot Cannon camera. While photographing, natural light was largely eliminated by blocking the nearby window. Samples were illuminated with a fiber optic ring lamp attached to a Zeiss CL 1500 ECO cold light source containing a type 6423FO halogen blub (15 V, 150 W). The overhead fluorescent lights were turned off during photographing. Images were measured using ImageJ v. 1.78 (National Institute of Health, Bathesda, Maryland). Images were saved as tif files and then converted to red, green, blue colour stacks in ImageJ. Total green and blue values were recorded for each cell; information regarding landmark placement used in defining wing cells is given in Figure 3. 3. Whole-wing green and blue values were calculated by multiplying the value for each cell by the percent area of that cell region and then summing.

Prior to measuring WIP values a repeatability analysis was conducted by imaging and measuring the right wing of 20 male flies twice. Repeatability was estimated as the ANOVA-based intraclass correlation coefficient (Lessells and Boag 1987). Repeatability was analyzed for total green and blue calculated by summing the values of the five 'cells' and weighting each by the area of its cell. Wings used in the repeatability analysis were photographed twice using the ZoomBrowser EX software. After the first picture the cover slip was removed and the wing was repositioned on the black cardboard background. The purpose of the repeatability was to capture the amount of variation due to changing lighting conditions,

orientation of the wing, and measurement error in defining the cells. The repeatability values were above 0.96 for each of the three traits (Table 3. 1).

Subsequent to the repeatability analysis, the same five wing cells were measured on the experimental males following the same protocol as above (but measuring each sample only once). Prior to the analysis, one outlier was removed using the Mahalanobis distance-based technique in the multivariate platform of JMP v. 12.1.0 (SAS Institute Inc., Cary, NC), possibly representing measurement or transcription errors during data collection.

iv) Statistical Analysis

Standardized linear (directional) selection gradients on males CHCs and wing colour were estimated by a first order multiple regression of relative mating success on the standardized (mean=0, standard deviation=1) eight logcontrast trait values and standardized total wing green and blue values. The model was fit by ordinary least squares (Lande and Arnold 1983). However, because mating was binomially distributed, significance testing of the individual gradients employed a logistic regression fit via restricted maximum likelihood (Preziosi and Fairbairn 1996; Rundle et al. 2008). The overall significance of selection on CHCs and wing colour were tested via a nested model comparison approach using likelihood ratio tests following Chenoweth et al. (2005) and (Chenoweth et al. 2012). The overall significance of sexual selection on male CHCs compared a full model that included the eight logcontrast CHCs to a reduced model that lacked them. Significance of

sexual selection on wing colour was tested similarly by comparing models that included vs. excluded these traits. This was done both when including and excluding the eight logcontrast CHCs in the two models, and was also done separately for whole-wing green and blue values and for the green and blue values for the five wing cells together in a single model. Finally, nonlinear sexual selection on whole-wing green and blue values was tested by comparing a full model that included both the first-order (i.e. linear) terms and the second-order terms (representing quadratic and correlational selection) with a reduced model that included only the linear terms. I did not test non-linear selection for the green and blue values for the five individual cells, nor for the eight logcontrast CHCs, due to limited replication. A past study also suggests that sexual selection on CHCs in males in this population is primarily directional (Sztepanacz and Rundle 2012).

Results

Overall there was significant selection on CHCs (likelihood ratio test: χ^2 = 16.053, df = 8, p = 0.042) and the model explained 1.69% of the variation in male mating success ($r^2_{adjusted}$) with coefficient of variation values for CHCs ranging between 0.0606 to 0.3020 and values for the wing colour ranging between 0.0745 and 0.0765 (Table A. 4). The selection gradients for individual logcontrast CHCs are given in Table 3. 2 with trait, 2-Me-C_{30}, being significant. In contrast, there was no evidence for sexual selection on wing colour. In particular, the addition of whole-wing green and blue values did not significantly improve the fit of the model that included the eight logcontrast CHCs (likelihood ratio test: χ^2 = 1.132, df = 2, p = 0.568), and whole-wing green and blue values were also not significant on their own (i.e. in a model that excluded the CHCs; Likelihood ratio test: χ^2 = 1.131, df = 2, p = 0.568). Furthermore, there was no evidence of sexual selection on the green or blue values for the five separate cells of the wing (likelihood ratio test: χ^2 = 5.65, df = 10, p = 0.844). Finally, there is no evidence of non-linear selection overall on whole wing green and blue values (likelihood ratio test: χ^2 = 2.004, df = 3, p= 0.572).

Discussion

Overall my results show that CHCs (likelihood ratio test: χ^2 = 16.053, df = 8, p = 0.042) are under sexual selection and also suggested that WIPs (Likelihood ratio test: χ^2 = 1.131, df = 2, p = 0.568) are not, with 1.69% ($r^2_{adjusted}$) of the variation in mating success being explained. My results for CHCs overall, and the magnitude and direction for the selection gradient on logcontrast 2-Me-C_{30}, are similar to results obtained in other recent experiments estimating sexual selection in males in this species (Delcourt et al. 2011; Sztepanacz and Rundle 2012; Gershman and Rundle 2016). Sample sizes in my experiment were smaller than those in previous studies and this may have reduced power, explaining why only one individual CHC was significant in my study whereas significant gradients were detected on five CHCs (i.e. (Z,Z)-5,9-$C_{25:2}$, (Z)-9-$C_{25:1}$, 2-Me-C_{26}, (Z,Z), 2-Me-C_{28}, (Z,Z)-5,9-$C_{29:2}$, and 2-Me-C_{30}) in these other studies.

My results contrast with those of Katayama et al. (2014) who found sexual selection on WIPs in *D. melanogaster*. It is important to note that male *D. melanogaster* display and vibrate their wings before mounting a female (Kyriacou and Hall 1984; Gleason 2005), while male *D. serrata* vibrate their wings after mounting a female (Hoikkala and Crossley 2000). This difference in courtship behaviour might affect the opportunity for sexual selection to act on WIPs in these two species, potentially making male wing colour patterns less visible to female *D. serrata*. In addition, the laboratory population I used has been maintained in an environment with a light background (ie. light beige *Drosophila* media) for approximately 13 years, while

WIPs are best observed on darker backgrounds (Shevtsova et al. 2011). My results could therefore reflect the fact that, after many generations of adaptation to an environment in which this signal is not visible, *D. serrata* females may have lost any preference for male WIPs. However, the DGRP *D. melanogaster* population from the Bloomington Stock Centre was created between 2008 and 2010 used by Katayama et al. (2014) has been adapted to the laboratory environment for almost an equal period of time as our stock population, yet apparent preferences for WIPs remained. Furthermore, in my study I did my best to control for confounding variables by always taking the right wing from the fly to control for left-right wing differences, before wing pictures were taken a measure of repeatability was done to ensure variation in wing measurements are due to biological differences instead of researcher error, and wing pictures were all taken in blocks (blocked the same way as the mating trials) to control for any light variation induced by environmental conditions (ie. sunny day versus cloudy day). Based on the methods in Katayama et al. (2014)it is unclear if they controlled for these variables.

Another difference between my experiment and the experiment done by Katayama et al. (2014) is that I analyzed green and blue colour values, while they analyzed hue, saturation and brightness (HSB). While HSB values are based on the red-green-blue (RGB) colour space, after discussions with a collaborator of the Rundle lab, Nathan Morehouse, it was decided that I should keep images in RGB form rather than converting them to HSB. The reason is that both saturation and brightness include the red spectral range in their calculation, but *Drosophila* are unable to see

red. HSB values are therefore a set of metrics designed around the human perceptual space and this space does not represent that of *Drosophila*. By keeping images in the RGB colour space, I could drop the red channel and analyze only green and blue, which in addition to UV are both relevant to *Drosophila* vision. Unfortunately, I was unable to measure UV because I lacked the necessary equipment to measure UV colouration. Neither myself nor Katayama et al. (2014) tested for sexual selection on UV colouration so it's possible that this trait is under sexual selection. This should be tested in future studies.

One of the major drawbacks of my experiment was the speed in which dissections could be completed following successful mating during a trial. The issue with delayed CHC extraction is that male CHC concentrations change for a time period after mating, after which they return to premating levels (Gershman and Rundle 2016). It is possible that the time delay in my experiment allowed changes in male CHC values, altering the estimates of the resulting sexual gradients. Before conducting the experiment we assessed if wings could be removed after CHC extraction, however our results showed very low repeatability values for green and blue (between 1% and 10%) and an increased probability in wings being damaged (sometimes 50% of wing samples were damaged). Based on those results were removed wings before CHC extraction, which greatly increased the time between mating and CHC extraction, as we were unable to remove wings at a pace equal to the mating rate. Further experiments would benefit from having additional support

to remove wings faster or by performing fewer trials each day by extending the experiment over more days.

My mating trials were designed to assay female mate preferences and they thus minimized male-male interactions by limiting the time males spent together before the female was added and there was no food resource for males to compete over. It is therefore possible that WIPs function as a signal during male-male competition and are thus a target of intrasexual selection instead of intersexual selection, but this was not captured by my assay. *D. serrata* males have a resource-defense mating system in which males will defend an food source/egg laying substrate, presumably as a way to gain access to females seeking out this resource. In a previous experiment, male mating success was shown to be associated with a male's success at defending such a territory (White and Rundle 2015). To assess a potential effect of WIPs on intrasexual selection, a territoriality assay like that of White and Rundle (2015) could be used and the outcome could be correlated with variation in male WIPs.

Currently WIPs are a trait that we do not fully understand and there is no clear consensus on how to best measure them. In my study I analyzed WIPs in five specific cell regions within the wing and computed the total wing WIP values based on the proportional totals from each cell region. Females may base their decision on full wing WIP values instead of on individual cell region values. Further studies would benefit form analyzing both the weighted sum of the individual cell regions

and the entire wing (including veins) to determine if there is a difference in significance between the two methods since females may assess males based on the entire wing oppose to the combination of the various cells.

Another area for future research concerning the potential importance of WIPs is to test for reproductive character displacement in them in *D. serrata*. *D. serrata* and the closely related *D. birchii* have partially overlapping ranges and premating isolation is stronger in areas of sympatry than allopatry. At least part of this is due to the reproductive character displacement of CHCs (i.e. CHCs of sympatric populations are more divergent than that of allopatric populations and this divergence has been shown to be the result of selection in *D. serrata* generated by the presence of *D. birchii* (Higgie and Blows 2008)). A similar pattern of reproductive character displacement of WIPs would suggest their involvement in mate choice/species recognition.

In conclusion, my results provide no evidence that WIPs in male *D. serrata* are a target of sexual selection from female mate preferences, although intrasexual selection and selection on UV patterns remains to be addressed. Insight into their potential role during mate choice and species recognition might be gained by characterizing differences between the sexes, among populations (e.g. sympatric vs allopatric), and among closely related species.

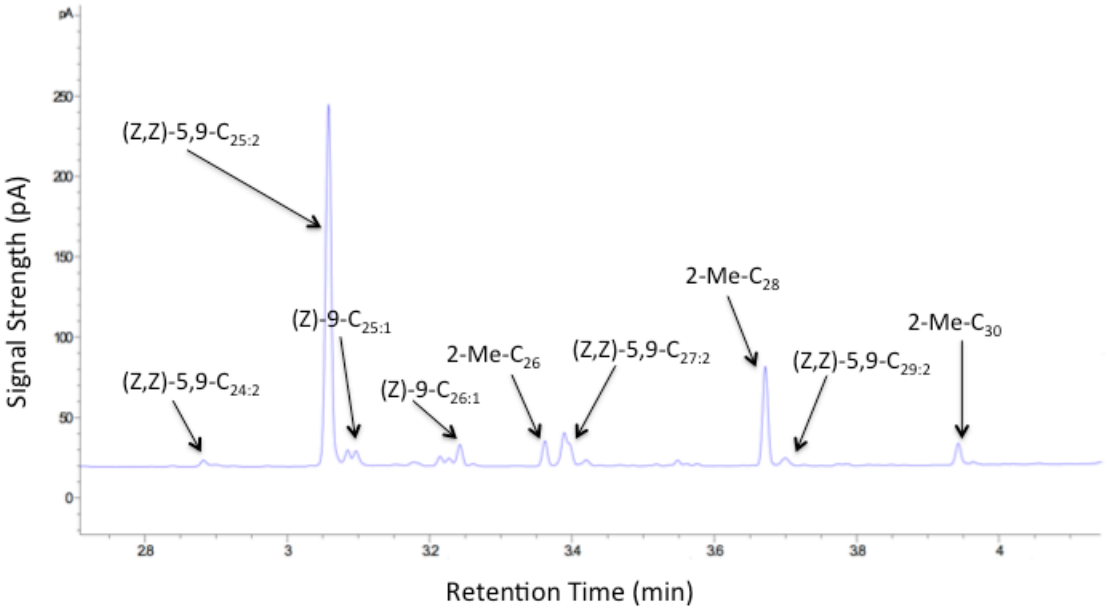

Figure 3. 1. Cuticular hydrocarbon profile of a single male *Drosophila serrata* with the nine labeled peaks used in sexual selection experiments.

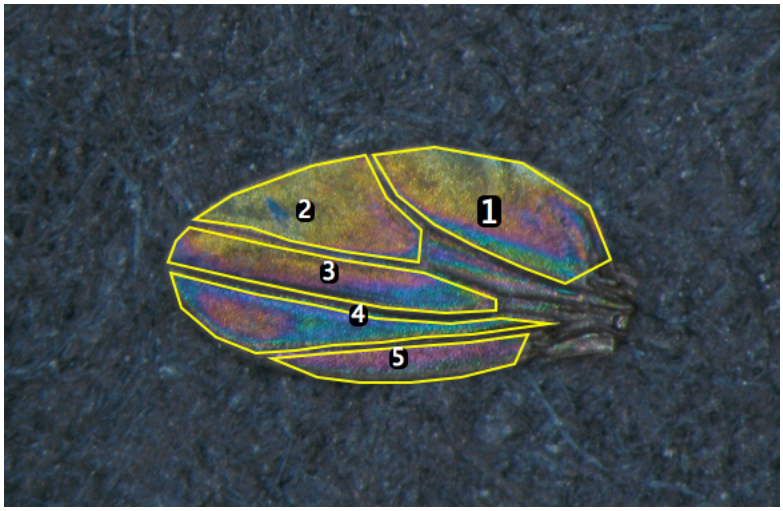

Figure 3. 2. Wing Interference Patterns in *D. serrata.* Area and green and blue values were collected for the five different wing cells shown.

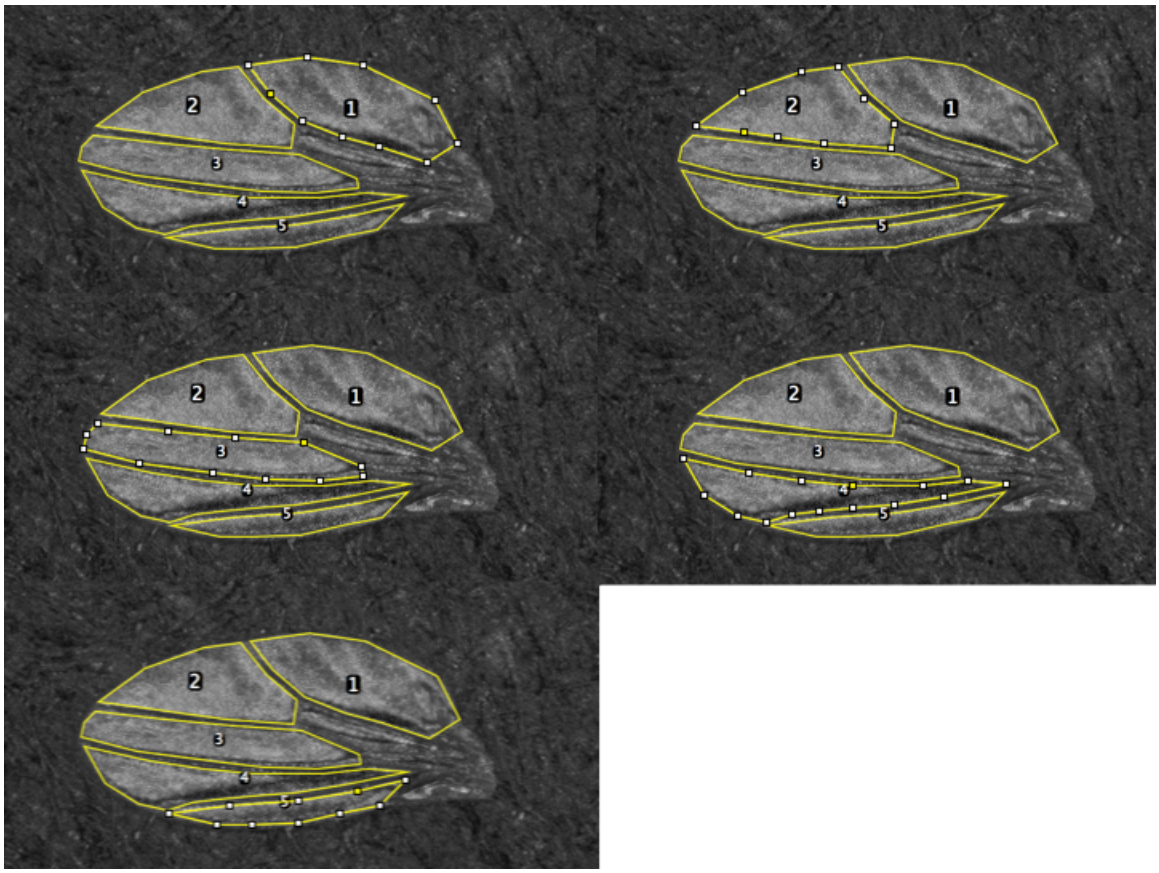

Figure 3. 3. The five different cell regions measured for blue and green colouring. Landmarks were chosen at locations where two veins intersected and at midway points between these vein intersections.

Table 3. 1. Repeatability estimates for whole-wing green and blue values for 20 male *D. serrata* wings.

Trait	Repeatability
Green	0.969
Blue	0.967

[a]measured via the ANOVA-based intraclass correlation coefficient.

Table 3. 2. Standardized linear selection gradients for logcontrast cuticular hydrocarbons and total wing blue and green values in male *D. serrata*.

Logcontrast trait	Selection gradient (β)	P-value
(Z,Z)-5,9-$C_{25:2}$	-0.012	0.8248
(Z)-9-$C_{25:1}$	0.006	0.9572
(Z)-9-$C_{26:1}$	0.099	0.4282
2-Me-C_{26}	0.092	0.4569
(Z,Z)-5,9-$C_{27:2}$	0.115	0.4409
2-Me-C_{28}	0.096	0.3486
(Z,Z)-5,9-$C_{29:2}$	0.027	0.7745
2-Me-C_{30}	**-0.210**	**0.0016**
whole-wing green	-0.043	0.3915
whole-wing blue	0.013	0.8006

Appendix

Table A. 1. Eigenvectors for the 17 principal components of CHC variation in male *P. litigata*.

	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8	Prin9	Prin10	Prin11	Prin12	Prin13	Prin14	Prin15	Prin16	Prin17
CLR1	-0.827	0.324	-0.049	-0.269	0.099	-0.173	-0.130	0.182	-0.142	-0.050	-0.136	-0.066	0.008	-0.004	-0.032	-0.009	-0.015
CLR2	0.656	0.299	0.609	-0.102	-0.065	-0.011	-0.160	0.085	-0.053	0.061	0.225	0.000	-0.012	0.015	-0.033	0.001	-0.057
CLR3	0.340	0.690	0.319	-0.222	-0.339	-0.029	0.043	0.044	-0.014	-0.295	0.007	0.173	0.038	0.072	0.042	0.080	0.077
CLR4	-0.554	0.371	-0.191	-0.304	0.425	-0.344	0.264	-0.126	0.152	0.067	0.111	0.023	-0.022	0.004	0.003	0.003	0.004
CLR5	0.120	0.455	-0.717	-0.206	-0.245	0.319	-0.018	0.053	-0.070	-0.012	0.155	-0.057	0.029	-0.036	0.075	-0.131	0.029
CLR6	-0.255	-0.089	0.452	-0.475	0.013	0.535	0.297	-0.017	-0.072	0.241	-0.161	0.166	-0.010	-0.039	-0.051	-0.023	0.004
CLR7	-0.365	0.470	0.071	0.665	0.375	0.231	0.007	-0.021	-0.042	-0.042	-0.006	0.002	-0.004	0.006	-0.006	0.002	0.001
CLR8	0.957	-0.064	0.167	-0.004	0.121	-0.042	-0.063	0.036	0.021	0.093	-0.025	-0.120	-0.032	-0.023	-0.016	0.010	0.061
CLR9	0.966	0.087	0.012	0.009	-0.008	-0.040	0.032	-0.046	0.150	-0.120	-0.107	0.008	-0.033	-0.048	-0.005	-0.034	-0.034
CLR10	0.515	0.034	-0.814	-0.028	-0.133	0.177	-0.001	-0.012	0.024	0.033	0.018	-0.023	0.028	0.032	-0.094	0.089	-0.023
CLR11	0.158	-0.308	0.167	-0.407	0.161	0.186	-0.110	-0.553	-0.323	0.030	-0.099	-0.205	0.120	-0.122	0.303	0.154	-0.077
CLR12	0.057	-0.450	-0.110	0.201	-0.342	-0.189	-0.044	-0.331	-0.185	0.113	-0.145	-0.026	-0.361	0.519	0.049	-0.075	-0.017
CLR13	-0.860	-0.043	0.214	0.085	-0.248	-0.053	-0.194	-0.248	0.138	0.021	-0.015	-0.029	0.133	0.004	-0.055	-0.029	0.015
CLR14	-0.758	-0.040	-0.216	0.240	-0.194	-0.094	-0.337	0.172	0.211	0.184	-0.027	0.139	-0.126	-0.090	0.076	0.034	0.000
CLR15	-0.129	-0.112	0.059	0.540	-0.540	-0.358	0.454	0.069	-0.177	0.059	0.007	-0.041	0.015	-0.087	-0.008	0.001	-0.006
CLR16	0.717	-0.539	-0.136	0.119	0.259	-0.155	-0.041	0.138	-0.035	0.044	-0.017	0.107	0.173	0.064	0.028	-0.024	-0.003
CLR17	-0.305	-0.860	-0.093	-0.057	0.172	-0.027	-0.108	-0.114	-0.173	-0.181	0.102	0.074	-0.102	-0.092	-0.055	-0.010	0.019
CLR18	-0.724	-0.539	0.182	-0.047	0.001	0.190	0.143	0.183	0.185	-0.100	0.041	-0.102	0.001	0.042	0.020	0.013	-0.007

Table A. 2. Eigenvalues and their total percent contribution for the 17 principal components of CHC variation in male *P. litigata.*

Number	Eigenvalue	Percent
1	0.4953	47.744
2	0.1511	14.56
3	0.1052	10.14
4	0.0772	7.442
5	0.0568	5.478
6	0.0405	3.907
7	0.0287	2.763
8	0.0195	1.88
9	0.0167	1.611
10	0.0126	1.212
11	0.0097	0.932
12	0.0072	0.693
13	0.0063	0.605
14	0.005	0.48
15	0.0027	0.262
16	0.0019	0.186
17	0.0011	0.106

Table A. 3. Coefficient of variation for the 18 CHC peaks (proportions) and the 10 morphometric traits investigated in *P. litigata.*

Trait	CV
P1	0.2883
P2	0.2151
P3	0.1655
P4	0.2775
P5	0.1797
P6	0.1985
P7	0.3663
P8	0.2526
P9	0.2847
P10	0.2521
P11	0.1165
P12	0.1356
P13	0.2956
P14	0.2118
P15	0.2310
P16	0.2571
P17	0.2618
P18	0.3167
Thorax	0.1117
Head Height	0.0908
Head Width	0.0901
Fore Tarsus	0.0909
Fore Tibia	0.099
Mid Tarsus	0.0871
Mid Tibia	0.0951
Hind Tarsus	0.0902
Hind Tibia	0.0953

Table A. 4. Coefficient of variation for the nine CHC compounds (proportions) and the two colour traits investigated in *D. serrata.*

Trait	CV
(Z,Z)-5,9-$C_{24:2}$	0.1186
(Z,Z)-5,9-$C_{25:2}$	0.0606
(Z)-9-$C_{25:1}$	0.1737
(Z)-9-$C_{26:1}$	0.1941
2-Me-C_{26}	0.3020
(Z,Z)-5,9-$C_{27:2}$	0.1903
2-Me-C_{28}	0.1499
(Z,Z)-5,9-$C_{29:2}$	0.2399
2-Me-C_{30}	0.2514
whole-wing green	0.0765
whole-wing blue	0.0745

Literature Cited

Aitchison, J. 1986. The Statistical Analysis of Composition Data. Chapman and Hall, London, United Kingdom.

Ancona, S., H. Drummond, and J. Zaldivar-Rae. 2010. Male whiptail lizards adjust energetically costly mate guarding to male–male competition and female reproductive value. Anim. Behav. 79:75–82.

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey.

Antony, C., and J.-M. Jallon. 1982. The chemical basis for sex recognition in *Drosophila melanogaster*. J. Insect Physiol. 28:873–880.

Auld, H. L., R. J. P. Pusiak, and J. J. Godin. 2016. Independent Mating Preferences for Male Body Size and Coloration in Female Trinidadian Guppies. Ethology 122:597–608.

Blows, M. W. 2002. Interaction between Natural and Sexual Selection during the Evolution of Mate Recognition. Proc. Biol. Sci. 269:1113–1118.

Blows, M. W., and R. A. Allan. 1998. Levels of Mate Recognition Within and Between Two *Drosophila* Species and Their Hybrids. Am. Nat. 152:826–837.

Blows, M. W., S. F. Chenoweth, E. Hine, and A. E. E. D. B. III. 2004. Orientation of the Genetic Variance ‐ Covariance Matrix and the Fitness Surface for Multiple Male Sexually Selected Traits. Am. Nat. 163:329–340.

Bonduriansky, R. 1995. A New Neartic Species of *Protopiophila Duda* (Diptera: Piophildae), with notes on its Behaviour and Comparison with *P. latipes* (Meigen). Entomol. Soc. Can. 127:859–863.

Bonduriansky, R. 2002. Leaping behaviour and responses to moisture and sound in larvae of piophilid carrion flies. Can. Entomol. 134:647–656.

Bonduriansky, R., and C. E. Brassil. 2005. Reproductive ageing and sexual selection on male body size in a wild population of antler flies (*Protopiophila litigata*). J. Evol. Biol. 18:1332–1340.

Bonduriansky, R., and C. E. Brassil. 2002. Senescence: Rapid and costly ageing in wild male flies. Nature 420:377.

Bonduriansky, R., and R. J. Brooks. 1998a. Copulation and Oviposition Behaviour of *Protopiophila litigata* (Dipera: Piophilidae). Can. Entomol. 130:399–405.

Bonduriansky, R., and R. J. Brooks. 1998b. Male antler flies (*Protopiophila litigata*; Diptera: Piophilidae) are more selective than females in mate choice - Canadian Journal of Zoology. Can. J. Zool. 76:1277–1285.

Bonduriansky, R., and R. J. Brooks. 1999. Why do male antler flies (*Protopiophila litigata*) fight? The role of male combat in the structure of mating aggregations on moose antlers. Ethol. Ecol. Evol. 11:287–301.

Bonduriansky, R., and A. E. and E. M. C. Whitlock. 2007. The Evolution of Condition‐Dependent Sexual Dimorphism. Am. Nat. 169:9–19.

Brooks, R., and J. A. Endler. 2001. Female Guppies Agree to Differ: Phenotypic and Genetic Variation in Mate-Choice Behavior and the Consequences for Sexual Selection. Evolution 55:1644–1655.

Candolin, U. 2003. The use of multiple cues in mate choice. Biol. Rev. 78:575–595.

Chenoweth, S. F., and M. W. Blows. 2003. Signal Trait Sexual Dimorphism and Mutual Sexual Selection in *Drosophila serrata*. Evolution 57:2326–2334.

Chenoweth, S. F., M. W. Blows, and A. E. D. E. L. Promislow. 2005. Contrasting Mutual Sexual Selection on Homologous Signal Traits in *Drosophila serrata*. Am. Nat. 165:281–289.

Chenoweth, S. F., J. Hunt, and H. Rundle. 2012. Analyzing and Comparing the Geometry of Individual Fitness Surfaces. P. *in* E. Svensson and R. Calsbeek, eds. The Adaptive Landscape in Evolutionary Biology. Oxford University Press, Oxford.

Chouteau, M., M. Arias, and M. Joron. 2016. Warning signals are under positive frequency-dependent selection in nature. Proc. Natl. Acad. Sci. 113:2164–2169.

Contreras-Garduño, J., A. V. Peretti, and A. Córdoba-Aguilar. 2006. Evidence that Mating Plug is Related to Null Female Mating Activity in the Scorpion *Vaejovis punctatus*. Ethology 112:152–163.

Córdoba-Aguilar, A., J. C. Salamanca-Ocaña, and M. Lopezaraiza. 2003. Female reproductive decisions and parasite burden in a calopterygid damselfly (Insecta: Odonata). Anim. Behav. 66:81–87.

Costanzo, K., and A. Monteiro. 2007. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. Proc. R. Soc. Lond. B Biol. Sci. 274:845–851.

Coyne, J. A., A. P. Crittenden, and K. Mah. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. Science 265:1461–1465.

Curtis, S., J. Sztepanacz, B. White, K. Dyer, H. Rundle, and P. Mayer. 2013. Epicuticular Compounds of *Drosophila subquinaria* and *D. recens*: Identification, Quantification, and Their Role in Female Mate Choice. J. Chem. Ecol. 39:579–590.

Darwin, C. 1859. The origin of species by means of natural selection. London, United Kingdom.

Dean, R., S. Nakagawa, T. Pizzari, A. E. E. Svensson, and E. R. G. Shaw. 2011. The Risk and Intensity of Sperm Ejection in Female Birds. Am. Nat. 178:343–354.

Delcourt, M., H. D. Rundle, A. E. T. Pizzari, and E. J. L. Bronstein. 2011. Condition Dependence of a Multicomponent Sexual Display Trait in *Drosophila serrata*. Am. Nat. 177:812–823.

Dickinson, J. L. 1992. Scramble competition polygyny in the milkweed leaf beetle: combat, mobility, and the importance of being there. Behav. Ecol. 3:32–41.

Dyer, K. A., B. E. White, J. L. Sztepanacz, E. R. Bewick, and H. D. Rundle. 2014. Reproductive Character Displacement of Epicuticular Compounds and their Contribution to Mate Choice in *Drosophila subquinaria* and *Drosophila Recens*. Evolution 68:1163–1175.

Egozcue, J., and V. Pawlowsky-Glahn. 2011. Compositional Data Analysis: Theory and Applications. J. Wiley, Chicherter, United Kingdom.

Endler, J. A., and A. E. Houde. 1995. Geographic variation in female preferences for male traits in *Poecilia reticulata*. Evolution 49:456–469.

Estrada, C., S. Schulz, S. Yildizhan, and L. E. Gilbert. 2011. Sexual Selection Drives the Evolution of Antiaphrodisiac Pheromones in Butterflies. Evolution 65:2843–2854.

Fedina, T. Y., T.-H. Kuo, K. Dreisewerd, H. A. Dierick, J. Y. Yew, and S. D. Pletcher. 2012. Dietary Effects on Cuticular Hydrocarbons and Sexual Attractiveness in *Drosophila*. PLOS ONE 7:e49799.

Gage, M. J. G. 1991. Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. Anim. Behav. 42:1036–1037.

García-González, F., and M. Gomendio. 2004. Adjustment of copula duration and ejaculate size according to the risk of sperm competition in the golden egg bug (*Phyllomorpha laciniata*). Behav. Ecol. 15:23–30.

Gershman, S., M. Delcourt, and H. D. Rundle. 2014a. Sexual selection on *Drosophila serrata* male pheromones does not vary with female age or mating status. J. Evol. Biol. 27:1279–1286.

Gershman, S. N., and H. Rundle. 2016. Level up: the expression of male sexually selected cuticular hydrocarbons is mediated by sexual experience. Anim. Behav. 112:169–177.

Gershman, S. N., E. Toumishey, and H. D. Rundle. 2014b. Time flies: time of day and social environment affect cuticular hydrocarbon sexual displays in *Drosophila serrata*. Proc. R. Soc. Lond. B Biol. Sci. 281:20140821.

Gershman, S. N., E. Toumishey, and H. D. Rundle. 2014c. Time flies: time of day and social environment affect cuticular hydrocarbon sexual displays in *Drosophila serrata*. Proc. R. Soc. Lond. B Biol. Sci. 281:20140821.

Gibbs, A. G., F. Fukuzato, and L. M. Matzkin. 2003. Evolution of water conservation mechanisms in *Drosophila*. J. Exp. Biol. 206:1183–1192.

Giglio, E. M., and K. A. Dyer. 2013. Divergence of premating behaviors in the closely related species *Drosophila subquinaria* and *D. recens*. Ecol. Evol. 3:365–374.

Gilbert, L. E. 1976. Postmating Female Odor in *Heliconius* Butterflies: A Male-Contributed Antiaphrodisiac? Science 193:419–420.

Gleason, J. M. 2005. Mutations and Natural Genetic Variation in the Courtship Song of *Drosophila*. Behav. Genet. 35:265–277.

Godin, J.-G. J., and H. E. McDonough. 2003. Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behav. Ecol. 14:194–200.

Gosden, T. P., H. D. Rundle, and S. F. Chenoweth. 2014. Testing the correlated response hypothesis for the evolution and maintenance of male mating preferences in *Drosophila serrata*. J. Evol. Biol. 27:2106–2112.

Higgie, M., and M. W. Blows. 2008. The evolution of Reproductive Character Displacement Conflicts with How Sexual Selection Operates within a Species. Evolution 62:1192–1203.

Higgie, M., M. W. Blows, A. E. M. J. Ryan, and E. M. C. Whitlock. 2007. Are Traits That Experience Reinforcement Also Under Sexual Selection? Am. Nat. 170:409–420.

Hine, E., S. Lachish, M. Higgie, and M. W. Blows. 2002. Positive Genetic Correlation between Female Preference and Offspring Fitness. Proc. Biol. Sci. 269:2215–2219.

Hoikkala, A., and S. Crossley. 2000. Copulatory Courtship in *Drosophila*: Behavior and Songs of *D. birchii* and *D. serrata*. J. Insect Behav. 13:71–86.

Hoikkala, A., S. Crossley, and C. Castillo-Melendez. 2000. Copulatory Courtship in *Drosophila birchii* and *D. serrata*, Species Recognition and Sexual Selection. J. Insect Behav. 13:361–373.

Howard, R. W., L. L. Jackson, H. Banse, and M. W. Blows. 2003. Cuticular Hydrocarbons of *Drosophila birchii* and *D. serrata*: Identification and Role in Mate Choice in D. serrata. J. Chem. Ecol. 29:961–976.

Hunt, J., R. R. Snook, C. Mitchell, H. S. Crudgington, and A. J. Moore. 2012. Sexual selection and experimental evolution of chemical signals in *Drosophila pseudoobscura*. J. Evol. Biol. 25:2232–2241.

Jaenike, J., K. A. Dyer, C. Cornish, and M. S. Minhas. 2006. Asymmetrical Reinforcement and Wolbachia Infection in *Drosophila*. PLOS Biol. 4:e325.

Johansson, B. G., and T. M. Jones. 2007. The role of chemical communication in mate choice. Biol. Rev. 82:265–289.

Katayama, N., J. K. Abbott, J. Kjærandsen, Y. Takahashi, and E. I. Svensson. 2014. Sexual selection on wing interference patterns in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 111:15144–15148.

Kemp, D. J., D. Jones, J. M. Macedonia, and A. K. Krockenberger. 2014. Female mating preferences and male signal variation in iridescent *Hypolimnas* butterflies. Anim. Behav. 87:221–229.

Kingsolver, J., S. Diamond, A. Siepielski, and S. Carlson. 2012. Synthetic analyses of phenotypic selection in natural populations: lessons, limitations and future directions. Evol. Ecol. 26:1101–1118.

Kingsolver, J. G., H. E. Hoekstra, J. M. Hoekstra, D. Berrigan, S. N. Vignieri, C. E. Hill, A. Hoang, P. Gibert, P. Beerli, and E. J. Travis. 2001. The Strength of Phenotypic Selection in Natural Populations. Am. Nat. 157:245–261.

Kuo, T.-H., J. Y. Yew, T. Y. Fedina, K. Dreisewerd, H. A. Dierick, and S. D. Pletcher. 2012. Aging modulates cuticular hydrocarbons and sexual attractiveness in *Drosophila melanogaster*. J. Exp. Biol. 215:814–821.

Kwan, L., H. D. Rundle, and J. Feder. 2010. Adaptation to Desiccation Fails to Generate Pre- and Posymating Isolation in Replicate *Drosophila melanogaster* Laboratory Populations. Evolution 64:710–723.

Kyriacou, C. P., and J. C. Hall. 1984. Learning and memory mutations impair acoustic priming of mating behaviour in *Drosophila*. Nature 308:62–65.

Lande, R., and S. J. Arnold. 1983. The Measurement of Selection on Correlated Characters. Evolution 37:1210–1226.

Lessells, C. M., and P. T. Boag. 1987. Unrepeatable Repeatabilities: A Common Mistake. The Auk 104:116–121.

Mackay, T. F. C., S. Richards, E. A. Stone, A. Barbadilla, J. F. Ayroles, D. Zhu, S. Casillas, Y. Han, M. M. Magwire, J. M. Cridland, M. F. Richardson, R. R. H. Anholt, M. Barrón, C. Bess, K. P. Blankenburg, M. A. Carbone, D. Castellano, L. Chaboub, L. Duncan, and Z. Harris. 2012. The *Drosophila melanogaster* Genetic Reference Panel. Nature 482:173–178.

McGuigan, K., A. Van Homrigh, and M. W. Blows. 2008. An Evolutionary Limit to Male Mating Success. Evolution 62:1528–1537.

Mérot, C., B. Frérot, E. Leppik, and M. Joron. 2015. Beyond magic traits: Multimodal mating cues in *Heliconius butterflies*. Evolution 69:2891–2904.

Merrill, R. M., Z. Gompert, L. M. Dembeck, M. R. Kronforst, W. O. McMillan, C. D. Jiggins, and L. Delph. 2011. Mate Preference Across the Speciation Continuum in a Clade of Mimetic Butterflies. Evolution 65:1489–1500.

Montooth, K. L., and A. G. Gibbs. 2003. Cuticular pheromones and water balance in the house fly, *Musca domestica*. Comp. Biochem. Physiol. - Part Mol. Integr. Physiol. 135:457–465.

Oliver, J. C., K. A. Robertson, and A. Monteiro. 2009a. Accommodating natural and sexual selection in butterfly wing pattern evolution. Proc. R. Soc. Lond. B Biol. Sci. 276:2369–2375.

Oliver, J. C., K. A. Robertson, and A. Monteiro. 2009b. Accommodating natural and sexual selection in butterfly wing pattern evolution. Proc. R. Soc. Lond. B Biol. Sci. 276:2369–2375.

Oudin, M. J., R. Bonduriansky, and H. D. Rundle. 2015. Experimental evidence of condition‐dependent sexual dimorphism in the weakly dimorphic antler fly *Protopiophila litigata* (Diptera: Piophilidae). Biol. J. Linn. Soc. 116:211–220.

Papke, R. S., D. J. Kemp, and R. L. Rutowski. 2007. Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). Animal Behaviour, 73, 47-54. Anim. Behav. 73:1083–1083.

Parkash, R., B. Kalra, and V. Sharma. 2008. Changes in cuticular lipids, water loss and desiccation resistance in a tropical drosophilid: Analysis of variation between and within populations. Fly (Austin) 2:189–197.

Partecke, J., A. von Haeseler, and M. Wikelski. 2002. Territory establishment in lekking marine iguanas, *Amblyrhynchus cristatus*: support for the hotshot mechanism. Behav. Ecol. Sociobiol. 51:579–587.

Préault, M., O. Chastel, F. Cézilly, and B. Faivre. 2005. Male bill colour and age are associated with parental abilities and breeding performance in blackbirds. Behav. Ecol. Sociobiol. 58:497–505.

Preziosi, R. F., and D. J. Fairbairn. 1996. Sexual size dimorphism and selection in the wild in the waterstrider *Aquarius remigis*: Body size, components of body size and male mating success. J. Evol. Biol. 9:317–336.

Robertson, K. A., and A. Monteiro. 2005. Female *Bicyclus anynana* Butterflies Choose Males on the Basis of Their Dorsal UV-Reflective Eyespot Pupils. Proc. Biol. Sci. 272:1541–1546.

Rowe, L., and D. Houle. 1996. The Lek Paradox and the Capture of Genetic Variance by Condition Dependent Traits. Proc. Biol. Sci. 263:1415–1421.

Rowland, W. J., K. J. Bolyard, J. J. Jenkins, and J. Fowler. 1995. Video playback experiments on stickleback mate choice: female motivation and attentiveness to male colour cues. Anim. Behav. 49:1559–1567.

Rundle, H. D., S. F. Chenoweth, and M. W. Blows. 2009. The diversification of mate preferences by natural and sexual selection. J. Evol. Biol. 22:1608–1615.

Rundle, H. D., S. F. Chenoweth, M. W. Blows, A. E. G. W. Gilchrist, and E. M. A. Geber. 2008. Comparing Complex Fitness Surfaces: Among‐Population Variation in Mutual Sexual Selection in *Drosophila serrata*. Am. Nat. 171:443–454.

Rundle, H. D., S. F. Chenoweth, P. Doughty, and M. W. Blows. 2005. Divergent Selection and the Evolution of Signal Traits and Mating Preferences. PLOS Biol. 3:1988–1995.

Rundle, H. D., S. F. Chenoweth, and M. Webster. 2011. Stronger Convex (Stabilizing) Selection on Homologous Sexual Display Traits in Females than in Males: A Multipopulation Comparison in *Drosophila serrata*. Evolution 65:893–899.

Rundle, H. D., and K. A. Dyer. 2015. Reproductive character displacement of female mate preferences for male cuticular hydrocarbons in *Drosophila subquinaria*. Evolution 69:2625–2637.

Sandkam, B., C. M. Young, and F. Breden. 2015. Beauty in the eyes of the beholders: colour vision is tuned to mate preference in the Trinidadian guppy (*Poecilia reticulata*). Mol. Ecol. 24:596–609.

Savage, K. E., J. Hunt, M. D. Jennions, and R. Brooks. 2005. Male attractiveness covaries with fighting ability but not with prior fight outcome in house crickets. Behav. Ecol. 16:196–200.

Senar, J. C., J. Figuerola, and J. Pascual. 2002. Brighter Yellow Blue Tits Make Better Parents. Proc. Biol. Sci. 269:257–261.

Shevtsova, E., C. Hansson, D. H. Janzen, and J. Kjærandsen. 2011. Stable structural color patterns displayed on transparent insect wings. Proc. Natl. Acad. Sci. 108:668–673.

Skroblin, A., and M. W. Blows. 2006. Measuring natural and sexual selection on breeding values of male display traits in *Drosophila serrata*. J. Evol. Biol. 19:35–41.

Steiger, S., G. D. Ower, J. Stökl, C. Mitchell, J. Hunt, and S. K. Sakaluk. 2013. Sexual selection on cuticular hydrocarbons of male sagebrush crickets in the wild. Proc. R. Soc. Lond. B Biol. Sci. 280:20132353.

Sztepanacz, J. L., and H. D. Rundle. 2012. Reduced Genetic Variance Aamong High Fitness Individuals: Inferring Stabilizing Selection on Male Sexual Displays in *Drosophila serrata*. Evolution 66:3101–3110.

Thomas, M. L., and L. W. Simmons. 2008. Sexual dimorphism in cuticular hydrocarbons of the Australian field cricket *Teleogryllus oceanicus* (Orthoptera: Gryllidae). J. Insect Physiol. 54:1081–1089.

Thurman, T. J., and B. M. Seymoure. 2016. A bird's eye view of two mimetic tropical butterflies: coloration matches predator's sensitivity. J. Zool. 298:159–168.

Warburton, N. M., P. W. Bateman, and P. A. Fleming. 2013. Sexual selection on forelimb muscles of western grey kangaroos (Skippy was clearly a female). Biol. J. Linn. Soc. 109:923–931.

Weddle, C. B., C. Mitchell, S. K. Bay, S. K. Sakaluk, and J. Hunt. 2012. Sex‐specific genotype‐by‐environment interactions for cuticular hydrocarbon expression in decorated crickets, *Gryllodes sigillatus*: implications for the evolution of signal reliability. J. Evol. Biol. 25:2112–2125.

White, A. J., and H. D. Rundle. 2015. Territory defense as a condition‐dependent component of male reproductive success in *Drosophila serrata*. Evolution 69:407–418.

Wittkopp, P. J., and P. Beldade. 2009. Development and evolution of insect pigmentation: Genetic mechanisms and the potential consequences of pleiotropy. Semin. Cell Dev. Biol. 20:65–71.

Yeh, S.-D., S.-R. Liou, and J. R. True. 2006. Genetics of divergence in male wing pigmentation and courtship behavior between *Drosophila elegans* and *D. gunungcola*. Heredity 96:383–395.

Zhu, Y. 2013. The *Drosophila* visual system. Cell Adhes. Migr. 7:333–344.