uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Lucia Kwan
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biology)
GRADE / DEGREE

Department of Biology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Adaptation of Desiccation Resistance Fails to Generate Pre- and Postmating
in *Dropsophilia Melanogaster*

TITRE DE LA THÈSE / TITLE OF THESIS

H. Rundle
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

R. Kassen

R. Sargent

T. Sherratt

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

ADAPTATION TO DESICCATION RESISTANCE FAILS TO GENERATE PRE- AND POSTMATING ISOLATION IN *DROSOPHILA MELANOGASTER*

LUCIA KWAN

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in the
Ottawa-Carleton Institute of Biology

Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-59463-6
Our file Notre référence
ISBN: 978-0-494-59463-6

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Many laboratory speciation experiments have raised allopatric populations in different environments to determine whether reproductive isolation evolves as a by-product of adaptation. Few, however, have controlled for the effects of genetic drift, addressed the evolution of both pre- and postmating isolation, or investigated the conditions that promote or hamper the process. I present results of a long-term evolution experiment in which 12 replicate populations of *Drosophila melanogaster* independently evolved for more than 57 generations under alternative desiccation treatments (six control and six desiccation-selected populations). Specifically, I demonstrate the divergence between the desiccation and control populations of cuticular hydrocarbons, key traits that have been implicated in mate choice and sexual isolation in *Drosophila*. Despite this divergence, there was no detectable pre- or postmating isolation between the desiccation and control populations. Novel environments are generally thought to promote the evolution of reproductive isolation. Understanding the conditions that favour or hamper this remains a key challenge for speciation research.

RÉSUMÉS

Plusieurs expériences de laboratoire ont élevé des populations allopatriques dans des environnements différents afin de déterminer si l'isolation reproductive évolue en tant qu'effet secondaire de l'adaptation. Cependant, peu de ces recherches ont contrôlé les effets de la dérive génétique, ont étudié l'évolution de l'isolation pré- et post- copulation, ou ont investigué les conditions favorisant ou limitant ce processus. Je présente les résultats d'une expérience d'évolution à long terme dans laquelle 12 populations répliquées de *Drosophila melanogaster* ont évolué indépendamment les unes des autres pendant plus de 57 générations sous un régime de déshydratation comportant six réplicas contrôles et six réplicas qui ont été sélectionnés pour résister à la déshydratation. Plus précisément, je démontre qu'il y a eu divergence des hydrates de carbone cuticulaires entre les populations sélectionnées pour la résistance à la déshydratation et les populations contrôles, un trait clé impliqué dans la sélection du partenaire sexuelle et dans l'isolation sexuelle chez *Drosophila*. Malgré cette divergence, aucune isolation pré- ou post-copulation n'a pu être détecté entre les populations contrôles et celles sélectionnées pour la résistance à la déshydratation. Les nouveaux environnements sont généralement considérés comme propices à l'évolution de l'isolation reproductive. Comprendre les conditions qui favorisent ou limitent l'isolation reproductive demeure un des principaux défis pour la recherche sur la spéciation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Howard D. Rundle. In the past two years, Howard has provided guidance and support in every aspect of my thesis. He has challenged me day in and day out and because of this, I am better researcher. I have learned more than I thought I would about anything and everything related to evolutionary biology. He has also been very patient with me, especially when it comes to statistics and the small things in research, which I have a tendency to make a big deal out it. Most importantly, Howard has always made time for me regardless of how preoccupied he was. Honestly, being a student of Howard's has been an honour and a privilege. Howard, thanks for one of the most challenging and rewarding experiences of my academic career so far.

Second, I would like to thank all the current and past members of the Rundle Lab, including Heather Auld, Marc Charette, Matthieu Delcourt, Shahira Khair, Kelsie MacLellan, David Punzalan, Vanessa Rotondo, Alex Wong, and Pamela Wu. In the past two years, these people have been my backbone, assisting with a number of large and often crazy assays, one of which included adding food colouring into at least 2000 vials until 4:00 am on a Saturday. Needless to say, I would not have made it through graduate school without them. I would like to especially thank Matthieu Delcourt and David Punzalan. Matthieu has helped me conquer my battle against the gas chromatography and everything related to it. David has provided endless and unconditional support since the first day I met him. More than anybody, he has changed the way I understand and approach research and for this, I thank him. There are only a handful of people that have made a big impact in my academic career, and David is definitely one of them.

I would also like to thank my committee members, Dr. Rees Kassen from the University of Ottawa and Dr. Tom Sherratt from Carleton University. They have provided guidance, when necessary, and great insight into my thesis. The discussions with Rees and Tom has defined my thesis and more importantly, driven me to see the bigger picture at hand.

Finally, throughout my M.Sc., I have received financial support from both the Natural Sciences and Engineering Research Council of Canada (NSERC) and University of Ottawa. In addition, much of the research expenses, as well as travel to a number of conferences, were generously covered by a NSERC grant to Howard.

Chapter 1

An Overview of Ecological Speciation

INTRODUCTION

Much of the diversity of life exists among species and understanding their evolutionary origins is thus a major goal of evolutionary biology. Speciation research has come a long way since Darwin's "On the Origin of Species" (1859), in which the origin of new species was referred to as that "mystery of mysteries". Today, we have a good understanding of what species are and how they form. Of the many species definitions (reviewed in Coyne and Orr 2004), Mayr's (1942) biological species concept provides the most straightforward approach to studying the origin of species and therefore is almost universally adopted (although often implicitly). I do so explicitly here. In this concept, species are groups of interbreeding natural populations that are reproductively isolated from other such groups. Reproductive isolation evolves when barriers to gene flow restrict or prevent alleles from moving between populations. Such barriers can occur prior to, or following, sperm or pollen transfer, generating pre- and postmating isolation, respectively (Coyne and Orr 2004). The ultimate outcome is the formation of two species. The study of speciation has largely focused on its genetic basis (e.g., the alleles causing reproductive isolation) and its biogeography (e.g., allopatry versus sympatry), including seminal work by Dobzhansky (1937) and Mayr (1942). In the past two decades, however, attention has shifted to the mechanisms causing reproductive isolation to evolve, and in particular the role of ecology in this process. The idea that ecology may be involved dates back at least to the Modern

Synthesis (e.g, Fisher 1930; Wright 1940; Mayr 1942; Muller; 1942; Dobzhansky 1951), but it received little attention in the intervening years.

Ecological speciation occurs when reproductive isolation evolves as a consequence of divergent natural selection acting on populations that inhabit different environments or exploit different niches (Mayr 1942; Dobzhansky 1951; Endler 1977; Schluter 2000, 2001; Rundle and Nosil 2005). Divergent selection arises from (a) the ecological interactions of organisms with their abiotic environment that occurs during resource acquisition, and (b) the ecological interactions among organisms during resource acquisition (e.g., competition, predation). Divergent selection can occur between allopatric populations due to differences in their environments, and may arise in sympatry from the exploitation of alternate resources as well as ecological interactions between the populations. Ecological speciation includes the special case of disruptive selection on a single population (e.g., arising from frequency-dependent intraspecific competition; Doebeli 1996) and therefore can generate pre- and/or postmating isolation under any geographical context.

In contrast, nonecological speciation occurs when reproductive isolation evolves ultimately due to chance events including genetic drift, founder events/population bottlenecks, hybridization and polyploidization (reviewed in Coyne and Orr 2004). Nonecological speciation can also be driven by selection that is independent of ecological differences (e.g., some models of sexual selection such as Fisherian runaway (Lande 1981; Kirkpatrick 1982) and sexual conflict (Rice 1998; Holland and Rice 1998)), or is not divergent between populations (e.g., fixation of alternative but genetically incompatible alleles in separate populations experiencing similar selection).

MECHANISMS OF ECOLOGICAL SPECIATION

Under ecological speciation, reproductive isolation can evolve indirectly as a by-product of adaptive divergence. Environmental differences and/or ecological interactions can generate divergent natural selection on phenotypic traits, including morphology, behaviour, and physiology, and as a by-product of these changes reproductive isolation evolves incidentally (Schluter 2000; Kirkpatrick and Ravigné 2002; Rundle and Nosil 2005). Divergent selection arising from environmental differences can occur between populations in allopatry (e.g., occupying different environments) or sympatry (e.g., occupying different niches or exploiting alternative resources within the same environment). Conversely, divergent selection from ecological interactions can only occur between populations in sympatry (e.g., interspecific competition).

In some instances, reproductive isolation itself can be adaptive and thus directly favored by selection. This occurs when individuals that mate heterospecifically (i.e., with an individual from another, divergent population) suffer reduced fitness compared to individuals that mate homospecifically (i.e., within their own population). Homospecific matings are therefore favored over heterospecific matings, generating selection that can strengthen premating isolation. Reduced fitness from heterospecific matings may arise directly (e.g., increase predation risk, individuals that are less resistant to an alternate suite of parasites) or indirectly, via the fitness of the resulting offspring. The latter process is known as reinforcement and is thought by some to be the final stage of speciation in the majority of cases (Dobzhansky 1937, 1940; Blair 1955; Servedio and Noor 2003).

When the fitness costs of heterospecific mating arise ultimately from ecological mechanisms, the process above may be considered a component of ecological speciation. Reinforcement can occur as a component of ecological speciation, for example, if hybrid offspring suffer reduced fitness because their intermediate phenotypes are poorly suited to either native environment (and no intermediate environment exists). In contrast, if reductions in hybrid fitness arise from nonecological mechanisms (e.g., genetic incompatibilities between the parental genomes that evolved by genetic drift), then reinforcement is not considered a component of ecological speciation.

EVIDENCE FOR ECOLOGICAL SPECIATION

There is evidence that reproductive isolation has evolved in both nature and the laboratory (reviewed in Schluter 2000; Coyne and Orr 2004; Nosil *et al.* 2005). Below, I will highlight some key case studies, starting with studies in nature.

Evidence from Nature

There is no shortage of studies of ecological speciation in nature, although the majority involves indirect evidence from pattern-based data (reviewed in Schluter 2000), including inferring ecological speciation when the traits conferring reproductive isolation are under divergent selection due to ecological mechanisms. A classic example comes from the remarkable long-term studies of Darwin's finches on the Galápagos Islands. These studies have clearly shown that size and shape of the beak and body are closely linked to feeding behaviour and diet (reviewed in Grant 1986). Ratcliffe and Grant (1983) and Podos (2001) have demonstrated that premating isolation between some of the species has evolved as a by-product of the adaptation of these traits to divergent environments. Ratcliffe and Grant (1983) studied the link between morphology and species recognition in Darwin's ground finches *Geospiza*. Using four of the six species, they found that the combined stimuli of the head and body size, traits that are strongly selected via their effects on resource acquisition, were important in discriminating between conspecific and heterospecific mates. Podos (2001) expanded on this result by exploring the link between morphology and vocal signal structure. He found that differences in beak and body sizes led to differences in songs, which are associated with mating signals, in nine species of Darwin's finches. Specifically, finches with large beaks and body sizes produced lower rates of syllable repetition and narrower frequency bandwidths, while the opposite pattern was observed in finches with small beaks and body sizes.

Evidence for ecological speciation is also provided when postmating isolation has an ecological basis, or in other words, when reductions in hybrid fitness are caused by a mismatch between their phenotype and environment. Such reproductive isolation is ecologically-dependent (also termed "environment-dependent"; Rice and Hostert 1993) and usually arises because the intermediate phenotypes of hybrids render them less effective at exploiting the native environments or resources of either native species. Alternatively, reproductive isolation can be ecologically-independent (also termed "unconditional"; Rice and Hostert 1993) if hybrids fare poorly in all environments because of genetic incompatibilities between the parental genomes that occur independent of the environment. Genetic incompatibilities can be produced by ecological and nonecological speciation, whereas ecologically-dependent postmating isolation is a unique prediction of ecological speciation (Rundle and Whitlock 2001). Determining the cause of any reductions in hybrid fitness is therefore key to evaluating the evidence for ecological speciation.

In nature, threespine sticklebacks (*Gasterosteus aculeatus*) from British Columbia, Canada, are one of the strongest cases of ecologically-dependent postmating isolation. In *G. aculeatus*, pairs of species, called 'benthics' and 'limnetics', coexist in several coastal lakes and occupy different niches in each lake: benthics forage on insects in the littoral zone and the limnetics on zooplankton in the open water. Using a direct, experimental approach, Hatfield and Schluter (1999) tested for ecological selection against hybrids by measuring the fitness of benthics, limnetics, and hybrids between them in both the littoral and open water environments in the lake, as well as a benign laboratory environment. They found that F1 and F2 hybrids had high fitness in the benign environment; however, F1 hybrids performed poorly in the wild, growing slower than benthics in the littoral zone and slower than limnetics in the open water. While consistent with ecologically-dependent postmating isolation, it has been suggested that the severity of genetic incompatibilities may depend on the stressfulness of the environment. To differentiate between these hypotheses, Rundle (2002) measured the fitness of benthic and limnetic backcrosses (i.e., F1 crossed with the parental type) in both environments in the wild. The idea is that the two backcrosses are phenotypically divergent but suffer equally on average from any genetic incompatibilities. Ecological speciation therefore makes a clear prediction: the backcross that more closely resembles the native species should be more fit in that particular native environment (i.e., benthic backcross > limnetic backcross in littoral zone; limnetic backcross > benthic backcross in open water; Rundle and Whitlock 2001). In his study, Rundle (2002) found that benthic backcrosses grew about twice as fast than limnetic backcrosses in the littoral zone, while the reverse pattern was observed in the open water, providing strong evidence that postmating isolation between these sticklebacks is ecological in origins.

Evidence from the Laboratory

The role of ecology in speciation has also been extensively studied in the laboratory. Here, research has largely focused on the evolution of reproductive isolation between allopatric populations experiencing divergent natural selection (but see Rice and Salt 1988, 1990). The basic approach is to use experimental evolution to allow replicate populations, derived from a common ancestor, to evolve and potentially adapt to environments differing in some characteristics (e.g., climates, habitats, resources). Under ecological speciation, reproductive isolation is expected to evolve between populations experiencing divergent selection (i.e., adapting to different environments) but not between populations under parallel selection (i.e., independently adapting to the same environment). Such a comparison controls for the effects of genetic drift in allopatry, which could cause reproductive isolation to evolve between any pair of populations, independent of their environment. Premating isolation is usually tested using mate choice trials to determine whether individuals from two populations mate assortatively. Postmating isolation is tested by determining whether the fitness of hybrids, created from crosses between populations adapted to different environments, is reduced relative to the parental populations. If postmating isolation is detected, its magnitude should be compared to that occurring in hybrids created from crosses between populations independently adapted to the same environment (i.e., to control for genetic drift in allopatry), although this has been rarely done.

Two classic studies demonstrated the evolution of premating isolation as a by-product of adaptation using the above experimental approach. Kilias *et al.* (1980) reared different populations of *Drosophila melanogaster* on cold-dry-dark environment or warm-damp-light environment for five years. Similarly, Dodd (1989) reared replicate populations of *Drosophila pseudoobscura* on maltose-based environment or starch-based environment for one year. In both cases, tests for premating isolation were performed using multiple-choice mating trials in which individuals can choose between conspecific and heterospecific mates. Assortative mating was detected between populations reared in the different environments, but not between populations independently reared in the same environment, demonstrating the evolution of premating isolation due to divergent natural selection in both cases.

An elegant study of postmating isolation using the above experimental approach was performed by Dettmann *et al.* (2007). Replicate populations of yeast, *Saccharomyces cerevisiae*, were allowed to evolve in high-salinity (S) or low-glucose minimal medium (M) for 500 generations. After adaptation, they tested for reproductive isolation, where the performance of hybrids (S-M) and native populations (S and M) were compared using two components of fitness: the rate of mitotic reproduction and efficiency of meiotic reproduction. S-M hybrids were shown to have a reduced rate of mitotic reproduction compared to the S populations in the high-salinity environment, and a reduced rate relative to the M populations in the minimal medium environment. To determine if this postmating isolation was driven by ecological or nonecological mechanisms, they compared the performance of S-M hybrids against that of S-S and M-M hybrids, created from parallel-adapted populations, in both environments, respectively. They found that the rate of mitotic reproduction was the same between hybrids of parallel-adapted populations (S-S and M-M) and native populations (S and M), providing direct evidence that reproductive isolation had an ecological basis. The efficiency of meiotic reproduction was also compared for the S and M populations, as well as the hybrids (S-M, S-S, and M-M). Similar to mitotic efficiency, S-M hybrids had lower meiotic efficiency than both the native types and hybrids of parallel-adapted populations, again providing direct evidence for the role of divergent selection in the evolution of this form of postmating isolation. Interestingly, the parallel-adapted populations fared worse than the native populations, demonstrating the evolution of some reproductive isolation between populations subjected to parallel selection.

FUTURE DIRECTIONS

Our understanding of ecological speciation is far from complete. In the laboratory, for example, few studies have controlled for the effects of genetic drift on the evolution of reproductive isolation. In addition to Kilias *et al.* (1980) and Dodd (1989), I am only aware of two other studies, also in *Drosophila*, that can disentangle the effects of divergent selection and genetic drift (Mooers *et al.* 1999; Rundle 2003). Reproductive isolation failed to evolve in both of these, yet we have little idea as to why. A comprehensive understanding of the processes that hamper or promote the evolution of reproductive isolation is therefore much needed (Florin and Ödeen 2002). Furthermore, most studies of ecological speciation have only addressed the evolution of premating isolation, with little attention paid to postmating isolation. The relative rates at which the various reproductive isolating barriers evolve therefore remain relatively unknown.

In this thesis, I performed a direct test of ecological speciation in the laboratory using 12 replicate populations of *Drosophila melanogaster* that have independently evolved for more than 57 generations under alternative desiccation treatments (six control, 'C', six desiccation-selected, 'D'). My experiment builds upon previous laboratory studies of ecological speciation in a number of ways that provide key insight into cases in which reproductive isolation fails to evolve. First, I tested for the evolution of both pre- and postmating isolation. To the best of my knowledge, a properly controlled experiment that explores the evolution of both forms of reproductive isolation has never been published. Second, the duration of the experiment was longer than a number of past studies that have detected significant premating isolation. For example, premating isolation evolved after only 16 generations of selection for geotaxis in the housefly, *Musca domestica* (Hurd and Eisenberg 1975), while Soans *et al.* (1974) observed the same result after only 38 generations. Similarly, Dodd (1989) found premating isolation after only 12 generations of selection for different carbohydrate sources in *D. pseudoobscura* (see Evidence from Laboratory). Third, effective population sizes in my experiment were likely relatively large in comparison to past experiments given the census sizes of the populations (approximately 1000 individuals per population after selection). Effective population size has been associated with the success or failure of past laboratory speciation experiments (Ödeen and Florin 2000). Fourth, desiccation-selection is generally observed to impact a large suite of traits, generating multifarious divergent selection that should maximize the opportunity for reproductive isolation to evolve as a side effect (Rice and Hostert 1993; Nosil *et al.* 2009).

Most important to interpreting any absence of reproductive isolation, I have previously shown that these populations have diverged between treatments and that these changes are adaptive in the desiccation environment (Kwan *et al.* 2008). Additional evidence is also provided in the current experiment pertaining to traits that have been implicated in sexual behaviour in *Drosophila*. Unlike a number of past studies, I also test whether selection was divergent between environments, a fundamental requirement of ecological speciation that has been linked to the success or failure of past laboratory experiments (Florin and Ödeen 2002).

The chapter that follows presents the results of this experiment. It is written in the style of a stand-alone scientific manuscript because producing such material is a crucial skill to learn for every academic and this chapter will soon be submitted to an evolutionary journal. This necessarily generated a small amount of overlap in the background material presented in this chapter and the opening of the next, but I endeavoured to keep this to a minimum.

Chapter 2

Adaptation to Desiccation Fails to Generate Reproductive Isolation

INTRODUCTION

Ecological speciation occurs when reproductive isolation evolves as a consequence of divergent natural selection acting on populations occupying different environments or exploiting alternative resources (Mayr 1942; Dobzhanksy 1951; Endler 1977; Schluter 2000, 2001; Rundle and Nosil 2005). Pre- and/or postmating isolation accrue as natural selection drives populations up separate adaptive peaks in the fitness landscape. When populations are allopatric, the evolution of reproductive isolation occurs indirectly, as a by-product of the adaptive divergence of phenotypic traits (e.g., morphology, behaviour, physiology). Laboratory experiments have confirmed the feasibility of this process (e.g., Kilias *et al.* 1980; Dodd 1989; Dettman *et al.* 2007; Dettman *et al.* 2008), and there is strong evidence that it occurs in nature (reviewed in Schluter 2000; Coyne and Orr 2004). This includes cases in which reproductive isolation has been shown to have evolved in parallel in independent populations inhabiting similar environments (i.e., so-called 'parallel speciation'; Schluter and Nagel 1995; Funk 1998; Rundle *et al.* 2000; Nosil *et al.* 2002; McKinnon *et al.* 2004).

Our understanding of ecological speciation is nevertheless incomplete (Rundle and Nosil 2005; Nosil *et al.* 2009). Laboratory experiments have focused on the evolution of premating isolation, for example, with much less attention being given to postmating isolation (but see Dettman *et al.* 2007; Dettman *et al.* 2008). We therefore lack a comprehensive understanding of the relative rates at which different forms of reproductive isolation evolve during the early stages of ecological speciation. In addition, many of the past laboratory experiments failed to control for the effects of genetic drift in allopatry (Rice and Hostert 1993; Coyne and Orr 2004). Of those that did, reproductive isolation failed to evolve in some cases (e.g., Mooers *et al.* 1999; Rundle 2003), yet we have limited insight into why. Exploring these issues is an important task for future laboratory speciation experiments.

Here, I take advantage of a long-term evolution experiment involving 12 replicate populations of *Drosophila melanogaster* that have independently evolved under alternative desiccation treatments (six control, 'C'; six desiccation-selected, 'D'). These treatments expose individuals to very different climates during a portion of their adult lives every generation: arid for the D populations and humid for the C populations. In *Drosophila*, the evolution of desiccation resistance has been shown to involve extensive phenotypic remodeling (e.g., Hoffmann and Parsons 1989; Gibbs *et al.* 1997; Chippindale *et al.* 1998) and the response to selection of these particular experimental populations has been characterized in detail in previous work (Kwan *et al.* 2008). Briefly, after 46 generations of experimental evolution, substantial adaptation to the desiccation environment had occurred in the D populations, generating a 68% increase in survival time under arid conditions relative to the C populations. The D populations also exhibited extended development and faster growth as larvae, contributing to a 20% increase on average in adult body weight relative to the controls. This adaptation to the desiccation environment appears to have come at a cost, reducing pre-adult viability and female fecundity relative to the C populations in both a stressful (desiccation) environment and a non-stressful environment characteristic of that experienced by the common ancestors of the D and C populations. However, fitness costs of desiccation adaptation in the control environment (which induces some starvation stress; see Materials and Methods) have not previously been estimated.

All replicate D and C populations occur in discrete cages and rearing vials with no gene flow between them, characteristic of island populations that have been evolving for more than 57 generations in allopatry. Reproductive isolation may therefore have evolved as a by-product of this adaptive divergence between environments, representing the early stages of ecological speciation. Here, I explore two aspects of this. First, I further characterize the evolutionary divergence of these populations with respect to a suite of traits, cuticular hydrocarbons (CHCs), that have potential links to both desiccation resistance and premating isolation. CHCs are long chain-length carbon compounds found on insect epicuticles. They have waterproofing capabilities (Nelson 1993) and their variation in *Drosophila* and other insects has been associated with desiccation resistance (Toolson and Kuper-Simbron 1989; Gibbs 1998; Howard and Bloomquist 2005), including a pattern in *D. melanogaster* of increasing carbon chain-length with latitude (Rouault *et al.* 2001) and in response to desiccation adaptation during a laboratory evolution experiment (Gibbs *et al.* 1997). (Longer chain-length CHCs are expected to be more effective at preventing water loss because they have higher melting temperatures; Gibbs 1998). CHCs also function as a pheromonal system that is involved in sexual communication in *Drosophila* (Jallon 1984; Markow and Toolson 1990; Ferveur *et al.* 1997; Blows and Allan 1998; Grillet *et al.* 2006). Their expression is plastic and individuals have been shown to rapidly alter these traits in relation to their social context in both *D. melanogaster* (Kent *et al.* 2008; Krupp *et al.* 2008) and *Drosophila serrata* (Petfield *et al.* 2005). CHCs have been shown to mediate sexual isolation among species in the *melanogaster* subgroup (e.g., Coyne 1996) and between races of *D.*

melanogaster (e.g., Fang *et al.* 2002). Detailed genetic and evolutionary experiments in *D. serrata* have also demonstrated an important role of CHCs in both mate choice within populations (Chenoweth and Blows 2003, 2005) and species recognition (Blows and Allan 1998; Higgie *et al.* 2000; Higgie and Blows 2007). All else being equal, the evolutionary divergence of CHCs in response to desiccation-selection would be expected to generate premating isolation eventually.

Second, in addition to investigating the divergence of a suite of traits implicated in desiccation resistance and mate choice, I directly test for the evolution of both pre- and postmating isolation between populations from these two environments. The test for premating isolation employs a powerful multiple-choice design in which 50 individuals of each sex and population are released into a cage and mating pairs are identified. Cages are treated as independent replicates to determine whether populations adapted to different environments mate assortatively. To test for postmating isolation, I employ a reciprocal transplant experiment in which the survival and fecundity of D and C individuals, as well as F1 hybrids between them, is estimated in both the desiccation and control environments experienced during experimental evolution. Postmating isolation would be indicated by a reduction in F1 hybrid fitness relative to the population adapted to that environment and could be caused by genetic incompatibilities that have arisen between the D and C genomes (i.e., so-called 'intrinsic genetic' or 'unconditional' postmating isolation; Rice and Hostert 1993; Schluter 2000; Coyne and Orr 2004), or by a mismatch between hybrid phenotype and environment (i.e., so-called 'ecologically-dependent' or 'environment-dependent' postmating isolation; Rice and Hostert 1993; Schluter 2000; Rundle and Whitlock 2001; Coyne and Orr 2004). Including individuals from both the D and C populations in these reciprocal transplants permits an additional test of a specific prediction of ecologically-dependent postmating isolation: in the absence of any genetic incompatibilities, the rank order of relative fitness in the desiccation environment should be D > F1 > C, and the reverse in the control environment. Inclusion of both D and C individuals also allows a test of whether selection was divergent between environments, a fundamental requirement of ecological speciation that has been linked to the success or failure of past laboratory experiments (Florin and Ödeen 2002).

MATERIALS AND METHODS

Derivation and Maintenance of Experimental Populations

A detailed description of the stock population and the evolution experiment is available in Kwan *et al.* (2008). Briefly, I created six replicate populations that were selected for desiccation resistance (D) and six matched control populations (C). Populations $D_{1\text{-}3}$ and $C_{1\text{-}3}$ were derived from the laboratory-adapted LH_M population (Chippindale and Rice 2001) that has wild-type (red) eyes; populations $D_{4\text{-}6}$ and $C_{4\text{-}6}$ were derived from a stock carrying the recessive brown-eye (*bw*) mutation in the LH_M genetic background (LH_M-*bw*). (In Kwan *et al.* (2008), populations $D_{1\text{-}3}$ and $C_{1\text{-}3}$ are labeled $DR_{1\text{-}3}$ and $CR_{1\text{-}3}$, respectively, while populations $D_{4\text{-}6}$ and $C_{4\text{-}6}$ are labeled $DB_{1\text{-}3}$ and $CB_{1\text{-}3}$, respectively.)

Treatments were implemented as follows. On day 12 of every generation (designating day 1 as when the eggs were laid), desiccation-selection occurred in each D population by introducing approximately 4,000 adult flies from their rearing vials into a sealed plastic cage (20 cm×20 cm×14 cm) lacking food or water and containing a 100 g packet of Drierite® desiccant. Selection was terminated when approximately 75% mortality had occurred in a population. Each C population was maintained contemporaneously with its matched D population (e.g., C_1 with D_1) by introducing approximately 1,250 individuals into a separate cage containing only a non-nutritional agar plate as a source of water (no food or desiccant was present), thereby inducing mild starvation stress. Starvation stress in a particular C population was terminated when desiccation-selection in its matched D population was ended. Once selection was terminated, survivors were supplied with fresh cornmeal-molasses food and live yeast for two days while in their cage, after which they were then allowed to oviposit on fresh medium for 14-16 h. On day 16, 100-125 of the eggs were introduced into each of the 8-dram rearing vials (40 for the D; 10 for the C) to create the next generation for each population. Except during the selection treatments, larvae and adults were reared at 25 °C, 50% relative humidity, and 12L:12D cycle.

The selection protocol paired the D and C populations bearing the same subscript by handling and time spent in their respective treatments. Because little to no mortality occurred in the C populations during starvation stress, census population sizes of adults after selection were similar in the two treatments (i.e., 75% mortality in a D population left approximately 1,000 individuals alive). Effective populations sizes of the two treatments may have differed for a number of reasons, however, including a strongly female-biased sex ratio in the D populations after selection (females survive desiccation stress much better than males; Kwan *et al.* 2008), the opportunity of the D females to have mated with a greater diversity of males prior to selection, and stronger selection in the desiccation treatment. Prior to all assays, approximately 1,000 individuals per population were reared for two generations in a cage (i.e., a common environment) under relaxed selection, with access to both food and water, to minimize environmental effects.

Response to Selection of Cuticular Hydrocarbons

After 69 generations of experimental evolution, cuticular hydrocarbons (CHCs) were extracted from individual flies from all 12 experimental populations. Extractions were performed under two treatments: non-stressed (benign conditions) and stressed (desiccation conditions). This factorial design allows us to determine whether there were evolved differences in CHC expressions between populations from the desiccation and control treatments, whether individuals altered their CHC expressions in direct response to desiccation stress (i.e., phenotypic plasticity), and whether there was an interaction between these factors (e.g., evolutionary divergence between the D and C populations in their plastic response to desiccation stress).

On day 11, individuals were lightly anesthetized with CO_2, separated by sex, and housed in holding vials containing food. For the non-stressed treatment, CHCs were extracted on day 12 from individuals taken directly from these holding vials. For the stressed treatment, prior to CHC extraction on day 12, individuals were desiccated for 4-5 h in vials containing approximately 6 g of Drierite® desiccant (separated from the flies by a thin foam stopper) and sealed with Parafilm® to prevent influx of water vapor from the ambient environment.

CHCs were extracted from 30 males and 30 females from each combination of population and stress treatment. Extractions were performed by washing individual flies in 100 μL of hexane for approximately 3 min and then vortexing for 1 min. CHC samples were then analyzed using a dual-channel Agilent Technologies 6890N gas chromatograph fitted with HP5 columns of 50 m×0.32 mm internal diameter, pulsed splitless inlets, and flame ionization detectors. The temperature program began by holding 57 °C for 1.10 min, increasing to 190 °C at a rate of 100 °C/min, holding at 190 °C for 1.20 min, increasing to 270 °C at a rate of 5 °C/min, and finally increasing to 300 °C at a rate 120 °C/min and then holding at 300 °C for 5.00 min. Individual CHC profiles were determined by integration of the area under 31 peaks in females and 28 peaks in males, representing all those that could be reliably identified in every individual of that sex. Although the pattern of peaks was broadly consistent with those chemically identified by Foley *et al.* (2007) in a different population of

D. melanogaster, the precise correspondence of some peaks was unclear. I therefore refer to the individual traits by their sequential number within the CHC profiles of each sex (Figs. S1 and S2).

Variation among individuals in quantifying absolute CHC abundances using gas chromatography can be large. To remove this technical error, the abundance of each CHC was expressed as a proportion of the total hydrocarbons extracted for that individual. Logcontrasts were then generated to break the unit-sum constraint associated with proportional data (Aitchison 1986; Blows and Allan 1998). Logcontrasts were calculated for each of the n CHCs for every individual using $\text{logcontrast}(\text{CHC}_n) = \log_{10}(\text{proportion}(\text{CHC}_n) / \text{proportion}(\text{CHC}_x))$, where CHC_x is an arbitrarily chosen common divisor (here, CHC_{11} in females and CHC_5 in males). Note that this transformation reduces the number of traits by one, resulting in 30 logcontrast CHCs in females and 27 logcontrast CHCs in males. Prior to the analysis, multivariate outliers were identified and removed using the Mahalanobis distance technique implemented in the software package JMP version 7.0.2 (SAS Institute Cary, NC).

Statistical analyses employed a non-additive mixed linear model for factorial randomized complete block designs (Newman *et al.* 1997; Quinn and Keough 2002) in which phenotype (D versus C) and treatment (stressed versus non-stressed) were fixed effects, and block (1-6) was a random effect representing the six D-C pairs of populations. Significance tests for the main effects of phenotype and treatment employed the respective interaction with block as the F ratio denominator (e.g., $F_{\text{phenotype}} = \text{MS}_{\text{phenotype}} / \text{MS}_{\text{phenotype}\times\text{block}}$), thereby recognizing that each D-C pair represents a single, evolutionary replicate. As with all unreplicated randomized complete block designs, there is no test of the three-way interaction (phenotype×treatment×block) because it cannot be estimated separately from the residual error variance (Quinn and Keough 2002).

The ideal analysis would have been a multivariate version of the above model that includes all CHCs as response variables. However, given the large number of traits relative to the modest number of replicate populations that can be accommodated in a long-term evolution experiment, this model could not be fitted. I therefore employed a univariate approach that analyzed each logcontrast CHC separately. Sequential Bonferroni (Rice 1989) and false discovery rate (FDR; Benjamini and Hochberg 1995) corrections were employed for multiple comparisons. Because both approaches gave the same results, only the former are presented. All models were fit using maximum likelihood implemented by the 'mixed' procedure in SAS version 9.1 (SAS Institute Cary, NC).

Test for Premating Isolation

Assortative mating between the D and C populations was tested after 57 generations of experimental evolution. Replicate multiple-choice assays were employed in which 50 virgin males and 50 virgin females from a single population of each treatment were placed together in a cage (200 individuals total). Tests in which individuals can choose between conspecific and heterospecific mates have been shown to be more sensitive at detecting assortative mating than no-choice tests (Coyne *et al.* 2005), and multiple-choice tests permit both male and female choice to contribute to assortative mating. Six separate combinations of the D and C populations were tested (Table 1), all involving unique populations. These combinations controlled for both population of origin, preventing individuals of either sex from having a choice between individuals from their own population versus individuals from a different population, and eye-color (previous assays indicated reduced mating success of LH_M-*bw* relative to LH_M males; L. Kwan, unpublished data). Eight replicate cages were performed for each of the six combinations using unique individuals in all cases.

Flies for use in the mating trials were collected as virgins on day 10 using light CO_2 anesthesia and housed in temporary holding vials containing food. Mating trials were performed on day 14. Replicate cages for each mating combination were run simultaneously. Cages were checked approximately every 11 minutes and mating pairs were removed and identified. Cages were terminated after 25 mating pairs or 3 h had elapsed (an average of 24.54 ± 0.27 matings occurred in each cage). To permit their identification, all individuals were marked by feeding them overnight (day 13-14) in vials with abundant yeast saturated with red or blue commercial food colouring. Replicate cages within a mating combination were reciprocally marked to balance any colour effects (two replicate cages using each of the four possible color combinations involving two population and two sexes). Consistent with past studies (Mooers *et al.* 1999; Rundle 2003), such effects were weak or absent (Tables S1 and S2).

Assortative mating was evaluated using the index Y (Bishop *et al.* 1975) based on the cross-product ratio (α) of a 2×2 contingency table containing the number of matings in each male and female combination. Y is defined as $(\sqrt{\alpha} - 1) / (\sqrt{\alpha} + 1)$, where α is the product of the number of C-by-C and D-by-D matings, divided by the product of the number of C-by-D and D-by-C matings. Y varies from -1 for perfect negative assortative mating to $+1$ for perfect positive assortative mating, with zero indicating random mating. *Y* is a margin-free index and is therefore not biased by varying propensities to mate (Bishop *et al.* 1975). *Y* was treated as a simple measure of assortative mating and was calculated for each replicate mating cage (because matings within a cage are not independent of one another). A single overall test for assortative mating between the desiccation and control treatments was performed using a one-sample *t*-test, treating the six mating combinations as replicates, to determine whether mean *Y* was significantly different from zero. Within each specific mating combination, assortative mating between that particular pair of populations was evaluated using a one-sample *t*-test that treated cages as replicates.

For each sex, the relative mating success of D individuals was calculated as the proportion (*p*) of total matings in a cage that involved D individuals. As for *Y* above, I am primarily interested in overall treatment effects on relative mating success, so a single one-sample *t*-test was performed to determine whether mean *p* differed significantly from 0.5 (indicating equal mating success of D and C individuals of that sex), treating the six mating combinations as replicates. Within each specific mating combination, deviations from random mating for a particular pair of populations were tested using a one-sample *t*-test that treated cages as replicates. All proportions were arcsine-square root transformed prior to the analysis (results are qualitatively unchanged using untransformed proportions).

Female Mating Rates – Mating rates of D and C virgin females were directly compared in a separate assay after 67 generations of experimental evolution by individually presenting both types of females with LH_M virgin males. Individuals were collected as virgins on day 10 using light CO_2 anesthesia and housed separately by sex in holding vials containing food.

On day 14, female mating rate was determined by the time (in seconds) it took a single female to copulate with a single male transferred into her vial. Females from a single D population and their matched C population were tested simultaneously (e.g., pair 1: D_1 females and C_1 females). Within each pair, there were 50 trials using D females and 50 trials using C females. All trials were terminated after copulation or 1 h had elapsed (copulation occurred in all but 23 of the 600 trials).

A single overall test for a difference in time to mating between D and C females was performed using a paired *t*-test, treating the six D-C pairs as replicates. Within each specific D-C pair, a two-sample *t*-test was used to determine whether D females mated significantly faster than C females. Times were log transformed prior to the analysis (results are qualitatively unchanged using untransformed times). *P*-values are one-tailed given an *a priori* expectation from the results of the premating isolation assay of faster mating in D than C females.

Male Inbreeding Depression – After 69 generations of experimental evolution, the potential effect of inbreeding depression on male mating success and assortative mating was explored. I created matched pairs of F1 'hybrids' between the D_1 and D_3 populations ($F1_{D1\text{-}3}$) and the C_1 and C_3 populations ($F1_{C1\text{-}3}$), and between the D_4 and D_6 populations ($F1_{D4\text{-}6}$) and the C_4 and C_6 populations ($F1_{C4\text{-}6}$) (Table 2). These F1's were created from an equal number of crosses in both directions (e.g., D_1 females mated to D_3 males and D_1 males mated to D_3 females) and were pooled prior to the mating assay below. From these F1 crosses, 50 males were then used in multiple-choice mating trials using the same protocol as before. Eight replicate cages were tested for each of the two mating combinations (Table 2). These combinations controlled for population of origin and eye-color. Food coloring was again applied in a balanced design and no effects were detected (Table S1 and S2).

Test for Postmating Isolation

I tested for postmating isolation between the D and C populations using a reciprocal transplant assay in which survival and female fecundity were evaluated for D and C individuals, as well as F1 'hybrids' between them, in both the desiccation and control environments. F1's were created by mating individuals from a particular D population with opposite sex individuals from their matched C population, thereby creating six F1 populations corresponding to the original six D-C pairs. For each D-C pair, crosses were performed using equal numbers of individuals in both directions (e.g., D_1 females mated to C_1 males and D_1 males mated to C_1 females) and the resulting F1 offspring were pooled.

Due to logistical reasons, the assay was performed over three generations, with two D-C pairs and their resulting F1 hybrids tested in both environments during each generation (generation 72: $D_1/C_1/F1_{D1\text{-}C1}$ and $D_4/C_4/F1_{D4\text{-}C4}$; generation 75: $D_2/C_2/F1_{D2\text{-}C2}$ and $D_5/C_5/F1_{D5\text{-}C5}$; generation 76: $D_3/C_3/F1_{D3\text{-}C3}$ and $D_6/C_6/F1_{D6\text{-}C6}$). The environments mirrored the selection treatments used during experimental evolution, involving flies of the same age (day 12) and held under the same conditions. For each of the three phenotypes (D, C, and F1), exactly 1000 males and 1000 females were desiccated in the desiccation environment and approximately 250 males and 250 females were mildly starved in the control environment. When 75% mortality had occurred in the F1 population in the desiccation environment, desiccation/starvation stress was removed for all flies in each environment. All survivors were then supplied with food and live yeast for two days in their cages, again matching the normal maintenance during experimental evolution. Any additional deaths occurring during this time were recorded. Surviving females (to a maximum of 100) were collected using light CO_2 anesthesia and then transferred to standard 8-dram food vials and allowed to oviposit for 14-16 h. Adult progeny were counted 12 days later.

I calculated the mean fitness of each phenotype (D, C, and F1) in each environment (desiccation and control) as the product of the average fecundity of surviving females of that phenotype in that environment and their observed probability of surviving in that environment. As with CHCs, results were analyzed using a non-additive mixed linear model for factorial randomized complete block designs (Newman *et al.* 1997; Quinn and Keough 2002) in which phenotype (D versus C) and environment (desiccation versus control) were fixed effects and D-C pair (and associated F1; 1-6) was a random block effect. This analysis recognizes that each D-C pair, along with the F1 hybrids between them, represents a single, evolutionary replicate. In this model, the phenotype×environment interaction is of particular interest because it tests whether the fitness of the phenotypes varies between the two environments. Given a significant phenotype×environment interaction, the effect of phenotype was subsequently tested separately within each environment (removing all environment terms from the model), followed by post hoc pairwise comparisons employing sequential Bonferroni (Rice 1989) and FDR (Benjamini and Hochberg 1995) correction for multiple tests. Because both approaches gave the same results, only the former are presented. All models were fit using maximum likelihood implemented by the 'mixed' procedure in SAS version 9.1 (SAS Institute Cary, NC).

RESULTS

Response to Selection of Cuticular Hydrocarbons

After 69 generations of experimental evolution, CHCs diverged between the desiccation and control populations (Table 3). In females, the relative concentrations of the eight longest chain-length CHCs were significantly higher in the D than C populations, along with one other short chain-length CHC (Table 3; Fig. 1A). This is significantly more than would be expected by chance alone (binomial probability of nine or more success from 30 trials with $\hat{p} = 0.05$; $P < 0.001$), and this does not account for the clustering of significant effects in the long chain-length CHCs nor the consistency in effect direction (D > C for all nine significant comparison). Six of these effects remain significant after a conservative sequential Bonferroni correction for multiple comparisons (Fig. 1A). In males, the relative concentration of seven CHCs differed significantly between the D and C populations (Table 3). In contrast to females, D males had lower relative concentrations of all seven of these CHCs compared to C males, and they tended to be concentrated among the shorter chain-length CHCs (Table 3; Fig. 1B). Although none of these differences remained significant after a conservative sequential Bonferroni correction, seven out of 27 traits showing significant effects is more than would be expected by chance alone (binomial probability with $\hat{p} = 0.05$; $P < 0.001$), and this again does not account for the clustering of significant effects in short chain-length CHCs nor the consistency in effect direction (C > D for all seven significant comparison).

Differences in CHC expressions in response to desiccation stress (i.e., phenotypic plasticity) were less pronounced, with significant effects being detected in four CHCs in females and only one CHC in males (Table 3). None of these remained significant after correction for multiple comparisons. In females, there was no clear pattern in chain-length and effect sizes (difference in mean logcontrast CHC values, D – C ± SE; CHC_6 = -0.05 ± 0.03; CHC_{17} = -0.11 ± 0.03; CHC_{18} = -0.08 ± 0.05; CHC_{27} = -0.05 ± 0.02) tended to be smaller than that of the evolved differences (Fig. 1A) in this sex. Non-stressed individuals, however, expressed higher relative concentrations as compared to stressed individuals in all cases for both males and females. The interaction between phenotype and treatment was significant in only one CHC in the two sexes (i.e., CHC_9 in females; Table 3) and significance was lost after correction for multiple comparisons, providing little indication that the plastic response to desiccation stress diverged between the D and C populations.

Test for Premating Isolation: Mating Trials

After 57 generations of experimental evolution, assortative mating scores (*Y*) between the D and C populations did not differ significantly from zero overall ($t_5 = 0.53$, $P = 0.618$; Fig. 2), indicating the absence of any premating isolation. Mean *Y* also did not differ significantly from zero within any of the six specific mating combinations (Table 1). Although combination 2 approached significance, mean *Y* was less than zero in this case, indicating a weak tendency towards negative assortative mating.

Treatment effects were evident, however, in the relative mating success of both females and males. D females achieved more matings than C females in all six mating combinations (Fig. 3), a difference that is significant overall ($t_5 = 4.01$, $P = 0.010$). This higher mating success of D than C females was significant in two of the six specific mating combinations (Table 1) and was irrespective of the type of male involved ($p = 0.57$ and 0.56 overall with D and C males, respectively). In males, the opposite pattern was evident in that C males achieved significantly more matings than D males in all six mating combinations (Fig. 3), a difference that is also significant overall ($t_5 = -5.63$, $P = 0.003$). This increased mating success of C males was significant in five of the six specific mating combinations (Table 1) and was also irrespective of the type of female involved ($p = 0.64$ and 0.66 overall with D and C males, respectively).

Female Mating Rates – In five of the six D-C pairs, D females were quicker on average than C females to mate individually with LH_M males (Fig. 4), a difference that was significant overall ($t_5 = -2.14$, $P_{\text{one-tailed}} = 0.043$). The shorter time to mating for D compared to C females was significant for two of the specific population pairs (Fig. 4).

Male Inbreeding Depression – Mating trials in which the males were outbred F1 crosses between populations within their respective selection treatments produced qualitatively similar results to the previous mating trials. Mean Y did not differ from zero in either specific combination (Table 2) and the point estimates fell within the previously observed range (Table 1), indicating that assortative mating between treatments remained absent when males were outbred. The mating success of D females also remained high relative to C females, with the difference being significant in one of the two mating combinations (Table 2). F1 males from the desiccation treatment again achieved fewer matings than F1 males from the control treatment, with the difference being significant in both mating combinations (Table 2).

Test for Postmating Isolation: Reciprocal Transplant

Mean fitness, estimated as the average fecundity of females multiplied by their observed probability of surviving, showed a significant phenotype×environment interaction ($F_{2,10}$ = 123.30, $P < 0.001$), indicating that the fitness of the phenotypes (D, C, and F1) was environment-dependent. Main effects of environment ($F_{1,5}$ = 138.27, $P < 0.001$) and phenotype ($F_{2,10}$ = 151.78, $P < 0.001$) were also significant. The phenotype effect remained significant when the analyses were performed separately in each environment (control environment: $F_{2,10}$ = 21.90, $P < 0.001$; desiccation environment: $F_{2,10}$ = 243.86, $P < 0.001$).

In the desiccation environment, D's outperformed F1 hybrids, which outperformed C's (Fig. 5). All three pairwise combinations of these phenotypes differed significantly in post-hoc multiple comparisons after sequential Bonferroni correction for multiple tests (Rice 1989), indicating that F1 hybrid fitness was reduced relative to the native D phenotype (i.e., the evolution of postmating isolation). The rank order of fitness (D > F1 > C) is also consistent with ecologically-dependent postmating isolation, demonstrating that performance increased with phenotypic similarity to the D populations. Results differed in the control environment, however, with F1's performing best and little difference between C's and D's (Fig. 5). In a post-hoc multiple comparisons, the only significant difference was that F1's outperformed C's. Hybrid fitness therefore did not differ significantly from the native D phenotype and the point estimate was higher, providing no indication of any postmating isolation. Evidence for divergent selection was also lacking, with D's performing as well as C's in the control environment, suggesting that adaptation to the desiccation environment came at no cost in terms of performance in the control environment.

DISCUSSION

Despite empirical demonstrations from both nature and the laboratory that ecological speciation can occur, our understanding of the details of the process is incomplete. For example, the relative importance of different forms of reproductive isolation is poorly understood, as are the sources of divergent selection generating them. In addition, conditions that promote or hamper ecological speciation have received limited attention, even in cases of 'failed' ecological speciation in the laboratory. Here, I took advantage of a long-term desiccation-selection experiment in *Drosophila melanogaster* to study the potential evolution of reproductive isolation as a by-product of adaptation. First, I demonstrated the evolutionary divergence of these populations in a number of cuticular hydrocarbons (CHCs), traits previously implicated in both desiccation resistance and mating behaviour in *D. melanogaster*. Second, I conducted direct tests for the evolution of both pre- and postmating isolation. These tests, however, provided little evidence of the early stages of ecological speciation: premating isolation was absent and postmating isolation was asymmetrical, present in one environment but not in the other. I discuss the implications of these results below.

Evolutionary Divergence of Cuticular Hydrocarbons

CHCs have been previously implicated to play an important role in desiccation resistance in *Drosophila* and other insects (e.g., Toolson and Kuper-Simbron 1989; Rouault *et al.* 2001) and patterns of CHC variation in nature support this (e.g, Gibbs 1998; Rouault *et al.* 2001; Ferveur 2005). I am only aware of a single study, however, in which an evolutionary manipulation has been used to provide a direct test of the relationship between CHC expression and desiccation resistance. Gibbs *et al.* (1997) selected replicate populations of *D. melanogaster* for desiccation resistance for more than 100 generations using a protocol similar to the one adopted here, and found that selected flies had higher relative concentrations of longer chain-length CHCs compared to control flies, and that females had longer mean chain-lengths than males. Consistent with this, lipid melting temperatures were higher in selected than control flies, and in females than males. Sample sizes were small, however, and resolution was limited because CHCs were pooled by carbon number (chain-length), yielding only six composite traits (CHCs with 21-31 carbon atoms, odd numbers only).

Here, I extracted CHCs from more than 1000 individual D and C flies that were exposed to both stressed and non-stressed treatments on day 12 of age, corresponding to the day that the flies were desiccated/starved during experimental evolution. Compared to the evolved responses, plastic changes in CHCs in response to desiccation stress involved fewer CHCs, were smaller in magnitude, and tended to involve different traits (Table 3). Whether such changes are adaptive (i.e., increased desiccation resistance) is unknown, although the lack of correspondence between evolved differences and plastic responses suggests otherwise. The degree to which plastic changes may depend on the timing of the environmental manipulation versus trait measurement, and the degree of desiccation stress (which was less than that experienced during experimental evolution out of necessity of not killing control flies) remains to be determined.

After 69 generations of experimental evolution, evolved responses in a number of CHCs were observed, with little evidence of any interaction with plastic responses (CHC_9 in females was the only peak showing a significant phenotype×treatment interaction; Table 3). In females, adaptation to desiccation resulted in relatively greater amounts of long chain-length CHCs in D's compared to C's, whereas in males it involved relatively lesser amounts of shorter chain-length CHCs in D's compared to C's (Table 3; Fig. 1). The correspondence with the results of Gibbs *et al.* (1997) is remarkable: in their experiment, desiccation-adapted females had higher relative concentrations of the four longest chain-length groups (C25-C31), with two of these being significant (C25 and C29), whereas desiccation-adapted males had significantly lower relative concentrations of the two shortest chain-length groups (C21 and C23).

The evolution of higher relative concentrations of long chain-length CHCs in females is consistent with the proposed mechanistic link in which longer chain-length hydrocarbons provide a better barrier to water loss because they melt at higher temperatures (Gibbs *et al.* 1997; Gibbs 1998; Ferveur 2005). D females must survive the desiccation stress to ensure their fitness, generating strong natural selection for stress resistant traits (e.g., body size; Kwan *et al.* 2008). The absence of significant increases in relative concentration of long chain-length CHCs in males may be the product of weak selection: few males survive desiccation stress and their fitness is therefore gained posthumously via matings that occur prior to selection (Kwan *et al.* 2008). This requires that the genetic correlation between the sexes for long chain-length CHCs is low to reduce any indirect response in males to changes in females (some evidence of a response in males is apparent in the four longest chain-length CHCs; Fig. 1B). Genetic factors affecting CHCs in *D. melanogaster* have been identified using a variety of approaches and tend to be sex-specific (Foley *et al.* 2007), although as far as I am aware, no direct estimates of the intersexual genetic correlations exist for shared

CHCs. In *D. serrata*, the genetic correlations between the longest CHCs tend to be weak to moderately positive (Chenoweth *et al.* 2008).

The evolutionary mechanism generating lower relative concentrations of short chain-length CHCs in D males is less clear, although the consistent occurrence in replicate populations in the current experiment and that of Gibbs *et al.* (1997) implies a selective influence of some sort. It is possible that shorter chain-length CHCs may serve an unknown, but potentially important role in desiccation resistance, although this would be inconsistent with the proposed mechanistic link between CHC chain-length and water loss. Alternatively, in theory, changes in male CHCs could have arisen as an indirect effect of increases in long chain-length CHCs in females if a negative genetic correlation exists between these traits. Such a correlation seems unlikely given the apparent lack of strong intersexual genetic correlations between CHCs of similar chain-length. Finally, differences in sexual selection between desiccation and control environments could be responsible. As noted previously, males in the desiccation treatment rarely survive selection and therefore must mate prior to this to ensure their fitness. All else being equal, a D male's mating success early in life may therefore be a more important component of his lifetime fitness than a C male's, thus generating stronger sexual selection on the underlying traits that affect mating success in D than C males. Previous studies have found that one of the short chain-length CHCs in *D. melanogaster*, 7-trioscene (7-T,23C, identified as 7:23C in Folely *et al.* 2007), prevents or reduces male homosexual courtship (Ferveur and Sureau 1996; Sureau and Ferveur 1999; Svetec and Ferveur 2005) and affects female receptivity and mating behaviour (Grillet *et al.* 2006). Although CHC_7 in this experiment appears to correspond with the retention time of 7:23C in Folely *et al.* (2007), no evolved differences were detected (Table 3; Fig. 1B).

However, the relative concentrations of CHC_9 and CHC_{10}, both located very close to CHC_7 (Fig. S2) and 7:23C (Foley *et al.* 2007), were significantly lower in D than C males (Table 3; Fig. 1B). Interestingly, Grillet *et al.* (2006) found that females mated faster and more often with males with larger amounts of 7:23C, opposite to expectation if early-life mating success is under stronger sexual selection in D than C males. In addition, D males in the mating trials achieved significantly fewer matings than C males overall (Fig. 3), also inconsistent with the evolution of more attractive D males. A weakening of sexual selection on males via the evolution of less choosy D females could explain both results, and I return to this possibility later.

The Evolution of Reproductive Isolation

Reproductive isolation has been shown to evolve as a by-product of adaptation in a number of laboratory speciation experiments (Kilias *et al.* 1980; Dodd 1989; Dettman *et al.* 2007; Dettman *et al.* 2008), demonstrating that the initial stages of ecological speciation can occur quite rapidly. In contrast, there was little evidence of any reproductive isolation between the D and C populations in the current experiment. Multiple-choice tests provided no indication of any premating isolation in the form of assortative mating (Table 1; Fig. 2), and reciprocal transplant experiments revealed asymmetrical postmating isolation in which F1 hybrid fitness was reduced relative to the D populations in the desiccation environment, but was higher than the C populations in the control environment (Fig. 5). Asymmetrical postmating isolation represents a weak barrier to gene flow because even low levels of migration from a D into C population would be unopposed by selection, preventing the evolutionary divergence of the populations.

Two general explanations exist for the absence of reproductive isolation in laboratory speciation experiments. First, populations may not have adapted to their different environments, preventing speciation from occurring as a side effect. Tests for adaptation are sometimes lacking in past experiments (e.g., Rundle 2003) and when conducted, have provided a likely explanation for the failure to observe any reproductive isolation (e.g., Mooers *et al.* 1999). The absence of such tests hampers interpretation of negative results because this possibility cannot be addressed. This is not the case, however, in the present experiment. Adaptation to the novel desiccation environment was extensive in these populations, generating a 68% increase in survival time under arid conditions relative to the control populations, and involved divergence in life-history (e.g., extended development and faster larval growth) and morphology (e.g., 20% increase in body size) between the D and C populations (Kwan *et al.* 2008), as well as a number of CHCs (Table 3; Fig. 1).

The second explanation for the absence of reproductive isolation is that adaptation occurred in a manner that did not produce reproductive isolation as a by-product. Whether reproductive isolation evolves likely depends on a range of factors, including the form and intensity of selection, duration of the experiment, genetic basis of the traits underlying selection, and effective population sizes (Ödeen and Florin 2000; Florin and Ödeen 2002; Nosil *et al.* 2009). Evaluating the relative roles of these factors is an important goal for laboratory speciation experiments. In this case, the experiment involved population sizes and a duration similar or greater than many past studies that have found reproductive isolation. Adaptation to desiccation also involved changes in a diverse set of traits (e.g., pheromones, body size, life-history), increasing the chance that reproductive isolation would evolve as a by-product (e.g., multifarious divergent selection; Rice and Hostert 1993; Nosil *et al.* 2009).

The form of selection, however, stands out here as a potentially key factor affecting the likelihood of speciation. Ecological speciation occurs when reproductive isolation evolves as a result of ecologically-based divergent selection, or in other words, when selection acts in contrasting directions on populations inhabiting different environments or exploiting alternative niches (Schluter 2000, 2001; Rundle and Nosil 2005). The extensive adaptation to the desiccation environment in this experiment, however, appears to have come at no cost to performance in the control environment, revealing the presence of directional but not divergent selection. The absence of a cost to adaptation can arise if increases in fitness in one environment produce a correlated increase in fitness in another environment (Whitlock 1996; Bell 1997; Kassen 2002). In this experiment, the application of desiccation stress necessitated the removal of any food source (since food also acts as a water source). The appropriate control environment for isolating the effects of desiccation (thereby, permitting an investigation of the physiological basis of adaptation to this particular stress – the original impetus behind this experiment) was therefore to provide access to water via a non-nutritional agar plate, but not food. This induced a starvation stress that, although seldom caused death, may have generated selection via variation among individuals in their subsequent fecundity or mating success (experiments with the LH_M population have shown that female fecundity is strongly dependent on their consumption of live yeast; Morrow *et al.* 2005). If the genetic basis of resistance to desiccation and starvation stress is largely shared, adaptation to desiccation may have increased fitness in the control environment as a correlated response. Consistent with this, after 37 generations of experimental evolution, the

C populations were significantly better able to resist desiccation stress than the ancestors (L. Kwan, unpublished data). The greater desiccation resistance of the D than C populations is then explained by much stronger selection in the former than the latter environment. The apparent higher fitness of F1 hybrids in the control environment may have been caused by heterosis.

Despite the absence of premating isolation, there is nevertheless some evidence of divergence in mating behaviour. Females from all six D populations achieved more matings than did C females, irrespective of the type of male involved (Table 1; Fig. 3), suggesting the evolution of either faster choosing or less discriminating D females. A no-choice mating rate assay employing standard LH_M males directly confirmed this (Fig. 4). The evolution of faster mating females in the D populations is likely an adaptation to the selection protocol. Given that very few D males survive desiccation, D females must ensure that they acquire sufficient quantities of sperm prior to selection to maximize their post-selection fecundity. This is not an issue for C females, however, because virtually all C males survive starvation stress. If D females achieved high mating rates by becoming less discriminating of males, this could have weakened sexual selection on male CHCs, causing the evolution of decreased expression of short chain-length CHCs in males.

Opposite to the pattern in females, males from all six D populations achieved fewer matings than C males, again irrespective of the type of female involved (Table 1; Fig. 3). There are two potential explanations for the evolution of lower overall mating success of D males. First, despite controlling for census population sizes during experimental evolution, strong selection for desiccation resistance may have reduced the effective population size of the D populations more than C populations, generating inbreeding depression. Inbreeding depression in male mating success has been previously reported in *D. melanogaster* (Averhoff and Richardson; 1974; Rundle *et al.* 1998). The fact that D females outperformed C females suggests that inbreeding depression in the desiccation treatment was not extensive, although it is possible that male mating success is more sensitive to inbreeding depression than female mating success, or that the evolution of less choosy D females is also a manifestation of inbreeding depression of female mate choice. Inconsistent with the inbreeding explanation, however, is the fact that outbred D males remained less successful than outbred C males at achieving matings with both D and C females (Table 2).

The second explanation for the lower overall mating success of D males is the evolution of unattractive males due to sexual conflict. Sexual conflict arises from differences in the evolutionary interests of males and females (Parker 1979). Since few D males survive selection and mating is not observed during selection (L. Kwan, unpublished data; Kwan *et al.* 2008; Chippindale *et al.* 1998), the sexes may have dissimilar fitness interests before desiccation stress: early maturation and mating in males versus resource acquisition to maximize survival and later reproduction in females. Strong selection on stress resistant traits in D females may have caused correlated responses in D males that lowered their mating success, in effect pulling males off their sexual selection fitness peak via intralocus sexual conflict (Rice and Chippindale 2001). It is therefore possible that adaptation to the desiccation environment may have had a cost in terms of lower male mating success. The shared trait(s) reducing male mating success and lying at the centre of such a potential conflict are unknown, although body size is one candidate. Whatever the cause, a reduced mating success of D males would hamper speciation by increasing the mating success of immigrant C males.

Chapter 3

Conclusions

In my thesis, I investigated ecological speciation via the by-product mechanism in replicate populations of *Drosophila melanogaster* selected for desiccation resistance for more than 57 generations. First, I explored whether cuticular hydrocarbons (CHCs) have diverged in response to selection. Interesting patterns were revealed in both female and male CHCs. In females, D females had higher relative concentrations of long chain-length CHCs than C females, which likely serves as a barrier to water loss since long chain-length CHCs melt at higher temperatures. On the other hand, D males had lower relative concentration of short chain-length CHCs than C males. The evolutionary mechanism for this pattern is unknown, although it is possible that the evolution of less choosey D females has weakened sexual selection on male CHCs.

Second, I directly tested for the evolution of pre- and postmating isolation. Premating isolation would be indicated by assortative mating by treatment, which was tested using a multiple-choice assay. Despite responses to selection in a number of traits, assortative mating was absent between D and C individuals. Postmating isolation would be indicated by reductions in hybrid fitness, which can be ecologically-independent and/or ecologically-dependent. I employed a reciprocal transplant assay, in which the fitness of D, F1, and C individuals in both desiccation and control environment was measured. Results indicate that postmating isolation was asymmetrical: reduced hybrid fitness and the predicted rank order of fitness were observed in the desiccation environment, but not in the control environment.

The absence of divergent selection (D and C individuals were equally fit in the control environment) likely explains this pattern.

Despite the absence of reproductive isolation, there was a divergence in mating behaviours. D females had a higher mating success than C females, which likely results from being less choosey at pre-selection. D males, on the other hand, had a lower mating success than C males, which cannot explained by inbreeding depression. It is possible for intralocus sexual conflict to play a role here, where strong selection for stress resistant traits in D females causes correlated responses in D males that render them less attractive.

Speciation has been extensively studied in the laboratory, although few experiments have addressed the evolution of pre- and postmating isolation and controlled for the effects of genetic drift. In my thesis, I explored the role of ecology in the evolution of reproductive isolation, while addressing both of these factors, in *D. melanogaster* selected for desiccation resistance. Despite divergence in various traits, including CHCs, premating isolation was absent between populations from the two treatments and postmating isolation was asymmetrical, constituting a weak barrier to gene flow. Although novel environments are generally thought to promote reproductive isolation, the likelihood of reproductive isolation depends on number of additional factors aside from environmental differences and/or ecological interactions. The ease at which ecological speciation occurs may be overestimated. Among the missing gaps, future speciation research should focus on understanding the conditions that promote or hamper reproductive isolation.

Table 1. Results of multiple-choice mating trials between six unique combinations of the D and C populations. For each combination, the assortative mating score (*Y*) and the relative mating success of D versus C individuals of each sex (*p*) were evaluated using a one-sample *t*-test (*t*), treating cages as replicates.

			Assortative mating			Relative mating success					
						D females			D males		
Combination	Females	Males	*Y* (SE)	*t*	P^a	*p* (SE)	*t*	P^a	*p* (SE)	*t*	P^a
1	D_1, C_1	D_2, C_2	0.15 (0.10)	1.61	0.152	0.51 (0.05)	0.25	0.807	0.42 (0.04)	-2.00	0.085
2	D_2, C_2	D_3, C_3	-0.11 (0.05)	-2.28	0.056	0.59 (0.03)	3.21	0.015*	0.39 (0.04)	-2.93	0.022*
3	D_3, C_3	D_1, C_1	0.10 (0.10)	1.09	0.313	0.55 (0.03)	1.49	0.180	0.40 (0.02)	-5.09	0.001*
4	D_4, C_4	D_5, C_5	0.00 (0.08)	-0.05	0.961	0.54 (0.03)	1.77	0.120	0.24 (0.03)	-8.40	< 0.001*
5	D_5, C_5	D_6, C_6	-0.08 (0.12)	-0.68	0.518	0.62 (0.03)	4.33	0.003*	0.33 (0.04)	-4.63	0.002*
6	D_6, C_6	D_4, C_4	0.07 (0.06)	1.14	0.292	0.56 (0.03)	2.08	0.076	0.33 (0.05)	-3.53	0.010*

[a] *df* = 7 in all cases

*Significant at $P < 0.05$

Table 2. Results of multiple-choice mating trials involving outbred F1 males created by matings between populations within the desiccation and control treatments. For each combination, the assortative mating score (Y) and the relative mating success of D versus C females and $F1_D$ versus $F1_C$ males (p) were evaluated using a one-sample *t*-test (t), treating cages as replicates.

			Assortative mating			Relative mating success					
						D females			$F1_D$ males		
Combination	Females	Males	Y (SE)	t	P^a	p (SE)	t	P^a	p (SE)	t	P^a
1	D_2, C_2	$F1_{D1\text{-}D3}$, $F1_{C1\text{-}C3}$	-0.00 (0.05)	-0.04	0.966	0.56 (0.04)	1.59	0.156	0.43 (0.02)	-3.86	0.006*
2	D_5, C_5	$F1_{D4\text{-}D6}$, $F1_{C4\text{-}C6}$	0.14 (0.07)	1.90	0.100	0.62 (0.02)	5.05	0.002*	0.43 (0.03)	-2.43	0.045*

[a] *df* = 7 in all cases

*Significant at $P < 0.05$

Table 3. Results of mixed linear models testing for differences in CHCs between the D and C populations (phenotype), stressed and non-stressed (treatment), and their interaction. In females, 31 traits were identified with CHC_{11} used as the common divisor in calculating logcontrasts. In males, 28 traits were identified with CHC_5 used as the common divisor in calculating logcontrasts. The chemical identities of female and male CHCs do not correspond numerically (see Figs. S1 and S2).

	Females						Males					
	Phenotype		Treatment		Phenotype×treatment		Phenotype		Treatment		Phenotype×treatment	
CHC	*F*	P^a	*F*	P^a	*F*	P^a	*F*	P^a	*F*	P^a	*F*	P^a
1	0.08	0.794	0.02	0.897	1.92	0.224	7.31	0.043*	0.21	0.668	2.91	0.149
2	0.93	0.379	0.02	0.900	2.77	0.157	5.16	0.072	0.14	0.724	3.37	0.126
3	11.01	0.021*	1.82	0.236	2.11	0.206	4.33	0.092	2.89	0.150	0.09	0.773
4	0.16	0.708	0.05	0.828	1.17	0.328	25.51	0.004*	0.06	0.821	2.65	0.165
5	5.28	0.070	0.11	0.755	1.56	0.267						
6	1.74	0.245	7.56	0.040*	4.60	0.085	1.53	0.271	1.61	0.260	< 0.01	0.967
7	2.62	0.166	0.06	0.822	1.39	0.292	2.98	0.145	1.15	0.333	3.25	0.131
8	0.78	0.418	0.13	0.733	1.93	0.223	7.43	0.042*	0.17	0.695	0.31	0.604

9	5.52	0.066	0.17	0.701	8.66	0.032*	9.72	0.026*	2.72	0.160	3.29	0.130
10	1.26	0.313	0.19	0.680	0.05	0.838	27.47	0.003*	0.04	0.841	0.51	0.508
11							1.40	0.290	2.39	0.183	2.25	0.194
12	0.71	0.438	1.41	0.289	0.92	0.381	17.52	0.009*	0.22	0.661	0.90	0.386
13	2.08	0.209	< 0.01	0.996	3.50	0.120	4.51	0.087	1.67	0.253	1.74	0.244
14	2.86	0.152	0.63	0.464	4.16	0.097	10.47	0.023*	8.27	0.035*	4.87	0.078
15	1.35	0.298	0.67	0.451	0.22	0.659	3.21	0.133	1.42	0.286	4.89	0.078
16	0.88	0.392	4.39	0.090	1.71	0.248	0.02	0.897	1.69	0.250	1.59	0.263
17	0.80	0.412	9.59	0.027*	1.52	0.272	0.08	0.788	3.52	0.120	1.60	0.261
18	4.84	0.079	8.39	0.034*	1.18	0.326	0.39	0.559	1.58	0.264	1.70	0.249
19	4.08	0.100	3.74	0.111	1.55	0.268	1.39	0.291	2.36	0.185	1.94	0.222
20	1.41	0.288	2.11	0.206	1.47	0.279	0.98	0.367	4.54	0.087	2.34	0.187
21	3.66	0.114	0.57	0.484	0.25	0.636	0.15	0.713	1.15	0.332	1.25	0.315
22	2.87	0.151	0.19	0.678	0.13	0.731	0.34	0.585	1.26	0.313	1.24	0.316
23	2.86	0.152	2.56	0.171	0.02	0.900	0.25	0.636	1.47	0.280	1.58	0.264
24	9.53	0.027*	5.31	0.070	1.51	0.274	0.05	0.829	2.23	0.195	2.04	0.212

25	46.39	0.001*	4.07	0.100	0.01	0.909	2.53	0.173	2.16	0.201	2.11	0.206
26	7.29	0.043*	1.60	0.261	0.05	0.829	4.23	0.095	1.58	0.265	1.69	0.250
27	42.62	0.001*	7.33	0.042*	0.03	0.863	1.47	0.280	1.65	0.256	1.45	0.282
28	54.22	0.001*	1.81	0.236	0.20	0.673	6.06	0.057	1.86	0.231	1.39	0.291
29	69.48	< 0.001*	4.53	0.087	0.05	0.829						
30	35.62	0.002*	3.25	0.131	1.81	0.236						
31	39.40	0.002*	1.69	0.250	0.20	0.674						

[a] *df* = 1,5 in all cases

*Significant at $P < 0.05$

Figure 1. Changes in logcontrast CHC expression between D and C females (A) and males (B) for all CHCs (Table 3). More positive values indicate higher mean relative concentrations of that CHC in D than in C individuals. Asterisks denote specific CHCs in which expressions between D and C individuals differ significantly after sequential Bonferroni correction for multiple tests. Error bars are ± 1 SE.

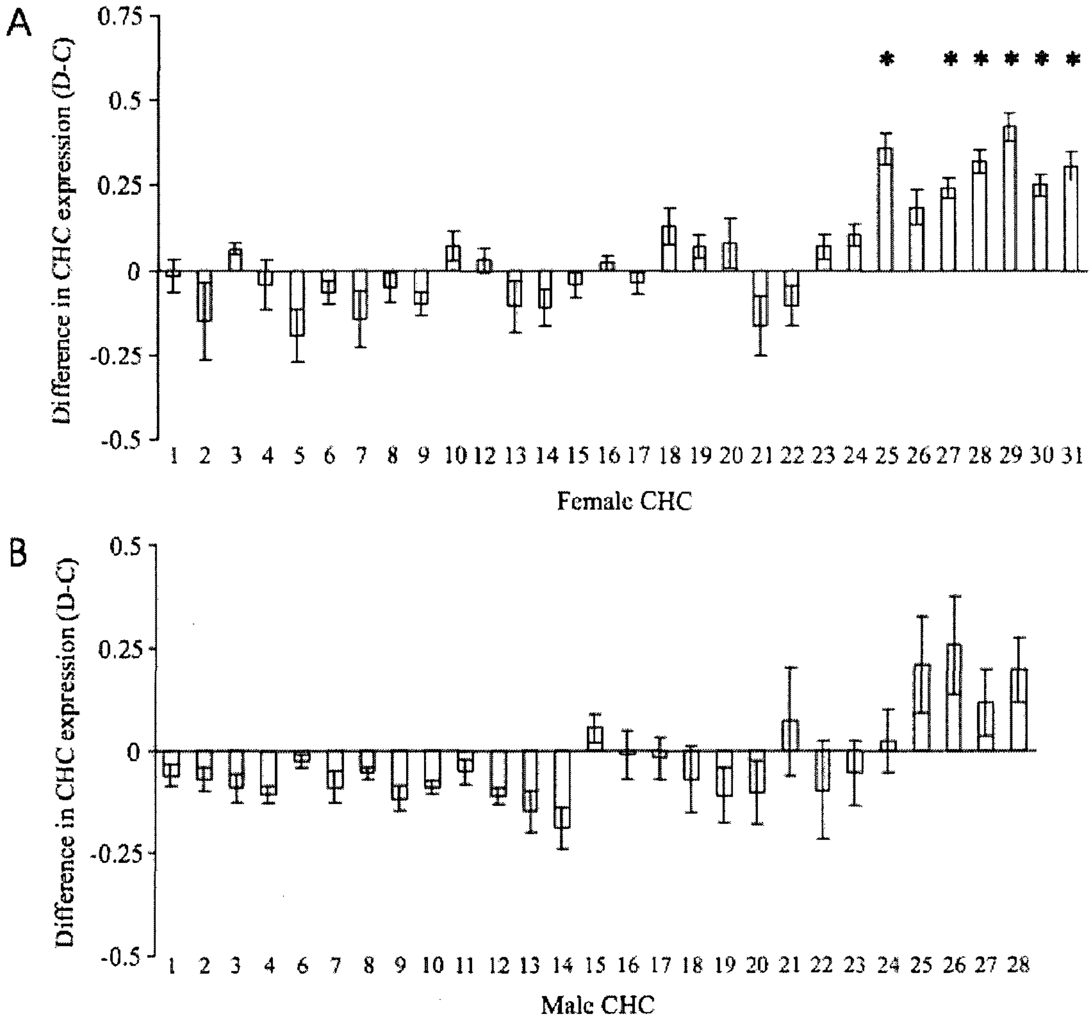


Figure 2. Assortative mating scores (*Y*) between the D and C populations. *Y* can vary between -1 (indicating perfect negative assortative mating) and +1 (indicating perfect positive assortative mating), with zero indicating random mating. Mating combinations are given in Table 1. Error bars are ± 1 SE.

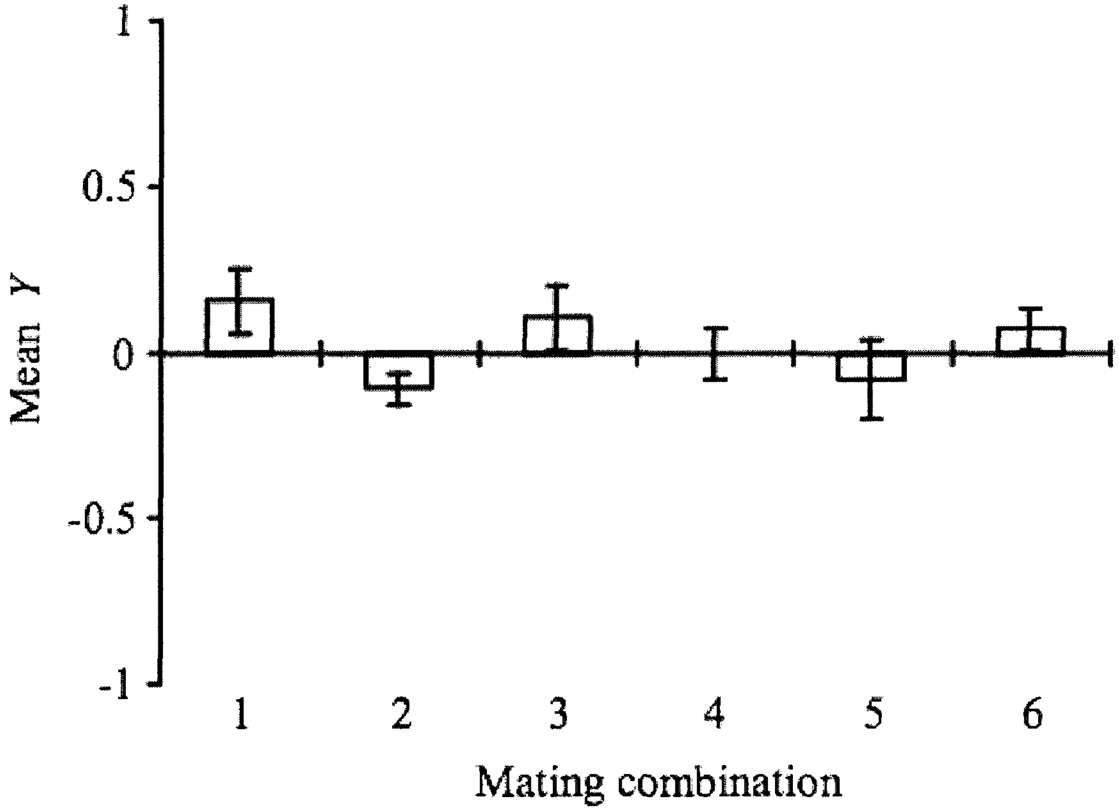


Figure 3. Mean proportion of total matings achieved by D females (filled bars) and males (open bars). A proportion of 0.5 indicates equal mating success of D and C individuals of that sex. Mating combinations are given in Table 1. Error bars are ± 1 SE.

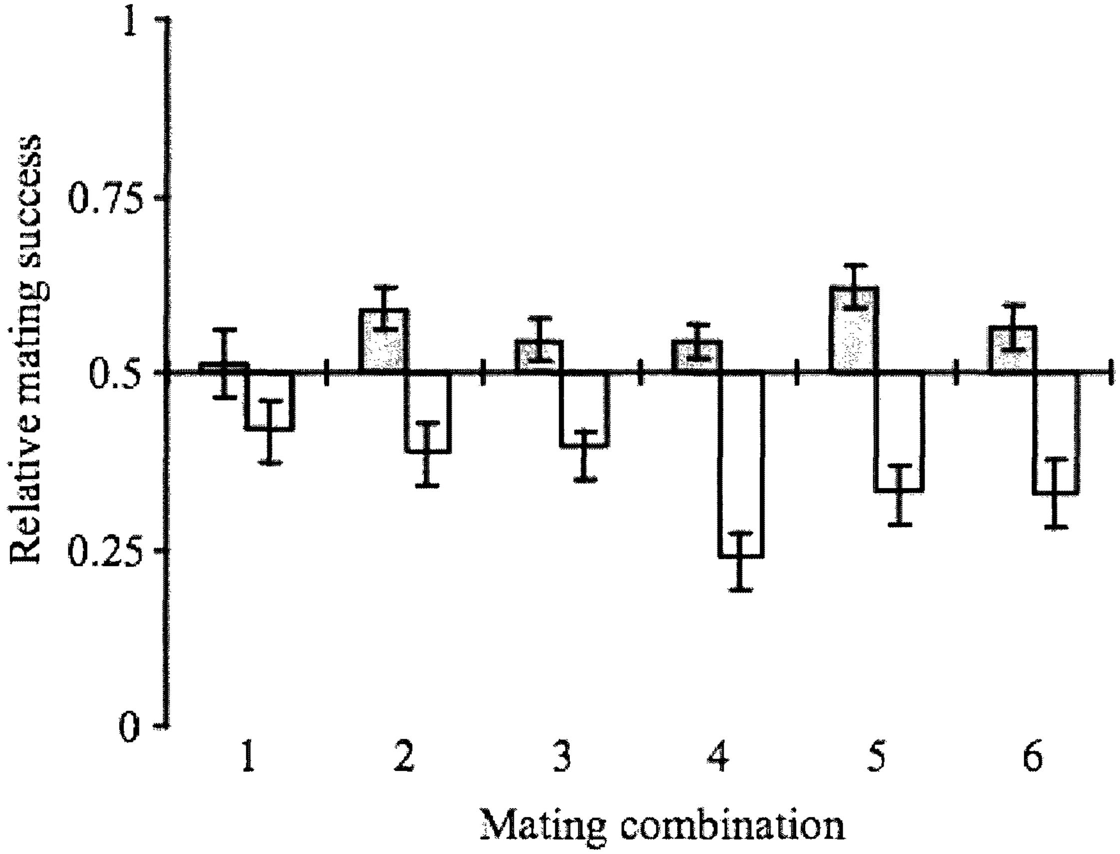


Figure 4. Time to mating of D (filled) and C (open) females when presented with ancestral LH_M males. Asterisks denote specific population pairs for which time to mating differs significantly in a two-sample *t*-test ($P < 0.05$, one-tailed). Error bars are ± 1 SE.

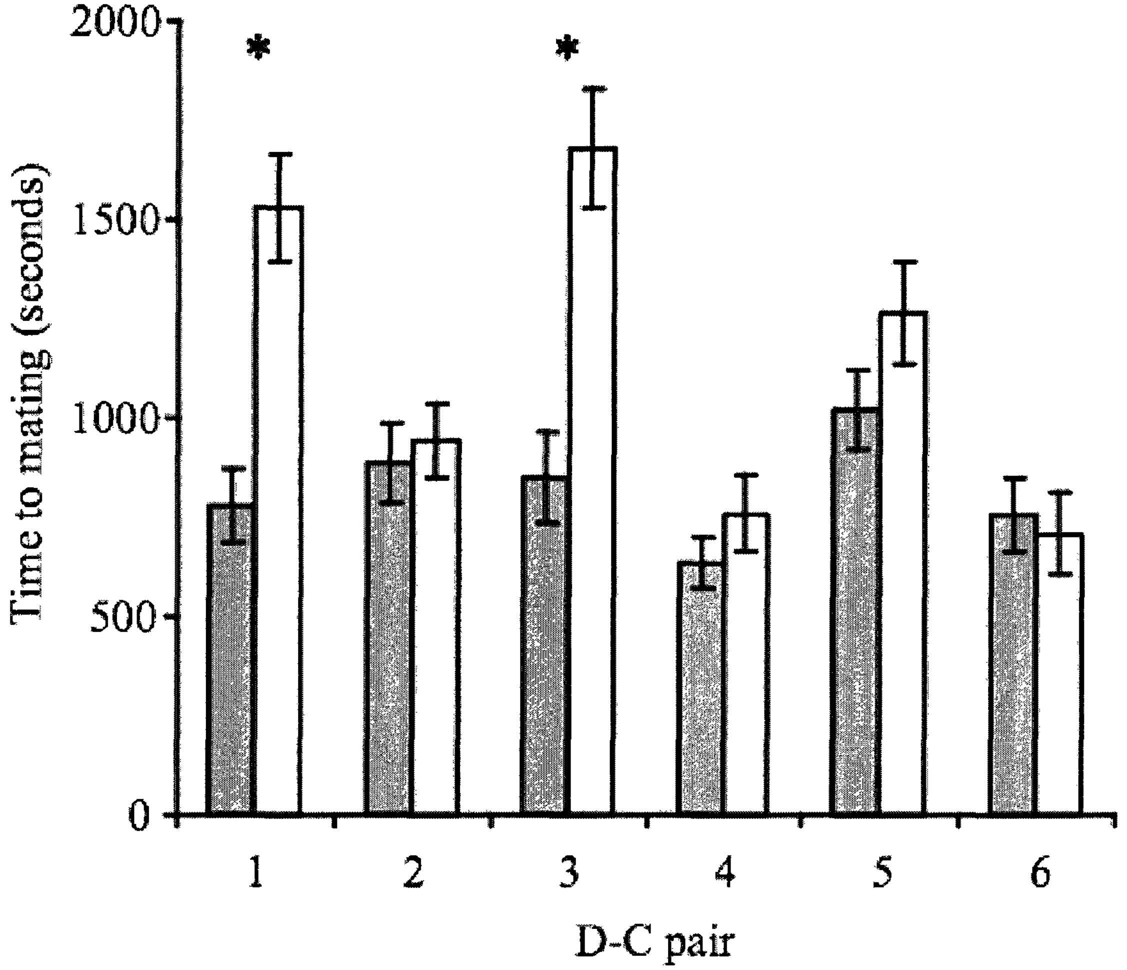
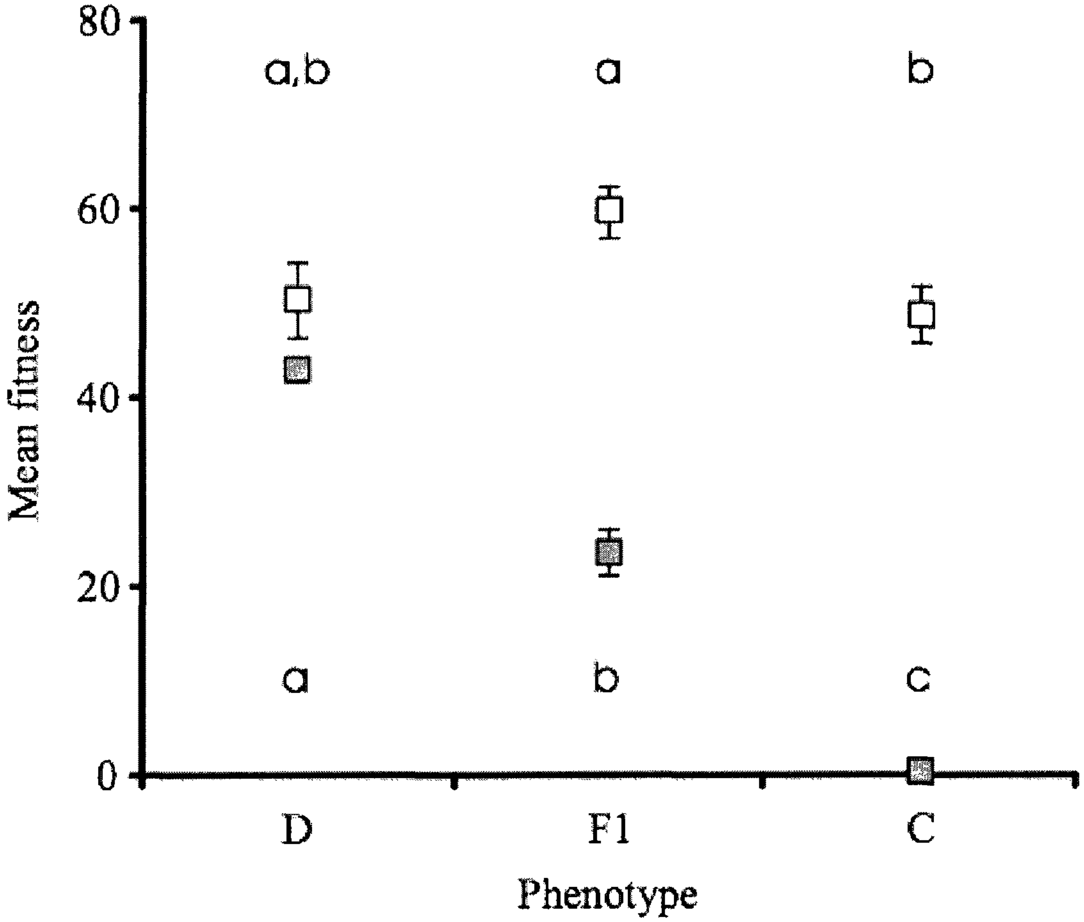


Figure 5. Mean fitness (probability of survival×fecundity) of various phenotypes when raised in the desiccation (filled squares) and control (open squares) environments. Within each environment, phenotypes with different letters were significantly different in post hoc comparisons after sequential Bonferroni correction for multiple tests. Error bars are ± 1 SE.



Supplementary Information

Table S1. Results of multiple-choice mating trials between unique combinations of red and blue individuals for the premating isolation and male inbreeding depression assays. For each assay, the assortative mating score (*Y*) and the relative mating success of red versus blue individuals of each sex (*p*) were evaluated using a single overall one-sample *t*-test (*t*), treating specific mating combinations as replicates.

	Assortative mating		Relative mating success			
			Red females		Red males	
Assay	t	P^a	t	P^a	t	P^a
Premating isolation	-1.11	0.316	-2.15	0.084	-1.26	0.262
Male inbreeding depression	-0.23	0.856	0.31	0.810	-1.67	0.344

[a] $df = 5$ in premating isolation; $df = 1$ in male inbreeding depression

Table S2. Results of multiple-choice mating trials between unique combinations of red and blue individuals for the premating isolation and male inbreeding depression assays. For each mating combination, the assortative mating score (*Y*) and the relative mating success of red versus blue individuals of each sex (*p*) were evaluated using a one-sample *t*-test (*t*), treating cages as replicates.

		Assortative mating			Relative mating success					
					Red females			Red males		
Assay	Combination	*Y* (SE)	*t*	P^a	*p* (SE)	*t*	P^a	*p* (SE)	*t*	P^a
Premating isolation	1	-0.16 (0.10)	-1.61	0.151	0.39 (0.03)	-3.80	0.007*	0.53 (0.05)	0.56	0.593
	2	-0.02 (0.06)	-0.33	0.748	0.47 (0.04)	-0.70	0.504	0.51 (0.06)	0.18	0.863
	3	0.16 (0.08)	1.88	0.102	0.48 (0.03)	-0.75	0.478	0.47 (0.04)	-0.82	0.440
	4	-0.15 (0.06)	-2.70	0.031*	0.46 (0.03)	-1.31	0.233	0.45 (0.10)	-0.48	0.647
	5	-0.07 (0.12)	-0.59	0.573	0.49 (0.05)	-0.22	0.831	0.50 (0.07)	0.05	0.959
	6	-0.07 (0.06)	-1.22	0.263	0.51 (0.04)	0.14	0.891	0.41 (0.07)	-1.25	0.252
Male inbreeding depression	1	0.07 (0.04)	1.93	0.094	0.56 (0.04)	1.59	0.156	0.49 (0.03)	-0.31	0.763
	2	-0.11 (0.08)	-1.39	0.206	0.47 (0.05)	-0.65	0.537	0.50 (0.04)	-0.07	0.949

[a] *df* = 7 in all cases

*Significant at $P < 0.05$

Figure S1. CHC profile of a typical female.

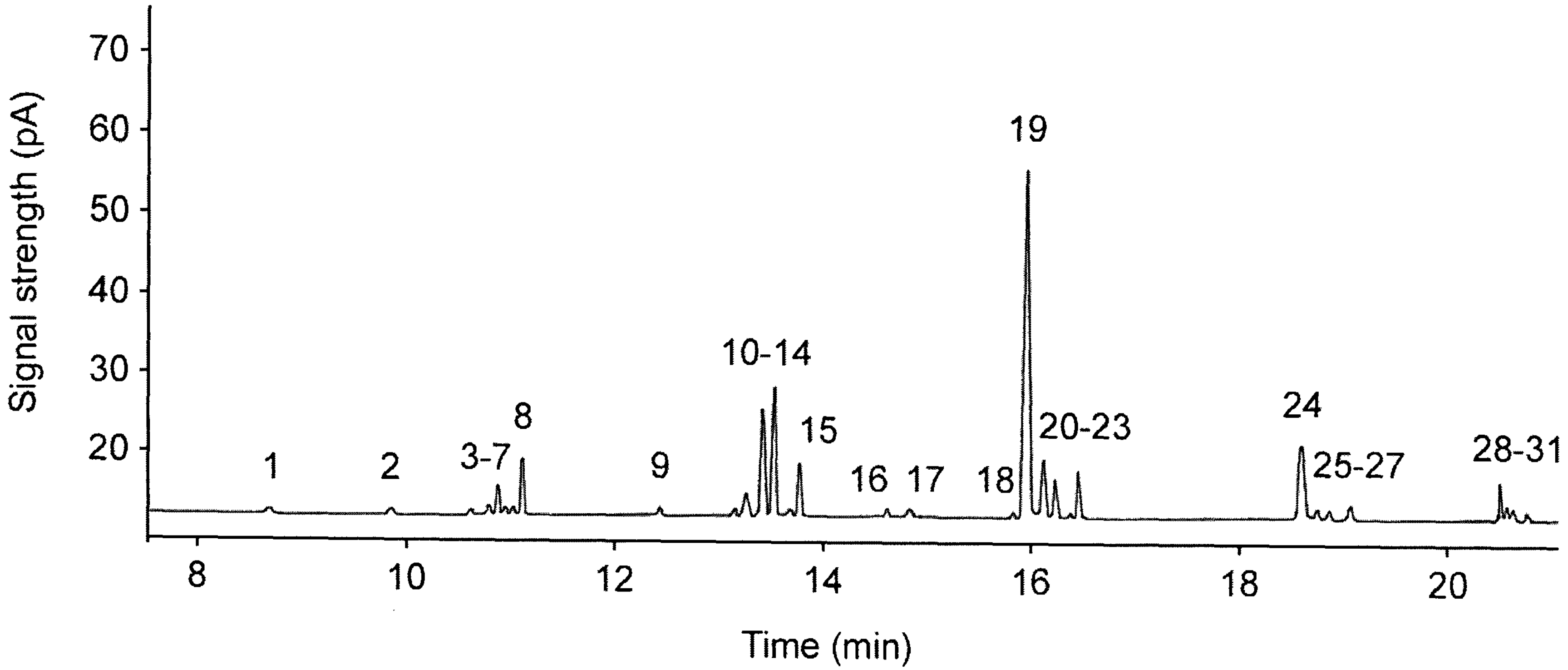
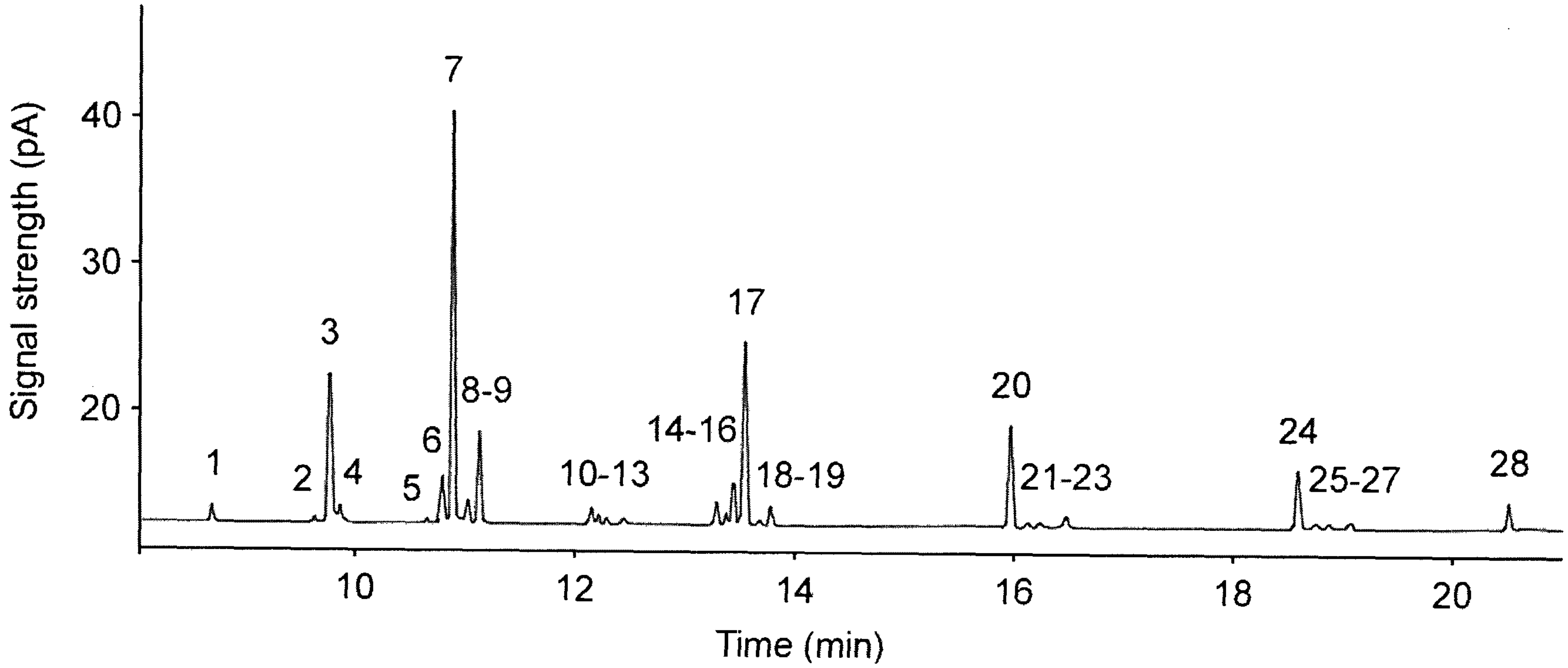


Figure S2. CHC profile of a typical male.



LITERATURE CITED

Aitchison, J. 1986. *The Statistical Analysis of Compositional Data.* Chapman and Hall, London.

Averhoff, W.W. and Richardson, R.H. 1974. Pheromonal control of mating patterns in *Drosophila melanogaster. Behavior Genetics* **4**: 207-225.

Bell, G.A.C. 1997. Experimental evolution in *Chlamydomonas.* I. Short-term selection for uniform and diverse environments. *Heredity* **78**: 490-497.

Benjamini, Y. and Hochberg, Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society **B*** 57: 289-300.

Bishop, Y.M.M., Fienberg, S.E. and Holland, P.W. 1975. *Discrete Multivariate Analysis: Theory and Practice.* MIT Press, Cambridge, Massachusetts.

Blair, W.F. 1955. Mating call and stage of speciation in the *Microhyla olivacea – M. carolinensis* complex. *Evolution* **9**: 469-480.

Blows, M.W. and Allan, R. A. 1998. Levels of mate recognition within and between two *Drosophila* species and their hybrids. *American Naturalist* **152**: 826-837.

Chenoweth, S.F. and Blows, M.W. 2003. Signal trait sexual dimorphism and mutual sexual selection in *Drosophila serrata. Evolution* **57**: 2326-2334.

Chenoweth, S.F. and Blows, M.W. 2005. Contrasting mutual sexual selection on homologous signal traits in *Drosophila serrata. American Naturalist* **165**: 281-289.

Chenoweth, S.F., Rundle, H.D. and Blows, M.W. 2008. Genetic constraints and the evolution of display trait sexual dimorphism by natural and sexual selection. *American Naturalist* **171**: 22-34.

Chippindale, A.K., Gibbs, A.G., Sheik, M., Yee, K.J., Djawdan, M., Bradley, T.J. and Rose, M.R. 1998. Resource acquisition and the evolution of stress resistance in *Drosophila melanogaster. Evolution* **52**: 1342-1352.

Chippindale, A.K. and Rice, W.R. 2001. Y chromosome polymorphism is a strong determinant of male fitness in *Drosophila melanogaster. Proceedings of the National Academy of Sciences (USA)* **98**: 5677-5682.

Coyne, J.A. 1996. Genetics of differences in pheromonal hydrocarbons between *Drosophila melanogaster* and *D. simulans. Genetics* **143**: 353-364.

Coyne, J.A. and Orr, H.A. 2004. *Speciation.* Sinauer, Sunderland, Massachusetts.

Coyne, J.A., Elwyn, S. and Rolán-Alvarez, E. 2005. Impact of experimental design on *Drosophila* sexual isolation studies: Direct effects and comparison to field hybridization data. *Evolution* **59**: 2588-2601.

Darwin, C. 1859. *On the Origin of Species by Means of Natural Selection on the Preservation of Favored Races in the Struggle for Life*. J. Murray, London.

Dettman, J.R., Sirjusingh, C., Kohn, L.M. and Anderson, J.B. 2007. Incipient speciation by divergent adaptation and antagonistic epitasis in yeast. *Nature* **447**: 585-588.

Dettman, J.R., Anderson, J.B. and Kohn, L.M. 2008. Divergent adaptation promotes reproductive isolation among experimental populations of the filamentous fungus *Neurospora*. *BMC Evolutionary Biology* **8**: 35.

Dobzhansky, T. 1937. *Genetics and the Origin of Species.* 1st ed. Columbia University Press, New York.

Dobzhansky, T. 1940. Speciation as a stage in evolutionary divergence. *American Naturalist* **74**: 302-321.

Dobzhansky, T. 1951. *Genetics and the Origin of Species.* 3rd ed. Columbia University Press, New York.

Dodd, D.M.B. 1989. Reproductive isolation as a consequence of adaptive divergence in *Drosophila pseudoobscura. Evolution* **43**: 1308-1311.

Doebeli, M. 1996. A quantitative genetic competition model for sympatric speciation. *Journal of Evolutionary Biology* **9**: 898-909.

Endler, J.A. 1977. *Geographic Variation, Speciation, and Clines.* Princeton University Press, New Jersey.

Fang, S., Takahashi, A. and Wu, C-I. 2002. A mutation in the promoter of desaturase 2 is correlated with sexual isolation between Drosophila behavioral races. *Genetics* **162**: 781-784.

Ferveur, J-F. and Sureau, G. 1996. Simultaneous influence on male courtship of stimulatory and inhibitory pheromones produced by live sex-mosaic *Drosophila melanogaster. Proceedings of the Royal Society of London B* **263**: 967-973.

Ferveur, J-F., Savarit, F., O'Kane, C.J., Sureau, G., Greenspan, R.J. and Jallon, J-M. 1997. Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males. *Science* **276**: 1555-1558.

Ferveur, J-F. 2005. Cuticular hydrocarbons: Their evolution and roles in *Drosophila* pheromonal communication. *Behavior Genetics* **35**: 279-295.

Fisher, R.A. 1930. *The Genetical Theory of Natural Selection.* Oxford University Press, Oxford.

Florin, A-B. and Ödeen, A. 2002. Laboratory environments are not conducive for allopatric speciation. *Journal of Evolutionary Biology* **15**: 10-19.

Foley, B., Chenoweth, S.F., Nuzhdin, S.V. and Blows, M.R. 2007. Natural genetic variation in cuticular hydrocarbon expression in male and female *Drosophila melanogaster. Genetics* **175**: 1465-1477.

Funk, D.J. 1998. Isolating a role for natural selection in speciation: Host adaptation and sexual isolation in *Neochlamisus bebianea* leaf beetles. *Evolution* **52**: 1744-1759.

Gibbs, A.G., Chippindale, A.K. and Rose, M.R. 1997. Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster. Journal of Experimental Biology* **200**: 1821-1832.

Gibbs, A.G. 1998. Water-proofing properties of cuticular lipids. *American Zoologist* **38**: 471-482.

Grant, P.R. 1986. *Ecology and Evolution of Darwin's Finches.* Princeton University Press, New Jersey.

Grillet, M., Dartevelle, L. and Ferveur, J-F. 2006. A *Drosophila* male pheromone affects female sexual receptivity. *Proceedings of the Royal Society of London B* **273**: 315-323.

Hatfield, T. and Schluter, D. 1999. Ecological speciation in sticklebacks: Environment-dependent hybrid fitness. *Evolution* **53**: 866-873.

Higgie, M., Chenoweth, S. and Blows, M.W. 2000. Natural selection and the reinforcement of mate recognition. *Science* **290**: 519-521.

Higgie, M. and Blows, M.W. 2007. Are traits that experience reinforcement also under sexual selection? *American Naturalist* **170**: 409-420.

Hoffmann, A.A. and Parsons, P.A. 1989. An integrated approach to environmental stress tolerance and life-history variation: Desiccation tolerance in *Drosophila. Biological Journal of the Linnean* Society **37**: 117-136.

Holland, B. and Rice, W.R. 1998. Chase-away selection: antagonistic selection versus resistance. *Evolution* **52**: 1-7.

Howard, R.W. and Blomquist, G.J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology* **50**: 371-393.

Hurd, L.E. and Eisenberg, R.M. 1975. Divergent selection for geotactic response and the evolution of reproductive isolation in sympatric and allopatric populations of houseflies. *American Naturalist* **109**: 353-358.

Jallon, J-M. 1984. A few chemical words exchanged by *Drosophila* during courtship and mating. *Behavior Genetics* **14**: 441-478.

Kassen, R. 2002. The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* **15**: 173-190.

Kent, C., Azanchi, R., Smith, B., Formosa, A. and Levine, J.D. 2008. Social context influences chemical communication in *D. melanogaster* males. *Current Biology* **18**: 1384-1389.

Kilias, G., Alahiotis, S.N. and Pelecanos, M. 1980. A multifactorial genetic investigation of speciation theory using *Drosophila melanogaster*. *Evolution* **34**: 730-737.

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. *Evolution* **36**: 1-12.

Kirkpatrick, M. and Ravigné, V. 2002. Speciation by natural and sexual selection: Models and experiments. *American Naturalist* **159**: S22-S35.

Krupp, J.J., Kent, C., Billeter, J.C., Azanchi, R., So, A.K.C., Schonfeld, J.A., Smith, B.P., Lucas, C. and Levine, J.D. 2008. Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. *Current Biology* **18**: 1373-1383.

Kwan, L., Bedhomme, S., Prasad, N.G. and Chippindale, A.K. 2008. Sexual conflict and environmental change: Tradeoffs within and between the sexes during the evolution of desiccation resistance. *Journal of Genetics* **87**: 383-394.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Sciences (USA)* **78**: 3721-3725.

Markow, T.A. and Toolson, E.C. 1990. Temperature effects on epicuticular hydrocarbons and sexual isolation in *Drosophila mojavensis*. In: *Ecological and Evolutionary Genetics of Drosophila* (Barker, J.S.F. ed.), pp. 315-331, Plenum Press, New York.

Mayr, E. 1942. *Systematics and the Origin of Species*. Columbia University Press, New York.

McKinnon, J.S., Mori, S., Blackman, B.K., David, L., Kingsley, D.M., Jamieson, L., Chou, J. and Schluter, D. 2004. Evidence for ecology's role in speciation. *Nature* **429**: 294-298.

Mooers, A. Ø., Rundle, H.D. and Whitlock, M.C. 1999. The effects of selection and bottlenecks on male mating success in peripheral isolates. *American Naturalist* **153**: 437-444.

Morrow, E.H., Steward, A.D. and Rice, W.R. 2005. Patterns of sperm precedence are not affected by female mating history in *Drosophila melanogaster*. *Evolution* **59**: 2608-2615.

Muller, H.J. 1942. Isolating mechanisms, evolution, and temperature. *Biological Symposiums* **6**: 71-125.

Nelson, D.R. 1993. Methyl-branched lipids in insects. In: *Insect Lipids: Chemistry, Biochemistry and Biology*. (Stanley-Samuelson, D.W., Nelson, D.R. eds), pp 271-315, University of Nebraska Press, Lincoln.

Newman, J.A., Bergelson, J. and Grafen, A. 1997. Blocking factors and hypothesis tests in ecology: Is your statistics text wrong? *Ecology* **78**: 1312-1320.

Nosil, P., Crespi, B.J. and Sandoval, C.P. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* **417**: 440-443.

Nosil, P., Vines, T.H. and Funk, D.J. 2005. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution* **59**: 705-719.

Nosil, P., Harmon, L.J. and Seehausen, O. 2009. Ecological explanations for (incomplete) speciation. 2009. *TRENDS in Ecology and Evolution* **24**: 145-156.

Ödeen, A. and Florin, A-B. 2000. Effective population size may limit the power of laboratory experiments to demonstrate sympatric and parapatric speciation. *Proceedings of the Royal Society of London B* **267**: 601-606.

Parker, G. 1979. Sexual selection and sexual conflict. In: *Sexual Selection and Reproductive Competition in Insects* (Blum, M. S., Blum, N. A. eds), pp. 123-166, Academic Press, New York.

Petfield, D., Chenoweth, S.F., Rundle, H.D. and Blows, M.W. 2005. Genetic variance in female condition predicts indirect genetic variances in male sexual display traits. *Proceedings of the National Academy of Sciences (USA)* **102**: 6045-6050.

Podos, J. 2001. Correlated evolution of morphology and vocal signal signature in Darwin's finches. *Nature* **409** : 185-188.

Quinn, G.P. and Keough, M.J. 2002. *Experimental Design and Data Analysis for Biologists.* Cambridge University Press, New York.

Ratcliffe, L.M. and Grant, P.R. 1983. Species recognition in Darwin's finches (*Geospiza*, Gould). I. Discrimination by morphological cues. *Animal Behaviour* **31**: 1139-1153.

Rice, W.R. and Salt, G.W. 1988. Speciation via disruptive selection on habitat preference: Experimental evidence. *American Naturalist* **131**: 911-917.

Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223-225.

Rice, W.R. and Salt, G.W. 1990. The evolution of reproductive isolation as a correlated character under sympatric conditions: Experimental evidence. *Evolution* **44**: 1140-1152.

Rice, W.R. and Hostert, E.E. 1993. Laboratory experiments on speciation: What have we learned in 40 years? *Evolution* **47**: 1637-1653.

Rice, W.R. 1998. Intergenomic conflict, interlocus antagonistic coevolution, and the evolution of reproductive isolation. In: *Endless Forms: Species and Speciation* (Howard, D.J., Berlocher, S.H. eds.), pp. 261-270, Oxford University Press, New York.

Rice, W.R. and Chippindale, A.K. 2001. Intersexual ontogenetic conflict. *Journal of Evolutionary Biology* **14**: 685-696.

Rouault, J, Capy, P. and Jallon, J-M. 2001. Variations of male cuticular hydrocarbons with geoclimatic variables: An adaptative mechanism in *Drosophila melanogaster*? *Genetica* **110**: 117-130.

Rundle, H.D., Mooers, A.Ø. and Whitlock, M.C. 1998. Single founder-flush events and the evolution of reproductive isolation. *Evolution* **52**: 1850-1855

Rundle, H.D., Nagel, L., Boughman, J.W. and Schluter, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287**: 306-308.

Rundle, H.D. and Whitlock, M.C. 2001. A genetic interpretation of ecologically dependent isolation. *Evolution* **55**: 198-201.

Rundle, H.D. 2002. A test of ecologically dependent postmating isolation between sympatric sticklebacks. *Evolution* **56**: 322-329.

Rundle, H.D. 2003. Divergent environments and population bottlenecks fails to generate premating isolation in *Drosophila pseudoobscura. Evolution* **57**: 2557-2565.

Rundle, H.D. and Nosil, P. 2005. Ecological speciation. *Ecology Letters* **8**: 336-352.

Schluter, D. and Nagel, L. 1995. Parallel speciation by natural selection. *American Naturalist* **146**: 292-301.

Schluter, D. 2000. *The Ecology of Adaptive Radiation.* Oxford University Press, New York.

Schluter, D. 2001. Ecology and the origin of species. *TRENDS in Ecology and Evolution* **16**: 372-380.

Servedio, M.R. and Noor, M.A.F. 2003. The role of reinforcement in speciation: Theory and data. *Annual Reviews of Ecology, Evolution, and Systematics* **34**: 339-364.

Soans, A.B., Pimentel, D. and Soans, J.S. 1974. Evolution of reproductive isolation in allopatric and sympatric populations. *American Naturalist* **108**: 117-124.

Sureau, G. and Ferveur, J-F. 1999. Co-adaptation of pheromone production and behavioural responses in *Drosophila melanogaster* males. *Genetical Research* **74**: 129-137.

Svetec, N. and Fervuer, J-F. 2005. Social experience and pheromonal perception can change male-male interactions in *Drosophila melanogaster. Journal of Experimental Biology* **208**: 891-898.

Toolson, E.C. and Kuper-Simbron, R. 1989. Laboratory evolution of epicuticular hydrocarbon composition and cuticular permeability in *Drosophila pseudoobscura*: Effects on sexual dimorphism and thermal-acclimation ability? *Evolution* **43**: 468-473.

Whitlock, M.C. 1996. The red queen beats jack-of-all-trades: The limitations on the evolution of phenotypic plasticity and niche breadth. *American Naturalist* **48**: S67-S77.

Wright, S. 1940. The statistical consequences of Mendelian heredity in relation to speciation. In: *The New Systematics* (Huxley, J.S. ed.), pp. 161-183, Oxford University Press, Oxford.