Diet, adaptation and the evolution of assortative mating in *Ceratitis capitata*

A thesis submitted to the School of Biological Sciences of the University of East Anglia for the degree of Doctor of Philosophy

By William John Nash

September 2015

Abstract

The action of natural selection in establishing barriers to gene flow between populations, or reproductive isolation, is increasingly understood to be a primary driver of speciation and thus biodiversity. 'Ecological speciation' is now supported by evidence from numerous studies in a range of natural populations. However, experimental tests of the role of divergent natural selection in the establishment of reproductive isolation are still scant. To address this omission, the role of larval diet in imposing divergent selection and causing ecological adaptation and reproductive isolation was tested. These tests were conducted on the Mediterranean fruit fly (*Ceratitis capitata*, Wiedemann) (medfly) model system, which has been relatively under-utilised in the experimental study of speciation. Using manipulative experiments and experimental evolution, the three major components of ecological speciation were examined. Firstly a source of divergent selection was established through quantification of the consequences of alteration in specific dietary nutrients during the development of medfly larvae. Following this, similar selective pressures were used as the basis of experimental evolution of medfly populations reared on divergent developmental diets. Divergence between these populations was assayed at several time points during evolution, in real time, using tests for sexual isolation. After 60 generations of experimental evolution a form of reproductive isolation between populations had evolved. The mechanism that may have led to the evolution of this isolation was also explored, through further mating tests, and also the quantification of male courtship behaviour. The genetic basis of the phenotypes associated with adaptation and sexual isolation was explored using transcriptomic sequencing and differential expression analysis of genes expressed in males from the two experimental regimes. A range of candidate genes was identified as differentially expressed, including genes associated with oxidative phosphorylation and chemosensation. Taken together, the results of this research present a novel example of how divergent ecological selection pressure can lead to the evolution of sexual isolation in experimental populations.

Acknowledgements

I owe an immense debt of gratitude to Tracey Chapman. Her skill as an evolutionary biologist and as a supervisor has guided my hand in the writing of this thesis on a multitude of levels. Beyond this, as a colleague and friend, the support, inspiration, and patience Tracey has shown me during this project have been invaluable. I also thank Matt Gage who, as a co-supervisor, has offered great insight and similar support for the duration of this work. To both, I am also grateful for the convening of a group of researchers who have enabled the germination of the thoughts tied up in this document. I am also particularly grateful to Irina Mohorianu, who has shown great patience in teaching me several of the key techniques used in this work. I have greatly enjoyed our collaboration, and look forward to its continuation.

The importance of the research group that I have had the privilege of working with at UEA is immeasurable. Of those who chose to study a slightly more sophisticated breed of fly, I thank first Phil Leftwich, who welcomed me to research at UEA, and has been a great mentor ever since. Also, those others to be found stalking the CEF, Mike, Lucy, and Naomi, I have enjoyed growing flies with you a lot. Past members of the group, Amanda, Claudia, Dom, and Alyson, offered me great inspiration when I first came to Norwich. Those who are still here, Damian, Wayne, Emily, Liz, Janet, Jessie, Ram, Jo, and Kris, continue to do so. The support and friendship offered by all of you during this project have really motivated its completion.

I feel that had I of been writing these thanks from another department or institution, my departmentally centred mentions may have been addressed by now. It is an extreme privilege not to be constrained in such a way, and to have been able to work in such an academically excellent and social department at UEA. I must offer thanks to those who have been present since I arrived, particularly Lewis, for countless excellent conversations (sober or not), Conrad, who is without doubt the most passionate entomohpile in existence (sober or not), and David, for combining the two qualities above in one exemplary package. Also to Amy, Tim, Alicia, Kitson, Dave W, Christiana, Karl, Cat, and Cata, thanks for making studying at UEA enjoyable from day one. To those who have joined during my time at UEA, thank you for continuing in this vein, Danni, Sarah, Liam, and Pierre (all office mates par excellence), Tom, Becca, Kat, Ellie, Claire, Ellen, Phil, Dave P, and Jamie, you have all been valued friends and colleagues. I specifically thank Iain Barr, who has offered me numerous opportunities to take science outside for a change. I have learnt a great deal in your tuition, and through your friendship.

It is commonly said that all work and no play make Will a dull boy. I have been truly lucky to have a skilled team of practitioners ensuring there has been a fair modicum of play whilst this thesis has been under construction. The bastion of truth and freethinking that 71 Pembroke Rd has come to represent is notable by its members. Ol, you've been the bestest bud imaginable and be it listening to records, mulling things over on the front step, or when watching Battlestar, you really have inspired this work. Iain, you're the most erudite dude I've ever met. I'll always strive to care about all matters, academic or not, as much as you do. Edwin, although your time with us was short, its impact was great. Never has a man worn a dressing gown with such

dignity. Phil, I can't communicate how glad I am you came to Norwich. The opportunity to continue on from Silwood has been more fun than I could of asked for, thanks for being my mate. Thanks must also go to Holly, Peter, and Jenny; you've been the greatest pals.

The foundation for the completion of this thesis came from my parents, Bill and Liz, who have supported me in every endeavour, ever since I started to have endeavours. The privilege of an upbringing in an environment where apologising to trees which you had damaged was the norm couldn't have done more to foster a deep connection with biology. Thank you for getting me here. Also, thanks go to my sister, Jo, who has always been the closest of friends, and recently an inspiration in her success. My final thanks go to my partner, Emily; who's kindness and support have made the writing of this thesis a pleasure, where it could have been tortuous. Your continued positivity and excitement at the world are infectious, and I'm indebted to you in so many ways.

Contents

List of tables

List of figures

Author Contributions

All parts of this thesis have been written by Will Nash (WJN) under the supervision of Prof Tracey Chapman (TC). At the time of submission, Chapter 2 of this thesis has been published and Chapter 3 forms the basis of a manuscript in revision following first review. Here the citation for the published work is provided as well as a description to the key author contributors to each chapter.

Chapter 2

Published as:

> Nash, W. J., and T. Chapman. 2014. Effect of dietary components on larval life history characteristics in the medfly (Ceratitis capitata: Diptera, Tephritidae). PLoS One 9:e86029.

WJN designed this study in consultation with TC and carried out all experimental work, analysed all the data, wrote the paper and was submitting and corresponding author on the manuscript.

Chapter 3

The research in this chapter was prepared as a manuscript under the Chapter title and submitted to *Evolution* in June 2015. It is currently in revision. This chapter represents a collaborative effort between WJN, TC, Dr Phil Leftwich (PL) and Dr L. Friend (LF). The estimated contributions are:

- TC provided conceptual guidance for all stages of this study and extensive comments on the manuscript.
- PL initiated the experimental evolution (EE) lines used in the study, in collaboration with TC. He designed the mating tests used at 5 and 30 generations of EE and conducted the experimental work in those generations collaboration with LF. He also commented on the manuscript.
- LF assisted with the experimental work conducted in generation 30. She also commented on the manuscript.
- WJN designed and conducted the generation 60 mating tests described in this chapter and conducted all of the analysis for all of the mating tests. He wrote the manuscript and was submitting and corresponding author.
- PL, LF and WJN all contributed to the up keep of the EE populations.

Chapter 4

This work in this chapter involved collaboration between WJN, TC, PL, LF and Dr Irina Mohorianu (IM). The estimated contributions are:

- TC provided conceptual guidance for all stages.
- LF assisted PL in conducting the experimental work described in this chapter and provided the flies for the behavioural analyses conducted by WJN.
- WJN designed and tested the experiment described in this chapter. He conducted all of video analysis and all statistical analyses. He will be lead author of the chapter as it progresses to publication.
- IM designed the normalised frequency distribution analysis used in this chapter and consulted with WJN extensively on the analysis.

Chapter 5

The concept of this chapter was a collaboration between WJN and TC. All experimental work was conducted by WJN in consultation with Dr Damian Smith. The novel Bioinformatics approaches used were designed by IM and were implemented by WJN under her guidance. All bioinformatics data analysis was conducted by WJN. WJN, IM, and TC collaborated to produce the manuscript that forms this chapter.

1 General Introduction.

The research described in this thesis investigated the evolutionary significance and consequences of ecological adaptation to different dietary environments. It first identified the selective pressures that can be imposed by qualitative and quantitative variation in nutrients during the developmental period. It then utilised similar selective pressures to create divergent selection between experimental populations. Population divergence based on this dietary selective pressure was then measured by testing for sexual isolation. Associated divergence in mating behaviour between populations was also investigated, in order to elucidate the mechanisms of any putative reproductive isolation observed. In the introduction below, the broader context for these experimental approaches is established through a description of the major influences of nutrition on life history, the role of nutrients in ecological adaptation and finally their role in driving the evolution of sexual isolation and ecological speciation.

1.1 Ecological significance of dietary nutrients

The combination of nutrients which an organism absorbs from its diet are essential for development and influence how fitness can be maximised (Roff 1992; Stearns 1992). Organisms require a specific combination of amino acids, carbohydrates, sterols, phospholipids, fatty acids, vitamins, minerals, trace elements and water in order to realise fitness (Stearns 1992). As nutrients are not uniformly spread throughout ecosystems, they can influence the geographic distribution of many organisms and the timing of major life history events such as reproduction (Simpson and Raubenheimer 2012). Within this 'heterogeneous resource landscape', it is rare that organisms achieve the ideal balance of all nutrients required for optimal biological function. This means that the nutrients available to an organism impose allocation decisions at a physiological level. Such trade-offs constrain microevolutionary optimisation – i.e. condition-based thresholds for reproduction, growth, and reproductive performance (Stearns 1992). Indeed, the relationship between the consumption of nutrients and the realisation of fitness can be seen as 'a network of interconnected trade-offs with a global optimum' (Illius et al. 2002). Although other biotic and abiotic factors may play an important role in determining fitness (e.g. Slansky and Rodriguez 1987; Schmitz 2008) dietary nutrients represent a key driver of natural selection (Raubenheimer et al. 2009).

The trade-offs imposed by a heterogeneous resource landscape may lead organisms to exploit different food sources non-randomly in order to maximise fitness (Waldbauer et al. 1984; Simpson et al. 2004). A feature of this 'dietary self selection' is that the behaviour an organism utilises when making food choices, or foraging, is modified

through time. These decisions can be represented in currency, often energy. The maximisation of gain in such currency is termed optimal foraging. This theory aims to explain and predict the choices made by organisms when foraging and the quality of food items that are foraged (Pyke et al. 1977; Stephens and Krebs 1986). Optimal foraging models have been successfully used to describe the interactions between organisms and diet over the past 50 years (Stephens et al. 2007) and have recently been employed over large spatial scales to understand organismal distribution and movement in relation to sources of nutrition (Owen-Smith et al. 2010; van Gils et al. 2015).

Recent advances in nutritional ecology have developed a 'nutritionally explicit' framework through which to interpret the trade-offs and behaviours associated with diet (Raubenheimer et al. 2009). Optimal foraging theory reduces nutrients to a single variable, energy currency, and in doing so fails to consider which particular nutrient within the diet may be the constraining factor governing a trade off. Seeking to address the 'unidirectional' lack of detail inherent to an optimal foraging view, the geometric framework of nutrition (GF) describes the relationship between all major dietary macronutrients and the development of traits in a heterogeneous resource landscape (Simpson and Raubenheimer 1993a,b, 2007, 2012; Simpson et al. 2004, 2015; Raubenheimer et al. 2009). In considering an individual's nutritional environment as a multidimensional space with individual nutrients as its axes, the GF establishes the optimal nutritional state for that individual as a 'nutritional target'. Within the GF, the trajectory at which an individual moves through nutritional space is referred to as a 'nutritional rail' (Figure 1.1). In an environment where diet components occur in a fixed ratio, progress towards the nutritional target is made along a single rail. However, if the ratio of nutrients in the environment is imbalanced or varies, progress towards the target is achieved by altering the intake of different nutrient components (Figure 1.1c, Simpson and Raubenheimer 1993a).

The GF was first used by (Raubenheimer and Simpson 1993) to examine compensatory feeding patterns in the desert locust (*Locusta migratoria*). As a proof of concept, the heterogeneity of the nutritional landscape was reduced to two macronutrients: protein and carbohydrates. By using a carefully defined range of experimental diets of differing nutrient ratios, 19 'nutritional rails' were studied simultaneously. The consumption of both nutrients was recorded over time, enabling a two-dimensional space to be plotted that located the position of the locusts along each rail over time. This allowed the nutritional target (Figure 1.1) to be estimated, and related to the physiology of the

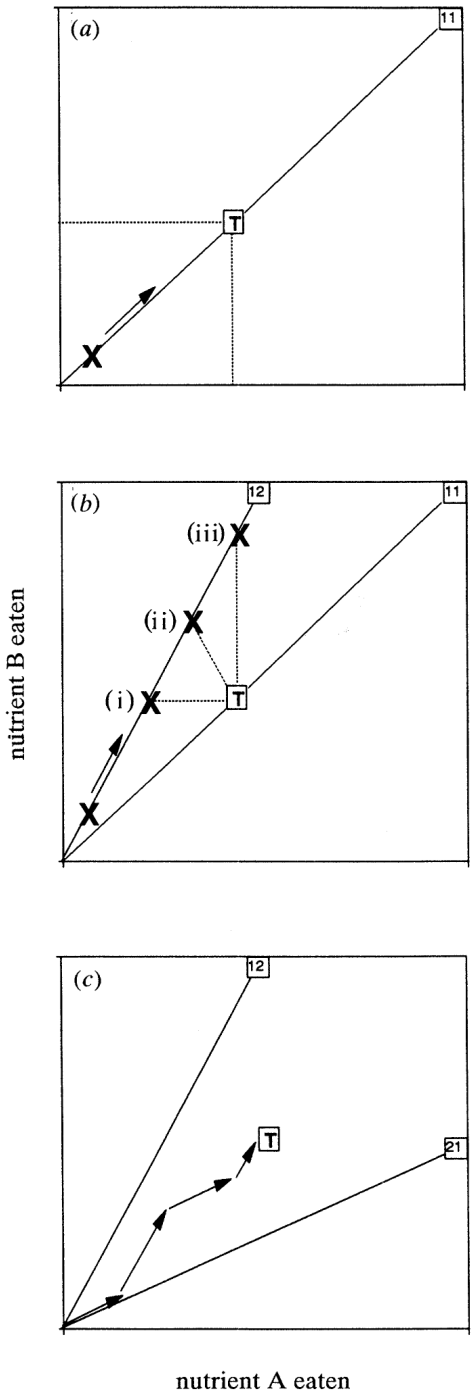

Figure 1.1. The geometric framework of nutrition. Axes represent nutritional planes for two functionally relevant nutrients, A and B. The intake target (T) lies on the 'rail' containing a 1:1 ratio of these two nutrients. X represents the current position of the organism in nutritional space. **a)** represents a food containing the optimum ratio of the two nutrients; the organism can reach T by moving directly along the rail. **b)** represents a food with a 2:1 ratio of the two nutrients, hence the organism cannot reach T. Three alternative strategies are shown are shown i) the organism can move along the rail until it reaches the target for B and suffers a shortfall of A, ii) it could eat until the target for A is achieved and thus over consume B, iii) it could eat until a intermediate pint is reach between the two. **c)** represents an organism with a choice between two foods, of 1:2 and 2:1 ratio of nutrients A and B, the arrows represent how it could feed on both to move most directly to T. Reproduced from Simpson and Raubenheimer (1993a) with permission

locust (survival, development time, and behaviour). This approach revealed the strategy by which the locust tailored its feeding to the heterogeneity of available protein and carbohydrate. Locusts were seen to regulate feeding so as to reduce their geometric distance in resource space from the intake target. The proximate mechanisms controlling intake of both protein and carbohydrate were suggested to be balanced via an adaptive feeding strategy to minimise costly overconsumption (Simpson and Raubenheimer 1993a). This approach to nutrient intake created a robust framework for nutritional ecology which could be applied to nutritional trade-offs in other arthropods (e.g. Simpson and Raubenheimer 1993a), birds (e.g. Köhler et al. 2012), fish (e.g. Simpson and Raubenheimer 2001; Ruohonen et al. 2007), and mammals (e.g. Felton et al. 2009). The GF has also been used to elucidate role of adaptive plasticity for nutrient intake in population and community structure (e.g. Behmer and Joern 2008; Raubenheimer et al. 2009). Overall, the GF can help us to gain a much more detailed view of how physiological, behavioural and metabolic plasticity is manifested in the face of resource heterogeneity (Behmer 2009; Simpson et al. 2015).

1.1.1 Patterns of diet utilisation in herbivorous insects

The detailed view of the ability of organisms to uptake specific nutrients from their environment that the GF describes was enabled by the study of herbivorous insects. The complexity of the relationship between herbivorous insects and the plants that they eat has led them to be central to the study of the physiological interaction of organisms with their nutritional environment and in addition the role of nutrition in population and community ecology. The co-evolutionary relationship between plant and insect has been on-going for 400 million years (Labandeira 2013; Bruce 2015) and exhibits great complexity (Bruce 2015). Herbivorous insects have undergone an extraordinary radiation during this time, with some estimates suggesting they represent one quarter of extant multicellular organisms (Strong et al. 1984). Coevolution between insect and host plant has long been suggested to be essential to this radiation (Ehrlich and Raven 1964) but there is scant supporting empirical evidence (Nyman 2010; Althoff et al. 2014; Suchan and Alvarez 2015). Recently, the role of the diverse array of nutritional resources represented by the plant species upon which herbivorous insects feed has been recognised as a key driver in this radiation (Jermy 1984; Janz et al. 2006; McKenna et al. 2009; Rabosky 2009; Nyman 2010).

Nutritional resources are a defining component of the niche that a species inhabits (e.g. Van Valen 1965; Soule and Stewart 1970; Roughgarden 1972). Narrowing of the

breadth of this niche, or specialisation, has been observed as a characteristic response to natural selection (Futuyma and Moreno 1988) as selection should favour individuals that achieve elevated fitness within a particular niche. Insect-plant relationships have become an important model in understanding specialisation. Although specialisation can be driven by many ecological factors, nutritional heterogeneity within and between plant hosts has provided numerous important examples (Futuyma and Moreno 1988; Forister et al. 2012). Insects exhibiting a specialised host relationship with a particular plant species suffer reductions in survival and growth when reared on different non-host plants (e.g. Wiklund 1975; Roininen and Tahvanainen 1989; Janz et al. 1994; Agosta 2008; Friberg and Wiklund 2009). However, many species that commonly co-occur with such specialists are able to utilise a range of hosts, exhibiting polyphagy or niche generalism (Novotny and Basset 2005; Singer 2008). Generalist species may not perform as well as a specialist on any single host, but do not suffer the deleterious effects of rearing across a range of hosts, (e.g. Roininen and Tahvanainen 1989; Janz et al. 1994; Friberg and Wiklund 2009). These two strategies exist on a gradient, with the intermediate being oligophagous species that utilise varying subsets of the available plant species in an environment (Jaenike 1990).

In considering the nature of adaptations to resource availability, it may be important to consider the underlying genetic architecture (Futuyma and Moreno 1988; Forister et al. 2012). This may influence the speed or capacity of organisms to respond. An herbivorous species that is specialised to a particular host will carry sets of alleles that optimise fitness for that specific host. In contrast, a generalist utilizing the same host plant is expected to lack specialised alleles and may therefore to show lower fitness, in comparison to a specialist, on that host. In general it is thought that, by occupying a broader niche, generalists maintain alleles associated with a range of environments ("a jack of all trades…") (Forister et al. 2012). Generalists are therefore expected to manifest sufficient behavioural and ecological plasticity to support movement between, and utilization of, a wider range of potential host plants within an environment (Loxdale et al. 2011).

Phylogenetic evidence shows that the transition between generalism and specialism is bidirectional, following a marginal overall trend towards specialisation across taxa (Nosil 2002; Nosil and Mooers 2005). Loxdale et al. (2011) suggest that 'biotypes' with generalist ecologies should not be maintained over evolutionary time in the face of selection for ecological specialisation. They suggest instead that generalist strategies may be an artefact of the 'time slice ecology' through which they are observed. In

contrast, the 'Oscillation' hypothesis (Janz and Nylin 2008) suggests instead that diverse allele sets associated with a generalist ecology may facilitate adaptive potential in the face of divergent selection provided by different hosts, with generalists acting as a sink for genetic diversity that can be recruited into specialisation, under the correct selective environment (Janz and Nylin 2008; Dennis et al. 2011). If the niche breadth of a generalist is wide, it has greater potential to encounter and survive in novel environments, which will counter the tendency for specialisation. This mobility across niches, and the resources they contain, allows generalists populating wide geographic scales to remain connected, thus maintaining gene flow of novel adaptations across the species as a whole (Dennis et al. 2011). This capacity is also evident in resilience, which is generally held to be greater for generalists than for a specialist linked to a specific host in a specific region, which, along with its plant host, is expected be more vulnerable to extinction (Dennis et al. 2011; Hardy and Otto 2014).

The observation that strategies of niche optimisation co-occur in great abundance (Novotny and Basset 2005; Singer 2008) and also that evolutionary transitions can be made between them (Nosil 2002; Nosil and Mooers 2005) highlights the diversity of strategies which herbivorous insects utilise to navigate the 'moving islands and archipelagos in multidimensional resource space' manifested by the plants on which they feed. Understanding of the divergent selection pressure that a population encounters when it colonises a novel host has become of central importance in the study of speciation (Nyman 2010). Indeed the impact upon fitness encountered during such niche shifts and the relationship with the instigation of reproductive isolation (RI) between populations forms the basis of 'ecological speciation' theory (Schluter 2000; Coyne and Orr 2004; Rundle and Nosil 2005; Nosil 2012).

1.2 Ecological Speciation

Our understanding of the role of natural selection in the formation of species has been greatly advanced in the past two decades. Prior to this, speciation theory was based upon two main themes of research, the geographic scenarios in which species could form (Mayr 1942, 1947; Futuyma and Mayer 1980; Coyne and Orr 2004) and the genetic architecture of speciation (Noor et al. 2001; Orr 2001; Rieseberg 2001; Ortiz-Barrientos et al. 2002). This work laid the foundation for the establishment of the understanding that divergent selection encountered across these geographic scenarios can cause RI between populations (Schluter 2000, 2001; Berlocher and Feder 2002; Drès and Mallet 2002; Coyne and Orr 2004; Rundle and Nosil 2005; Funk and Nosil 2008; Nosil 2012). In recent years, the addition of information drawn from next-

generation sequencing (NGS) technologies has allowed a more detailed, genome-wide description of the action of divergent selection (Seehausen et al. 2014). Herbivorous insects have provided essential models for studies of adaptation and speciation (Mitter et al. 1988; Funk et al. 2002; Simon et al. 2015) and have facilitated the emergence of ecological speciation theory (Nosil 2012).

1.2.1 Foundations of ecological speciation

Over 150 years have passed since Charles Darwin first suggested that natural selection in different environments might be responsible for the formation of species (Darwin 1859). During this time, the link between ecological diversity and species richness has guided the study of speciation. Adaptive radiations, such as those seen in the Galápagos finches crucial to Darwin's original work (Lack 1947), informed our early understanding of speciation (Simpson 1953; Ehrlich and Raven 1964; Van Valen 1965; Stanley 1979; Schluter 2000). The 'key innovations', in which radiating species evolve in order to access resources in new 'adaptive zones', were suggested to support diversity as adaptive radiations progressed (Simpson 1953). Interspecific competition within new niches was suggested to lead to species being partitioned by coexistence and coevolution (e.g. Ehrlich and Raven 1964; Mitter et al. 1988) thus supporting the existence of observed species diversity.

The definition of the biological species concept (Mayr 1942) and the advent of the modern evolutionary synthesis (Muller 1942; Mayr 1947; Dobzhansky 1951) led to the first considerations of the role of ecology in speciation within adaptive radiations. The RI between populations central to Mayr's species concept fitted well with instances of speciation in populations divided by geographic barriers. In such allopatric scenarios, the action of divergent selection on traits or their genetic correlates would affect RI, as there would be no homogenising effects of gene flow (Muller 1942; Mayr 1963). However, in the absence of geographic barriers, in sympatric scenarios, the probability of speciation was thought to be 'neither established nor even possible' (Mayr 1963), despite long standing observation of host races in sympatry (e.g. Walsh 1864).

A unifying theory of speciation was not developed in depth until the role of ecology in the formation of species was consolidated as the 'the ecological hypothesis of speciation' (Schluter 2001). In centralising the role of divergent selection between populations or subpopulations in differing environments in creating barriers to gene flow, this theory allowed provided a general mechanism for species formation that was not constrained by geographical context.

1.2.2 Ecological speciation

Populations that exist in differing ecological environments will experience different selection pressures. The need to attain resources and maximise fitness under such divergent selection will mean that populations undergo evolutionary change along different trajectories according to their environment. The effect of adaptation to different environments can cause populations to become reproductively isolated from each other. Regardless of whether the selection imposed by an environment falls directly on a trait that prevents one population from reproducing with another, or affects such traits as a side effect of through pleiotropy (e.g. Nagel and Schluter 1998; McKinnon et al. 2004), isolation between populations is still expected to occur. Selection can also act on traits that affect RI if they are physically linked with those associated with other traits subject to selection (Barton 1995; Hawthorne and Via 2001). This 'hitchhiking' prevents recombination during reproduction dividing the traits under selection from traits involved in RI, thus driving evolution in the absence of direct selection. Hitchhiking can also be enacted by non-random statistical associations between alleles, or linkage disequilibrium (Rundle and Nosil 2005) and can lead to 'divergence hitchhiking' which is though to spread the effects of divergent selection across genomic regions surrounding alleles under selection and eventually to the whole genome (Feder et al. 2012; Nosil and Feder 2012).

This concept forms the basis of the contemporary view of ecological speciation (ES). Defined as "the process by which barriers to gene flow evolve between populations as a result of ecologically based divergent selection between environments" (Nosil 2012), ES has become a main focus of speciation research over the past two decades. Although populations in different environments may develop RI through the random appearance of incompatible mutations by genetic drift, or by the action of sexual selection on traits involved in RI (Lande 1981), speciation under divergent natural selection is though to be a common mechanism in the instigation of RI (Nosil 2012).

Since it was formalised (Schluter 2001) several important predictions have arisen from ES theory which extend our understanding of how divergent selection can instigate RI between populations. If an organism that has undergone adaptation to a particular environment moves to an environment to which it is not adapted, it will be poorly equipped to survive in that new environment. Termed 'immigrant inviability', this effect will decrease the effective encounter rate between individuals from populations which are diverging between environments, effectively increasing levels of RI (Funk 1998; Via et al. 2000; Nosil 2004; Nosil et al. 2005). Also, if mating does occur between

individuals from differently adapted populations, immigrant females are less likely to survive to reproduce successfully, again strengthening RI between populations (Nosil et al. 2005).

RI can also be manifested even if sexual isolation is not apparent, acting postzygotically after reproduction is occurred. If mating does occur between individuals from populations that have begun to adapt to divergent environments, hybrid offspring will be formed. Such a hybrid will carry a mixture of the adapted sets of alleles represented by each parent. This mixture will not provide optimum fitness in either of the parental environments, leading the hybrid offspring to suffer from an environmental mismatch known as 'intrinsic hybrid inviability' (Via et al. 2000; Rundle and Whitlock 2001; Rundle 2002). Hybrid inviability may not arise, or hybrids may even be at an advantage in one or both environments due to the novelty generated by combining parental alleles (e.g. Grant and Grant 2002). However, where hybrid inviability does occur, it is predicted to strengthen RI.

Consideration of these predictions has led to the formation of a comprehensive theory of ES (Rundle and Nosil 2005; Schluter 2009; Nosil 2012). This has allowed speciation theory to transition from a focus on disparate geographic contexts of speciation towards a unified view of speciation as a continuum of divergence in the face of gene flow (Butlin et al. 2008). Although the geographic context in which divergence occurs is still crucial (Nosil 2012), in focusing of the divergent selection pressures driving adaptation, ES provides a theory of speciation that interacts with other potential mechanisms of speciation such as sexual selection (Maan and Seehausen 2011). Nosil (2012) reviews a framework for ES based on its three integral components:

1. A source of divergent selection: either manifested by differences between environments, interactions between populations or by ecologically based sexual selection.
2. A form of RI - split into three main classes: 1) barriers which are intrinsic to ES: immigrant inviability and ecologically dependent post-mating isolation, 2) barriers which are 'inherently ecological': habitat and temporal isolation, 3) all other barriers that are not 'inherently ecological': barriers which can evolve by many processes e.g. divergent mate or pollinator preferences.
3. A genetic mechanism to link selection to isolation - two main mechanisms: 1) Direct selection acting on the same genes that cause isolation, 2) Selection on genes not associated with isolation causing RI to evolve via non-random associations (linkage disequilibrium) in genes associated with isolation.

1.2.3 Ecological speciation in herbivorous insects

Many of the key findings that have contributed to the establishment of ES theory have been derived from ecologically specialised, phylogentically diverse and experimentally tractable herbivorous insects (Funk et al. 2002). Such systems have been carefully studied to gain a detailed understanding of each of the three components of ES suggested by (Nosil 2012). Although a small subsample of an ever-growing array of examples of ES in herbivorous insects, the species described below have provided key insights into ES.

The apple maggot fly, *Rhagoletis pomonella*, has long been a model species for speciation with gene flow (Bush 1969b; Coyne and Orr 2004). The native host of *R. pomonella* is thought to be downy hawthorn (*Crataegus mollis*). A host switch between hawthorn and cultivated apples (*Malus domestica*) at some point in the 1800s led to the formation of two partially isolated host races (Walsh 1867; Bush 1969a,b). Study of the divergence of these two host races in sympatry has been key to our understanding of speciation with gene flow (e.g. Feder et al. 1988; McPheron et al. 1988; Bush 1994; Berlocher and Feder 2002). *R. pomonella* is now understood to represent a species group ranging across North America (Xie et al. 2008; Hood et al. 2012; Powell et al. 2012) with each race or subspecies occupying a different host species. Hence, the *R. pomonella* species group provides an example of host shifts leading to new species in sympatry. *R. pomonella* exhibits strong host fidelity, as adults mate nearly exclusively on the fruit surface of their host, leading host races to remain spatially isolated (Feder et al. 1994). This host fidelity has been shown be matched by divergence in host fruit odour preference (Berlocher 2000; Linn et al. 2012; Powell et al. 2012). Variation in the fruiting times of different R. pomonella hosts also drives temporal divergent selection between host races. Hence flies of different host races have been selected to match their phenology to that of the host (Feder et al. 1993; Dambroski and Feder 2007). Adaptation to hosts which do not have overlapping fruiting periods has lead divergence of diapause duration between host races, matched to host fruiting time, with apple race flies emerging from their pupae 3-4 weeks earlier than hawthorn flies (Dambroski and Feder 2007).

Recent studies have begun to describe the genetic architecture of the host races present in the *Rhagoletis pomonella* species group. Divergence between apple and hawthorn races has been shown to be associated with a genome-wide signature of divergence. Further to this, 'newer' host races have been shown to represent a more exaggerated signature divergence over similar genomic regions, showing that novel

genomic regions have not been recruited to the areas of divergence as new hosts have been colonised (Powell et al. 2013, 2014). Also, studies have begun to examine the nature of the natural selection that may have contributed to the original formation of the apple and hawthorn host races, by assaying the potential resource benefits of the novel apple host (Ragland et al. 2012). Most recently, an elegant experimental approach has been employed to recreate a hypothetical first generation of the host shift from hawthorn to apple. Coupled with NGS this approach has provided an image of the 'extent and magnitude of the footprint of ecological selection' associated with a host switch. Large areas of the genome were seen to fall under selection during the single generation host switch, and allele frequency shifts are recorded in 30,000+ SNPs (Egan et al. 2015). This supports the role of linkage disequilibrium across the whole genome in manifesting RI between host races during ES.

The *Enchenopa binotata* species complex represents a key example of ecologically based sexual selection on divergent mating signals following host shifts in sympatry (Coyne and Orr 2004; Cocroft et al. 2008). Primarily, host shifts have caused temporal isolation through adaptation to specific host phenology, as the eggs of *Enchenopa sp.* only begin to develop when the host circulates sap at the start of the growing season (Wood and Keese 1990; Wood et al. 1990). However, species within the *E. binotata* complex also exhibit strong assortative mating by host plant (Wood 1980; Wood and Guttman 1982). This behavioural isolation is manifested via vibrational communication through the surface of the host plant. This form of communication is highly developed, with females being able to recognise males from specific host plants and also recognise levels of relatedness (Rodríguez et al. 2004; Cocroft et al. 2010). The evolution of these signals is strongly affected by female choice (Rodríguez et al. 2006). *E. binotata* vibrational signals have also been shown to be closely evolutionarily linked to host plant (Cocroft et al. 2010) and appear to have adapted to confer optimum efficiency of travel through the host-specific substrate (McNett and Cocroft 2008).

Recently this system has been used to show the effects of specific host genotypes on sexually selected signals, highlighting the role of interspecific indirect genetic effects of environment within host shift scenarios. Rearing on clonal lines of different host genotypes (of *Viburnum lentago*) caused marked differences in several aspects of male and female courtship behaviour and on mate preference (Rebar and Rodríguez 2014a,b, 2015). This shows the importance of host, even at the level of genotype, in manifesting divergent selection between environments.

The stick insect, *Timema cristinae,* exhibits partially reproductively isolated ecotypes between two hosts, *Adenostoma fasciculatum* and *Ceanothus spinosus* (Nosil 2007; Nosil et al. 2012). These ecotypes are morphologically divergent, particularly in traits related to crypsis, with their coloration adapted to be closer to that of their host. These adaptations confer lower mortality on the adapted host plant (Sandoval 1994). The camouflage exhibited by *T. cristinae* has been used to quantify the importance of immigrant inviability during ecological speciation, as transplant experiments reveal highly elevated levels of avian predation on non adapted individuals (Nosil 2004; Nosil and Crespi 2006). Parallel evolution between the morphs has provided a key example of the parallel genomic changes across the genome caused by adaptation under natural selection on the two host plants (Soria-Carrasco et al. 2014).

Recently this system has been used to quantify the nature of phenotypic selection imposed by the environment (alongside drift) across the whole genome. Individuals of each morph were genotyped, then transplanted (in the field) to opposing hosts. After ten days, remaining individuals were recaptured and genotyped, revealing the genomic signature of the selection imposed by host switching. The signature of this environmental selection was manifested by changes in allele frequency in numerous regions of the genome. A substantial and significant effect was observed on population genetic variation over an extremely short timescale (Gompert et al. 2014).

The stick insect system has also recently provided insight into the role of gene flow in preventing the completion of speciation. The observation of a third melanistic morph in *T. cristinae* with camouflage adapted to neither host, but that was present on both hosts, led to the identification of a single locus controlling melanism (Comeault et al. 2015). The camouflaged phenotype is under less predation pressure than either of the adapted morphs and exhibited a mating advantage over other morphs as well as a homotypic mating preference. This third morph was described as a 'genetic bridge' between the two morphs under divergent selection (Comeault et al. 2015). It provides an important example of how selection and gene flow maintain genetic variation in a population, preventing the completion of speciation (Nosil et al. 2009). Indeed, the melanistic morph of *T. cristinae* has been termed an 'anti-speciation' phenotype (Rogers 2015).

The pea aphid, *Acryrthosiphon pisum* is a well-known example of a phytophagous insect associated with a wide range of host plants, which appears to have gone through repeated ecological speciation (Peccoud and Simon 2010). This cosmopolitan species is now considered as a complex of species and host races. Across its European

range, divergent selection between aphid strains associated with multiple leguminous host plants appears to have resulted in considerable genetic differentiation between host races (Ferrari et al. 2006, 2008, 2012; Peccoud et al. 2009a, 2015a). Despite the host specialisation seen with the complex, the legume *Vicia faba* provides a host which is universally suitable and is thought to act as a 'bridge species' facilitating gene flow between host races (Ferrari et al. 2008). Between biotypes, isolating barriers primarily occur in premating traits due to biotype-specific host selection during the sexual phase of the aphid life cycle (Powell et al. 2006). Post-mating barriers between host races also exist, with hybrid individuals experiencing decreased performance on the parental host plant (Via et al. 2000).

The *Acryrthosiphon pisum* species complex has also highlighted the role that symbiotic organisms can have in ecological speciation. The maternally-inherited endosymbiotic bacteria *Buchnera aphidicola* (Baumann et al. 1995) and a range of related species (Oliver et al. 2010), confer host specific advantages across host races and have been shown to diverge alongside their aphid hosts (Peccoud et al. 2009b; Ferrari et al. 2012; Gauthier et al. 2015). Recently-described host races of *A. pisum* have been shown to recruit novel symbionts as they colonise new hosts (Peccoud et al. 2015b).

The genetic architecture of divergence across a range of hosts is well described in the *Acryrthosiphon pisum* species complex and many regions of the genome have been shown to fall under divergent selection between host races, with candidates showing association with adaptation to hosts including olfactory and gustatory receptors and salivary proteins (Jaquiéry et al. 2012; Smadja et al. 2012; Nouhaud et al. 2014). These detailed studies, utilising a candidate lead sequence capture approach, have recently allowed copy number variation (CNV) within these candidate regions to be investigated (Duvaux et al. 2015). CNV, or the presence of different numbers of copies of the same DNA sequence between individuals, can occur through gene duplication and deletion (Innan and Kondrashov 2010) and is an important source of genetic variation. CNV within candidate regions for divergence is widespread among 8 host races of *A. pisum* and CNV within olfactory and gustatory receptor gene families is suggested to be an important source of adaptive variation utilised during the formation of *A. pisum* host races (Duvaux et al. 2015).

***Drosophila* species** represent a wealth of examples of closely related populations at varying stages divergence along the speciation continuum. Study of *Drosophila* species pairs has provided the basis of key comparative studies of RI and ecological speciation (Coyne and Orr 1989, 1997; Funk et al. 2006; Funk and Nosil 2008; Yukilevich 2012;

Rabosky and Matute 2013). These studies analysed levels of pre- and post-zygotic isolation in an extensive set of *Drosophila* species pairs in relation to genetic (Coyne and Orr 1989, 1997) and ecological divergence (Funk et al. 2006; Funk and Nosil 2008) between pairs. This approach has been key in understanding the consistency of the relationship between divergence at the genetic or ecosystem level and the build up of RI in speciation. The well recorded diversity of *Drosophila* first facilitated this approach (Coyne and Orr 1989), which has now been extended to a wide variety of taxa including plants, birds, and fish (Funk et al. 2006). The dataset first used by (Coyne and Orr 1989) consists of many intriguing examples of speciation scenarios in *Drosophila*, many of which have provided important advances in the study of speciation under divergent selection.

The *Drosophila* melanogaster species subgroup has provided a wealth of information for almost all fields within biology, particularly contributing to understanding of the genetic architecture of reproductive isolation and dynamics of hybridisation (Mallet 2006). The island radiation of the *D. simulans* complex involves a trio of closely related species that have speciated under ecological selection (Kliman et al. 2000). Within this complex, the divergence of *D. sechellia* its sister species *D. simulans* is strongly linked to host specialisation (McBride 2007). *D. simulans* is a generalist (Lachaise and Silvain 2004) whereas *D. sechellia* has specialised to the fruit of *Morinda citrifolia* (Louis and David 1986). The fruit of *M. citrifolia* contains a range of alkanoic acids which are toxic to, and actively repel, other drosophilids (Farine et al. 1996). However, *D. sechellia* has evolved resistance to these chemicals and is attracted to them (R'Kha et al. 1991; Legal et al. 1992; Moreteau et al. 1994; Farine et al. 1996). Underlying this pattern, *D. sechellia* shows extreme divergence in olfactory and gustatory receptor genes, with loss of function mutations leading it to lose olfactory gene function at a rate that is 9-10 times faster than for *D. simulans* (McBride 2007). Strong behavioural isolation also exists between these species mediated by the chemical communication through cuticular hydrocarbons (e.g. Coyne 1996; Gleason et al. 2005).

Recent genomic studies of this system have suggested that, despite such host-related barriers and behavioural isolation, there has been gene flow between *Drosophila simulans* and *D. sechellia* over the last 5000 years. This may therefore be an example of speciation with gene flow (Garrigan et al. 2012; Brand et al. 2013). The association between olfactory receptors and host specialisation in *D. sechellia* has also led the characterisation of one of few recognised 'speciation genes' (Nosil 2012). The olfactory binding proteins OBP57d/e have been shown responsible for in the attraction of *D.*

sechellia to *Morinda citrifolia* fruit, and also to be responsible for behavioural differences underlying RI (driven by host choice) between *D. sechellia* and *D. melanogaster* (Matsuo et al. 2007).

Drosophila mojavensis represents an example of a *Drosophila* species that exhibits incipient speciation between hosts. Across their range, *D. mojavensis* populations utilise various cactus hosts (Etges et al. 1999). Different cactus hosts exert selection on life history traits across the range, including on adult mortality rates (Jaureguy and Etges 2007). There are significant genetic shifts associated with life history between populations (Etges 1990). This has resulted in pre-mating isolation between allopatric populations across the range (e.g. Markow 1991). Life history adaptation associated with host shifts is also genetically correlated with CHC-mediated mate choice decisions (Etges et al. 2010). The sexual isolation between populations is significantly affected by rearing substrate (Etges 1992) and rearing on cactus also significantly increases sexual isolation from the sister species, *D. arizonae,* in laboratory tests (Jennings and Etges 2009). The cactus host upon which a male is reared has also been shown to have important effects on courtship song and CHC profile (Etges et al. 2007, 2009) which are essential to mating success between populations (Etges and Ahrens 2001; Etges et al. 2006). These findings show that larval rearing environment is an important factor in the early stages of speciation in this system (Etges 2014).

Recent work tested for gene expression differences associated with rearing on different hosts and at different stages of life history, in genes thought to influence reproductive isolation (Etges 2014; Etges et al. 2015). This allowed the capture of expression differences throughout life history of an olfactory binding protein associated with host recognition between races (Etges et al. 2015) and also a number of candidate genes associated with successful copulation in populations adapted to different hosts (Smith et al. 2013). These results are important examples of the utility of transcriptomics in understanding the nature of genome-organism-environment interactions for speciation (Etges 2014).

In summary, even in the small selection of species described here, it is clear that herbivorous insects are invaluable to speciation research. Their study is driving the transition from a gene-based perspective of speciation to the genomic scale. The more recent studies described have brought classic model systems into an era increasingly dominated by NGS technologies. With the new technologies incorporated into ever stronger experimental designs, herbivorous insects can be used answer questions about the genomic architecture and dynamics of speciation, as well as to provide

important insights into newer areas of speciation research such as in transcriptomics and copy number variation (e.g. Duvaux et al. 2015; Etges et al. 2015). NGS techniques are also being used investigate the action of selection at the initiation of divergence (e.g. Egan et al. 2015). The manipulative approach used in such studies follows in the footsteps of experimental work in laboratory populations and may herald the dawn of an era of 'experimental genomics' (Nosil 2015).

1.3 Experimental evolution

Experimental evolution (EE) allows the study of evolutionary change in real time. By imposing specific conditions on a controlled set of populations, evolutionary processes occurring within populations associated with these conditions can be studied (Kawecki et al. 2012). As EE studies are initiated under controlled conditions, it is possible to capture the response of sets of experimental populations, and the genotypes therein, to the selection imposed by the experimental conditions, generation upon generation. As experimental populations are often created from a common ancestral population or genotype, experimental evolution can offer strong inference concerning the role of adaptation in differentiation between populations (Bailey and Bataillon 2015).

The controlled scenario which EE offers for study represents a unique window into evolutionary processes, one which runs 'almost orthogonal' to conventional studies of natural populations (Bailey and Bataillon 2015). The very nature of natural populations, i.e. that they are uncontrolled, makes the study of adaptation from an unbiased starting point almost impossible. Without out the ability to view a population from it's adaptive 'starting point' studies of natural populations must interpret either the fitness consequences of phenotypic variation and it's genetic basis ("top-down" approaches (e.g. Hoekstra 2006) to infer the nature of selection, or to look for the purely genetic signatures of selection within the genomes of such populations, manifested by patterns of polymorphism and nucleotide divergence ("bottom up" approaches, (Vitti et al. 2013). Evidence gleaned from EE goes hand in hand with theoretical studies to provide "proof of concept" and allows the subsequent study of the traits and signals identified as important in natural populations (Servedio et al. 2014). This allows EE to occupy a uniquely powerful position between nature and theory (Bailey and Bataillon 2015).

1.3.1 Choice of system

The power of EE has been used across a wide range of model systems to gain deep insights into evolutionary processes. As model systems can be selected for their particular strengths, the experimenter can choose an organism appropriate for the

study of a wide range of traits. Many EE studies employ microbial study systems reviewed in detail by (Bailey and Bataillon 2015). The extremely short generation time of microbes allows adaptation to be studied rapidly in real time. Indeed, a famous study, initiated in 1988, has now run for over 60,000 generations (Fox and Lenski 2015). Such studies have revealed important information regarding the distribution of fitness effects induced by novel mutations (e.g. Blount et al. 2012; Bailey et al. 2015; Lind et al. 2015), the role of frequency dependent selection in adaptation (e.g. Rosenzweig et al. 1994; Rozen and Lenski 2000; Lang et al. 2011; Maharjan et al. 2015) and specific mechanisms of adaptation under environmental selection (e.g. MacLean et al. 2004; Hall and Colegrave 2008). However, the asexuality of the majority of microbes used in EE limits their utility in relation to understanding adaptation in sexual species as, without recombination the predominant source of genetic variation becomes *de novo* mutation, altering the importance of standing genetic variation over short evolutionary time scales (Bailey and Bataillon 2015). This limits their use in relation the study of adaptation in sexual species as standing genetic variation is increasingly understood to be of key importance (e.g. Barrett et al. 2008; Egan et al. 2015).

In selecting sexual organisms for study under EE, the experimenter must sacrifice the short generation time of microbes. This may reduce the ability to retrieve information on some facets of adaptation, for example the emergence and fixation of novel mutations (Bailey and Bataillon 2015). The budding yeast *Saccharomyces cerevisiae* offers among the most rapid generation times available for easy study in a eukaryotic model species, and it has been used to great effect to study adaptation using EE (e.g. Segrè et al. 2006; Gresham et al. 2008; Araya et al. 2010; Spor et al. 2014; Metzger et al. 2015). The ubiquitous biological model species *Drosophila melanogaster* has also formed the basis of many important EE studies. The relatively short generation time of *D. melanogaster* (9 days, egg to adult at 25˚C) has allowed some long term experimental populations to reach over 600 generations (Burke et al. 2010). EE based on *D. melanogaster* populations has been used to study adaptation to environmental stresses (e.g. Kolss et al. 2009), density dependent selection (e.g. Santos et al. 1997; Fitzpatrick et al. 2007), artificial selection for postponed senescence e.g.(Rose 1984) and aging (e.g. Rose et al. 2002; Burke et al. 2010). An ingenious *D. melanogaster* EE design has also been used to show how environmental selection can cause adaptation in learning ability (Mery and Kawecki 2004). Recent advances in NGS technologies coupled with EE designs, 'evolve and resequence' studies (Schlötterer et al. 2014), are revolutionising studies of adaptation and speciation in model systems. Such techniques

have been implemented in *D. melanogaster* to capture genome wide signatures of adaptation (e.g. Tobler et al. 2014). The utility of *Drosophila* species as laboratory models for studying adaptation under EE has also been important to the study of the role ecological adaptation in species formation.

1.3.2 Experimental evolution in the study of ecological speciation

Experimental studies using *Drosophila* have provided key insights for ecological speciation theory, such as the action of pleiotropy and linkage in establishing RI reviewed in (Rice and Hosert 1993). A key study by (Dodd 1989) showed that behavioural isolation developed as a pleiotropic consequence of adaptation to divergent selection pressure between dietary regimes. This was the first observation of such an effect and was important in proving that divergent selection between environments could rapidly (in around a year) generate behavioural differentiation. Subsequent, related EE studies have yielded similar findings in other *Drosophila* species and have suggested a mechanism for the evolution of divergent mate choice. (Rundle et al. 2005) studied twelve replicate populations of *D. serrata*, dividing them between three dietary treatments for 29-37 generations. They observed divergence in both male CHC profiles (which determine mating success) and in female preference between the treatments. The clear interaction between natural and sexual selection in this study led to further EE in the *D. serrata* system, with designs aiming to manipulate both forms of selection (e.g. Rundle et al. 2006). An interesting recent finding from EE in *D. serrata* suggests sexual conflict may negate natural selection during adaptation to novel environments (Chenoweth et al. 2015). By imposing a factorial manipulation of levels of natural and selection during the early generations of exposure to a novel diet, Chenoweth et al. (2015) were able to show that the effects of sexual selection were antagonistic to single nucleotide polymorphisms which increased in frequency under natural selection alone, effectively 'cancelling out' the fitness gain associated with adaptation.

EE studies of *Drosophila* species have provided an experimental framework for the experimental study of the effects of divergent selection. However important ecological and genetic dissimilarities are present between even closely related species (e.g. Capy and Gibert 2004). Hence, though EE is a powerful tool, it needs to be implemented over a wide range of study systems to identify idiosyncratic features of particular systems (due to intrinsic differences) versus truly general principles (Kawecki et al. 2012).

1.4 Study system: The Mediterranean fruit fly

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), is a *Tephritid* fruit fly with a global range. It is notorious, 'virulent' crop pest (Carey 1991). Exhibiting high levels of polyphagy, the medfly has been recorded infesting over 350 hosts (Liquido et al. 1991). The financial importance of the medfly as a pest to agriculture has led to a vast research effort into its distribution, population history, life history dynamics, host interactions, sexual behaviour and more recently it's genome. Although the main focus of the majority of these studies has been on developing effective control measures, they highlight the tractability of the medfly as a laboratory model. The depth of knowledge surrounding the medfly detail an intriguingly robust generalist life history which has the potential to contribute understanding to many fields of biology. Also, as the closest non-drosophilid relative to *Drosophila* to be subject to a wide range of research, and to have a recently sequenced genome (https://www.hgsc.bcm.edu/arthropods/mediterranean-fruit-fly-genome-project) the medfly represents an ideal model to test concepts that have exclusively been tested in the *Drosophilidae* and to extend them to a species with a markedly different biology.

1.4.1 Global distribution

The global distribution of the medfly is facilitated by its highly invasive nature. The invasion history has been inferred from biochemical and molecular markers (Gasperi et al. 2002; Malacrida et al. 2007) and more recently from population genetic structure (Karsten et al. 2015). The native range of the medfly is thought to be in central Africa as, of all global populations surveyed, these maintain the highest genetic variability (Gasperi et al. 2002; Bonizzoni et al. 2004; Malacrida et al. 2007; Karsten et al. 2013, 2015). Decay in the genetic variability of medfly populations, due bottlenecking during colonisation, suggests that the medfly first invaded Europe from Africa, before a secondary invasion of Australasia from the European population (Gasperi et al. 2002; Malacrida et al. 2007; Karsten et al. 2015). In a separate invasion, the medfly was later introduced to the New World from the Mediterranean basin (Karsten et al. 2015). This remarkable global invasion has occurred in only the past ~150 years (Gasperi et al. 2002; Karsten et al. 2015). Populations across this global distribution have traditionally been thought to be connected human trade and transport and thus subject to gene flow (Gasperi et al. 2002; Malacrida et al. 2007). However, recent studies of population structure suggest this is not the case, which may result from increasingly effective containment measures (Karsten et al. 2015).

Genetically distinct global populations of the medfly (Karsten et al. 2015) have been shown to exhibit signatures of divergent adaptation in several life history traits (Diamantidis et al. 2008a). Adult longevity and reproductive schedule vary between global populations, suggesting the employment of different resource allocation strategies (Diamantidis et al. 2009). Similar global populations have also been shown to exhibit divergent abilities to adapt to domestication (a proxy for novel host colonisation) both in developmental traits during a single generation (Diamantidis et al. 2011b) and on a demographic scale over a number of generations (Diamantidis et al. 2011a). Across these studies, a unifying factor is that the ancestral populations retain the most 'robust' life history and have higher invasive potential. This suggests that as genetic variability has been lost during invasion, so have other elements of a robust generalism phenotype (Diamantidis et al. 2011a).

This global divergence in demographic parameters of life history has also been observed in male courtship traits (Briceño et al. 2002, 2007; Lux et al. 2002; Diamantidis et al. 2008b). As one of the main control methods utilised against the medfly relies upon the release of sterilised males (Sterile Insect Technique, SIT) it has been important to build a detailed picture of male courtship behaviour and it's global variation (Robinson et al. 2002). The male medfly utilises a multimodal courtship display as part of a lek-based mating system (reviewed in detail in Chapter 4). However, global variation in acoustic signals (Briceño et al. 2002), courtship behaviour (Lux et al. 2002; Briceño et al. 2007) and the incidence of pheromonal signalling (Papanastasiou et al. 2011) give little evidence of reproductive isolation, manifested by assortative mating by population, between geographically distinct global populations (Cayol 2000; Cayol et al. 2002).

1.4.2 Niche Generalism

The rapid global invasion of the medfly has been facilitated by its ability to express high levels of plasticity in response to several key environmental variables, making it an important example of a niche generalist. The medfly exhibits a highly plastic generation time and life history (Carey 1984) as well as a high tolerance to fluctuations in temperature (Nyamukondiwa et al. 2010). These traits combine to allow the medfly to occupy a fluid temporal niche, opening the opportunity to exploit the fruiting period of a wider range host plants (Yuval and Hendrichs 2000). Host selection and associated oviposition behaviour by medfly females also exhibit high levels of plasticity (Prokopy et al. 1984; Katsoyannos 1989). Potential hosts are identified by a range of volatile cues general to ripening fruit and leaves (Light et al. 1988; Levinson et al. 1990) as

well as visual cues dominated by the spherical shapes of fruit (Katsoyannos 1989). This leads to little differentiation between viable hosts. Despite a range of >350 viable hosts (Liquido et al. 1991) females will often lay eggs into fruits beyond their standard host range (Carey 1984) or into unripe fruits (Yuval and Hendrichs 2000).

As a result of this lack of host fidelity, the eggs of the medfly may be oviposited into a wide range of nutritional conditions. Larval medfly display high levels of developmental plasticity in the face of the nutritional challenges different host may offer. Larvae are seen to lengthen their development time in unfavourable hosts and can also successfully develop when artificially reared on fruits beyond their natural host range (Krainacker et al. 1987). Larvae can respond to the nutritional quality of the fruit in which they are developing, actively seeking out its most nutritious parts (Fernandes-da-Silva and Zucoloto 1993). Experimental evidence has revealed that the medfly can show considerable flexibility in its larval life history in response to specific changes in nutritional quality (Canato and Zucoloto 1993; Zucoloto 1993a,b; Chang et al. 2001; Chang 2004; Nestel et al. 2004; Nestel and Nemny-Lavy 2008).

1.4.3 Mating System

The mating system of the medfly is based upon loose substrate leks (Prokopy and Hendrichs 1979; Arita and Kaneshiro 1985; Whittier et al. 1992; Shelly et al. 1994; Benelli et al. 2014a,b). Leks are non-resource based mating aggregations (Höglund and Alatalo 1995) that allow females to choose males based on condition-dependent signals displayed at the lek site (Rowe and Houle 1996; Kokko and Heubel 2008). In medfly, condition is an important predictor of the mating success of males participating in leks and is strongly affected by developmental environment e.g.(Zucoloto 1993b; Kaspi et al. 2002; Anjos-Duarte et al. 2011; Navarro-Campos et al. 2011), nutrients (e.g. Blay and Yuval 1997; Kaspi and Yuval 2000; Kaspi et al. 2000; Shelly et al. 2002; Yuval et al. 2002; Maor et al. 2004; Joachim-Bravo et al. 2009; Costa et al. 2012) and semiochemicals consumed during adulthood (e.g. Papadopoulos et al. 2001; Juan-Blasco et al. 2013).

Medfly leks are convened on host trees by males, using long-range pheromonal signals that attract males and females to aggregate (Prokopy and Hendrichs 1979; Arita and Kaneshiro 1985). The pheromonal blend dispersed by the medfly contains at least five active compounds which elicit female response, but over fifty compounds in total (Heath et al. 1991; Jang et al. 1994; Light et al. 1999). Attendance of females to male-convened leks is dependent on lek size, with larger aggregations of males attracting more females (Shelly 2001). This highlights the role of female choice in the lek,

suggesting sexual selection is a strong driver of evolution in the medfly (Field et al. 2002).

Once females are attracted to a lek site, copulation success is governed by a number of factors. Males employ visual, auditory, and additional pheromonal cues as part of an extended courtship ritual (described in detail in Chapter 4). Courtship success has been related to courtship vigour (Whittier et al. 1994). The relationship between male size and courtship success is variable - some studies suggest larger males are more successful (e.g. Blay and Yuval 1997; Kaspi et al. 2000, 2001) whereas in others smaller males mate more frequently (Arita and Kaneshiro 1988). In support of the importance of condition in male display, males with fewer nutritional reserves engage less in courtship and secure fewer matings (e.g. Blay and Yuval 1997; Warburg and Yuval 1997; Yuval et al. 1998; Kaspi et al. 2002; Aluja et al. 2008; Joachim-Bravo et al. 2009).

In summary, the medfly is an important model for many aspects of biology. Its well-studied global invasion and extreme niche generalism allow many ecological and evolutionary questions to be addressed both in natural populations and in the laboratory. It is a tractable laboratory system and through the emphasis on successful rearing for control, much is now understood about how specific manipulations of its environment affect the life history of the medfly. Displaying a wide range of courtship behaviours, and a mating system strongly driven by sexual selection, it also provides an important model for behavioural ecology.

1.5 Thesis outline

1.5.1 Objectives

The overarching aim of this research was to use laboratory populations of the medfly to investigate the adaptive response of medfly to changes in larval rearing diet. Using single generational experiments, and replicated EE, this research aimed to expand the utility of the medfly as a model for evolutionary biology. Particularly, this works aimed to test for the evolution of RI in response to divergent selection provided by differing rearing diets, test the mechanism of any isolation that was observed, and to suggest candidates for it's genetic basis by analysis of transcriptomes.

Specific objectives were:

1. To test the developmental response of a laboratory population to manipulation specific nutritional components of larval diet.

2. To test for the evolution of RI between replicate EE populations developing on divergent larval diets.
3. To quantify the behavioural mechanisms associated with patterns of mating observed in the EE populations.
4. To identify genes which may be associated with adaptation to divergent developmental environment or differences in mate choice between the EE populations.

1.5.2 Chapter Outline

To address the specific objectives of this research the chapters of this thesis are arranged as follows.

Chapter 2 describes a single generation experiment in which medfly larvae drawn from a single laboratory population of medfly were reared on diets with altered protein levels and sources and, in a separate treatment, altered carbohydrate sources. The specific alterations to diet included the addition of a protein and carbohydrate source outside of the host range of the medfly. The development of these larval populations was described in detail, with the duration between developmental stages and survival at each stage being recorded. This data were used to analyse the proportion of individuals surviving from egg to pupae, from pupae to adult and in total from egg to adult. The duration of each of these developmental stages was also analysed. As a measure of the impact of larval diet on individual quality, pupal weight was also analysed. This experiment tested the hypothesis that a laboratory-adapted population of medfly would retain the ability to survive in a wide range of nutritional conditions as observed in natural populations (e.g. Krainacker et al. 1987). It also tested the ability of medfly larvae to alter developmental duration in response to different host environments (e.g. Carey 1984). Chapter 2 formed the basis of work published in Nash and Chapman (2014).

In Chapter 3, replicate populations of an EE study were tested for assortative mating indicative of RI, at three time points during the lifetime of the experiment. The EE design divided a single outbred population of medfly between two divergent larval diets, one based on sucrose the other on starch. The approximate calorific value of the two diets was calculated. Three replicate populations of each dietary treatment were tested against each other following 3-5, 30 and 60 generations of EE. Two experimental designs were used. The first utilised a limited choice quartet mating test design. The second a multiple-choice mating test. Proximate and maternal effects of

diet were minimised by rearing both treatments on a common garden diet. The identity of mating pairs at all time points was recorded and these data used to calculate three isolation coefficients described by Rolan-Alvarez and Caballero (2000). These estimated sexual isolation, sexual selection, and total isolation on the basis of mate choice exhibited between replicate lines. In order to statistically test for sexual isolation between populations an isolation index was also calculated. Asymmetry of mate choice was analysed as well as an estimator of the sexual fitness of individuals of each sex. This chapter tested the hypothesis that assortative mating indicative of RI would evolve between medfly populations evolving under divergent larval dietary regimes, when gene flow was excluded. This chapter formed the basis of a manuscript submitted to *Evolution*, which is currently in revision.

Chapter 4 further dissected the mate choice exhibited in the 30^{th} generation of the EE populations studied in Chapter 3. Single choice mating tests were used to test whether the results of Chapter 3 were replicable under different testing conditions. Mating tests were conducted with, and without, the proximate effects of diet in males. A video analysis methodology was employed to build a detailed description of male courtship behaviour leading to successful copulation. The frequency of occurrence of 4 key courtship behaviours and 7 non-courtship behaviours was recorded, as well as the total time spent in each of these behaviours prior to copulation. Data were analysed using generalised linear mixed models. This chapter tested the hypothesis that males from different EE regimes would exhibit divergent courtship behaviour. Also, it considered whether patterns of courtship behaviour could explain patterns of mate choice between populations.

Chapter 5 presents the results of RNA sequencing of males drawn form the 62^{nd} generation of the EE populations described in Chapter 3 and 4. Individuals from three replicate populations of both dietary regimes were reared on a common garden diet until sexual maturity, then flash frozen. Total RNA was extracted and divided into head/thorax and abdomen samples to allow identification of some degree of tissue specificity. The resulting RNA sequence data from these samples was subjected to rigorous quality control and a novel resampling based bootstrap normalisation to control for inter-sample variability. Genes exhibiting DE above 2 $\log_2$ offset fold change were annotated and grouped using manually assigned gene ontology terms. These data were used to the hypothesis that EE on divergent larval diets caused a replicated difference in the expression of genes across the genome. The hypothesis that genes

exhibiting DE would also be related to dietary adaptation or mate choice patterns was also investigated.

In Chapter 6 a summary of the key findings of the thesis research is presented. The limitations are reviewed and ways in which to extend the work in future considered. Perspectives are provided on the implications of the work and specific consideration is given to the potential for the medfly to be used as a model for testing ecological speciation hypotheses in the laboratory.

1.6 References

Agosta, S. J. 2008. Fitness consequences of host use in the field: temporal variation in performance and a life history tradeoff in the moth *Rothschildia lebeau* (Saturniidae). Oecologia 157:69–82.

Althoff, D. M., K. A. Segraves, and M. T. J. Johnson. 2014. Testing for coevolutionary diversification: linking pattern with process. Trends Ecol. Evol. 29:82–9.

Aluja, M., D. Perez-Staples, J. Sivinski, A. Sanchez, J. Pinero, D. Pérez-Staples, A. Sánchez, and J. Piñero. 2008. Effects of male condition on fitness in two tropical tephritid flies with contrasting life histories. Anim. Behav. 76:1997–2009.

Anjos-Duarte, C. S., A. M. Costa, and I. S. Joachim-Bravo. 2011. Sexual behaviour of the Mediterranean fruit fly (Diptera: Tephritidae): the influence of female size on mate choice. J. Appl. Entomol. 135:367–373.

Araya, C. L., C. Payen, M. J. Dunham, and S. Fields. 2010. Whole-genome sequencing of a laboratory-evolved yeast strain. BMC Genomics 11:88.

Arita, L. H., and K. Y. Kaneshiro. 1988. Body size and differential mating success between males of two populations of the Mediterranean fruit fly. Pacific Sci. 42:173–177.

Arita, L. H., and K. Y. Kaneshiro. 1985. The Dynamics of the Lek System and Mating Success in Males of the Mediterranean Fruit Fly, *Ceratitis capitata* (Wiedemann). Proc. Hawaiian Entomol. Soc. 25:39–48.

Bailey, S. F., and T. Bataillon. 2015. Can the experimental evolution program help us elucidate the genetic basis of adaptation in nature? Mol. Ecol. n/a–n/a.

Bailey, S. F., N. Rodrigue, and R. Kassen. 2015. The effect of selection environment on the probability of parallel evolution. Mol. Biol. Evol. 32:1436–48.

Barrett, R. D. H., S. M. Rogers, and D. Schluter. 2008. Natural selection on a major armor gene in threespine stickleback. Science 322:255–7.

Barton, N. H. 1995. Linkage and the limits to natural selection. Genetics 140:821–41.

Baumann, P., L. Baumann, C. Y. Lai, D. Rouhbakhsh, N. A. Moran, and M. A. Clark. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annu. Rev. Microbiol. 49:55–94.

Behmer, S. T. 2009. Insect Herbivore Nutrient Regulation. Annu. Rev. Entomol. 54:165–187.

Behmer, S. T., and A. Joern. 2008. Coexisting generalist herbivores occupy unique nutritional feeding niches. Proc. Natl. Acad. Sci. U. S. A. 105:1977–82.

Benelli, G., K. M. Daane, A. Canale, C.-Y. Niu, R. H. Messing, and R. I. Vargas. 2014a. Sexual communication and related behaviours in *Tephritidae*: current knowledge and potential applications for Integrated Pest Management. J. Pest Sci. (2004). 87:385–405.

Benelli, G., G. Giunti, A. Canale, and R. H. Messing. 2014b. Lek dynamics and cues evoking mating behavior in tephritid flies infesting soft fruits: implications for behavior-based control tools. Appl. Entomol. Zool. 49:363–373.

Berlocher, S. H. 2000. Radiation and divergence in the *Rhagoletis pomonella* species group: Inferences from allozymes. Evolution. 54:543–557.

Berlocher, S. H., and J. L. Feder. 2002. Sympatric speciation in phytophagous insects: moving beyond controversy? Annu. Rev. Entomol. 47:773–815.

Blay, S., and B. Yuval. 1997. Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). Anim. Behav. 54:59–66.

Blount, Z. D., J. E. Barrick, C. J. Davidson, and R. E. Lenski. 2012. Genomic analysis of a key innovation in an experimental *Escherichia coli* population. Nature 489:513–8.

Bonizzoni, M., C. R. Guglielmino, C. J. Smallridge, M. Gomulski, A. R. Malacrida, and G. Gasperi. 2004. On the origins of medfly invasion and expansion in Australia. Mol. Ecol. 13:3845–55.

Brand, C. L., S. B. Kingan, L. Wu, and D. Garrigan. 2013. A selective sweep across species boundaries in *Drosophila*. Mol. Biol. Evol. 30:2177–86.

Briceño, R. D., W. G. Eberhard, J. C. Vilardi, P. Liedo, and T. E. Shelly. 2002. Variation in the intermittent buzzing songs of male medflies (Diptera : Tephritidae) associated with geography, mass-rearing, and courtship success. Florida Entomol. 85:32–40.

Briceño, R. D., W. G. Eberhard, J. Vilardi, J. Cayol, and T. Shelly. 2007. Courtship behavior of different wild strains of *Ceratitis capitata* (Diptera: Tephritidae). Florida Entomol. 90:15–18.

Bruce, T. J. A. 2015. Interplay between insects and plants: dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. J. Exp. Bot. 66:455–65.

Burke, M. K., J. P. Dunham, P. Shahrestani, K. R. Thornton, M. R. Rose, and A. D. Long. 2010. Genome-wide analysis of a long-term evolution experiment with *Drosophila*. Nature 467:587–90.

Bush, G. L. 1969a. Mating behavior, host specificity, and the ecological significance of sibling species in frugivorous flies of the genus *Rhagoletis* (Diptera-Tephritidae). Am. Nat. 103:669–672.

Bush, G. L. 1969b. Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera, Tephritidae). Evolution. 23:237–251.

Bush, G. L. 1994. Sympatric speciation in animals: new wine in old bottles. Trends Ecol. Evol. 9:285–8.

Butlin, R. K., J. Galindo, and J. W. Grahame. 2008. Sympatric, parapatric or allopatric: the most important way to classify speciation? Philos. Trans. R. Soc. Lond. B. Biol. Sci. 363:2997–3007.

Canato, C. M., and F. S. Zucoloto. 1993. Diet selection by *Ceratitis capitata* larvae (Diptera, Tephritidae): Influence of the rearing diet and genetic factors. J. Insect Physiol. 39:981–985.

Capy, P., and P. Gibert. 2004. *Drosophila melanogaster, Drosophila simulans*: so Similar yet so Different. Genetica 120:5–15.

Carey, J. R. 1991. Establishment of the Mediterranean fruit fly in California. Science (80-.). 253:1369–1373.

Carey, J. R. 1984. Host-specific demographic studies of the Mediterranean fruit fly *Ceratitis capitata*. Ecol. Entomol. 9:261–270.

Cayol, J. P. 2000. Changes in sexual behavior and life history traits of tephritid species caused by mass-rearing processes. Fruit Flies Phylogeny Evol. Behav. 843–860.

Cayol, J. P., P. Coronado, M. Taher, and Cayol. 2002. Sexual compatibility in medfly (Diptera : Tephritidae) from different origins. Florida Entomol. 85:51–57.

Chang, C. L. 2004. Effect of amino acids on larvae and adults of *Ceratitis capitata* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 97:529–535.

Chang, C. L., C. Albrecht, S. S. A. El-Shall, and R. Kurashima. 2001. Adult reproductive capacity of *Ceratitis capitata* (Diptera: Tephritidae) on a chemically defined diet. Ann. Entomol. Soc. Am. 94:702–706.

Chenoweth, S. F., N. C. Appleton, S. L. Allen, and H. D. Rundle. 2015. Genomic Evidence that sexual selection impedes adaptation to a novel environment. Curr. Biol. 25:1860–1866.

Cocroft, R. B., R. L. Rodriguez, and R. E. Hunt. 2010. Host shifts and signal divergence: mating signals covary with host use in a complex of specialized plant-feeding insects. Biol. J. Linn. Soc. 99:60–72.

Cocroft, R. B., R. L. Rodríguez, and R. E. Hunt. 2008. Host shifts, the evolution of communication, and speciation in the *Enchenopa binotata* species complex of treehoppers. Pp. 88–100 *in* K. J. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press.

Comeault, A. A., S. M. Flaxman, R. Riesch, E. Curran, V. Soria-Carrasco, Z. Gompert, T. E. Farkas, M. Muschick, T. L. Parchman, T. Schwander, J. Slate, and P. Nosil. 2015. Selection on a genetic polymorphism counteracts ecological speciation in a stick insect. Curr. Biol. 25:1975–81.

Costa, A. M., C. S. Anjos-Duarte, A. K. P. Roriz, V. S. Dias, and I. S. Joachim-Bravo. 2012. Male diet and age influence to inhibit female remating in *Ceratitis capitata* (Diptera: Tephritidae). J. Appl. Entomol. 136:456–463.

Coyne, J. A. 1996. Genetics of a difference in male cuticular hydrocarbons between two sibling species, *Drosophila simulans* and *D. sechellia*. Genetics 143:1689–1698.

Coyne, J. A., and H. A. Orr. 1997. "Patterns of speciation in *Drosophila*" revisited. Evolution. 51:295–303.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Coyne, J., and H. Orr. 1989. Patterns of speciation in *Drosophila*. Evolution. 43:362–381.

Dambroski, H. R., and J. L. Feder. 2007. Host plant and latitude-related diapause variation in *Rhagoletis pomonella*: A test for multifaceted life history adaptation on different stages of diapause development. J. Evol. Biol. 20:2101–2112.

Darwin, C. 1859. The origin of species by means of natural selection: or, the preservation of favored races in the struggle for life. John Murray, London.

Dennis, R. L. H., L. Dapporto, S. Fattorini, and L. M. Cook. 2011. The generalism-specialism debate: the role of generalists in the life and death of species. Biol. J. Linn. Soc. 104:725–737.

Diamantidis, A. D., J. R. Carey, C. T. Nakas, and N. T. Papadopoulos. 2011a. Ancestral populations perform better in a novel environment: Domestication of Mediterranean fruit fly populations from five global regions. Biol. J. Linn. Soc. 102:334–345.

Diamantidis, A. D., J. R. Carey, C. T. Nakas, and N. T. Papadopoulos. 2011b. Population-specific demography and invasion potential in medfly. Ecol. Evol. 1:479–488.

Diamantidis, A. D., J. R. Carey, and N. T. Papadopoulos. 2008a. Life-history evolution of an invasive tephritid. J. Appl. Entomol. 132:695–705.

Diamantidis, A. D., N. T. Papadopoulos, and J. R. Carey. 2008b. Medfly populations differ in diel and age patterns of sexual signalling. Entomol. Exp. Appl. 128:389–397.

Diamantidis, A. D., N. T. Papadopoulos, C. T. Nakas, S. Wu, H. G. MÜller, and J. R. Carey. 2009. Life history evolution in a globally invading tephritid: Patterns of survival and reproduction in medflies from six world regions. Biol. J. Linn. Soc. 97:106–117.

Dobzhansky, T. G. 1951. Genetics and the Origin of Species. Columbia University Press.

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

Drès, M., and J. Mallet. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 357:471–92.

Duvaux, L., Q. Geissmann, K. Gharbi, J.-J. Zhou, J. Ferrari, C. M. Smadja, and R. K. Butlin. 2015. Dynamics of copy number variation in host races of the pea aphid. Mol. Biol. Evol. 32:63–80.

Egan, S. P., G. J. Ragland, L. Assour, T. H. Q. Powell, G. R. Hood, S. Emrich, P. Nosil, and J. L. Feder. 2015. Experimental evidence of genome-wide impact of ecological selection during early stages of speciation-with-gene-flow. Ecol. Lett. 18:817–825.

Ehrlich, P. R., and P. H. Raven. 1964. Butterflies and plants: a study in coevolution. Evolution. 18:586–608.

Etges, W. 1992. Premating isolation is determined by larval substrates in cactophilic *Drosophila mojavensis*. Evolution. 46:1945–1950.

Etges, W. J. 1990. Direction of life history evolution in *Drosophila mojavensis*. Pp. 37–56 *in* J. S. F. Barker, W. T. Starmer, and R. J. MacIntyre, eds. Ecological and evolutionary genetics of Drosophila. Springer, US.

Etges, W. J. 2014. No boundaries: genomes, organisms, and ecological interactions responsible for divergence and reproductive isolation. J. Hered. 105 Suppl :756–70.

Etges, W. J., and M. A. Ahrens. 2001. Premating isolation is determined by larval-rearing substrates in cactophilic *Drosophila mojavensis*. V. Deep geographic variation in epicuticular hydrocarbons among isolated populations. Am. Nat. 158:585–598.

Etges, W. J., C. C. de Oliveira, E. Gragg, D. Ortíz-Barrientos, M. a F. Noor, and M. G. Ritchie. 2007. Genetics of incipient speciation in *Drosophila mojavensis*. I. Male courtship song, mating success, and genotype X environment interactions. Evolution. 61:1106–1119.

Etges, W. J., C. C. De Oliveira, M. A. F. Noor, and M. G. Ritchie. 2010. Genetics of incipient speciation in *Drosophila mojavensis*. III. Life-history divergence in allopatry and reproductive isolation. Evolution 64:3549–69.

Etges, W. J., C. C. de Oliveira, M. G. Ritchie, and M. A. F. Noor. 2009. Genetics of incipient speciation in *Drosophila mojavensis*: II. Host plants and mating status influence cuticular hydrocarbon QTL expression and G x E interactions. Evolution 63:1712–30.

Etges, W. J., W. R. Johnson, G. A. Duncan, G. Huckins, and W. B. Heed. 1999. Ecological genetics of cactophilic *Drosophila*. Pp. 164–214. *in* R. Robichaux, ed. Ecology of Sonoran Desert plants and plant communities. University of Arizona Press, Tucson, Arizona.

Etges, W. J., M. V Trotter, C. C. de Oliveira, S. Rajpurohit, A. G. Gibbs, and S. Tuljapurkar. 2015. Deciphering life history transcriptomes in different environments. Mol. Ecol. 24:151–79.

Etges, W. W. J., C. L. C. Veenstra, and L. L. Jackson. 2006. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. VII. Effects of larval dietary fatty acids on adult epicuticular hydrocarbons. J. Chem. Ecol. 32:2629–46.

Farine, J.-P., L. Legal, B. Moreteau, and J.-L. Le Quere. 1996. Volatile components of ripe fruits of *Morinda citrifolia* and their effects on *Drosophila*. Phytochemistry 41:433–438.

Feder, J. L., C. A. Chilcote, and G. L. Bush. 1988. Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. Nature 336:61–64.

Feder, J. L., S. P. Egan, and P. Nosil. 2012. The genomics of speciation-with-gene-flow. Trends Genet. 28:342–50.

Feder, J. L., T. A. Hunt, and L. Bush. 1993. The effects of climate, host plant phenology and host fidelity on the genetics of apple and hawthorn infesting races of *Rhagoletis pomonella*. Entomol. Exp. Appl. 69:117–135.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. U. S. A. 91:7990–4.

Felton, A. M., A. Felton, D. Raubenheimer, S. J. Simpson, W. J. Foley, J. T. Wood, I. R. Wallis, and D. B. Lindenmayer. 2009. Protein content of diets dictates the daily energy intake of a free-ranging primate. Behav. Ecol. 20:685–690.

Fernandes-da-Silva, P., and F. Zucoloto. 1993. The influence of host nutritive value on the performance and food selection in *Ceratitis capitata* (Diptera, Tephritidae). J. Insect Physiol. 39:883–887.

Ferrari, J., H. C. J. Godfray, A. S. Faulconbridge, K. Prior, and S. Via. 2006. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. Evolution 60:1574–84.

Ferrari, J., S. Via, and H. C. J. Godfray. 2008. Population differentiation and genetic variation in performance on eight hosts in the pea aphid complex. Evolution. 62:2508–24.

Ferrari, J., J. A. West, S. Via, and H. C. J. Godfray. 2012. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. Evolution. 66:375–90.

Field, S. A., R. Kaspi, and B. Yuval. 2002. Why do calling medflies (Diptera: Tephritidae) cluster? Assessing the Empirical Evidence for models of medfly lek evolution. Florida Entomol. 85:63–72.

Fitzpatrick, M. J., E. Feder, L. Rowe, and M. B. Sokolowski. 2007. Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene. Nature 447:210–2.

Forister, M. L., L. A. Dyer, M. S. Singer, J. O. Stireman III, J. T. Lill, and Singer, M S. 2012. Revisiting the evolution of ecological specialization, with emphasis on insect-plant interactions. Ecology 93:981–991.

Fox, J. W., and R. E. Lenski. 2015. From Here to Eternity - The theory and practice of a really long experiment. PLoS Biol. 13:e1002185.

Friberg, M., and C. Wiklund. 2009. Host plant preference and performance of the sibling species of butterflies *Leptidea sinapis* and *Leptidea reali*: a test of the trade-off hypothesis for food specialisation. Oecologia 159:127–37.

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

Funk, D. J., K. E. Filchak, and J. L. Feder. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. Pp. 251–267 *in* Genetics of Mate Choice: From Sexual Selection to Sexual Isolation. Springer, Netherlands.

Funk, D. J., and P. Nosil. 2008. Comparative analyses of ecological speciation. Pp. 117–135 *in* K. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Oakland, CA.

Funk, D. J., P. Nosil, and W. J. Etges. 2006. Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. Proc. Natl. Acad. Sci. U. S. A. 103:3209–13.

Futuyma, D. J., and G. C. Mayer. 1980. Non-Allopatric Speciation in Animals. Syst. Zool. 29:254–271.

Futuyma, D. J., and G. Moreno. 1988. The evolution of ecological specialization. Annu. Rev. Ecol. Syst. 19:207–233.

Garrigan, D., S. B. Kingan, A. J. Geneva, P. Andolfatto, A. G. Clark, K. R. Thornton, and D. C. Presgraves. 2012. Genome sequencing reveals complex speciation in the *Drosophila simulans* clade. Genome Res. 22:1499–511.

Gasperi, G., M. Bonizzoni, L. M. Gomulski, V. Murelli, C. Torti, A. R. Malacrida, and C. R. Guglielmino. 2002. Genetic differentiation, gene flow and the origin of infestations of the medfly, *Ceratitis capitata*. Genetica 116:125–135.

Gauthier, J.-P., Y. Outreman, L. Mieuzet, and J.-C. Simon. 2015. Bacterial communities associated with host-adapted populations of pea aphids revealed by deep sequencing of 16S ribosomal DNA. PLoS One 10:e0120664.

Gleason, J. M., J.-M. Jallon, J.-D. Rouault, and M. G. Ritchie. 2005. Quantitative trait loci for cuticular hydrocarbons associated with sexual isolation between *Drosophila simulans* and *D. sechellia*. Genetics 171:1789–98.

Gompert, Z., A. A. Comeault, T. E. Farkas, J. L. Feder, T. L. Parchman, C. A. Buerkle, and P. Nosil. 2014. Experimental evidence for ecological selection on genome variation in the wild. Ecol. Lett. 17:369–79.

Grant, P. R., and B. R. Grant. 2002. Unpredictable evolution in a 30-year study of Darwin's finches. Science 296:707–11.

Gresham, D., M. M. Desai, C. M. Tucker, H. T. Jenq, D. A. Pai, A. Ward, C. G. DeSevo, D. Botstein, and M. J. Dunham. 2008. The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. PLoS Genet. 4:e1000303.

Hall, A. R., and N. Colegrave. 2008. Decay of unused characters by selection and drift. J. Evol. Biol. 21:610–7.

Hardy, N. B., and S. P. Otto. 2014. Specialization and generalization in the diversification of phytophagous insects: tests of the musical chairs and oscillation hypotheses. Proc. R. Soc. B 281:20132960.

Hawthorne, D. J., and S. Via. 2001. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. Nature 412:904–7.

Heath, R. R., P. J. Landolt, J. H. Tumlinson, D. L. Chambers, R. E. Murphy, R. E. Doolittle, B. D. Dueben, J. Sivinski, and C. O. Calkins. 1991. Analysis, synthesis, formulation, and field testing of three major components of male mediterranean fruit fly pheromone. J. Chem. Ecol. 17:1925–40.

Hoekstra, H. E. 2006. Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity (Edinb). 97:222–34.

Höglund, J., and R. V. Alatalo. 1995. Leks. Princeton University Press.

Hood, G. R., S. P. Egan, and J. L. Feder. 2012. Evidence for sexual isolation as a prezygotic barrier to gene flow between morphologically divergent species of *Rhagoletis* fruit flies. Ecol. Entomol. 37:521–528.

Illius, A. W., B. J. Tolkamp, and J. Yearsley. 2002. The evolution of the control of food intake. Proc. Nutr. Soc. 61:465–472.

Innan, H., and F. Kondrashov. 2010. The evolution of gene duplications: classifying and distinguishing between models. Nat. Rev. Genet. 11:4.

Jaenike, J. 1990. Host Specialization in Phytophagous Insects. Annu. Rev. Ecol. Syst. 21:243–273.

Jang, E. B., D. M. Light, R. G. Binder, R. A. Flath, and L. A. Carvalho. 1994. Attraction of female mediterranean fruit flies to the five major components of male-produced pheromone in a laboratory flight tunnel. J. Chem. Ecol. 20:9–20.

Janz, N., and S. Nylin. 2008. The Oscillation hypothesis of host-plant range and speciation. Pp. 203 – 215 *in* K. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Oakland, CA.

Janz, N., S. Nylin, and N. Wahlberg. 2006. Diversity begets diversity: host expansions and the diversification of plant-feeding insects. BMC Evol. Biol. 6:4.

Janz, N., S. Nylin, and N. Wedell. 1994. Host plant utilization in the comma butterfly: sources of variation and evolutionary implications. Oecologia 99:132–140.

Jaquiéry, J., S. Stoeckel, P. Nouhaud, L. Mieuzet, F. Mahéo, F. Legeai, N. Bernard, A. Bonvoisin, R. Vitalis, and J.-C. Simon. 2012. Genome scans reveal candidate regions involved in the adaptation to host plant in the pea aphid complex. Mol. Ecol. 21:5251–64.

Jaureguy, L. M., and W. J. Etges. 2007. Assessing patterns of senescence in *Drosophila mojavensis* reared on different host cacti. Evol. Ecol. Res. 9:91–107.

Jennings, J. H., and W. J. Etges. 2009. Species hybrids in the laboratory but not in nature: a reanalysis of premating isolation between *Drosophila arizonae* and *D. mojavensis*. Evolution 64:587–98.

Jermy, T. 1984. Evolution of insect/host plant relationships. Am. Nat. 124:609–630.

Joachim-Bravo, I. S., C. S. Anjos, and A. M. Costa. 2009. The role of protein in the sexual behaviour of males of *Ceratitis capitata* (Diptera: Tephritidae): mating success, copula duration and number of copulations. Zool. (Curitiba, Impresso) 26:407–412.

Juan-Blasco, M., V. San Andrés, M. A. Martínez-Utrillas, R. Argilés, I. Pla, A. Urbaneja, and B. Sabater-Muñoz. 2013. Alternatives to ginger root oil aromatherapy for improved mating performance of sterile *Ceratitis capitata* (Diptera: Tephritidae) males. J. Appl. Entomol. 137:244–251.

Karsten, M., B. Jansen van Vuuren, P. Addison, and J. S. Terblanche. 2015. Deconstructing intercontinental invasion pathway hypotheses of the Mediterranean fruit fly (*Ceratitis capitata*) using a Bayesian inference approach: are port interceptions and quarantine protocols successfully preventing new invasions? Divers. Distrib. 21:813–825.

Karsten, M., B. J. van Vuuren, A. Barnaud, and J. S. Terblanche. 2013. Population genetics of *Ceratitis capitata* in South Africa: implications for dispersal and pest management. PLoS One 8:e54281.

Kaspi, R., I. Feitelson, T. Drezner, and B. Yuval. 2001. A novel method for rearing the progeny of wild mediterranean fruit flies using artificial fruit. Phytoparasitica 29:15–22.

Kaspi, R., S. Mossinson, T. Drezner, B. Kamensky, and B. Yuval. 2002. Effects of larval diet on development rates and reproductive maturation of male and female Mediterranean fruit flies. Physiol. Entomol. 27:29–38.

Kaspi, R., P. P. W. Taylor, and B. Yuval. 2000. Diet and size influence sexual advertisement and copulatory success of males in Mediterranean fruit fly leks. Ecol. Entomol. 25:279–284.

Kaspi, R., and B. Yuval. 2000. Post-teneral protein feeding improves sexual competitiveness but reduces longevity of mass-reared sterile male mediterranean fruit flies (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 93:949–955.

Katsoyannos, B. I. 1989. Response to shape, size and color. Pp. 307–324. *in* A. S. Robinson and G. Hooper, eds. Fruit flies: Their biology, natural Enemies and control. Elsevier Science Publishers, Amsterdam.

Kawecki, T. J., R. E. Lenski, D. Ebert, B. Hollis, I. Olivieri, and M. C. Whitlock. 2012. Experimental evolution. Trends Ecol. Evol. 27:547–60.

Kliman, R. M., P. Andolfatto, J. A. Coyne, F. Depaulis, M. Kreitman, A. J. Berry, J. McCarter, J. Wakeley, and J. Hey. 2000. The Population Genetics of the Origin and Divergence of the *Drosophila simulans* Complex Species. Genetics 156:1913–1931.

Köhler, A., D. Raubenheimer, and S. W. Nicolson. 2012. Regulation of nutrient intake in nectar-feeding birds: insights from the geometric framework. J. Comp. Physiol. B. 182:603–11.

Kokko, H., and K. Heubel. 2008. Condition-dependence, genotype-by-environment interactions and the lek paradox. Genetica 132:209–16.

Kolss, M., R. K. Vijendravarma, G. Schwaller, and T. J. Kawecki. 2009. Life-history consequences of adaptation to larval nutritional stress in *Drosophila*. Evolution 63:2389–401.

Krainacker, D. A., J. R. Carey, and R. I. Vargas. 1987. Effect of larval host on life history traits of the Mediterranean fruit fly, *Ceratitis capitata*. Oecologia 73:583–590.

Labandeira, C. C. 2013. A paleobiologic perspective on plant-insect interactions. Curr. Opin. Plant Biol. 16:414–21.

Lachaise, D., and J.-F. Silvain. 2004. How two Afrotropical endemics made two cosmopolitan human commensals: the *Drosophila melanogaster* - *D. simulans* palaeogeographic riddle. Genetica 120:17–39.

Lack, D. 1947. Darwin's Finches. Cambridge University Press, Cambridge.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proc. Natl. Acad. Sci. 78:3721–3725.

Lang, G. I., D. Botstein, and M. M. Desai. 2011. Genetic variation and the fate of beneficial mutations in asexual populations. Genetics 188:647–661.

Legal, L., J. R. David, and J. M. Jallon. 1992. Toxicity and attraction effects produced by *Morinda citrifolia* fruits on the *Drosophila melanogaster* complex of species. Chemoecology 3:125–129.

Levinson, H. Z., A. R. Levinson, and K. Müller. 1990. Influence of some olfactory and optical properties of fruits on host location by the Mediterranean fruit fly (*Ceratitis capitata* Wied.). J. Appl. Entomol. 109:44–54.

Light, D. M., E. B. Jang, R. G. Binder, R. A. Flath, and S. Kint. 1999. Minor and intermediate components enhance attraction of female Mediterranean fruit flies to natural male odor pheromone and its synthetic major components. J. Chem. Ecol. 25:2757–2777.

Light, D. M., E. B. Jang, and J. C. Dickens. 1988. Electroantennogram responses of the mediterranean fruit fly, *Ceratitis capitata*, to a spectrum of plant volatiles. J. Chem. Ecol. 14:159–80.

Lind, P. A., A. D. Farr, and P. B. Rainey. 2015. Experimental evolution reveals hidden diversity in evolutionary pathways. Elife 4:e07074.

Linn, C. E., W. L. Yee, S. B. Sim, D. H. Cha, T. H. Q. Powell, R. B. Goughnour, and J. L. Feder. 2012. Behavioral evidence for fruit odor discrimination and sympatric host races of *Rhagoletis pomonella* flies in the Western United States. Evolution 66:3632–41.

Liquido, N. J., L. A. Shinoda, and R. T. Cunningham. 1991. Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): An annotated world review. Entomological Society of America, Lanham.

Louis, J., and J. R. David. 1986. Ecological specialization in the *Drosophila melanogaster* species subgroup: a case study of *D. sechellia*. Acta oecologica. Oecologia Gen. 7:215–229.

Loxdale, H. D., G. Lushai, and J. A. Harvey. 2011. The evolutionary improbability of "generalism" in nature, with special reference to insects. Biol. J. Linn. Soc. 103:1–18.

Lux, S. A., F. N. Munyiri, J. C. Vilardi, P. Liedo, A. Economopoulos, O. Hasson, and S. Quilici. 2002. Consistency in courtship pattern among populations of Medfly (Diptera: Tephritidae): comparisons among wild strains and strains mass reared for SIT Operations. Florida Entomol. 85:113.

Maan, M. E., and O. Seehausen. 2011. Ecology, sexual selection and speciation. Ecol. Lett. 14:591–602.

MacLean, R. C., G. Bell, and P. B. Rainey. 2004. The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. Proc. Natl. Acad. Sci. U. S. A. 101:8072–7.

Maharjan, R. P., B. Liu, L. Feng, T. Ferenci, and L. Wang. 2015. Simple phenotypic sweeps hide complex genetic changes in populations. Genome Biol. Evol. 7:531–44.

Malacrida, A. R., L. M. Gomulski, M. Bonizzoni, S. Bertin, G. Gasperi, and C. R. Guglielmino. 2007. Globalization and fruitfly invasion and expansion: The medfly paradigm. Genetica 131:1–9.

Mallet, J. 2006. What does *Drosophila* genetics tell us about speciation? Trends Ecol. Evol. 21:386–93.

Maor, M., B. Kamensky, S. Shloush, and B. Yuval. 2004. Effects of post-teneral diet on foraging success of sterile male Mediterranean fruit flies. Entomol. Exp. Appl. 110:225–230.

Markow, T. A. 1991. Sexual isolation among populations of *Drosophila mojavensis*. Evolution. 45:1525–1529.

Matsuo, T., S. Sugaya, J. Yasukawa, T. Aigaki, and Y. Fuyama. 2007. Odorant-binding proteins OBP57d and OBP57e affect taste perception and host-plant preference in *Drosophila sechellia*. PLoS Biol. 5:e118.

Mayr, E. 1963. Animal Species and Evolution. Harvard University Press, Cambridge, MA.

Mayr, E. 1947. Ecological factors in speciation. Evolution. 1:263–288.

Mayr, E. 1942. Systematics and the Origin of Species, from the Viewpoint of a Zoologist. Columbia University Press, New York.

McBride, C. S. 2007. Rapid evolution of smell and taste receptor genes during host specialization in *Drosophila sechellia*. Proc. Natl. Acad. Sci. U. S. A. 104:4996–5001.

McKenna, D. D., A. S. Sequeira, A. E. Marvaldi, and B. D. Farrell. 2009. Temporal lags and overlap in the diversification of weevils and flowering plants. Proc. Natl. Acad. Sci. U. S. A. 106:7083–8.

McKinnon, J. S., S. Mori, B. K. Blackman, L. David, D. M. Kingsley, L. Jamieson, J. Chou, and D. Schluter. 2004. Evidence for ecology's role in speciation. Nature 429:294–8.

McNett, G. D., and R. B. Cocroft. 2008. Host shifts favor vibrational signal divergence in Enchenopa binotata treehoppers. Behav. Ecol. 19:650–656.

McPheron, B. A., D. C. Smith, and S. H. Berlocher. 1988. Genetic differences between host races of *Rhagoletis pomonella*. Nature 336:64–66.

Mery, F., and T. J. Kawecki. 2004. The effect of learning on experimental evolution of resocurce preference in *Drosopihla melanogaster*. Evolution. 58:757–767.

Metzger, B. P. H., D. C. Yuan, J. D. Gruber, F. Duveau, and P. J. Wittkopp. 2015. Selection on noise constrains variation in a eukaryotic promoter. Nature 521:344–347.

Mitter, C., B. Farrell, and B. Wiegmann. 1988. The phylogenetic study of adaptive zones: Has phytophagy promoted insect diversification? Am. Nat. 132:107–128.

Moreteau, B., S. R'Kha, and J. R. David. 1994. Genetics of a nonoptimal behavior: Oviposition preference of *Drosophila mauritiana* for a toxic resource. Behav. Genet. 24:433–441.

Muller, H. J. 1942. Isolating mechanisms, evolution and temperature. Biol. Symp. 6:71–125.

Nagel, L., and D. Schluter. 1998. Body Size, natural selection, and speciation in sticklebacks. Evolution. 52:209–218.

Nash, W. J., and T. Chapman. 2014. Effect of dietary components on larval life history characteristics in the medfly (*Ceratitis capitata*: Diptera, Tephritidae). PLoS One 9:e86029.

Navarro-Campos, C., M. T. Martínez-Ferrer, J. M. Campos, J. M. Fibla, J. Alcaide, L. Bargues, C. Marzal, and F. Garcia-Marí. 2011. The influence of host fruit and temperature on the body size of adult *Ceratitis capitata* (Diptera: Tephritidae) under laboratory and field conditions. Environ. Entomol. 40:931–938.

Nestel, D., and E. Nemny-Lavy. 2008. Nutrient balance in medfly, *Ceratitis capitata*, larval diets affects the ability of the developing insect to incorporate lipid and protein reserves. Entomol. Exp. Appl. 126:53–60.

Nestel, D., E. Nemny-Lavy, and C. L. Chang. 2004. Lipid and protein loads in pupating larvae and emerging adults as affected by the composition of Mediterranean fruit fly (*Ceratitis capitata*) meridic larval diets. Arch. Insect Biochem. Physiol. 56:97–109.

Noor, M. A., K. L. Grams, L. A. Bertucci, and J. Reiland. 2001. Chromosomal inversions and the reproductive isolation of species. Proc. Natl. Acad. Sci. U. S. A. 98:12084–8.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking sticks. Am. Nat. 169:151–62.

Nosil, P. 2012. Ecological Speciation. Oxford University Press, Oxford.

Nosil, P. 2015. Evolution: Sex limits adaptation. Curr. Biol. 25:R613–R616.

Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proc. R. Soc. B 271:1521–8.

Nosil, P. 2002. Transition rates between specialization and generalization in phytophagous insects. Evolution. 56:1701–1706.

Nosil, P., and B. J. Crespi. 2006. Experimental evidence that predation promotes divergence in adaptive radiation. Proc. Natl. Acad. Sci. 103:9090–9095.

Nosil, P., and J. L. Feder. 2012. Genomic divergence during speciation: causes and consequences. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367:332–42.

Nosil, P., Z. Gompert, T. E. Farkas, A. A. Comeault, J. L. Feder, C. A. Buerkle, and T. L. Parchman. 2012. Genomic consequences of multiple speciation processes in a stick insect. Proc. R. Soc. B 279:5058–65.

Nosil, P., L. J. Harmon, and O. Seehausen. 2009. Ecological explanations for (incomplete) speciation. Trends Ecol. Evol. 24:145–156.

Nosil, P., and A. Ø. Mooers. 2005. Testing hypotheses about ecological specialization using phylogenetic trees. Evolution. 59:2256–2263.

Nosil, P., T. H. Vines, and D. J. Funk. 2005. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution. 59:705–19.

Nouhaud, P., J. Peccoud, F. Mahéo, L. Mieuzet, J. Jaquiéry, and J.-C. Simon. 2014. Genomic regions repeatedly involved in divergence among plant-specialized pea aphid biotypes. J. Evol. Biol. 27:2013–20.

Novotny, V., and Y. Basset. 2005. Host specificity of insect herbivores in tropical forests. Proc. Biol. Sci. 272:1083–90.

Nyamukondiwa, C., E. Kleynhans, and J. S. Terblanche. 2010. Phenotypic plasticity of thermal tolerance contributes to the invasion potential of Mediterranean fruit flies (*Ceratitis capitata*). Ecol. Entomol. 35:565–575.

Nyman, T. 2010. To speciate, or not to speciate? Resource heterogeneity, the subjectivity of similarity, and the macroevolutionary consequences of niche-width shifts in plant-feeding insects. Biol. Rev. 85:393–411.

Oliver, K. M., P. H. Degnan, G. R. Burke, and N. A. Moran. 2010. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu. Rev. Entomol. 55:247–66.

Orr, H. 2001. The genetics of species differences. Trends Ecol. Evol. 16:343–350.

Ortiz-Barrientos, D., J. Reiland, J. Hey, and M. Noor. 2002. Recombination and the divergence of hybridizing species. Genetica 116:167–178.

Owen-Smith, N., J. M. Fryxell, and E. H. Merrill. 2010. Foraging theory upscaled: the behavioural ecology of herbivore movement. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365:2267–78.

Papadopoulos, N. T., B. I. Katsoyannos, N. a. Kouloussis, and J. Hendrichs. 2001. Effect of orange peel substances on mating competitiveness of male *Ceratitis capitata*. Entomol. Exp. Appl. 99:253–261.

Papanastasiou, S. A., A. D. Diamantidis, C. T. Nakas, J. R. Carey, and N. T. Papadopoulos. 2011. Dual reproductive cost of aging in male medflies: Dramatic decrease in mating competitiveness and gradual reduction in mating performance. J. Insect Physiol. 57:1368–1374.

Peccoud, J., F. Mahéo, M. de la Huerta, C. Laurence, and J.-C. Simon. 2015a. Genetic characterisation of new host-specialised biotypes and novel associations with bacterial symbionts in the pea aphid complex. Insect Conserv. Divers. 8:484–492.

Peccoud, J., F. Mahéo, M. de la Huerta, C. Laurence, and J.-C. Simon. 2015b. Genetic characterisation of new host-specialised biotypes and novel associations with bacterial symbionts in the pea aphid complex. Insect Conserv. Divers. n/a–n/a.

Peccoud, J., A. Ollivier, M. Plantegenest, and J.-C. Simon. 2009a. A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. Proc. Natl. Acad. Sci. U. S. A. 106:7495–7500.

Peccoud, J., and J.-C. Simon. 2010. The pea aphid complex as a model of ecological speciation. Ecol. Entomol. 35:119–130.

Peccoud, J., J.-C. Simon, H. J. McLaughlin, and N. A. Moran. 2009b. Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. Proc. Natl. Acad. Sci. U. S. A. 106:16315–20.

Powell, G., C. R. Tosh, and J. Hardie. 2006. Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. Annu. Rev. Entomol. 51:309–30.

Powell, T. H. Q., D. H. Cha, C. E. Linn, and J. L. Feder. 2012. On the scent of standing variation for speciation: behavioral evidence for native sympatric host races of *Rhagoletis pomonella* (Diptera: Tephritidae) in the southern United States. Evolution. 66:2739–56.

Powell, T. H. Q., A. A. Forbes, G. R. Hood, and J. L. Feder. 2014. Ecological adaptation and reproductive isolation in sympatry: genetic and phenotypic evidence for native host races of Rhagoletis pomonella. Mol. Ecol. 23:688–704.

Powell, T. H. Q., G. R. Hood, M. O. Murphy, J. S. Heilveil, S. H. Berlocher, P. Nosil, and J. L. Feder. 2013. Genetic divergence along the speciation continuum: the transition from host race to species in *Rhagoletis* (Diptera: tephritidae). Evolution 67:2561–76.

Prokopy, R. J., and J. Hendrichs. 1979. Mating behavior of *Ceratitis capitata* on a field-caged host tree. Ann. Entomol. Soc. Am. 72:642–648.

Prokopy, R. R. J., P. T. McDonald, and T. T. Y. Wong. 1984. Inter-population variation among *Ceratitis capitata* flies in host acceptance pattern. Entomol. Exp. Appl. 35:65–69.

Pyke, G. H., H. R. Pulliman, and E. L. Charnov. 1977. Optimal foraging: a selective review of theory and tests. Q. Rev. Biol. 137–154.

R'Kha, S., P. Capy, and J. R. David. 1991. Host-plant specialization in the *Drosophila melanogaster* species complex: a physiological, behavioral, and genetical analysis. Proc. Natl. Acad. Sci. 88:1835–1839.

Rabosky, D. L. 2009. Ecological limits and diversification rate: alternative paradigms to explain the variation in species richness among clades and regions. Ecol. Lett. 12:735–43.

Rabosky, D. L., and D. R. Matute. 2013. Macroevolutionary speciation rates are decoupled from the evolution of intrinsic reproductive isolation in *Drosophila* and birds. Proc. Natl. Acad. Sci. U. S. A. 110:15354–9.

Ragland, G. J., S. B. Sim, S. Goudarzi, J. L. Feder, and D. A. Hahn. 2012. Environmental interactions during host race formation: host fruit environment moderates a seasonal shift in phenology in host races of *Rhagoletis pomonella*. Funct. Ecol. 26:921–931.

Raubenheimer, D., and S. J. Simpson. 1993. The geometry of compensatory feeding in the locust. Anim. Behav. 45:953–964.

Raubenheimer, D., S. J. Simpson, and D. Mayntz. 2009. Nutrition, ecology and nutritional ecology: toward an integrated framework. Funct. Ecol. 23:4–16.

Rebar, D., and R. L. Rodríguez. 2014a. Genetic variation in host plants influences the mate preferences of a plant-feeding insect. Am. Nat. 184:489–99.

Rebar, D., and R. L. Rodríguez. 2015. Insect mating signal and mate preference phenotypes covary among host plant genotypes. Evolution. 69:602–610.

Rebar, D., and R. L. Rodríguez. 2014b. Trees to treehoppers: genetic variation in host plants contributes to variation in the mating signals of a plant-feeding insect. Ecol. Lett. 17:203–10.

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

Rieseberg, L. H. 2001. Chromosomal rearrangements and speciation. Trends Ecol. Evol. 16:351–358.

Robinson, A. S. Robinson, J. P. Cayol, and J. Hendrichs. 2002. Recent findings on medfly sexual behavior: Implications for sit. Florida Entomol. 85:171–181.

Rodríguez, R. L., K. Ramaswamy, and R. B. Cocroft. 2006. Evidence that female preferences have shaped male signal evolution in a clade of specialized plant-feeding insects. Proc. Biol. Sci. 273:2585–93.

Rodríguez, R. L., L. E. Sullivan, and R. B. Cocroft. 2004. Vibrational communication and reproductive isolation in the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). Evolution. 58:571–578.

Roff, D. A. 1992. Evolution Of Life Histories: Theory and Analysis. Springer, US.

Rogers, S. M. 2015. Evolution: Anti-speciation in walking sticks. Curr. Biol. 25:R726–8.

Roininen, H., and J. Tahvanainen. 1989. Host selection and larval performance of two willow-feeding sawflies. Ecology 70:129.

Rolan-Alvarez, E., and A. Caballero. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution. 54:30–36.

Rose, M. R. 1984. Laboratory Evolution of Postponed Senescence in *Drosophila melanogaster*. Evolution. 38:1004–1010.

Rose, M. R., M. D. Drapeau, P. G. Yazdi, K. H. Shah, D. B. Moise, R. R. Thakar, C. L. Rauser, and L. D. Mueller. 2002. Evolution of late-life mortality in *Drosophila melanogaster*. Evolution. 56:1982–1991.

Rosenzweig, R. F., R. R. Sharp, D. S. Treves, and J. Adams. 1994. Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. Genetics 137:903–917.

Roughgarden, J. 1972. Evolution of niche width. Am. Nat. 106:683–718.

Rowe, L., and D. Houle. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc. R. Soc. B 263:1415–1421.

Rozen, D., and R. Lenski. 2000. Long-term experimental evolution in *Escherichia coli*. VIII. Dynamics of a balanced polymorphism. Am. Nat. 155:24–35.

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

Rundle, H. D., S. F. Chenoweth, and M. W. Blows. 2006. The roles of natural and sexual selection during adaptation to a novel environment. Evolution. 60:2218–2225.

Rundle, H. D., S. F. Chenoweth, P. Doughty, and M. W. Blows. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLoS Biol. 3:e368.

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336–352.

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

Ruohonen, K., S. J. Simpson, and D. Raubenheimer. 2007. A new approach to diet optimisation: A re-analysis using European whitefish (*Coregonus lavaretus*). Aquaculture 267:147–156.

Sandoval, C. P. 1994. The effects of the relative geographic scales of gene flow and selection on morph frequencies in the walking-stick *Timema cristinae*. Evolution. 48:1866–1879.

Santos, M., D. J. Borash, A. Joshi, N. Bounlutay, and L. D. Mueller. 1997. Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. Evolution. 51:420–432.

Schlötterer, C., R. Kofler, E. Versace, R. Tobler, and S. U. Franssen. 2014. Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation. Heredity. 114:431–440.

Schluter, D. 2001. Ecology and the origin of species. Trends Ecol. Evol. 16:372–380.

Schluter, D. 2009. Evidence for ecological speciation and its alternative. Science 323:737–41.

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

Schmitz, O. J. 2008. Herbivory from Individuals to Ecosystems. Annu. Rev. Ecol. Evol. Syst.

Seehausen, O., R. K. Butlin, I. Keller, C. E. Wagner, J. W. Boughman, P. A. Hohenlohe, C. L. Peichel, G.-P. Saetre, C. Bank, A. Brännström, A. Brelsford, C. S. Clarkson, F. Eroukhmanoff, J. L. Feder, M. C. Fischer, A. D. Foote, P. Franchini, C. D. Jiggins, F. C. Jones, A. K. Lindholm, K. Lucek, M. E. Maan, D. A. Marques, S. H. Martin, B. Matthews, J. I. Meier, M. Möst, M. W. Nachman, E. Nonaka, D. J. Rennison, J. Schwarzer, E. T. Watson, A. M. Westram, and A. Widmer. 2014. Genomics and the origin of species. Nat. Rev. Genet. 15:176–92.

Segrè, A. V, A. W. Murray, and J.-Y. Leu. 2006. High-resolution mutation mapping reveals parallel experimental evolution in yeast. PLoS Biol. 4:e256.

Servedio, M. R., Y. Brandvain, S. Dhole, C. L. Fitzpatrick, E. E. Goldberg, C. A. Stern, J. Van Cleve, and D. J. Yeh. 2014. Not just a theory—The utility of mathematical models in evolutionary biology. PLoS Biol. 12:e1002017.

Shelly, T. E. 2001. Lek size and female visitation in two species of tephritid fruit flies. Anim. Behav. 62:33–40.

Shelly, T. E., S. S. Kennelly, and D. O. McInnis. 2002. Effect of adult diet on signaling activity, mate attraction, and mating success in male mediterranean fruit flies (Diptera : Tephritidae). Florida Entomol. 85:150–155.

Shelly, T. E., T. S. Whittier, and K. Y. Kaneshiro. 1994. Sterile insect release and the natural mating system of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 87:470–481.

Simon, J.-C., E. D'Alençon, E. Guy, E. Jacquin-Joly, J. Jaquiéry, P. Nouhaud, J. Peccoud, A. Sugio, and R. Streiff. 2015. Genomics of adaptation to host-plants in herbivorous insects. Brief. Funct. Genomics, doi: 10.1093/bfgp/elv015.

Simpson, G. G. 1953. The major features of evolution. Columbia University Press, New York.

Simpson, S. J., F. J. Clissold, M. Lihoreau, F. Ponton, S. M. Wilder, and D. Raubenheimer. 2015. Recent advances in the integrative nutrition of arthropods. Annu. Rev. Entomol. 60:293–311.

Simpson, S. J., and D. Raubenheimer. 2001. A framework for the study of macronutrient intake in fish. Aquac. Res. 32:421–432.

Simpson, S. J., and D. Raubenheimer. 1993a. A multi–level analysis of feeding behaviour: the geometry of nutritional decisions. Phil Trans R Soc B 342:381–402.

Simpson, S. J., and D. Raubenheimer. 2007. Caloric restriction and aging revisited: the need for a geometric analysis of the nutritional bases of aging. J. Gerontol. Ser. A Biol. Sci. Med. Sci. 62:707–713.

Simpson, S. J., and D. Raubenheimer. 1993b. The central role of the haemolymph in the regulation of nutrient intake in insects. Physiol. Entomol. 18:395–403.

Simpson, S. J., and D. Raubenheimer. 2012. The nature of nutrition: A unifying framework from animal adaptation to human obesity. Princeton University Press, Princeton, NJ.

Simpson, S. J., R. M. Sibly, K. P. Lee, S. T. Behmer, and D. Raubenheimer. 2004. Optimal foraging when regulating intake of multiple nutrients. Anim. Behav. 68:1299–1311.

Singer, M. S. 2008. Evolutionary ecology of polyphagy. Pp. 29–42 *in* K. J. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Oakland, CA.

Slansky, F., and J. G. Rodriguez. 1987. Nutritional ecology of insects, mites, spiders, and related invertebrates: an overview. Pp. 1–69 *in* F. Slansky and J. G. Rodriguez, eds. Nutritional ecology of insects, mites, spiders, and related invertebrates. Wiley, New York.

Smadja, C. M., B. Canbäck, R. Vitalis, M. Gautier, J. Ferrari, J.-J. Zhou, and R. K. Butlin. 2012. Large-scale candidate gene scan reveals the role of chemoreceptor genes in host plant specialization and speciation in the pea aphid. Evolution 66:2723–38.

Smith, G., Y. Fang, X. Liu, J. Kenny, A. R. Cossins, C. C. de Oliveira, W. J. Etges, and M. G. Ritchie. 2013. Transcriptome-wide expression variation associated with environmental plasticity and mating success in cactophilic *Drosophila mojavensis*. Evolution 67:1950–63.

Soria-Carrasco, V., Z. Gompert, A. A. Comeault, T. E. Farkas, T. L. Parchman, J. S. Johnston, C. A. Buerkle, J. L. Feder, J. Bast, T. Schwander, S. P. Egan, B. J. Crespi, and P. Nosil. 2014. Stick insect genomes reveal natural selection's role in parallel speciation. Science 344:738–42.

Soule, M., and B. R. Stewart. 1970. The "Niche-Variation" hypothesis: A test and alternatives. Am. Nat. 104:85–97.

Spor, A., D. J. Kvitek, T. Nidelet, J. Martin, J. Legrand, C. Dillmann, A. Bourgais, D. de Vienne, G. Sherlock, and D. Sicard. 2014. Phenotypic and genotypic convergences are influenced by historical contingency and environment in yeast. Evolution 68:772–90.

Stanley, S. M. 1979. Macroevolution, pattern and process. W.H. Freeman, San Fransisco.

Stearns, S. C. 1992. The Evolution of Life Histories. Oxford University Press, Oxford.

Stephens, D. W., J. S. Brown, and R. C. Ydenberg. 2007. Foraging: Behavior and ecology. University of Chicago Press, Oakland, CA.

Stephens, D. W., and J. R. Krebs. 1986. Foraging theory. Princeton University Press.

Strong, D. R., J. H. Lawton, and S. R. Southwood. 1984. Insects on plants. Community patterns and mechanisms. Blackwell Scientific Publicatons, New York.

Suchan, T., and N. Alvarez. 2015. Fifty years after Ehrlich and Raven, is there support for plant-insect coevolution as a major driver of species diversification? Entomol. Exp. Appl. 157:98–112.

Tobler, R., S. U. Franssen, R. Kofler, P. Orozco-Terwengel, V. Nolte, J. Hermisson, and C. Schlötterer. 2014. Massive habitat-specific genomic response in *D. melanogaster* populations during experimental evolution in hot and cold environments. Mol. Biol. Evol. 31:364–75.

Van Gils, J. A., M. van der Geest, B. De Meulenaer, H. Gillis, T. Piersma, and E. O. Folmer. 2015. Moving on with foraging theory: incorporating movement decisions into the functional response of a gregarious shorebird. J. Anim. Ecol. 84:554–64.

Van Valen, L. 1965. Morphological variation and the width of the ecological niche. Am. Nat. 99:377–390.

Via, S., A. C. Bouck, and S. Skillman. 2000. Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution. 54:1626–1637.

Vitti, J. J., S. R. Grossman, and P. C. Sabeti. 2013. Detecting natural selection in genomic data. Annu. Rev. Genet. 47:97–120.

Waldbauer, G. P., R. W. Cohen, and S. Friedman. 1984. Self-selection of an optimal nutrient mix from defined diets by larvae of the corn earworm, *Heliothis zea* (Boddie). Physiol. Zool. 57:590–597.

Walsh, B. D. 1864. On Phytophagic Varieties and Phytophagic Species. Proc. Entomol. Soc. Philadelphia 3:403–430.

Walsh, B. D. 1867. The apple-worm and the apple maggot. J. Hortic. 2:338–343.

Warburg, M. S., and B. Yuval. 1997. Effects of energetic reserves on behavioral patterns of Mediterranean fruit flies (Diptera: Tephritidae). Oecologia 112:314–319.

Whittier, T. S., K. Y. Kaneshiro, and L. D. Prescott. 1992. Mating behavior of Mediterranean Fruit Flies (Diptera: Tephritidae) in a natural environment. Ann. Entomol. Soc. Am. 85:214–218.

Whittier, T. S., F. Y. Nam, T. E. Shelly, and K. Y. Kaneshiro. 1994. Male courtship success and female discrimination in the Mediterranean fruit fly (Diptera: Tephritidae). J. Insect Behav. 7:159–170.

Wiklund, C. 1975. The evolutionary relationship between adult oviposition preferences and larval host plant range in *Papilio machaon* L. Oecologia 18:185–197.

Wood, T. K. 1980. Divergence in the *Enchenopa binotata* Say complex (Homoptera: Membracidae) effected by host plant adaptation. Evolution. 34:147–160.

Wood, T. K., and S. I. Guttman. 1982. Ecological and behavioral basis for reproductive isolation in the sympatric *Enchenopa binotata* complex (Homoptera: Membracidae). Evolution. 36:233–242.

Wood, T. K., and M. C. Keese. 1990. Host-plant-induced assortative mating in *Enchenopa* treehoppers. Evolution. 44:619–628.

Wood, T. K., K. L. Olmstead, and S. I. Guttman. 1990. Insect phenology mediated by host-plant water relations. Evolution. 44:629–636.

Xie, X., A. P. Michel, D. Schwarz, J. Rull, S. Velez, A. A. Forbes, M. Aluja, and J. L. Feder. 2008. Radiation and divergence in the *Rhagoletis pomonella* species complex: inferences from DNA sequence data. J. Evol. Biol. 21:900–13.

Yukilevich, R. 2012. Asymmetrical patterns of speciation uniquely support reinforcement in *Drosophila*. Evolution 66:1430–46.

Yuval, B., and J. Hendrichs. 2000. Behavior of flies in the genus *Ceratitis* (Dacinae: Ceratitidine). Pp. 429–457 *in* M. Aluja and A. L. Norrbom, eds. Fruit flies (Tephritidae): phylogeny and evolution of behavior. CRC Press LLC, Boca Raton, FL.

Yuval, B., R. Kaspi, S. A. Field, S. Blay, and P. Taylor. 2002. Effects of post-tenral nutrition on reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). Florida Entomol. 85:165–170.

Yuval, B., R. Kaspi, S. Shloush, and M. S. Warburg. 1998. Nutritional reserves regulate male participation in Mediterranean fruit fly leks. Ecol. Entomol. 23:211–215.

Zucoloto, F. S. 1993a. Adaptation of a *Ceratitis capitata* population Diptera, Tephritidae to an animal protein-based diet. Entomol. Exp. Appl. 67:119–127.

Zucoloto, F. S. 1993b. Nutritive value and selection of diets containing different carbohydrates by larvae of *Ceratitis capitata* (Diptera: Tephritidae). Rev. Bras. Biol. 53:611–618.

2 Effect of dietary components on larval life history characteristics in the medfly (*Ceratitis capitata*: Diptera, Tephritidae).

Published as:
Nash, W. J., and T. Chapman. 2014. Effect of dietary components on larval life history characteristics in the medfly (Ceratitis capitata: Diptera, Tephritidae). PLoS One 9:e86029.

Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0086029

2.1 Abstract

Background

The ability to respond to heterogeneous nutritional resources is an important factor in the adaptive radiation of insects such as the highly polyphagous Medfly. Here we examined the breadth of the Medfly's capacity to respond to different developmental conditions, by experimentally altering diet components as a proxy for host quality and novelty.

Methodology/Principal Findings

We tested responses of larval life history to diets containing protein and carbohydrate components found in and outside the natural host range of this species. A 40% reduction in the quantity of protein caused a significant increase in egg to adult mortality by 26.5% ± 6% in comparison to the standard baseline diet. Proteins and carbohydrates had differential effects on larval versus pupal development and survival. Addition of a novel protein source, casein (i.e. milk protein), to the diet increased larval mortality by 19.4% ± 3% and also lengthened the duration of larval development by 1.93 ± 0.5 days in comparison to the standard diet. Alteration of dietary carbohydrate, by replacing the baseline starch with simple sugars, increased mortality specifically within the pupal stage (by 28.2% ± 8% and 26.2% ± 9% for glucose and maltose diets, respectively). Development in the presence of the novel carbohydrate lactose (milk sugar) was successful, though on this diet there was a decrease of 29.8 ± 1.6μg in mean pupal weight in comparison to pupae reared on the baseline diet.

Conclusions

The results confirm that laboratory reared Medfly retain the ability to survive development through a wide range of fluctuations in the nutritional environment. We highlight new facets of the responses of different stages of holometabolous life histories to key dietary components. The results are relevant to colonisation scenarios and key to the biology of this highly invasive species.

2.2 Introduction

The nutrients that an organism absorbs from its diet are essential for development, and determine how organisms can maximise their fitness (Roff 1992; Stearns 1992). In holometabolous insects, alteration in diet quality during development has wide ranging effects upon many life history characteristics (Chapman et al. 2013). The two major nutritive components of diet that contribute to development are proteins and carbohydrates. Proteins provide essential amino acids necessary for viability. Imbalances in dietary amino acids can have significant effects upon development and fitness (Dadd 1985) and may underlie the effect of dietary restriction on lifespan (Grandison et al. 2009). Carbohydrates provide energy to fuel development and represent the mechanism by which energy is stored for future use (Dadd 1985). The availability of different nutrients during the developmental phase determines characteristics such as growth rate (Nijhout 2003a), developmental survival and also impacts upon adult traits such as body size (Nijhout 2003b).

The relationship between dietary macronutrients and the development of traits in heterogeneous nutritional environments can be described by the geometric framework (GF) (Simpson and Raubenheimer 1993, 2007; Piper et al. 2011). In considering an individual's nutritional environment as a multidimensional space with individual nutrients as its axes, the GF establishes the optimal nutritional state for that individual as a 'nutritional target'. Within the GF, the trajectory at which an individual moves through nutritional space is referred to as a 'nutritional rail'. In an environment where diet components occur in a fixed ratio, progress towards the nutritional target is made along a single rail. However, if the ratio of nutrients in the environment is imbalanced or varies, progress towards the target is achieved by altering the intake of different nutrient components (Simpson and Raubenheimer 1993).

Holometabolous insects maintain robust mechanisms to ensure that development is successful in the environment in which their larvae develop, and hence that the nutritional target is obtained. The interaction between larval growth rate, critical weight and the endocrinological control of larval development offers the possibility of significant plasticity in the determination of adult size and energy stores (Nijhout 1999; Davidowitz et al. 2003, 2005; Davidowitz and Nijhout 2004; Edgar 2006). Critical weight is a point reached during the exponential growth rate of the final larval instar, which determines when the process of pupation can begin. This critical weight is influenced by diet quality and is relatively insensitive to external environmental factors (Davidowitz et al. 2003; Davidowitz and Nijhout 2004). Critical weight thus allows an

insect to adapt the rate of its development to diverse nutritional environments in order to optimise key adult traits, such as body size (Andersson 1994).

The present study focuses on a highly successful generalist species and its ability to adapt its developmental life history to changes in specific nutrients within the larval environment. The Mediterranean fruit fly (Medfly), *Ceratitis capitata*, is highly polyphagous, infesting over 350 hosts (Liquido et al. 1991) and can successfully utilise oviposition sites beyond its natural host range (Krainacker et al. 1987). Experimental evidence has revealed that the Medfly can show considerable flexibility in its life history, permitting the use of a diverse range of larval diets (Canato and Zucoloto 1993; Zucoloto 1993b; Chang et al. 2001; Chang 2004; Nestel et al. 2004; Nestel and Nemny-Lavy 2008). These findings suggest striking variability in the genes underlying diet selection and utilisation. Indeed, following artificial selection, Medfly can even be reared successfully on diets derived entirely from a non-herbivorous source (Zucoloto 1993a).

The Medfly is a globally important agricultural pest, and effective mass rearing strategies have been developed as part of sterile insect technique (SIT) programmes (Robinson et al. 2002). These have highlighted the importance of the larval diet in determining adult mating success, and show that adults reared on poor diets suffer reduced fitness (Zucoloto 1993b; Kaspi et al. 2002; Anjos-Duarte et al. 2011; Navarro-Campos et al. 2011). Protein deficiency in the larval environment also reduces body size in wild (Krainacker et al. 1987; Navarro-Campos et al. 2011) and laboratory (Kaspi et al. 2002) populations. This is important as small body size is associated with reduced male mating success (Kaspi et al. 2000, 2002). Reduced protein can also delay larval development and reduce survival to adult eclosion (Cangussu and Zucoloto 1997; Plácido-Silva et al. 2006; Nestel and Nemny-Lavy 2008). Large, protein fed males are more likely to have their sperm stored in the female and to have more sperm stored (Taylor and Yuval 1999). Dietary effects on body size could be mediated through alterations in the quantity of nutrients stored as lipids and as proteins prior to pupariation (Nestel et al. 2004; Nestel and Nemny-Lavy 2008). In females, the nutritional quality of larval diet affects ovarian development and egg production (Cangussu and Zucoloto 1993, 1995; Zucoloto and Fernandes-da-Silva 1997).

An important omission from existing studies of diet on development, however, is the effect of nutrient quality (via use of existing and novel hosts) as well as quantity on different developmental stages. This is relevant to our understanding how the Medfly can tailor its developmental progress towards a nutritional target, as well as for further

development of husbandry in SIT programmes. We addressed this omission by testing the effect of standard and novel protein and carbohydrate components on the developmental life history of Medflies. We altered diet components to provide variation in both host quality and host novelty, using protein and carbohydrate sources both inside and outside the natural host range.

2.3 Materials and Methods

2.3.1 Origin and maintenance of fly stocks

The study was conducted using *Ceratitis capitata* from the Toliman wild type strain, sourced from the Guatemalan Mass rearing facility, and raised under laboratory conditions since 1990 (Morrison et al. 2009). Prior to experimentation flies were reared in 1L cages with 50 individuals per cage at an approximate 1:1 sex ratio. Adult flies were fed a 3:1 sucrose:yeast hydrolysate diet and water *ad libitum*. Cages were maintained on a 12:12 light dark photoperiod at 25°C and experiments were also conducted under these same conditions. Stock lines were reared on a bran-based larval diet (Brewer's Yeast 147.3g/L, Sucrose 295g/L, Citric Acid 10.1g/L, Sodium benzoate 9.1g/L, Wheat 440g/L, Water 1L). Eight generations prior to the experiments, stock populations were placed on a starch-based larval diet (Agar 5g/L, Starch 30g/L, Brewer's Yeast 30g/L, Propionic Acid 5ml/L, Water 1L). Each generation, approximately 500 eggs were placed on 100ml of starch diet in a glass bottle. When 3rd Instar larvae started to 'jump' from the larval medium, the bottles were laid on sand and pupae allowed to emerge for 7 days.

2.3.2 Dietary treatments

Wild type flies from the Toliman mass rearing strain were used in these experiments (see Supplementary Methods for details). Six diet treatments were used in addition to the standard starch larval diet upon which the flies were maintained (Table 2.1). The first three larval diets altered protein sources. The 'High Protein' diet contained 40% more protein (yeast hydrolysate) than the standard starch larval diet, 'Low Protein' contained 40% less yeast and the 'casein' diet replaced the yeast with an equal quantity of casein, one of the two main proteins in cow's milk. We supplemented the casein diet with multivitamin powder (Boots) and table salt (Saxa) (Table 2.1) to compensate for non-protein differences in comparison to the high/low protein diets. The second set of three diets altered carbohydrate sources. In the 'glucose' diet, the polysaccharide of the starch diet was substituted by an equal quantity of its monosaccharide glucose base, in the 'maltose' diet starch was replaced by the disaccharide sugar maltose (two glucose molecules joined by α(1→4) bond; (Quigley

et al. 1970)); and finally in the 'lactose' treatment, the starch was replaced by the disaccharide milk sugar lactose (glucose + galactose joined by β(1→4) bond; (Fries et al. 1971)). Note that these carbohydrate diets contained a standard quantity of yeast (50g/L), which itself contains other sources of carbohydrate in small amounts. Therefore only the major carbohydrate source was varied in these diets.

Table 2.2. Larval diets used in this study.

Ingredient	Starch	High Protein	Low Protein	Casein (Milk Protein)	Glucose	Maltose	Lactose	
Water	1000	1000	1000	1000	1000	1000	1000	ml
Agar	15	15	15	15	15	15	15	g
Starch	30	30	30	30	-	-	-	g
Glucose	-	-	-	-	30	-	-	g
Maltose	-	-	-	-	-	30	-	g
Lactose	-	-	-	-	-	-	30	g
Yeast	50	70	30	-	50	50	50	g
Casein	-	-	-	50	-	-	-	g
Propionic Acid	5	5	5	-	5	5	5	ml
Multivitamin powder	-	-	-	0.3	-	-	-	g
Salt	-	-	-	0.3	-	-	-	g
Nipagin	-	-	-	25	-	-	-	ml

2.3.3 Experimental protocol

The experiment was conducted in two blocks, over consecutive generations, first the protein then the carbohydrate experiment. For each replicate 100 eggs were selected at random using a light microscope and placed on 55mm disc of Whatman filter paper soaked in dH_2O. This disk was then placed on 20g of the appropriate diet in a Petri dish. This process was repeated for each dietary treatment in a block, as well as for 20g of the standard Starch diet, which acted as a baseline control for each block of the experiment. Egg samples were allocated at random to the diet treatments. The four Petri dishes within each block were then treated as one replicate for each diet. Five replicates were conducted for the protein experiment (n = 5 replicates of 100 eggs for each of the 4 diet treatments), and six replicates for the carbohydrate experiment (n = 6 replicates of 100 eggs for each of the 4 treatments). Petri dishes were sealed using 'Parafilm' for 11 days, at which point the film was removed and the plates placed in larger boxes to allow larvae access to sand for pupation. Larvae were allowed 5 days, following the emergence of the first pupae, to exit the larval diet to pupate. It was uncommon for larvae to remain in the food at the end of this period, and any remaining larvae at this point were discarded from the experiment.

2.3.4 Larval and pupal development time

The number of pupae emerging each day was recorded on each of the 5 days allowed for pupation, allowing calculation of mean larval development time. The daily cohort of emerging pupae was sieved from the sand and transferred to a Petri dish. These Petri dishes were then checked daily for adult emergence. Adults were counted and their sex recorded, allowing mean pupal development time to be calculated for each replicate. Individuals that only partially emerged were discarded from the experiment. Overall, development time was calculated by summing the mean larval and pupal development time of each replicate.

2.3.5 Larval and pupal survival

The total number of pupae present at the end of the 5 days allowed for pupation was recorded as a measure of larval survival. The total number of fully emerging adults was used as the measure of pupal survival. Overall survival for each replicate was calculated by subtracting the number of surviving adults from the original replicate population size of 100 eggs.

2.3.6 Pupal weight

Each cohort of pupae were weighed on the day of emergence, and the total weight of the cohort divided by the number of pupae per cohort in order to give the mean pupal weight per treatment per day. Pupal weight was used as a proxy for adult size.

2.3.7 Statistical analysis

Data analysis was conducted in R v2.13.2 (R Development Core Team 2015). The data for the protein and carbohydrate experiments were analysed separately. Development time was measured as a count of the number of days between each developmental period and analysed by generalised linear model (GLM) using the Poisson distribution. Survival was treated as proportion data (proportion of individuals entering the developmental stage that successfully completed it) and analysed by GLMs using the binomial distribution. Weight data were analysed using ANOVA, and GLM ANCOVA to incorporate emergence day. Data that were overdispersed were analysed using quasipoisson and quasibinomial distributions. Binomial data that displayed heteroscedasticity were weighted according to the inverse proportion of the dispersion of the data. After each model was fitted, significance of treatment comparisons was assessed using Tukey HSD multiple comparison tests ('multcomp' package; Hothorn et al. 2008) in R. Bonferroni correction was applied to the multcomp::glht results object using the 'summary' function in R{base}. This scaled the resulting p values by the number of comparisons conducted, in order to control for false positives incurred by

multiple comparisons. It is noted that this is a highly conservative correction, but it was employed to rule out the possibility of type one error.

2.4 Results

2.4.1 Egg to adult survival

The number of individuals surviving from egg to adult was significantly altered by both the protein and carbohydrate diet manipulations. There was an overall effect of protein treatment ($F_{3,16}$ = 7.878, P = 0.002; Figure 2.1a). A significantly lower proportion of eggs reared on low protein survived to adulthood than did those reared on starch (P = 0.008) and high protein diets (P = 0.01). Also, a significantly lower proportion of eggs reared on the casein diet survived to adulthood than those reared on starch (P = 0.002) and high protein diets (P = 0.003). There was also a significant effect of carbohydrate treatment on overall egg to adult survival ($F_{3,20}$ = 13.962, P < 0.001; Figure 2.1a). A significantly lower proportion of eggs reared on the glucose diet survived to adult eclosion than did those reared on lactose (P < 0.001) and starch (P = 0.02). Survival was also lower on the maltose in comparison to the lactose (P < 0.001) and starch (P < 0.001) diets.

2.4.1.1 Larval survival

Protein treatment had a significant effect on larval survival, i.e. the number of individuals surviving from egg to pupae ($F_{3,16}$ = 10.742, P < 0.001; Figure 2.1b). A significantly lower proportion of larvae reared on low protein and casein diets survived to pupation than those reared on the high protein diet (post hoc tests, P < 0.001). Larval survival was also significantly lower on the casein in comparison to starch-based diet (P = 0.028). In contrast, variation in carbohydrates had no significant effect on larval survival ($F_{3,20}$ = 1.8253, P = 0.175; Figure 2.1b).

2.4.1.2 Pupal survival

Pupal survival (i.e. the proportion of pupae eclosing as adults) was also significantly altered by protein treatment ($F_{3,16}$ = 3.6825, P = 0.03; Figure 2.1c). The proportion of pupae surviving to adult eclosion was significantly lower for the casein in comparison to the starch diet (post hoc tests, P = 0.02). In contrast to larval survival, carbohydrate treatment did have a significant effect on pupal survival ($F_{3,20}$ = 9.1262, P < 0.001; Figure 2.1c). A significantly lower proportion of pupae reared on the glucose diet survived to adulthood than did those raised on lactose (P < 0.001). Also, the proportion of pupae surviving to adult eclosion on the maltose diet was significantly lower than for pupae reared on lactose (P < 0.001) and starch (P = 0.03).

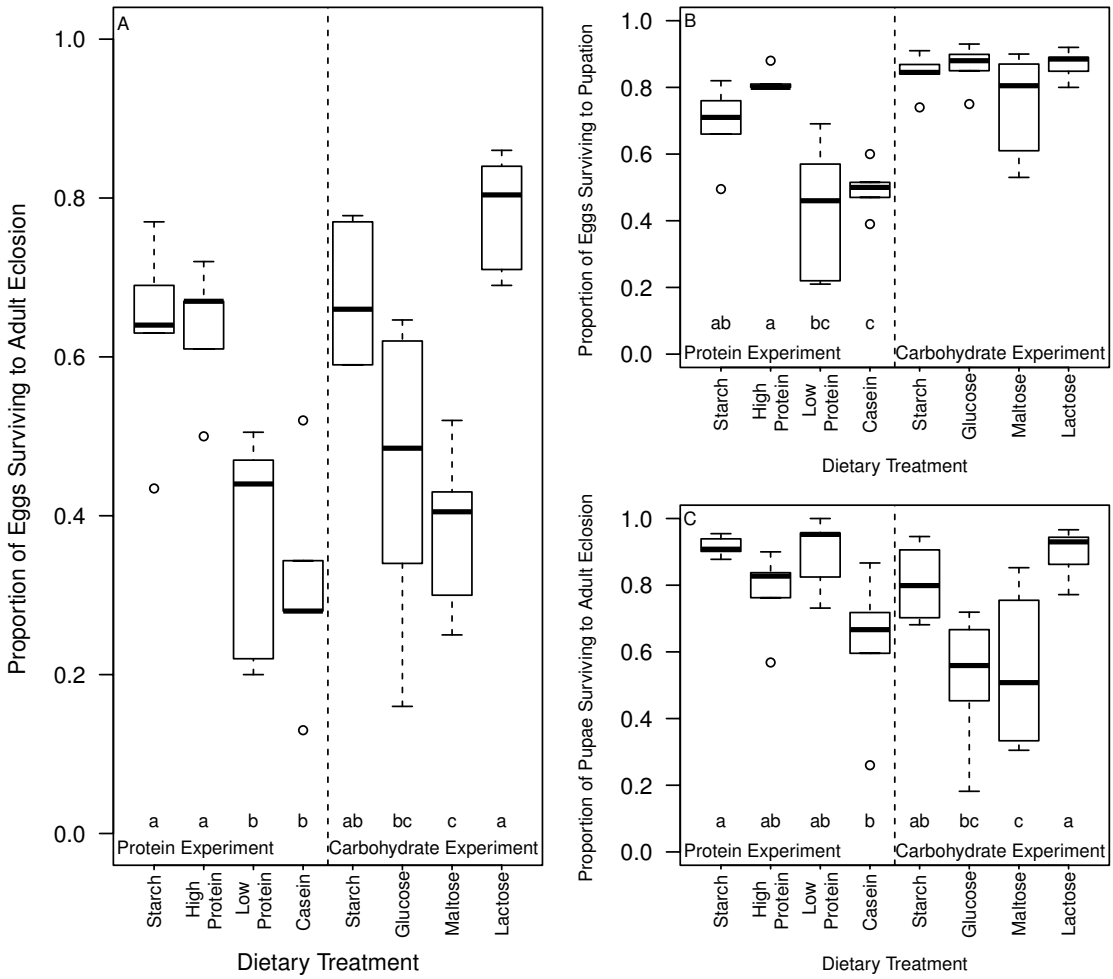

Figure 2.1. Proportion of Medfly individuals surviving between each developmental stage when reared upon different dietary treatments. For each panel, on the left are the 4 diets with altered protein content and on the right the 4 diets with altered carbohydrate content. (a) Overall proportion of eggs surviving from egg laying to adult eclosion. (b) Proportion of eggs surviving to pupal formation. (c) Proportion of pupae surviving from initial pupal formation to adult eclosion. Dotted lines represent the range of the data; outer limits of the boxes indicate inter quartile range and the black line at the centre of each box represents the median value. Circles represent outliers. Letters indicate groupings significantly different following post hoc tests (see text for details).

Overall, diet components had contrasting effects on survival through the different life history stages of development, with a large effect of protein on survival during the larval growth phase and of carbohydrate on survival during the pupal phase.

2.4.2 Development time

Development time was significantly altered by protein treatments ($F_{3,16}$ = 11.548, P < 0.001; Figure 2.2a). Eggs reared on casein took significantly longer to develop to adulthood than did eggs reared on starch (post hoc tests, P = 0.009) and high protein

(P < 0.001). Interestingly, carbohydrate treatment had no significant effect on the overall duration of development ($F_{3,20}$ = 0.5405, P = 0.660; Figure 2.2a).

2.4.2.1 Larval development time

Protein had a significant effect on larval development (i.e. duration of development from egg to pupa; $F_{3,16}$ = 9.5858, P < 0.001; Figure 2.2b). The development time of larvae reared on casein was significantly longer than for starch (post hoc tests, P = 0.018) and high protein (P < 0.001). Carbohydrate treatment had no significant effect on the mean duration of larval development ($F_{3,20}$ = 0.9082, P = 0.455; Figure 2.2b).

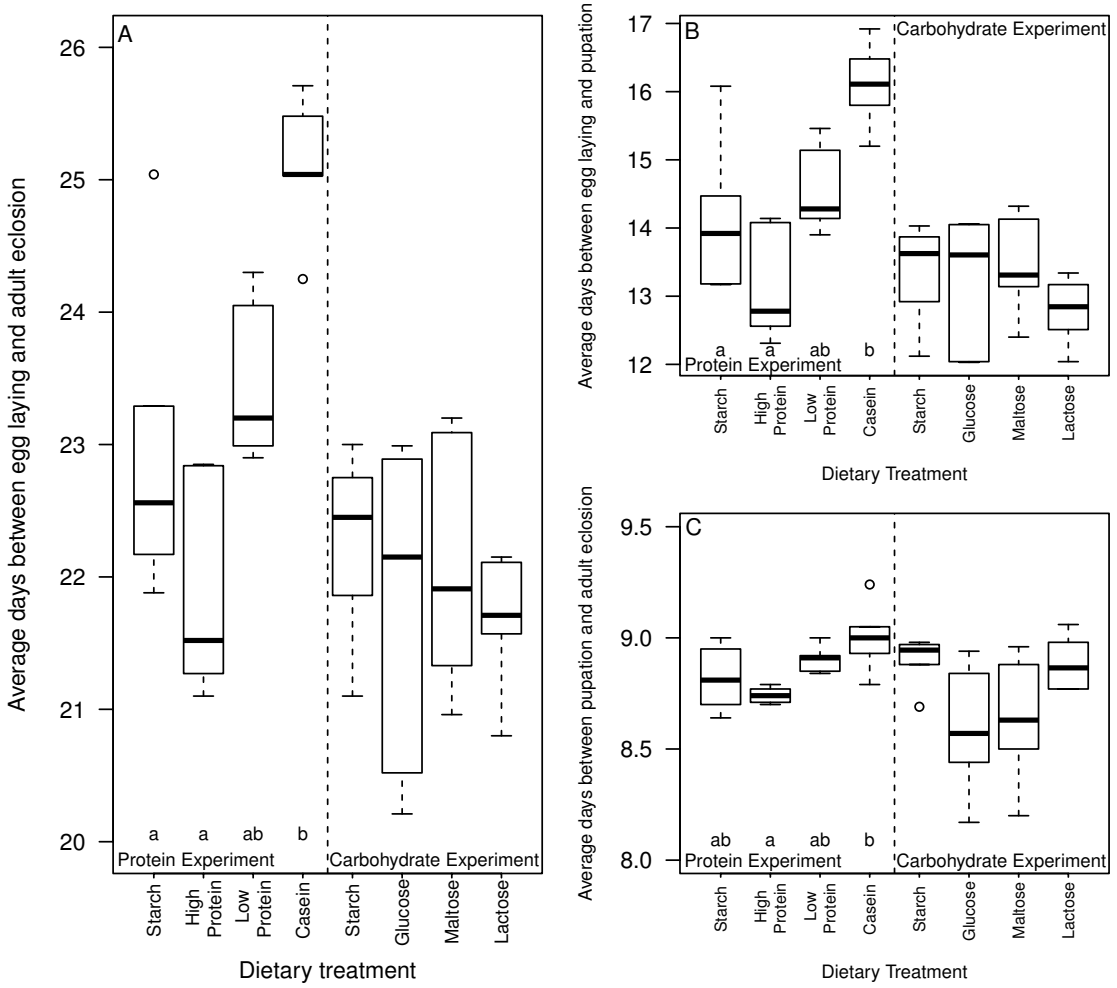

Figure 2.2. Average duration of each stage of development of Medfly reared upon different dietary treatments. For each panel, the protein experiment is on the left hand side, carbohydrate experiment on the right hand side. (a) Average duration of overall development (median days) from egg laying to adult eclosion. (b) Average duration of development (median days) of the larval stage, from egg to pupal formation. (c) Average duration of the pupal stage (median days) from pupal formation to adult eclosion. Boxplots are as defined in Fig. 2.1.

2.4.2.2 Pupal development time

Protein treatment had a significant effect on pupal development (time from pupa to adult eclosion; $F_{3,16} = 4.3837$, $P = 0.02$; Figure 2.2c). The development time of pupae reared on casein was significantly longer than that of pupae reared on high protein (post hoc tests, $P = 0.02$). Carbohydrate treatment had a marginally significant effect on the duration of pupal development ($F_{3,20} = 3.5694$, $P = 0.032$; Figure 2.2c). However, this effect was non significant following post hoc tests, potentially due to the severity of the Bonferroni correction implemented.

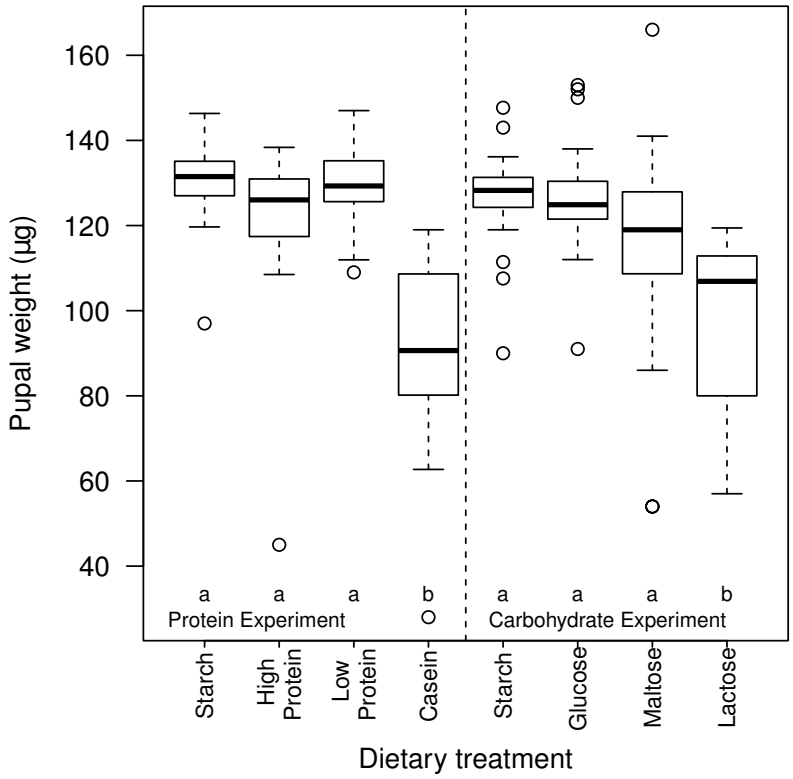

Figure 2.3 Pupal weight (median weight in µg) of Medfly following rearing upon different dietary treatments. The protein experiment is on the left hand side, carbohydrate experiment on the right hand side. Boxplot is as defined in Figure 2.1.

2.4.3 Pupal weight

Pupal weight was significantly affected by protein ($F_{3,94} = 35.218$, $P < 0.001$; Figure 2.3) and was significantly lower for casein than all other diets (post hoc tests, $P < 0.001$, all comparisons). Carbohydrate also had a significant effect on pupal weight ($F_{3,93} = 14.162$, $P < 0.001$; Figure 2.3). Pupae reared on lactose had significantly lower mean weights than all other treatments (starch and lactose based diets $P < 0.001$; maltose $P = 0.015$). To further analyse this finding, an ANCOVA, which considered pupal emergence day as a covariate of mean pupal weight, was fitted. This showed a significant interaction between protein and day of pupal emergence ($F_{3,90} = 8.2832$, P

< 0.001; Appendix 2.1, Figure 2.A1a). Both diet ($F_{3,90}$ = 48.2115, P < 0.001), and pupal emergence day ($F_{1,90}$ = 13.8325, P < 0.001) also had a significant effect upon mean pupal weight. The significant interaction was driven by the negative gradient in the casein treatment (-11.28, t = -4.401, P < 0.001). The same analysis performed for the carbohydrate experiment revealed a significant effect of diet ($F_{3,89}$ = 15.7155, P < 0.001) but not day ($F_{1\ 89}$ = 2.4118, P = 0.124) on pupal weight. The interaction between diet and emergence day was significant ($F_{3,89}$ = 3.9304, P = 0.011; Figure 2.A1b), and was driven by the interaction between the lactose and emergence day, for which the gradient was significantly negative (-11.29, t = -2.719, P = 0.008).

2.5 Discussion

The results reveal that different dietary nutrients had significant but divergent effects on different stages of development in the medfly. Decreases in protein quantity and quality had pronounced effects on larval development, increasing mortality and the duration of development. Alteration of carbohydrate quality affected mortality within the pupal stage. The results confirm that the Medfly can develop successfully on a wide range of different protein and carbohydrate sources. As a generalist, the Medfly therefore retains sufficient genetic variation to allow the expression of adaptive plasticity across a range of developmental environments (Forister et al. 2012). This plasticity can buffer the adult phenotype against the effects of environmental variation during larval development. Such plasticity is thought to be advantageous to generalist species during colonisation, but may also have negative effects on overall adaptive radiation (Thibert-Plante and Hendry 2011).

Our study highlights the potential importance of the holometabolous lifestyle to generalist species. The larval phase of development, where 90% of adult body mass is accrued (Chapman et al. 2013) represents the defined growth phase. This allows the duration of development to be tailored to optimise progress along a nutritional rail towards the nutritional target (Simpson and Raubenheimer 1993, 2007) to achieve a stable adult phenotype. This suggests that protein is the key nutrient during the larval phase, as duration of development increased in larvae reared on diets with reduced protein content or quality. However, individuals reared on a low protein diet did not show reduced adult body size. This trade-off between development time and body size is consistent with the endocrinological control model of holometabolous development (Davidowitz and Nijhout 2004). Also, decreased numbers of individuals survived on diets with reduced protein. This shows protein to be a limiting resource during the larval phase. These results are in agreement with previous studies that manipulated

protein in order to optimise the mass-rearing process (Nestel et al. 2004; Nestel and Nemny-Lavy 2008).

Individuals that successfully completed the larval growth phase and entered the metamorphic pupal phase were not affected, in the traits assayed here, by the protein content of their diets. Indeed, it was during the metamorphic phase that the effects of the carbohydrate components of diet became apparent. Larvae reared on diets containing simple carbohydrates (glucose, maltose) exhibited lower survival during metamorphosis. No effects of those carbohydrates were seen during larval development, suggesting that glucose and maltose are less efficient energy sources, or are less able to facilitate the provision of storable energy, e.g. as lipids in the fat body or as glycogen (Tolmasky et al. 2001; Nestel et al. 2003). If energy stores such as in the fat body can be influenced significantly by carbohydrate quality (Nestel et al. 2004; Nestel and Nemny-Lavy 2008), it will interesting to consider the wider effects this may have on adult phenotype beyond body size - for example on early life reproductive potential (Aguila et al. 2013). However, variation in stored lipids and proteins in larvae about to pupate can potentially be compensated for during metamorphosis (Nestel et al. 2004). The lack of effects of carbohydrates on growth rate or development time during the larval phase suggests that the larvae have a limited ability to compensate for poor quality carbohydrates in the diet by, for example, slowing growth rate in order to maximise carbohydrate energy storage for future development. The effects of the two major diet components protein and carbohydrate are therefore relatively independent of one another.

The only treatments that significantly altered adult size were the novel diets that fell outside of the Medfly's natural host range (casein, lactose). Such diets can be used to simulate encounters with 'alien' hosts, for example during colonisation events. For a highly invasive, generalist species such as the medfly (Gasperi et al. 2002), which can exhibit great plasticity in the degree of host oviposition preference (Carey 1984; Prokopy et al. 1984; Katsoyannos et al. 1986; Katsoyannos 1989), the ability to maximise developmental success in 'alien' hosts is predicted to be an important trait. In our study, Medflies developed successfully on both novel host treatments, though adult body size was decreased. When the protein source was novel, all elements of development were compromised. Novel carbohydrate, on the other hand, caused no significant changes to the developmental traits assayed. However, more individuals survived than on the baseline diet and surviving pupae eclosed as adults at a faster rate.

The novel protein diet exhibited the same kinds of effects on developmental traits as for the standard diet where protein content was reduced by 40%, but expressed them to a greater degree. This suggests that the individuals surviving to pupariation could not maintain a stable adult phenotype, and paid a cost in terms of body size. The pattern seen in the novel carbohydrate treatment (see also Krainacker et al. 1987) may reflect the impact of novel carbohydrates on the metabolic control of development (Davidowitz and Nijhout 2004), or on the efficiency of metabolism during development (Tolmasky et al. 2001; Nestel et al. 2003).

Considering responses to diet in the context of colonisation and invasion, responses to novel proteins may be less important than for other nutrients, as protein content of host fruit is generally low (0.86 ± 0.59%; Jurkevitch 2011) and invariant (McCance et al. 2002). The carbohydrate content in fruits is, however, higher and more variable (13.7 ± 13.7%; McCance et al. 2002; Jurkevitch 2011). The availability of carbohydrates will also vary across the range of hosts into which individuals may oviposit over the fruiting season, and also within hosts during the course of ripening and decay. The plasticity we observed is likely to be crucial in coping with such fluctuations and facilitating successful development. This is particularly so when considering the role that factors such as fruit structure (Papachristos et al. 2008) and secondary metabolites (Katsoyannos et al. 1997; Salvatore et al. 2004; Papachristos et al. 2009) may have within novel hosts.

Developmental plasticity reflects the ability of the medfly to adopt a range of nutritional rails (Simpson and Raubenheimer 1993) dependent upon the nutrients it encounters. Medflies exhibit behavioural adaptations to heterogeneous nutrients during development, such as larval migration to areas of higher nutritional quality within a host (Fernandes-da-Silva and Zucoloto 1993). In dietary conditions that are nutritionally homogenous, these behavioural adaptations become obsolete. The results of this study are consistent with the hypothesis that the Medfly exhibits developmental plasticity, manifested as the ability to travel down different nutritional rails. Unsurprisingly, less efficient and less successful nutritional rails are the only options to follow when the nutritional space comprises novel components. We suggest that the application of methods designed to define and control intake of nutrients will be extremely useful and may offer insight into the apparently atypical responses of medfly lifespan to dietary restriction (Carey et al. 2005).

Overall, the results of this study highlight the potential flexibility of phytophagous insects such as the Medfly. The plasticity seen in developmental traits gives insight into

the ability of this wide-ranging generalist to adapt to variation within its nutritional environment. A key extension to the present work would be to test the effects on the adult phenotype. If, as other studies suggest (e.g. Kaspi et al. 2002), there are significant effects of larval diets in adult flies, then a detailed understanding of the effects of the availability of specific dietary nutrients during development on adult life history could have relevance for mass rearing strategies in this globally invasive pest. The swift global radiation of the Medfly has presumably favoured the spread of alleles that facilitate developmental success in many nutritional environments. This is reflected in the alteration of survival and developmental duration, and the maintenance of a relatively stable adult body mass in those individuals that do survive. For a generalist species, this allows resilience to fluctuation in nutrient availability both within and across hosts. This adaptive ability has also fostered resilience to harsh nutritional environments and maximises fitness even in radically different, novel host environments.

2.6 Acknowledgements

We thank the NERC for funding (quota PhD studentship) and Dr. Phil Leftwich for help and advice with experiments.

2.7 References

Aguila, J. R., D. K. Hoshizaki, and A. G. Gibbs. 2013. Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. J. Exp. Biol. 216:399–406.

Andersson, M. B. 1994. Sexual selection. Princeton University Press.

Anjos-Duarte, C. S., A. M. Costa, and I. S. Joachim-Bravo. 2011. Sexual behaviour of the Mediterranean fruit fly (Diptera: Tephritidae): the influence of female size on mate choice. J. Appl. Entomol. 135:367–373.

Canato, C. M., and F. S. Zucoloto. 1993. Diet selection by *Ceratitis capitata* larvae (Diptera, Tephritidae): Influence of the rearing diet and genetic factors. J. Insect Physiol. 39:981–985.

Cangussu, J. A., and F. S. Zucoloto. 1997. Effect of protein sources on fecundity, food acceptance, and sexual choice by *Ceratitis capitata* (Diptera, Tephritidae). Rev. Bras. Biol. 57:611–618.

Cangussu, J. A., and F. S. Zucoloto. 1993. Influence of partial malnutrition on egg production by *Ceratitis capitata* (Diptera, Tephritidae). Rev. Bras. Biol 53:155–158.

Cangussu, J. A., and F. S. Zucoloto. 1995. Self-selection and perception threshold in adult females of *Ceratitis capitata* (Diptera, Tephritidae). J. Insect Physiol. 41:223–227.

Carey, J. R. 1984. Host-specific demographic studies of the Mediterranean fruit fly *Ceratitis capitata*. Ecol. Entomol. 9:261–270.

Carey, J. R., P. Liedo, H.-G. Müller, J.-L. Wang, Y. Zhang, and L. Harshman. 2005. Stochastic dietary restriction using a Markov-chain feeding protocol elicits complex, life history response in medflies. Aging Cell 4:31–9.

Chang, C. L. 2004. Effect of amino acids on larvae and adults of *Ceratitis capitata* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 97:529–535.

Chang, C. L., C. Albrecht, S. S. A. El-Shall, and R. Kurashima. 2001. Adult Reproductive Capacity of *Ceratitis capitata* (Diptera: Tephritidae) on a Chemically Defined Diet. Ann. Entomol. Soc. Am. 94:702–706.

Chapman, R. F., S. J. Simpson, and A. E. Douglas. 2013. The insects: structure and function. Cambridge University Press.

Dadd, R. H. 1985. Nutrition: organisms. Compr. Insect Physiol. Biochem. Pharmacol. Vol. 4. Regul. Dig. Nutr. Excretion 313–390.

Davidowitz, G., L. J. D'Amico, and H. F. Nijhout. 2003. Critical weight in the development of insect body size. Evol. Dev. 5:188–197.

Davidowitz, G., and H. F. Nijhout. 2004. The physiological basis of reaction norms: the interaction among growth rate, the duration of growth and body size. Integr. Comp. Biol. 44:443–449.

Davidowitz, G., D. A. Roff, and H. F. Nijhout. 2005. A physiological perspective on the response of body size and development time to simultaneous directional selection. Integr. Comp. Biol. 45:525–531.

Edgar, B. A. 2006. How flies get their size: genetics meets physiology. Nat. Rev. Genet. 7:907–916.

Fernandes-da-Silva, P., and F. Zucoloto. 1993. The influence of host nutritive value on the performance and food selection in *Ceratitis capitata* (Diptera, Tephritidae). J. Insect Physiol. 39:883–887.

Forister, M. L., L. A. Dyer, M. S. Singer, J. O. Stireman III, J. T. Lill, and Singer, M S. 2012. Revisiting the evolution of ecological specialization, with emphasis on insect-plant interactions. Ecology 93:981–991.

Fries, D. C., S. T. Rao, and M. Sundaralingam. 1971. Structural chemistry of carbohydrates. III. Crystal and molecular structure of 4-O--D-galactopyranosyl--D-glucopyranose monohydrate (-lactose monohydrate).". Acta Crystallogr. Sect. B Struct. Crystallogr. Cryst. Chem. 27:994–1005.

Gasperi, G., M. Bonizzoni, L. M. Gomulski, V. Murelli, C. Torti, A. R. Malacrida, and C. R. Guglielmino. 2002. Genetic differentiation, gene flow and the origin of infestations of the medfly, *Ceratitis capitata*. Genetica 116:125–135.

Grandison, R. C., M. D. W. Piper, and L. Partridge. 2009. Amino-acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. Nature 462:1061–1064.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous Inference in General Parametric Models. Biometrical J. Biometrische Zeitschrift 50:346–363.

Jurkevitch, E. 2011. Insect "Symbiology" Is Coming of Age, Bridging Between Bench and Field. Pp. 27–41 *in* Beneficial Microorganisms in Multicellular Life Forms. Springer.

Kaspi, R., S. Mossinson, T. Drezner, B. Kamensky, and B. Yuval. 2002. Effects of larval diet on development rates and reproductive maturation of male and female Mediterranean fruit flies. Physiol. Entomol. 27:29–38.

Kaspi, R., P. P. W. Taylor, and B. Yuval. 2000. Diet and size influence sexual advertisement and copulatory success of males in Mediterranean fruit fly leks. Ecol. Entomol. 25:279–284.

Katsoyannos, B. I. 1989. Response to shape, size and color. Pp. 307–324. *in* Fruit flies: their biology, natural enemies and control 3.

Katsoyannos, B. I., N. A. Kouloussis, and N. T. Papadopoulos. 1997. Response of *Ceratitis capitata* to citrus chemicals under semi-natural conditions. Entomol. Exp. Appl. 82:181–188.

Katsoyannos, B. I., K. Panagiotidou, and I. Kechagia. 1986. Effect of color properties on the selection of oviposition site by *Ceratitis capitata*. Entomol. Exp. Appl. 42:187–193.

Krainacker, D. A., J. R. Carey, and R. I. Vargas. 1987. Effect of larval host on life history traits of the Mediterranean fruit fly, *Ceratitis capitata*. Oecologia 73:583–590.

Liquido, N. J., L. A. Shinoda, and R. T. Cunningham. 1991. Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): an annotated world review. Entomological Society of America.

McCance, R. A., E. M. Widdowson, F. S. Agency, G. B. F. S. Agency, and A. I. of F. Research. 2002. McCance and Widdowson's The Composition of Foods. Royal Society of Chemistry and the Food Standards Agency.

Morrison, N. I., D. F. Segura, K. C. Stainton, G. Fu, C. A. Donnelly, and L. S. Alphey. 2009. Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*. Entomol. Exp. Appl. 133:146–153.

Navarro-Campos, C., M. T. Martínez-Ferrer, J. M. Campos, J. M. Fibla, J. Alcaide, L. Bargues, C. Marzal, and F. Garcia-Marí. 2011. The influence of host fruit and temperature on the body size of adult *Ceratitis capitata* (Diptera: Tephritidae) under laboratory and field conditions. Environ. Entomol. 40:931–938.

Nestel, D., and E. Nemny-Lavy. 2008. Nutrient balance in medfly, *Ceratitis capitata*, larval diets affects the ability of the developing insect to incorporate lipid and protein reserves. Entomol. Exp. Appl. 126:53–60.

Nestel, D., E. Nemny-Lavy, and C. L. Chang. 2004. Lipid and protein loads in pupating larvae and emerging adults as affected by the composition of Mediterranean fruit fly (*Ceratitis capitata*) meridic larval diets. Arch. Insect Biochem. Physiol. 56:97–109.

Nestel, D., D. S. Tolmasky, and E. Nemny-Lavy. 2003. Lipid, carbohydrates and protein patterns during metamorphosis of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 96:237–244.

Nijhout, H. F. 1999. Control mechanisms of polyphenic development in insects. Bioscience 49:181–192.

Nijhout, H. F. 2003a. Development and evolution of adaptive polyphenisms. Evol. Dev. 5:9–18.

Nijhout, H. F. 2003b. The control of body size in insects. Dev. Biol. 261:1–9.

Papachristos, D. P., A. C. Kimbaris, N. T. Papadopoulos, and M. G. Polissiou. 2009. Toxicity of citrus essential oils against *Ceratitis capitata* (Diptera: Tephritidae) larvae. Ann. Appl. Biol. 155:381–389.

Papachristos, D. P., N. T. Papadopoulos, and G. D. Nanos. 2008. Survival and Development of Immature Stages of the Mediterranean Fruit Fly (Diptera: Tephritidae) in Citrus Fruit. J. Econ. Entomol. 101:866–872.

Piper, M. D. W., L. Partridge, D. Raubenheimer, and S. J. Simpson. 2011. Dietary restriction and aging: a unifying perspective. Cell Metab. 14:154–160.

Plácido-Silva, M. do C., A. M. da Silva Neto, F. S. Zucoloto, and I. S. Joachim-Bravo. 2006. Effects of different protein concentrations on longevity and feeding behavior of two adult populations of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae). Neotrop. Entomol. 35:747–752.

Prokopy, R. R. J., P. T. McDonald, and T. T. Y. Wong. 1984. Inter-population variation among *Ceratitis capitata* flies in host acceptance pattern. Entomol. Exp. Appl. 35:65–69.

Quigley, G. J., A. Sarko, and R. H. Marchessault. 1970. Crystal and molecular structure of maltose monohydrate. J. Am. Chem. Soc. 92:5834–5839.

R Development Core Team, R. 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.

Robinson, A. S. Robinson, J. P. Cayol, and J. Hendrichs. 2002. Recent findings on medfly sexual behavior: Implications for SIT. Florida Entomol. 85:171–181.

Roff, D. A. 1992. Evolution Of Life Histories: Theory and Analysis. Springer.

Salvatore, A., S. Borkosky, E. Willink, and A. Bardón. 2004. Toxic effects of lemon peel constituents on *Ceratitis capitata*. J. Chem. Ecol. 30:323–333.

Simpson, S. J., and D. Raubenheimer. 1993. A multi–level analysis of feeding behaviour: the geometry of nutritional decisions. Phil Trans R Soc B 342:381–402.

Simpson, S. J., and D. Raubenheimer. 2007. Caloric restriction and aging revisited: the need for a geometric analysis of the nutritional bases of aging. journals Gerontol. Ser. A Biol. Sci. Med. Sci. 62:707–713. Oxford University Press.

Stearns, S. C. 1992. The Evolution of Life Histories. OUP Oxford.

Taylor, P. W., and B. Yuval. 1999. Postcopulatory sexual selection in Mediterranean fruit flies: advantages for large and protein-fed males. Anim. Behav. Anim Behav 58:247–254.

Thibert-Plante, X., and A. P. Hendry. 2011. The consequences of phenotypic plasticity for ecological speciation. J. Evol. Biol. 24:326–342.

Tolmasky, D. S., A. Rabossi, and L. A. Quesada-Allué. 2001. Synthesis and mobilization of glycogen during metamorphosis of the medfly Ceratitis capitata. Arch. Biochem. Biophys. 392:38–47.

Zucoloto, F. S. 1993a. Adaptation of a *Ceratitis capitata* (Diptera, Tephritidae) population to an animal protein-based diet. Entomol. Exp. Appl. 67:119–127.

Zucoloto, F. S. 1993b. Nutritive value and selection of diets containing different carbohydrates by larvae of *Ceratitis capitata* (Diptera: Tephritidae). Rev. Bras. Biol. 53:611–618.

Zucoloto, F. S., and G. Fernandes-da-Silva. 1997. Effect of host nutritive value on egg production by *Ceratitis capitata* (Diptera, Tephritidae). J. Insect Physiol. 43:939–943.

2.8 Appendix 2.1 - Supporting Figures

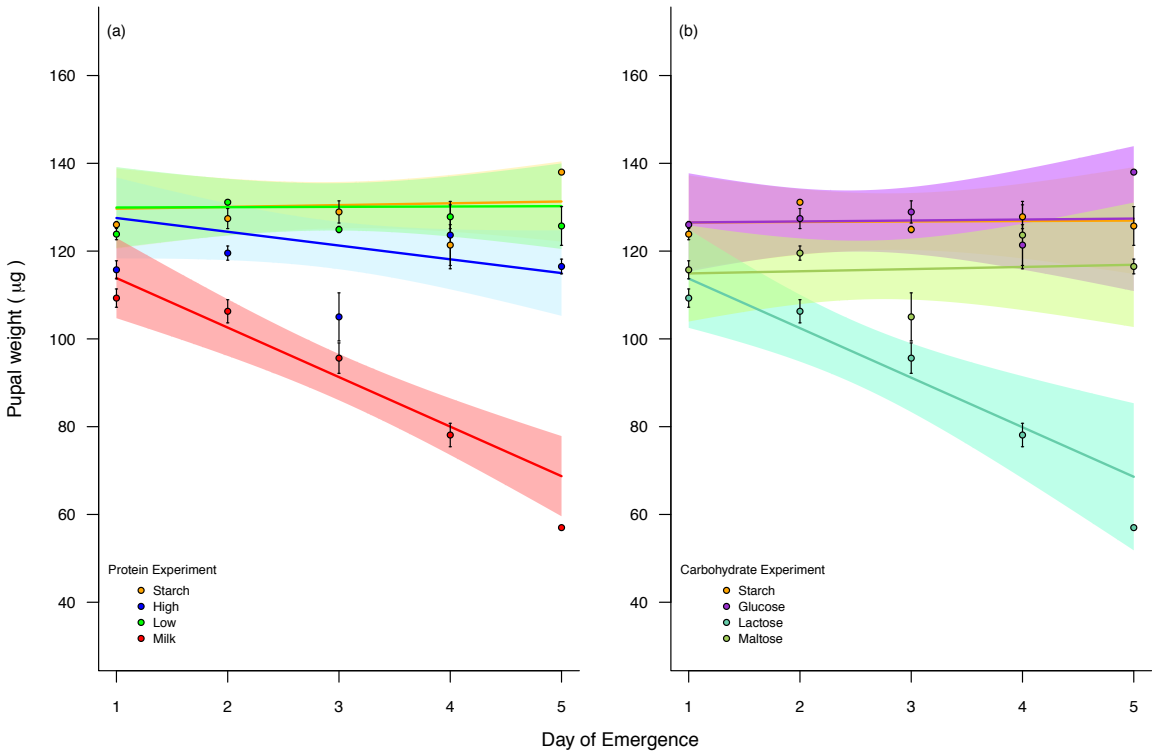

Figure 2.A1. Mean pupal weight (µg) in relation to day of emergence. Points represent mean data; solid lines represent ANCOVA models fitted with mean pupal weight as a main effect and emergence day as a covariate. Shaded regions indicate 95% confidence interval of the model. Error bars represent 1 standard error. (a) Protein experiment, (b) Carbohydrate experiment.

3 Sexual isolation evolves following experimental evolution under divergent developmental diet regimes in the medfly (*Ceratitis capitata*: Diptera, Tephritidae).

3.1 Abstract

Experimental evolution provides a powerful tool with which to view evolution in action, particularly for fundamental events in the initiation of reproductive isolation (RI). Here the hypothesis that maintenance of populations of medfly (*Ceratitis capitata*), on divergent larval diet regimes would result in dietary adaptation and the evolution of RI through assortative mating. This study subjected a single, laboratory adapted population to replicated experimental evolution on two larval diets, based on sugar ('ASG') and starch ('S'), respectively. Assortative mating by diet was investigated at generations 3 & 5, 30 and 60. The major finding was the existence, in generation 60, of significant, male-driven, assortative mating by larval diet treatment. Adaptation to the different diets was therefore associated with incipient RI, within 60 generations. Prior to the generation 60 tests, there was a strong pattern of more frequent matings by ASG males. In the earliest tests this was due to a strong proximate effect of diet, which diminished over time with the emergence of significant assortative mating preference according to rearing treatment of origin.

3.2 Introduction

Ecological adaptation is frequently key to the evolution of reproductive isolation (RI), and hence speciation, with divergent selection mediated by differing environments leading to the diversification of populations through reduced gene flow (Schluter 2000; Coyne and Orr 2004; Rundle and Nosil 2005). This process, based upon local adaptation, is termed 'ecological speciation' (Rundle and Nosil 2005; Nosil 2012). Much is still unknown concerning the exact nature of barriers to gene flow and the how such barriers are instigated (Butlin et al. 2012). It is also not yet clear how the divergent selection that is central to ecological speciation interacts with other selective forces linked to speciation such as sexual selection (Panhuis et al. 2001; Maan and Seehausen 2011; Butlin et al. 2012; Safran et al. 2013).

Within phytophagous (plant eating) insects, there are well-known examples of isolation associated with divergent selection, or ecological speciation, following shifts to different hosts. For example, in *Rhagoletis* fruit flies (Feder et al. 1994) and *Timema* stick insects (Nosil 2007), strong associations with particular plant hosts exist, and genetic divergence between populations is reported following adaptation associated with host plant shifts. If the host shift occurs in sympatry with the original host plant, this leads to the subsequent formation of host races (Linn et al. 2003; Nosil et al. 2012; Powell et al. 2014; Soria-Carrasco et al. 2014). This adaptation, and host race formation, can have a significant impact on traits affecting the probability of RI. The RI itself can be manifested in space, time, or through mate preference, with the latter potentially being subject to selection against maladaptive hybrid matings (Feder et al. 1994; Dambroski and Feder 2007; Nosil 2007; Nosil et al. 2012). It is therefore generally thought that host plant specialisms can play an important role in the evolution of RI and therefore speciation.

A phytophagous species that is specialised to a particular host will carry sets of alleles that optimise fitness for that specific host. In contrast, a generalist utilizing the same host plant is expected to lack specialised alleles and may therefore show lower fitness in comparison to a specialist, on that host. In general it is thought that, by occupying a broader niche, generalists maintain alleles associated with a range of environments ("a jack of all trades …") (Forister et al. 2012). Generalists are therefore expected to manifest sufficient behavioural and ecological plasticity to support movement between, and utilization of, potentially divergent host plants within an environment (Loxdale et al. 2011). Phylogenetic evidence shows that the transition between generalism and specialism is bidirectional, following a marginal overall trend towards specialisation

across taxa (Nosil 2002; Nosil and Mooers 2005). In contrast to the role of specialists, the relevance of generalist ecologies in speciation in general remains unclear (Dennis et al. 2011; Loxdale et al. 2011). A well-known example of a phytophagous insect associated with a wide range of host plants, which appears to have gone through repeated ecological speciation, is the pea aphid *Acryrthosiphon pisum* (Peccoud and Simon 2010). This cosmopolitan species is now considered as a complex of species and biotypes. Across its European range, divergent selection between aphid strains that are associated with different legume host plants appears to have resulted in considerable genetic differentiation in aphids and their bacterial endosymbionts (Peccoud et al. 2009, 2015; Ferrari et al. 2012). Between biotypes, isolating barriers are predominantly manifested as premating isolation due to biotype-specific host selection during the sexual phase of the aphid life cycle (Powell et al. 2006). Postmating barriers between biotypes also exist, with hybrid individuals experiencing decreased performance on the parental host plant (Via et al. 2000).

Loxdale et al. (2011) suggest that biotypes with generalist ecologies (such as found in the pea aphid complex) should not be maintained over evolutionary time in the face of selection for ecological specialisation. Instead, they propose that generalist strategies may be an artefact of the 'time slice ecology' through which they are observed. In contrast, the 'Oscillation' hypothesis (Janz and Nylin 2008) suggests that diverse allele sets associated with a generalist ecology may facilitate adaptive potential in the face of divergent selection, with generalists acting as a sink for the recruitment of genetic diversity into specialisation, and thus speciation, under the appropriate selective environment (Janz and Nylin 2008; Dennis et al. 2011). If a generalist have a wide niche width, it has greater potential to encounter novel environments, which will counter the tendency for specialisation. Mobility across niches allows generalists populating wide geographic scales to remain connected, thus maintaining gene flow and facilitating the evolution of novel adaptations (Dennis et al. 2011). This capacity is also evident in resilience, which is generally held to be greater for generalists than specialists (Dennis et al. 2011; Hardy and Otto 2014).

More information is urgently needed on the significance of RI in generalists. In addition, the interplay between the strength of sexual selection and ecological adaptation on the likelihood of RI is not yet well understood. Theory suggests that the honest advertisement of local adaptation via sexual signalling may provide an interface between natural and sexual selection and may facilitate population divergence through the formation of host races or ecotypes (van Doorn et al. 2009). This relies on traits

involved in premating isolation being indicators of quality because of condition dependence (Rowe and Houle 1996; Hill 2011). However, selection focused on phenotypic traits associated with generalist ecology is less likely to lead to specialisation, as a broad niche breadth will be maintained, and connectivity of populations within this broad niche will cause gene flow to therefore reduce selection for divergence. Accordingly, a generalist may effectively experience weaker selection on a trait, or suites of traits, than is needed to result in evolutionary responses that are sufficient to lead to incipient RI, and hence ultimately speciation (Nosil et al. 2009). This study addressed this omission by testing whether incipient RI could indeed result from the novel specialisation of a notable generalist.

The research in this chapter was based upon the study of divergent and replicated allopatric experimental evolution (EE) originating from a single population. The EE populations were assayed for changes in mating preferences over time. Directional selection between populations was created using divergent larval diets, to represent different hosts - adult diet was standardised. Previously, RI has been observed following experimental evolution on different diets in several *Drosophila* species (Dodd 1989; Rundle et al. 2005). The RI is thought to have evolved in the *D. serrata* system through changes to signal traits important in mate choice (Rundle et al. 2005). The medfly provides an excellent model to examine whether adaptation in response to divergent selection exists in a generalist species reported to show little global RI. It exhibits a wide range of male signal traits (Eberhard 1999), which are manifested in a lek mating system (Field et al. 2002). It is of interest to test the medfly's adaptive potential, as although it occupies a global range (Gasperi et al. 2002), RI has not yet been observed between individuals from geographically isolated populations (Cayol et al. 2002). The suite of behaviours and mating system of this species also represent an ideal model for testing the effect of divergent selection on rapid expression of local adaptation on mate choice, due to the importance of condition dependence in lek-based mating systems (Rowe and Houle 1996).

The medfly is a generalist and exhibits great plasticity in its host selection, utilization (Levinson et al. 1990; Yuval and Hendrichs 2000) and oviposition behaviour (Prokopy et al. 1984; Yuval and Hendrichs 2000). Larvae are viable in a wide range of fruits, from both inside and outside of the known host range (Carey 1984; Krainacker et al. 1987). Larvae also maintain a high level of developmental plasticity when domesticated as laboratory strains (Krainacker et al. 1987; Zucoloto 1993; Nash and Chapman 2014). This highly plastic host choice behaviour is evident in reports that medfly can

infest over 350 different host fruits globally (Liquido et al. 1991). As such, the medfly has become of huge economic importance as a damaging and invasive agricultural pest. The highly plastic generation time, life history (Carey 1984), and thermal tolerance (Nyamukondiwa et al. 2010) are thought to have facilitated its global invasion (Gasperi et al. 2002; Malacrida et al. 2007). Global populations vary in a wide range of demographic (Diamantidis et al. 2008a,b, 2009, 2011a) and behavioural traits (Briceño et al. 2002, 2007), as well as in genetic structure (Gasperi et al. 2002; Karsten et al. 2015). Despite this variation across geographically isolated populations, no premating RI has been reported even across geographically distant populations (Cayol et al. 2002).

To test if local adaptation can act to initiate RI in the medfly in the absence of gene flow, experimental evolution was conducted under two divergent larval dietary treatments. An outbred base population was divided into two sets of three independent replicates, each selected on a different larval diet. In order to test the hypothesis that RI would evolve between these two sets of replicated populations as a by-product of adaptation to divergent selection on larval diets, mating tests were used to detect assortative mating by diet regime. The three replicate populations were tested in parallel to test the hypothesis that, if incipient RI does evolve due adaptation to divergent larval diets, it would be consistent across line replicates. The results of these tests were used to calculate a set of 3 coefficients (Rolan-Alvarez and Caballero 2000) to fully describe RI - the pair sexual isolation index (PSI), the pair sexual selection index (PSS), and the pair total isolation index (PTI). These coefficients gave the strength of sexual isolation, the effect of sexual selection and a measure of overall isolation, respectively. The results were consistent with an initial pattern of sexual selection advantage for flies reared on the sugar-based ASG diet followed by later evidence for RI by larval diet of origin. However, this finding was limited by the lack of testing between replicate populations within dietary regime, meaning the assortative mating observed could not be fully attributed to divergence due to divergent selection.

3.3 Methodology

This study tested for evidence of assortative mating by diet between replicated sets of experimental evolution lines adapting to differing larval diet regimes. Mating tests were conducted at generations 3-5, 30 and 60 of the experimental evolution. At each of the three time points, tests were conducted for assortative mating by diet type. Tests were conducted for both regimes on both diets (generations 3-5 and 30) or for both regimes on their own and a common garden diet (generation 60). At generation five and

generation 30 a quartet mating test design (with a single male and female from each of the two lines to be tested) was used. At generation 60, a multiple-choice design was employed in which 25 males and females of each line were simultaneously exposed to each other.

3.3.1 Origin and maintenance of fly stocks

The TOLIMAN type strain originating from Guatemala and reared in the laboratory since 1990 (Morrison et al. 2009) was used as the base stock. For at least two years prior to the start of these experiments this strain was reared on a wheat bran diet (24% wheat bran, 16% sugar, 8% yeast, 0.6% citric acid, 0.5% sodium benzoate). To initiate the experimental evolution, flies were established on modified versions of the larval diets used by Sharon et al. (2010), (i) sucrose-based 'ASG' medium (1% agar, 7.4% sugar, 6.7% maize, 4.75% yeast, 2.5% Nipagin (10% in ethanol), 0.2% propionic acid) or (ii) 'Starch' (S) medium (1.5% agar, 3% starch, 5% yeast, 0.5% propionic acid). The caloric value of both larval diets was estimated from published sources. Three independent biological replicates of each of the two regimes were maintained under allopatry. All experiments and culturing were conducted at 25°C, 50% relative humidity, on a 12:12 light dark photoperiod. Adults emerging from each replicate were maintained in groups of roughly 30 males and 30 females in plastic cages (9cm x 9cm x 9cm). Adults from all lines received the same standard adult diet (*ad libitum* access to sucrose-yeast food; 3:1 w/w yeast hydrolysate: sugar paste and water). Each generation, approximately 500 eggs were placed on 100ml of the appropriate diet in a glass bottle. When third instar larvae started to 'jump' from the larval medium, the bottles were laid on sand and pupae allowed to emerge for seven days. Pupae were then sieved from the sand and held in 9mm petri dishes until eclosion of adults began.

To test for evidence of ecological adaptation and any associated RI, mating preferences of the replicate populations were tested at three generational time points following establishment. At the early (3-5) and middle (30) generation assays, flies were tested following rearing on their own regime larval diet and on the opposite larval diet. Generation three flies were reared on their own larval food regime for testing (i.e. regime food/test food: ASG/ASG and S/S treatments). For testing at generation five, flies were reared for two generations on the opposite food (i.e. regime food/test food: ASG/S, S/ASG treatments). For the latter, eggs were seeded onto the opposite food to their parents for two generations to remove any nutritional biases / parental effects. At generation 30, all four treatments (ASG/ASG, S/S, ASG/S, S/ASG) were conducted

simultaneously. For the tests at generation 60, a common garden design was employed. Eggs were seeded onto the common garden diet two generations prior to testing. This diet was based on glucose (1.5% agar, 3% glucose, 5% yeast, 0.5% propionic acid). All mating tests (on own diets and the common garden diet) were conducted simultaneously. Sample sizes for all tests are given in Appendix 3.1, Table 3.A1.

3.3.2 Mating tests

Flies were sorted by sex within 24 hours of eclosion to ensure virginity. Experimental flies were reared in standard 0.8L rearing cages. To enable identification, one male and one female from each population in each mating test were marked with a spot of red paint on the dorsal side of the thorax, while anaesthetised on ice. Treatments were fully controlled for handling effects and marking.

Assortative mating tests

At generations 3 & 5 and 30 a quartet mating test design was used. In this, four 5-7 day-old males and females were placed together in a mating chamber. The quartets were composed of either four flies reared on their own larval regimes, or of a male and female reared in their own larval regimes together with a male and female reared for two generations on the opposite larval diet. This created five types of mating quartet, composed of pairs reared in two different conditions (regime food / test food | regime food: ASG/ASG|S, ASG/S|ASG, ASG/S|S, S/ASG|ASG, S/ASG|S). For example, ASG/S|ASG represents a quartet of a male and female from the ASG regime reared on the Starch larval diet for two generations (ASG/S), placed with a male and female from the ASG larval diet (|ASG). The two females in each quartet were aspirated into the 250ml transparent plastic mating arenas at lights on (09:00). The two males were then aspirated into the arenas 30 minutes later. The identity of the first male and female to mate was recorded along with time of male introduction and the time that mating started and ceased. Cages were monitored for three and a half hours or until the first mating pair ceased copulation.

In generation 60, a multiple-choice design was employed in order to provide increased opportunity for mate choice. Five days post eclosion and 48 hours prior to the mating tests, 25 females of each of the two treatments to be compared, and similarly 25 males of each treatment, were placed into two, single sex, 0.8L rearing cages. The two cages were connected via a sliding door of corrugated card. Both cages were supplied with ad libitum 3:1 sugar yeast hydrolysate diet and water. Mating tests were conducted when the flies were 7-8 days post eclosion, with sexes and treatments

exactly balanced for age composition. Mating tests were initiated at 09:30, 30 minutes after lights on by slowly raising the card door. Mating pairs that subsequently formed were gently removed and placed in numbered 1.5ml eppendorf tubes for later identification. Three replicates were conducted on each test day, starting at 30 minute intervals. The order in which the replicate line pairs were tested was alternated in order to control for any effects of different start times. The experiment continued until 25 mated pairs had been collected, or until 30 minutes had elapsed. The collection of 25 pairs amounted to half of the total population in the cages. Therefore, any effect of diminishing choice due to removal of flies from the cage was minimized (Casares et al. 1998). Four replicates were conducted for each combination of tests. The identity of both individuals in each mating pair was recorded. As only 50% of matings were sampled, mating pairs were treated as independent (Casares et al. 1998; Coyne et al. 2005) and results were pooled by line replicate prior to further analysis.

3.3.3 Statistical analysis

The number of observed and total possible pairings for each pair type was calculated for each replicate. These raw data were then analysed using JMATING ver 1.0 (Carvajal-Rodriguez and Rolan-Alvarez 2006). This software allows the calculation of descriptive coefficients based on modifications to a standard cross product estimator of isolation (Rolan-Alvarez and Caballero 2000). The coefficients (see Rolan-Alvarez and Caballero 2000) are the pair sexual isolation index (PSI), the pair sexual selection index (PSS), and the pair total isolation index (PTI). The equations for the PSI, PSS, and PTI coefficients are presented in Table 3.1. PSI was calculated from the number of observed matings for each pair type divided by the expected number of matings within these mating pairs. Assuming random mating, it measures *sexual isolation*. PSS was calculated by dividing the expected number of mating pairs within the observed mating frequencies by the expected number of pair types from the total potential mates. In comparing between copulating and non-copulating samples from every pair type, under the assumption of random mating, PSS measures *the effect of sexual selection*. PTI is the product of PSI and PSS (PSI × PSS = PTI), and was calculated from the number of observed mating pairs for each pair type divided by expected numbers of mating pairs from the total potential mates. It combined the effects of sexual isolation and sexual selection to describe *overall isolation*. A detailed description of these coefficients is given by Rolan-Alvarez and Caballero (2000). Non-parametric G tests, also calculated in JMATING, were used to test for deviations from random mating across the whole coefficient dataset for each mating test. As the G test is additive, it allows the significance of the contributions of sexual isolation (GI, testing PSI

coefficients) and sexual selection (GS, testing PSS coefficients) to total isolation (GT, testing PTI coefficients) to be calculated.

JMATING was also used to calculate I_{PSI}, a joint isolation index. This measure replaced the observed mating pair numbers with the PSI coefficient, described above, as recommended to avoid several statistical drawbacks (Rolan-Alvarez and Caballero 2000). It was calculated, as demonstrated below, using the PSI coefficients for homotypic (i.e. larval diet assortative mating 'AA' or 'BB') pairs, the PSI coefficients for heterotypic (disassortative mating, 'AB' or 'BA') pairs, and the total PSI coefficient for all pairs:

$$I_{PSI} = \frac{(PSI_{AA} + PSI_{BB}) - (PSI_{BA} + PSI_{AB})}{(PSI_{AA} + PSI_{BA} + PSI_{AB} + PSI_{BB})}$$

I_{PSI} values vary from -1 to 1, with +1 being total assortative mating and -1 total dissasortative mating. I_{PSI} of 0 therefore denotes random mating. Following Coyne et al. (2005), I_{PSI} was used to describe total isolation and the PTI coefficient to describe positive and negative preferences for mating pairs within each line pair, at each of the three generational time points.

An index of mating asymmetry (IA_{PSI}) was calculated to capture the difference in frequency between homotypic and heterotypic pairs (AA/BB or BA/AB) based on the PSI coefficient, and was calculated as PSI_{AA}/PSI_{BB} and PSI_{BA}/PSI_{AB}, respectively (Rolán-Alvarez 2004). Values of IA_{PSI} centre around 1 (no asymmetry), with values below one reflecting asymmetry towards the first pair type, and values greater than one representing asymmetry towards the second. This index was calculated for the mating tests conducted on flies reared on their own larval diet across all three generational time points. IA_{PSI} was calculated in JMATING using the following equation:

$$IA_{\mathrm{PSI}} = |\ \mathrm{PSI}_{aa} - \mathrm{PSI}_{ab}\ |$$

Table 3.1. Equations for PSI, PSS, and PTI coefficients after Rolan-Alvarez and Caballero (2000). The top right panel describes the total population (*T*), divided between two types. The number of males representing each type in a mating test are *A* and *B*, and the number of females of the corresponding types are *A′* and *B′*. The number of copulating pairs (*t*) is divided between the number of observed pairs of every combination of male and female types (*aa*, *ab*, *ba*, *bb*). *S* is the expected number of copulating pairs, given the population frequencies.

The main panel (below) shows the equations used to derive PSI, PSS, and PTI coefficients. These are based on the model of the overall population and outcome of the mating test described in the right hand panel. Detailed description of these equations is provided in the text.

		Females		
		A′	*B′*	
Males	*A*	***aa***	***ab***	*aa + ab*
	B	***ba***	***bb***	*ba + bb*
		aa + ba	*ab + bb*	*t*

t = aa + ab + ba + bb
T = A + B + A′ + B′
S = (*AA′*) + (*AB′*) + (*BA′*) + (*BB′*)

Observed Pairs		Expected pair types from total numbers		Expected pair types from mates	
aa	*ab*	$\frac{AA'}{S} = t$	$\frac{AB'}{S} = t$	$\frac{(aa + ab)(aa + ba)}{t}$	$\frac{(aa + ab)(ab + bb)}{t}$
ba	*bb*	$\frac{BA'}{S} = t$	$\frac{BB'}{S} = t$	$\frac{(aa + ba)(ba + bb)}{t}$	$\frac{(ba + bb)(ab + bb)}{t}$

Pair total index (PTI)		Pair sexual isolation index (PSI)		Pair sexual selection index (PSS)	
$PTI_{aa} = \frac{(aa)S}{AA't}$	$PTI_{ab} = \frac{(ab)S}{AB't}$	$PSI_{aa} = \frac{(aa)t}{(aa + ab)(aa + ba)}$	$PSI_{ab} = \frac{(ab)t}{(aa + ab)(ab + bb)}$	$PSS_{aa} = \frac{(aa + ab)(ab + bb)S}{(AA')t^2}$	$PSS_{ab} = \frac{(aa + ab)(ab + bb)S}{(AB')t^2}$
$PTI_{ba} = \frac{(ba)S}{BA't}$	$PTI_{bb} = \frac{(bb)S}{BB't}$	$PSI_{ba} = \frac{(ba)t}{(aa + ba)(ba + bb)}$	$PSI_{bb} = \frac{(ab)t}{(ba + bb)(ab + bb)}$	$PSS_{ba} = \frac{(aa + ba)(ba + bb)S}{(BA')t^2}$	$PSS_{bb} = \frac{(ba + bb)(ab + bb)S}{(BB')t^2}$

Finally, to compare the sexual fitness of males and females, JMATING was employed to calculate *W*, the cross product estimator of sexual selection (Carvajal-Rodriguez and Rolan-Alvarez 2006). This was calculated individually for males and females, and gave the relative fitness of each treatment, in comparison to the fittest treatment within each line pairing. *W* was calculated for *A* (males of one of two types in a population) in JMATING using the following equation, where lowercase letters are the observed mating pairs in the mating test (See Table 3.1):

$$W_A = \frac{(PSS_{aa} + PSS_{ab})}{(PSS_{ba} + PSS_{bb})}$$

The significance of PSI, PSS, and PTI coefficients was calculated as the bootstrap probability of rejecting the null hypothesis of random distribution, after 10,000 iterations of resampling observed and expected frequencies for PTI, and from mated data when estimating PSI and PSS (Rolan-Alvarez and Caballero 2000). When applied to the isolation index (I_{PSI}), the asymmetry index (IA_{PSI}), and the estimator of sexual selection (*W*), this is the two-tail bootstrap probability that the value is significantly different from one (which would be equivalent to random mating, or zero asymmetry) after 10,000 iterations of resampling. All bootstrapping was conducted in JMATING. To test for the overall effect of diet in RI as indicated by each / any of these three metrics (I_{PSI}, IA_{PSI}, *W*), probability values generated for each line replicate comparison were combined using Fisher's sum of logs method, implemented with the 'metap' package in R (Dewey 2016). All other data handling and statistical analysis was conducted in R ver 3.1.1 (R Development Core Team 2015).

Table 3.2. Ingredients, quantities and caloric value of the two experimental evolution diets (ASG and Starch). Superscript represents source of information, shown below the table.

Diet	Ingredient	Quantity	Kcal/g	Kcal in diet	Total kcal/litre
Starch	Water	1000ml	-	-	291
	Agar	15g	0.26[1]	3	
	Potato Starch	30g	4.2[2]	126	
	Yeast	50g	3.25[1]	162	
	Propionic Acid	5ml	-	-	
ASG	Water	850ml	-	-	684
	Agar	12.5g	0.26[1]	3	
	Yeast	47.5g	3.25[1]	154	
	Corn Meal	67g	3.62[1]	243	
	Sugar	73.5g	3.87[1]	284	
	Propionic Acid	2ml	-	-	
	Nipagin	25ml	-	-	

Sources: [1]http://ndb.nal.usda.gov/, [2] Southgate and Durnin (1970)

3.4 Results

3.4.1 Caloric value of larval diets

Table 3.2 describes the caloric value of the two larval rearing diets, ASG and S. This descriptive analysis showed that the ASG larval diet contained over twice the amount of available KCal/L than the S, and was therefore potentially a more energy-rich diet.

3.4.2 Testing for non random mating – general patterns

After three generations of experimental evolution, individuals from the three replicates of each treatment were tested using the quartet mating test design. ASG males were over-represented in mating pairs across all replicates (Figure 3.1A & Appendix 3.1, Table 3.A1). Mating frequencies for the crossed environment tests conducted at generation five (Figure 3.1B-E & Appendix 3.1, Table 3.A1) revealed a dominant proximate effect of the current diet. At generation 30, as in generation three, ASG males mated at highest frequency in tests of flies reared on their own regime (Figure 3.2A, Appendix 3.1, Table 3.A1). Mating frequencies for the crossed environment tests (Figure 3.2B-E & Appendix 3.1, Table 3.A1) suggested that the dominant proximate diet effect had diminished, suggesting some dietary adaptation. In generation 60, mating tests were performed using the multiple-choice design. At this stage, homotypic (ASG/ASG male + ASG/ASG female, S/S male + S/S female) pairs predominated, although a residual bias towards matings with ASG males remained (Figure 3.3A, Appendix 3.1, Table 3.A1). Again, the pattern of homotypic pairings occurring at highest frequency was observed. Overall, S males mated most frequently (Figure 3.3B, Appendix 3.1, Table 3.A1). These general patterns are explored statistically in detail, below.

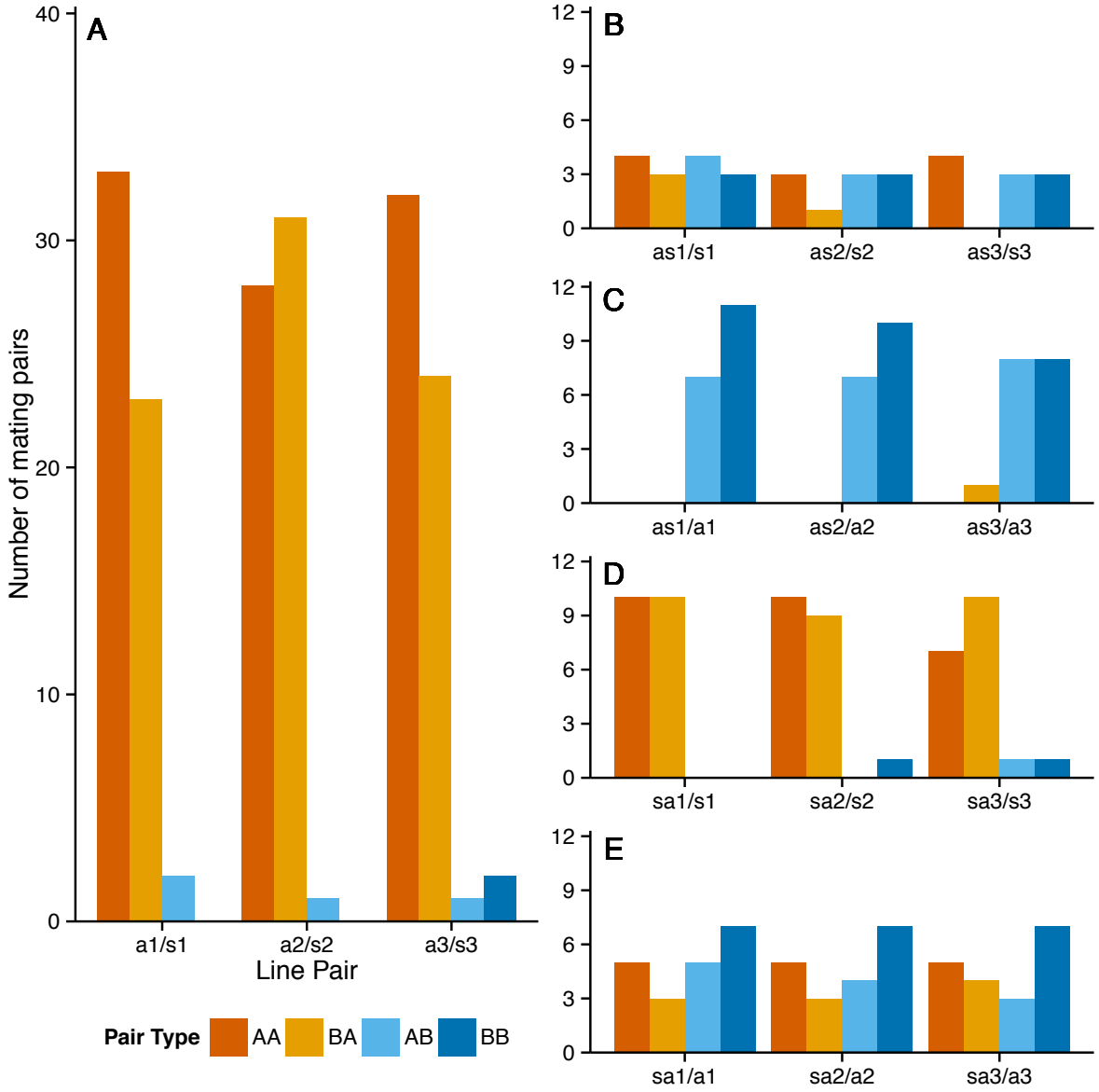

Figure 3.1 The number of mating pairs formed in quartet mating tests between ASG and Starch dietary selection lines after 3-5, generations of selection. Each plot shows three replicates, with lowercase letters representing diet (ASG = a or Starch = s) and replicate information (1-3). Upper case letters represent 'pair types' formed within that replicate (homotypic = AA, BB; heterotypic = AB, BA). Dark Orange bars represent homotypic pairings between the first regime in the replicate title (i.e. a1/s1). Light orange bars represent heterotypic pairings composed of a male from this treatment, and a female from the second regime in the replicate title. Light blue bars represent the opposite heterotypic pairing. Dark blue bars represent homotypic matings between the second regime in the replicate title (i.e. a1/s1). **A)** Flies reared on their own larval diet (ASG/ASG vs. S/S), tested at generation three. **B)** Quartets composed of male and female ASG individuals tested on larval Starch diets versus male and female Starch individuals (ASG/S|S), at generation five. **C)** Quartet composed of male and female ASG individuals, tested on larval Starch diet versus male and female ASG individuals (ASG/S|ASG), at generation five. **D)** Quartet composed of male and female Starch individuals tested on larval ASG diet versus male and female Starch individuals (S/ASG|S), tested at generation five. E) Quartet composed of male and female Starch individuals tested on larval ASG diet versus male and female ASG individuals (S/ASG|ASG), tested at generation five.

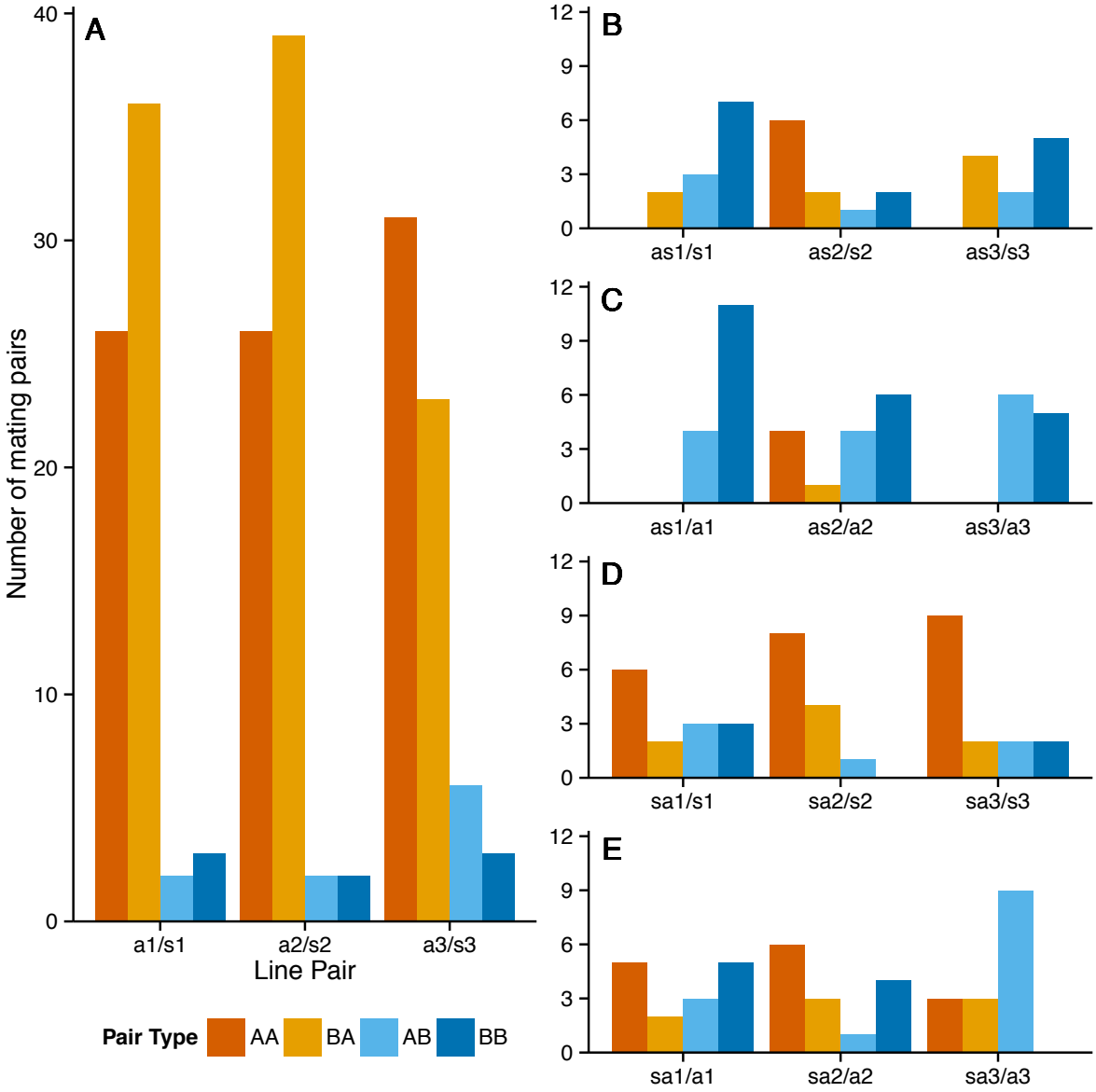

Figure 3.2. The number of mating pairs formed in quartet mating tests between ASG and Starch dietary selection lines after 30 generations of selection. Plot layout, construction, and labelling as described for Figure 3.1. All tests conducted at generation 30.

3.4.3 Testing for differences in sexual isolation (PSI), sexual selection (PSS) and total isolation (PTI) indices

Initial tests - generations 3-5

At the initiation of experimental evolution, all three replicates (a1-3; s1-3) showed significant deviation from random preference when tested on their own larval food regimes (ASG/ASG & S/S), as shown by the PTI coefficient (a1/s1, GT = 67.57, df = 3, $P < 0.001$; a2/s2, GT = 74.54, df = 3, $P < 0.001$; a3/s3, GT = 59.56, df = 3, $P < 0.001$, Appendix 3.1, Table 3.A2). The major contributor to this effect came from the PSS coefficient (a1/s1, GS = 65.51, df = 2, $P < 0.001$; a2/s2, GS = 73.07, df = 2, $P <$

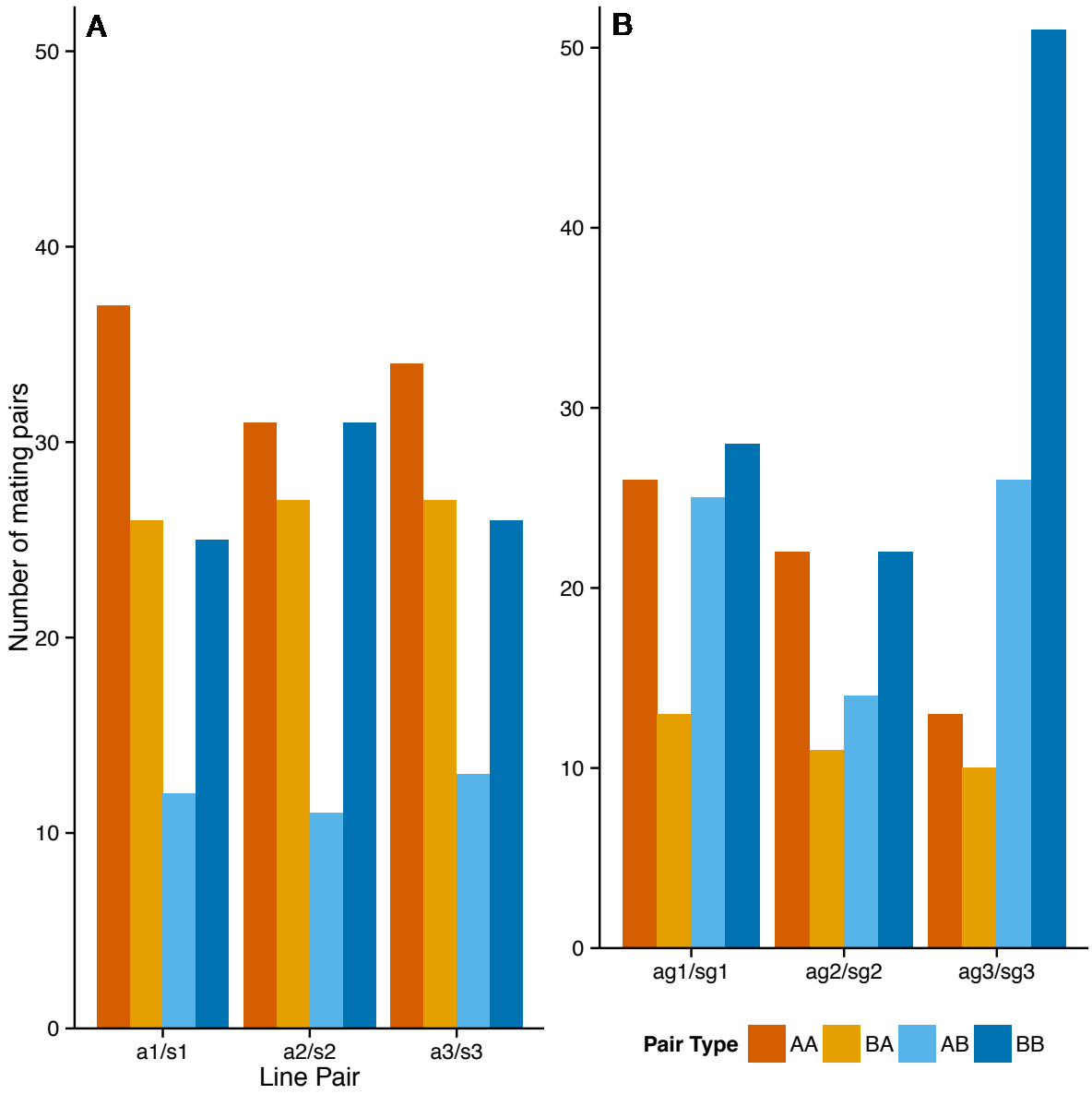

Figure 3.3. The number of mating pairs formed in multiple-choice mating tests between ASG and Starch dietary selection lines after 60 generations of selection. Plot construction and labelling as described in Figure 3.1. **A)** Mating pairs formed when lines were tested when reared in their own regime background (ASG/ASG vs. S/S). **B)** Mating pairs tested on the common garden glucose diet (ASG/G vs. S/G).

0.001; a3/s3, GS = 58.91, df = 2, $P < 0.001$). The PSS coefficients showed strong sexual selection on ASG males, as both types of mating pairs containing ASG males ('AA' mating type = ASG/ASG♀ + ASG/ASG♂, 'BA' type = S/S♀ + ASG/ASG♂) were significantly higher than one (ranging from 1.55 to 2.31). This was also reflected in sexual selection against pairs containing S males ('AB' type = ASG/ASG♀ + S/S♂, 'BB' type = S/S♀ + S/S♂; PSS coefficients 0.05 - 0.12).

For the tests on the opposite type of larval food (Appendix 3.1, Table 3.A3 – A6), ASG flies tested on S diets placed with S competitors (ASG/S|S) showed no or only a weak deviation in PSI (as3/s3, GI = 3.9, df = 1, $P = 0.048$, Figure 3.4B). Against ASG competitors (ASG/S|A) there was a highly significant deviation from random PTI

(as1/a1, GT = 25.85, df = 3, $P < 0.001$; as2/a2, GT = 24.1, df = 3, $P < 0.001$; as3/a3, GT = 17.35, df = 3, $P < 0.001$). This effect was explained predominately by the sexual selection coefficient, PSS (as1/a1, GS = 25.85, df = 2, $P < 0.001$; as2/a2, GT = 24.1, df = 2, $P < 0.001$; as3/a3, GS = 16.02, df = 2, $P < 0.001$). PSS coefficients for pairs containing ASG males ('AB' mating type = ASG/S♀ + ASG/ASG♂, 'BB' type = ASG/ASG♀ + ASG/ASG♂) were all above one (1.5 - 2.29) with 'BB' type pairs significantly higher than one in 2/3 replicates. Pairs containing ASG males tested on the S diet ('AA' type = ASG/S♀ + ASG/S♂, 'BA' = ASG♀ + ASG/S♂) had PSS coefficients significantly lower than one in all line replicates (0.08 - 0.18). This pattern was mirrored in the PTI coefficients. S individuals reared on ASG diets against S competitors (S/ASG|S) also showed a strong deviation from random PTI (sa1/s1, GT = 27.73, df = 3, $P < 0.001$; sa2/s2, GT = 21.22, df = 3, $P < 0.001$; sa3/s3, GT = 14.08, df = 3, $P = 0.003$). This was again predominately explained by the PSS (sa1/s1, GS = 27.73, df = 2, $P < 0.001$; sa2/s2, GT = 19.79, df = 2, $P < 0.001$; sa3/s3, GS = 14.03, df = 2, $P < 0.001$). However, here the sexual selection advantage occurred in the crossed diet individuals. Pairs containing S males tested on ASG ('AA' mating type = S/ASG♀ + S/ASG♂, 'BA' = S/S♀ + S/ASG♂) exhibited PSS coefficients significantly higher than one in 2/3 replicates (1.59 - 2.18). Pairs containing S males ('AB' type = S/ASG♀ + S/S♂, 'BB' = S/S♀ + S/S♂) exhibited PSS coefficients significantly lower than 1 (0.09 to 0.26). Again these values are reflected in the PTI coefficients. S flies tested on ASG diet with ASG competitors (S/ASG|ASG) showed no significant deviation from random across all coefficients.

The results showed a competitive advantage for males reared in an ASG background over males reared on S. Even two generations of ASG rearing was enough to 'rescue' the disadvantage of the S diet. This was also true in reverse – two generations of S rearing was enough to remove the advantage of ASG flies reared on ASG. This suggested that, by three generations of experimental evolution there was no genetic adaptation to the two dietary treatments that had any significant effect on mate choice.

Middle (generation 30) tests

The PSI, PSS, and PTI coefficients (Appendix 3.1, Table 3.A7 – A11) were again analysed using non-parametric G tests to test for any deviations from random. Flies reared and tested on their own food showed similar patterns to above. There was a highly significant deviation from random in PTI (a1/s1, GT = 59.13, df = 3, $P < 0.001$; a2/s2, GT = 67.73, df = 3, $P < 0.001$; a3/s3, GT = 36.47, df = 3, $P < 0.001$) again

explained by strong differences in PSS (a1/s1, GS = 59.13, df = 2, $P < 0.001$; a2/s2, GS = 67.57, df = 2, $p < 0.001$; a3/s3, GS = 36.31, df = 2, $P < 0.001$). As seen above, PSS coefficients for pairs containing ASG males ('AA' mating type = ASG/ASG♀ + ASG/ASG♂, 'BA' = S/S♀ + ASG/ASG♂) were significantly greater than one (1.34 - 2.24) in 2/3 replicates. Pairs containing S males ('AB' type = ASG/ASG♀ + S/S♂, 'BB' = S/S♀ + S/S♂) all returned PSS values significantly lower than one (0.09 and 0.36). There was significant positive preference for males reared on ASG ('AA' and 'BA' mating type pairs), with PTI coefficients greater than one (1.38 - 2.26), significantly so for 2/3 replicates. S male mating pairs ('AB' and 'BB' mating type) returned PTI values significantly lower than one (0.11 to 0.39). In contrast, the results from the mating tests of flies reared on the opposite diets were inconsistent and no general pattern emerged (Appendix 3.1, Table 3.A8 - A11). In general, the proximate effects of diet were diminished in comparison to the patterns seen at the initiation of the experimental evolution.

Overall, at generation 30 the general trend, particularly for mating tests conducted with males reared on their selection regime diets, was similar to that observed earlier in experimental evolution. Males maintained on ASG maintained a competitive advantage over S males. The 'rescue' effect seen as a response to proximate food treatment at generation five was present, though to a much lesser degree, and was also more variable among replicates. S males tested on ASG remained more successful when in competition with S males. ASG males tested on S remained less competitive than ASG males. Significant heterogeneity in preference was also observed when both types of crossed diet flies were tested with flies maintained on the diet to which they were crossed (a pairing that, in the absence of adaptation, should effectively be neutral, as in generation five). The findings suggest that by generation 30 lines were beginning to respond to experimental evolution.

Late (generation 60) tests

To remove parental effects flies were reared on a 'common garden' diet for two generations prior to mating tests. A multiple-choice mating test design was used to increase choice for individuals of both sexes and treatments. Mating pairs were collected as they formed until 50% of the possible pairs had formed. This avoided the effects of diminishing mate choice.

The mating tests on individuals tested on their own diets showed significant deviations from random PTI (a1/s1, GT = 13.44, df = 3, $P = 0.004$; a2/s2, GT = 12.77, df = 3, $P = 0.005$; a3/s3, GT = 3.5, df = 1, $P = 0.061$). This effect was driven by significant

deviations from random PSI (a1/s1, GI = 6.56, df = 1, *P* = 0.010; a2/s2, GI = 7.63, df = 1, *P* = 0.006; a3/s3, GI = 4.86, df = 1, *P* = 0.027) with 2/3 replicates also showing significant deviations from random PSS (a1/s1, GS = 6.88, df = 2, *P* = 0.032; a2/s2, GS = 5.14, df = 2, *P* = 0.077). There was a clear pattern for homotypic (diet assortative) pairings ('AA' mating type = ASG/ASG♀+ ASG/ASG♂, 'BB' = S/S♀ + S/S♂) to exhibit PSI coefficients above one, and heterotypic (diet dissasortative) pairings ('BA' type = S/S♀ + ASG/ASG♂, 'AB' = ASG/ASG♀ + S/S♂) to show negative values. The resulting pair total isolation index (PTI), returned values significantly higher than one for 'AA' mating type pairs ('AA' = ASG/ASG♀ + ASG/ASG♂) in 2/3 replicates (a1/s1, a3/s3). Also, PTI coefficients for the 'AB' mating pair types (Figure 3.6A, panel 3, 'AB' = ASG/ASG♀ + S/S♂) were significantly lower than one for all lines (0.44 - 0.52). Hence by generation 60 there was, in the flies tested on their own diets, evidence for significant assortative mating by diet.

When tested in the common garden, the patterns were similar, though with some interesting differences. In 2/3 replicates (ag1/sg1, ag3/sg3) the common garden environment removed any significant effect of treatment on the PSI distribution. However, ag2/sg2 showed significant deviation from random in PSI (ag2/sg2, GI = 5.4, df = 1, *P* = 0.020). The third replicate responded differently, showing a strong deviation from random in PTI, explained almost exclusively by sexual selection (PSS) (ag3/sg3, GT = 39.43, df = 3, *P* < 0.001; GS = 35.65, df = 2, *P* < 0.001). A similar pattern in PSI as observed above in the 'on treatment' tests, with homotypic pairs ('AA' mating type = ASG/ASG♀ + ASG/ASG♂, 'BB' = S/S♀ + S/S♂, Appendix 3.1, Table 3.A13) returning values above one, and heterotypic ('BA' type = S/S♀ + ASG/ASG♂, 'AB' = ASG/ASG♀ + S/S♂) scoring below one. The PSS coefficient showed no significant differences from one in 2/3 replicates (ag1/sg1, ag2/sg2), but the third (ag3/sg3) responded differently. The PTI coefficients were significantly lower than one for 'BA' mating types ('BA' = S♀ + ASG♂) in all line replicates (0.4 - 0.64). Reflecting the high values seen in PSS, the ag3/sg3 line replicate also returned significant PTI for the two homotypic pair types, with 'AA' pairs ('AA' = ASG♀ + ASG♂) being significantly lower than one, and 'BB' pairs ('BB' = S♀ + S♂) significantly greater than one.

At generation 60, under conditions in which greater expression of choice was allowed, a signal of significant assortative mating by diet was detected, in both the 'on diet' and common garden tests. The competitive advantage of ASG diet remained only in the 'on diet' tests and was again mediated by sexual selection advantages. Hence, the removal of the environmental differences from the experimental evolution removed any sexual

selection advantage for ASG males. The maintenance of the assortative mating pattern in the common garden suggests that genetic adaptation within the lines according to their different environments resulted in the evolution of incipient RI.

3.4.4 Testing for significant isolation - I_{PSI} - Isolation index

The joint isolation index was used to test whether the patterns of matings showed significant positive or negative assortment. At the initiation of experimental evolution there were no significant deviations from random (Appendix 3.1, Table 3.A14). The same was also true for flies tested on their own or the opposite diets at generation 30 (Appendix 3.1, Table 3.A15), with just one replicate of the S tested on ASG showing significant negative assortment against ASG individuals (Appendix 3.1, Table 3.A15). At generation 60 in the multiple-choice scenario, all line pairings showed significant assortative mating when tested on their own and on the common garden diets, though the effects observed on the common garden were marginally significant (Table 3.3).

To test the main effect of dietary background on assortative mating, Fisher's combined probability test was used to assess the significance of patterns of assortment across replicates. In generation 60, when flies were reared in their own dietary background the combined significance of assortative mating was highly significant (χ^2 = 27.03, d.f. = 6, $P < 0.001$). This was also true when flies were reared in on common garden diet for two generations (χ^2 = 19.7, d.f. = 6, $P = 0.003$).

Table 3.3. I_{PSI} isolation index values (Coyne et al. 2005) for each line pairing after 60 generations of selection. Values presented are bootstrap means with standard deviations; P values are the two-tail bootstrap probability that the value is significantly different from one (equivalent to random mating), after 10,000 iterations of resampling.

Line Pair	I_{PSI} value	St dev.	P	Sig.
a1/s1	0.27	0.10	0.013	*
a2/s2	0.28	0.10	0.004	**
a3/s3	0.23	0.10	0.026	*
ag1/sg1	0.20	0.11	0.059	.
ag2/sg2	0.28	0.12	0.016	*
ag3/sg3	0.23	0.12	0.056	.

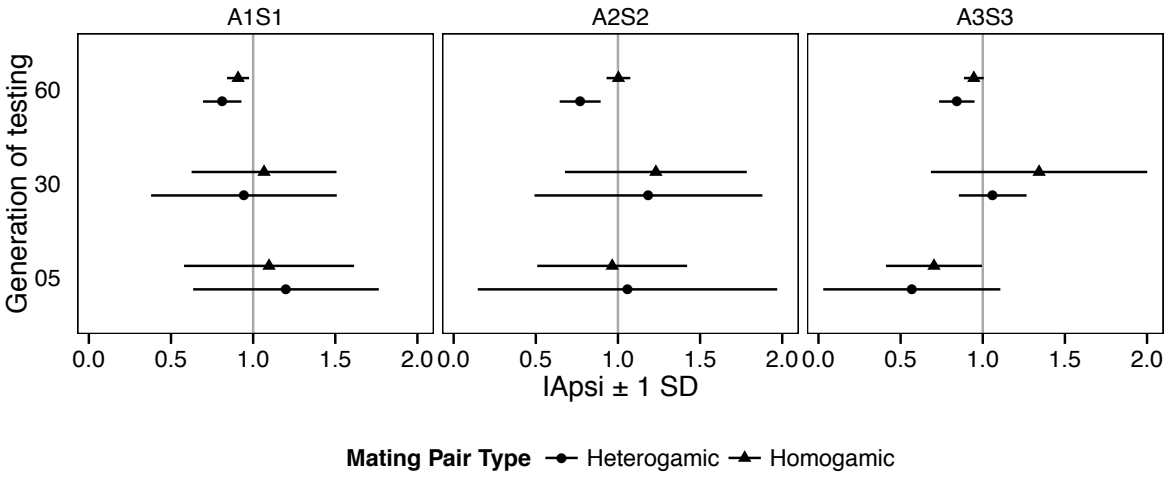

Figure 3.4. Index of Asymmetry based on PSI coefficient (IA_{PSI}). The 3 panels represent data from flies reared in selective background for each line pairing from three biological replicates, compared over the three generational time points at which mating tests were conducted.

3.4.5 Testing for significant asymmetry in matings - IA_{PSI} - Asymmetry index

For mating assays conducted on individuals tested in their own selective background IA_{PSI} was used to examine whether there was asymmetry in the proportion of homo- versus heterotypic matings. IA_{PSI} was consistently significantly lower than from 1 for heterotypic pairs ('BA' mating type = S/S♀ + ASG/ASG♂, 'AB' = ASG/ASG♀ + S/S♂) in generation 60, showing a consistent effect of diet across replicates (Fisher's combined probability test, X^2 = 23.22, d.f. = 6, P < 0.001), but not for earlier generation tests (Figure 3.4). The frequency of heterotypic pairs was skewed towards BA type matings, i.e. those comprising S females and ASG males.

3.4.6 Testing for divergence in *W* - Cross product estimator of sexual selection

In the final analysis *W* was calculated, as described above, for mating tests conducted on individuals tested on their own selective background across all three time points. W is fixed at one for the fittest individual of each sex. There was no significant difference between treatments in female specific *W* at any generational time point (Figure 3.5A). However, *W* for S males was lower than for ASG males at every time point. This effect was significant in all cases (generation 3 and generation 30: 0/10,000 bootstrap probabilities non significant; generation 60: Fishers Combined test, X^2 = 24.28, d.f. = 6, P < 0.001).

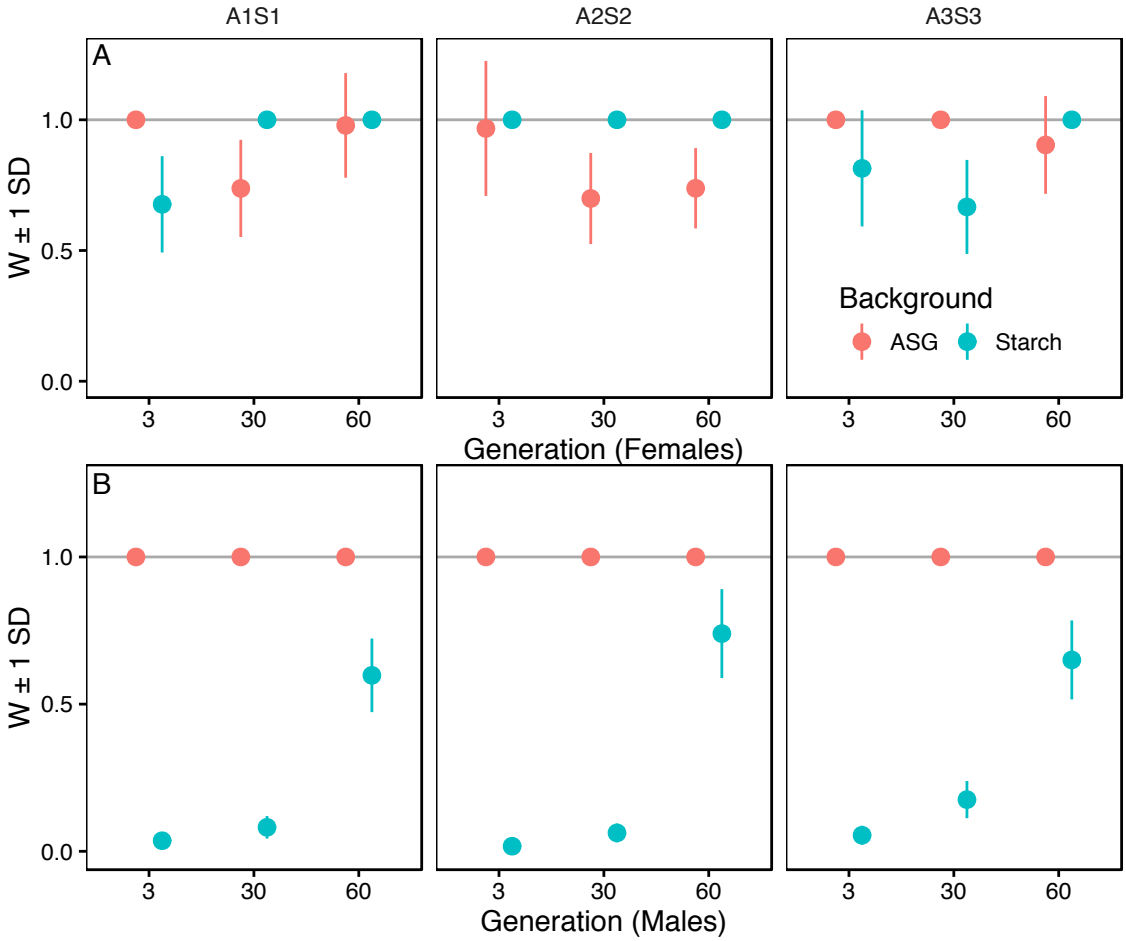

Figure 3.5. *W*, the cross product estimator of sexual selection. The panels represent data from flies reared in selective background for each line pairing from three biological replicates, compared over the three generational time points at which mating tests were conducted.

3.5 Discussion

The major finding was the existence of significant, male-driven, assortative mating following replicated experimental evolution on divergent larval rearing diets. Adaptation to the different diets was therefore associated with incipient RI, within 60 generations. Prior to the final tests, there was a strong pattern of higher frequency of matings involving ASG males, with a proximate diet effect in the earliest generation tested, which itself then decayed by generation 30. Hence, over the early, middle, and late generational tests a diminution in the strength of the competitive advantage in male fitness associated with the ASG diet was observed, as significant assortative preference for rearing treatment of origin emerged. Significant asymmetry at generation 60 between the frequencies of the two types of heterotypic pairs was observed, indicating a significant advantage of ASG males on their own diet and for S males on the common garden diet.

However, the experimental evidence presented in the Chapter is not by itself fully sufficient to attribute the observed assortative mating to RI arising from adaptation to diet, as the effects of genetic drift have not yet been directly tested for. In order to rule out the possibility of random genetic drift (Coyne and Orr 2004) between allopatric replicates it would be necessary to conduct the same mating tests on crosses between the replicates within each diet regime. Such 'same host' tests are essential to fully ascribe the effects of divergent selection in initiating RI (Funk and Nosil 2008). That said, genetic drift-generated RI has not previously been captured in any laboratory-based study (Rundle 2003). However, as yet I cannot exclude the possibility that founder effects associated with the establishment of the EE generate the assortative mating patterns observed here.

The uniformity of the patterns of assortative mating observed across the replicates (including at the underlying transcriptomic level, see Chapter 5) suggests that in principle the two larval rearing diets provided sufficiently distinct selective environments to drive the evolution of parallel divergence. This effect appeared to be male-driven, as shown by differences among the regimes in male sexual selection (PSS coefficient) and male fitness (*W* coefficient) advantage. At the initiation of experimental evolution (in a limited choice scenario) these effects gave one treatment (ASG) a strong competitive advantage over another. The medfly is a lekking species (Field et al. 2002) and reproductive success is strongly dependent on a male's competitive ability in a sexual context. Many studies have highlighted the importance of adult nutrition (mainly protein) on male mating success in the medfly (Blay and Yuval 1997; Kaspi and Yuval 2000; Shelly et al. 2002; Joachim-Bravo et al. 2009; Costa et al. 2012). However, in this study it was larval, not adult diet that was varied. Larval dietary nutrition is also reported as essential for reproductive maturation and copulation success in the medfly (Kaspi et al. 2002; Anjos-Duarte et al. 2011). This study is, however, the first to provide evidence consistent with the idea that maintaining medfly populations on different larval diets (effectively different hosts) can lead to assortative mating and potentially RI.

The two larval diets were highly different in terms of caloric value. Although this is only an indication of total nutritional value, calories do give an insight into the likely selection pressures to which the founding population was challenged. The ASG larval diet had over twice the Kcal/L than the Starch, and this was sufficient to lead to the sexually competitive advantage observed. The specific nutritional content of diets, rather than caloric content per se affects life history traits such as lifespan (Mair et al.

2005). Here, the addition of corn meal to the ASG diet offered an additional source of carbohydrates, proteins, and other dietary nutrients (http://ndb.nal.usda.gov/). The competitive advantage seen by ASG males may have been mediated by this increase in diet content and complexity. It is also possible other non-nutritional factors associated with the diet, such as its consistency or other additives, could also have affected development or life history.

Reproductive isolation generated by divergent selection based on diet was previously observed in experimental evolution studies (Dodd 1989; Rundle et al. 2005; Greig 2009). However, this is the first study to isolate the effects of larval diet. In a system that has strong sexual dimorphism, manifested in lekking behaviour, there is strong sexual selection on a suite of costly traits in males, allowing females the opportunity for choice prior to mating (Whittier et al. 1994). The relationship between these sexually selected traits and the overall condition of the male is essential to mate choice. Genic capture hypothesises that female preference for costly male traits will result in the evolution of a genetic covariance between condition and expression of a sexually selected trait. Therefore traits will only be optimally expressed when a male is in good condition (Rowe and Houle 1996). The results show that the diet experienced by a holometabolous insect during development can directly influence mate choice, even if adult nutrition is controlled. Condition-dependence of ornamental traits can be thought of as a correlate of the maintenance of optimal functionality of vital body systems (Hill 2011). This suggests that developmental conditions are vital in programming either the pattern of resource allocation in adult life history, or in shaping the pathways through which condition is manifested. As both larval diet regimes in this study had equal resources to maintain somatic state as adults, modifications to cellular function that manifested the competitive advantage seen on the ASG diet were selected upon during development. Sexual signal traits have been shown to diverge as a result of adaptation to experimental dietary selection (Rundle et al. 2005), and larval dietary nutrients have significant effects on adult size in holometabolous insects (Nijhout 2003; Edgar 2006), the expression of adult sexual signals (Delcourt and Rundle 2011; Havens and Etges 2013), and secondary sexual characters (Bonduriansky et al. 2015). The results link the importance of developmental nutrients to the expression of sexually selected traits in a nutritionally homogeneous adult environment. The divergent natural selection imposed by larval diet may have been linked to sexual selection in the adult population through condition dependence, in line with the theoretical model of disruptive ecological selection suggested by van Doorn et al. (2009), although it is noted this model cannot operate in allopatry. The decay of the

competitive advantage of ASG males could represent an association between mate preference and local adaptation, fuelled by interaction with sexual selection. It has suggested that such 'internal' interactions between natural and sexual selection, where factors of the environment may effect the ability to produce sexual signals, are important in the instigation of RI between populations (Safran et al. 2013).

Sexual selection has long been recognised as an important driver in population divergence (Lande 1981; Panhuis et al. 2001). In allopatric scenarios, as studied here, population divergence is often accompanied by rapid change in male secondary sexual characters important for copulatory success in line with the rapid action of sexual selection, as exemplified by the Hawaiian *Drosophila* adaptive radiation (e.g. Hoikkala and Kaneshiro 1993; Magnacca and Price 2015). However, it is hard to ascribe the driving selective force behind such differentiation, as allopatry leaves a population vulnerable to selection from many angles, and also to the action of isolation caused by random genetic drift (Turelli et al. 2001). Although genetic drift has the potential to contribute to such alteration of population traits (e.g. Whitlock et al. 2002), I note that such drift-based change has not been seen to contribute to RI over similar evolutionary time scales (Rice and Hosert 1993; Rundle 2003; Kawecki et al. 2012).

Experimental evolution of assortative mating and putative incipient RI in medfly populations is an interesting and novel result in the context of comparisons to studies of wild populations. Geographically isolated populations of wild caught flies have not returned any significant RI between populations on a global scale (Cayol et al. 2002; Lux et al. 2002). Despite this, significant behavioural differences are seen between similarly geographically isolated populations, in courtship song (Briceño et al. 2002), and also courtship behaviour (Briceño et al. 2007,Diamantidis et al. 2008b). Further differentiation has been observed between global populations in other life history traits such as growth rate, longevity, and sexual maturation (Diamantidis et al. 2008a, 2009), as well as pre-adult traits (Diamantidis et al. 2011b), and also resilience to a domestication process (a potential proxy for an enforced change of host) (Diamantidis et al. 2011a).

The variance seen across these traits in global populations of medfly reflects overall genetic diversity of these populations, which is closely linked to the medfly's invasion history (Gasperi et al. 2002; Karsten et al. 2015). Lack of divergence (isolation) between populations that vary globally is likely due to the effects of gene flow between populations (Malacrida et al. 1998, 2007; Karsten et al. 2015), potentially mediated by invasions due to human transport (Wilson et al. 2009). This migration between

populations has likely suppressed the capacity for specialisation shown here, and maintained the plasticity exhibited by the medfly as a generalist, in both host and temporal space (Yuval and Hendrichs 2000). Despite this, this study demonstrates that a laboratory population (over 20 years isolated from the wild) maintained sufficient genetic variation to respond to experimentally implemented divergent selection resulting in significant assortative mating, and putative behavioural isolation, after only 60 generations of allopatry. Relating this to natural populations, as quarantine measures become increasingly effective and reduce gene flow between global medfly populations (Karsten et al. 2015), the adaptive potential observed here may lead global populations to diverge further.

The mechanisms through which divergent selection is acting on mating preferences in this generalist species remain to be elucidated, but the results raise interesting questions regarding the adaptive potential of the medfly. It is likely that adaptation to developmental dietary environment drives selection across a suite of traits in the medfly, but whether the strength of this selection is strong enough to cause divergence in the face of gene flow is uncertain outside of a laboratory setting. 'An important extension to the work conducted here will be to account for the effects of genetic drift between the populations studied, as described above. Without these data, it is not possible to wholly attribute the findings to divergence-based environmental selection pressure. However, the potential for dietary (host) specialisation within one of the most notorious generalists has implications for the efficacy of programmes that seek to control its populations using mass-reared laboratory strains. As well as this, these findings offer opportunities for advances in the understanding of the role of developmental environment in the generation of isolation between populations, and it's place within the speciation continuum.

3.6 References

Anjos-Duarte, C. S., A. M. Costa, and I. S. Joachim-Bravo. 2011. Sexual behaviour of the Mediterranean fruit fly (Diptera: Tephritidae): the influence of female size on mate choice. J. Appl. Entomol. 135:367–373.

Blay, S., and B. Yuval. 1997. Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). Anim. Behav. 54:59–66.

Bonduriansky, R., M. A. Mallet, D. Arbuthnott, V. Pawlowsky-Glahn, J. J. Egozcue, and H. D. Rundle. 2015. Differential effects of genetic vs. environmental quality in *Drosophila melanogaster* suggest multiple forms of condition dependence. Ecol. Lett. 18:317-326.

Briceño, R. D., W. G. Eberhard, J. C. Vilardi, P. Liedo, and T. E. Shelly. 2002. Variation in the intermittent buzzing songs of male medflies (Diptera : Tephritidae) associated with geography, mass-rearing, and courtship success. Florida Entomol. 85:32–40.

Briceño, R. D., W. G. Eberhard, J. Vilardi, J. Cayol, and T. Shelly. 2007. Courtship behavior of different wild strains of *Ceratitis capitata* (Diptera: Tephritidae). Florida Entomol. 90:15–18.

Butlin, R., A. Debelle, C. Kerth, R. R. Snook, L. W. Beukeboom, R. F. Castillo Cajas, W. Diao, M. E. Maan, S. Paolucci, F. J. Weissing, L. van de Zande, A. Hoikkala, E. Geuverink, J. Jennings, M. Kankare, K. E. Knott, V. I. Tyukmaeva, C. Zoumadakis, M. G. Ritchie, D. Barker, E. Immonen, M. Kirkpatrick, M. Noor, C. Macias Garcia, T. Schmitt, and M. Schilthuizen. 2012. What do we need to know about speciation? Trends Ecol. Evol. 27:27–39.

Carey, J. R. 1984. Host-specific demographic studies of the Mediterranean fruit fly *Ceratitis capitata*. Ecol. Entomol. 9:261–270.

Carvajal-Rodriguez, A., and E. Rolan-Alvarez. 2006. JMATING: a software for the analysis of sexual selection and sexual isolation effects from mating frequency data. BMC Evol. Biol. 6.

Casares, P., M. C. Carracedo, B. Del Rio, R. Pieiro, L. Garca-Florez, and a R. Barros. 1998. Disentangling the effects of mating propensity and mating choice in *Drosophila*. Evolution. 52:126–133.

Cayol, J. P., P. Coronado, M. Taher, and Cayol. 2002. Sexual compatibility in medfly (Diptera : Tephritidae) from different origins. Florida Entomol. 85:51–57.

Costa, A. M., C. S. Anjos-Duarte, A. K. P. Roriz, V. S. Dias, and I. S. Joachim-Bravo. 2012. Male diet and age influence to inhibit female remating in *Ceratitis capitata* (Diptera: Tephritidae). J. Appl. Entomol. 136:456–463.

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

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Dambroski, H. R., and J. L. Feder. 2007. Host plant and latitude-related diapause variation in *Rhagoletis pomonella*: A test for multifaceted life history adaptation on different stages of diapause development. J. Evol. Biol. 20:2101–2112.

Delcourt, M., and H. D. H. Rundle. 2011. Condition dependence of a multicomponent sexual display trait in *Drosophila serrata*. Am. Nat. 177:812–823.

Dennis, R. L. H., L. Dapporto, S. Fattorini, and L. M. Cook. 2011. The generalism-specialism debate: the role of generalists in the life and death of species. Biol. J. Linn. Soc. 104:725–737.

Dewey, M. 2016. metap: meta-analysis of significance values.

Diamantidis, A. D., J. R. Carey, C. T. Nakas, and N. T. Papadopoulos. 2011a. Ancestral populations perform better in a novel environment: Domestication of Mediterranean fruit fly populations from five global regions. Biol. J. Linn. Soc. 102:334–345.

Diamantidis, A. D., J. R. Carey, C. T. Nakas, and N. T. Papadopoulos. 2011b. Population-specific demography and invasion potential in medfly. Ecol. Evol. 1:479–488.

Diamantidis, A. D., J. R. Carey, and N. T. Papadopoulos. 2008a. Life-history evolution of an invasive tephritid. J. Appl. Entomol. 132:695–705.

Diamantidis, A. D., N. T. Papadopoulos, and J. R. Carey. 2008b. Medfly populations differ in diel and age patterns of sexual signalling. Entomol. Exp. Appl. 128:389–397.

Diamantidis, A. D., N. T. Papadopoulos, C. T. Nakas, S. Wu, H. G. MÜller, and J. R. Carey. 2009. Life history evolution in a globally invading tephritid: Patterns of survival and reproduction in medflies from six world regions. Biol. J. Linn. Soc. 97:106–117.

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

Eberhard, W. 1999. Sexual behavior and sexual selection in the medfly, *Ceratitis capitata*. *in* M. Aluja and A. Norrbom, eds. Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior. CRC Press, Delray Beach, Florida.

Edgar, B. A. 2006. How flies get their size: genetics meets physiology. Nat. Rev. Genet. 7:907–916.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. U. S. A. 91:7990–4.

Ferrari, J., J. A. West, S. Via, and H. C. J. Godfray. 2012. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. Evolution. 66:375–90.

Field, S. A., R. Kaspi, and B. Yuval. 2002. Why do calling medflies (Diptera: Tephritidae) cluster? Assessing the Empirical Evidence for models of medfly lek evolution. Florida Entomol. 85:63–72.

Forister, M. L., L. A. Dyer, M. S. Singer, J. O. Stireman III, J. T. Lill, and Singer, M S. 2012. Revisiting the evolution of ecological specialization, with emphasis on insect-plant interactions. Ecology 93:981–991.

Funk, D. J., and P. Nosil. 2008. Comparative analyses of ecological speciation. Pp. 117–135 *in* K. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Oakland, CA.

Gasperi, G., M. Bonizzoni, L. M. Gomulski, V. Murelli, C. Torti, A. R. Malacrida, and C. R. Guglielmino. 2002. Genetic differentiation, gene flow and the origin of infestations of the medfly, Ceratitis capitata. Genetica 116:125–135.

Greig, D. 2009. Reproductive isolation in *Saccharomyces*. Heredity. 102:39–44.

Hardy, N. B., and S. P. Otto. 2014. Specialization and generalization in the diversification of phytophagous insects: tests of the musical chairs and oscillation hypotheses. Proc. R. Soc. B 281:20132960.

Havens, J. A., and W. J. Etges. 2013. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. IX. Host plant and population specific epicuticular hydrocarbon expression influences mate choice and sexual selection. J. Evol. Biol. 26:562–576.

Hill, G. E. 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. Ecol. Lett. 14:625–34.

Hoikkala, A., and K. Kaneshiro. 1993. Change in the signal-response sequence responsible for asymmetric isolation between *Drosophila planitibia* and *Drosophila silvestris*. Proc. Natl. Acad. Sci. 90:5813–5817.

Janz, N., and S. Nylin. 2008. The Oscillation hypothesis of host-plant range and speciation. Pp. 203 – 215 *in* K. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Oakland, CA.

Joachim-Bravo, I., C. Anjos, and A. Costa. 2009. The role of protein in the sexual behaviour of males of *Ceratitis capitata* (Diptera: Tephritidae): mating success, copula duration and number of copulations. Zool. 26:407–412.

Karsten, M., B. Jansen van Vuuren, P. Addison, and J. S. Terblanche. 2015. Deconstructing intercontinental invasion pathway hypotheses of the Mediterranean fruit fly (*Ceratitis capitata*) using a Bayesian inference approach: are port interceptions and quarantine protocols successfully preventing new invasions? Divers. Distrib. 1–13.

Kaspi, R., S. Mossinson, T. Drezner, B. Kamensky, and B. Yuval. 2002. Effects of larval diet on development rates and reproductive maturation of male and female

Mediterranean fruit flies. Physiol. Entomol. 27:29–38.

Kaspi, R., and B. Yuval. 2000. Post-teneral protein feeding improves sexual competitiveness but reduces longevity of mass-reared sterile male Mediterranean fruit flies (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 93:949–955.

Kawecki, T. J., R. E. Lenski, D. Ebert, B. Hollis, I. Olivieri, and M. C. Whitlock. 2012. Experimental evolution. Trends Ecol. Evol. 27:547–60.

Krainacker, D. A., J. R. Carey, and R. I. Vargas. 1987. Effect of larval host on life history traits of the Mediterranean fruit fly, *Ceratitis capitata*. Oecologia 73:583–590.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proc. Natl. Acad. Sci. 78:3721–3725.

Levinson, H. Z., A. R. Levinson, and K. Müller. 1990. Influence of some olfactory and optical properties of fruits on host location by the Mediterranean fruit fly (*Ceratitis capitata*, Wied.). J. Appl. Entomol. 109:44–54.

Linn, C., J. L. Feder, S. Nojima, H. R. Dambroski, S. H. Berlocher, and W. Roelofs. 2003. Fruit odor discrimination and sympatric host race formation in *Rhagoletis*. Proc. Natl. Acad. Sci. U. S. A. 100:11490–11493.

Liquido, N. J., L. A. Shinoda, and R. T. Cunningham. 1991. Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): an annotated world review. Entomological Society of America, Lanham.

Loxdale, H. D., G. Lushai, and J. A. Harvey. 2011. The evolutionary improbability of "generalism" in nature, with special reference to insects. Biol. J. Linn. Soc. 103:1–18.

Lux, S. A., F. N. Munyiri, J. C. Vilardi, P. Liedo, A. Economopoulos, O. Hasson, and S. Quilici. 2002. Consistency in courtship pattern among populations of Medfly (Diptera: Tephritidae): comparisons among wild strains and strains mass reared for SIT Operations. Florida Entomol. 85:113.

Maan, M. E., and O. Seehausen. 2011. Ecology, sexual selection and speciation. Ecol. Lett. 14:591–602.

Magnacca, K. N., and D. K. Price. 2015. Rapid adaptive radiation and host plant conservation in the Hawaiian picture wing *Drosophila* (Diptera: Drosophilidae). Mol. Phylogenet. Evol. 92:226–42.

Mair, W., M. D. W. Piper, and L. Partridge. 2005. Calories do not explain extension of life span by dietary restriction in *Drosophila*. PLoS Biol. 3:e223.

Malacrida, A. R., L. M. Gomulski, M. Bonizzoni, S. Bertin, G. Gasperi, and C. R.

Guglielmino. 2007. Globalization and fruitfly invasion and expansion: The medfly paradigm. Genetica 131:1–9.

Malacrida, A. R., F. Marinoni, C. Torti, L. M. Gomulski, F. Sebastiani, C. Bonvicini, G. Gasperi, and C. R. Guglielmino. 1998. Genetic aspects of the worldwide colonization process of *Ceratitis capitata*. J. Hered. 89:501–7.

Morrison, N. I., D. F. Segura, K. C. Stainton, G. Fu, C. A. Donnelly, and L. S. Alphey. 2009. Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*. Entomol. Exp. Appl. 133:146–153.

Nash, W. J., and T. Chapman. 2014. Effect of dietary components on larval life history characteristics in the medfly (*Ceratitis capitata*: Diptera, Tephritidae). PLoS One 9:e86029.

Nijhout, H. F. 2003. The control of body size in insects. Dev. Biol. 261:1–9.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking sticks. Am. Nat. 169:151–62.

Nosil, P. 2012. Ecological Speciation. Oxford University Press, Oxford.

Nosil, P. 2002. Transition rates between specialization and generalization in phytophagous insects. Evolution. 56:1701–1706.

Nosil, P., Z. Gompert, T. E. Farkas, A. A. Comeault, J. L. Feder, C. A. Buerkle, and T. L. Parchman. 2012. Genomic consequences of multiple speciation processes in a stick insect. Proc. R. Soc. B 279:5058–65.

Nosil, P., L. J. Harmon, and O. Seehausen. 2009. Ecological explanations for (incomplete) speciation. Trends Ecol. Evol. 24:145–156.

Nosil, P., and A. Ø. Mooers. 2005. Testing hypotheses about ecological specialization using phylogenetic trees. Evolution. 59:2256–2263.

Nyamukondiwa, C., E. Kleynhans, and J. S. Terblanche. 2010. Phenotypic plasticity of thermal tolerance contributes to the invasion potential of Mediterranean fruit flies (*Ceratitis capitata*). Ecol. Entomol. 35:565–575.

Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. Trends Ecol. Evol. 16:364–371.

Peccoud, J., F. Mahéo, M. de la Huerta, C. Laurence, and J.-C. Simon. 2015. Genetic characterisation of new host-specialised biotypes and novel associations with bacterial symbionts in the pea aphid complex. Insect Conserv. Divers. 8:484–492.

Peccoud, J., A. Ollivier, M. Plantegenest, and J.-C. Simon. 2009. A continuum of

genetic divergence from sympatric host races to species in the pea aphid complex. Proc. Natl. Acad. Sci. U. S. A. 106:7495–7500.

Peccoud, J., and J.-C. Simon. 2010. The pea aphid complex as a model of ecological speciation. Ecol. Entomol. 35:119–130.

Powell, G., C. R. Tosh, and J. Hardie. 2006. Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. Annu. Rev. Entomol. 51:309–30.

Powell, T. H. Q., A. A. Forbes, G. R. Hood, and J. L. Feder. 2014. Ecological adaptation and reproductive isolation in sympatry: genetic and phenotypic evidence for native host races of Rhagoletis pomonella. Mol. Ecol. 23:688–704.

Prokopy, R. R. J., P. T. McDonald, and T. T. Y. Wong. 1984. Inter-population variation among *Ceratitis capitata* flies in host acceptance pattern. Entomol. Exp. Appl. 35:65–69.

R Development Core Team, R. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

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

Rolán-Alvarez, E. 2004. Evolution of asymmetry in sexual isolation: A criticism of a test case. Evol. Ecol. Res. 6:1099–1106.

Rolan-Alvarez, E., and A. Caballero. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution. 54:30–36.

Rowe, L., and D. Houle. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc. R. Soc. B 263:1415–1421.

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

Rundle, H. D., S. F. Chenoweth, P. Doughty, and M. W. Blows. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLoS Biol. 3:e368.

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336–352.

Safran, R. J., E. S. C. Scordato, L. B. Symes, R. L. Rodríguez, and T. C. Mendelson. 2013. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: A research agenda. Trends Ecol. Evol. 28:643–650.

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

Sharon, G., D. Segal, J. M. Ringo, A. Hefetz, I. Zilber-Rosenberg, and E. Rosenberg. 2010. Commensal bacteria play a role in mating preference of *Drosophila*

melanogaster. Proc. Natl. Acad. Sci. 107:20051–20056.

Shelly, T. E., S. S. Kennelly, and D. O. McInnis. 2002. Effect of adult diet on signaling activity, mate attraction, and mating success in male Mediterranean fruit flies (Diptera : Tephritidae). Florida Entomol. 85:150–155.

Soria-Carrasco, V., Z. Gompert, A. A. Comeault, T. E. Farkas, T. L. Parchman, J. S. Johnston, C. A. Buerkle, J. L. Feder, J. Bast, T. Schwander, S. P. Egan, B. J. Crespi, and P. Nosil. 2014. Stick insect genomes reveal natural selection's role in parallel speciation. Science 344:738–42.

Southgate, D. A., and J. V Durnin. 1970. Calorie conversion factors. An experimental reassessment of the factors used in the calculation of the energy value of human diets. Br. J. Nutr. 24:517–35.

Turelli, M., N. H. Barton, and J. A. Coyne. 2001. Theory and speciation. Trends Ecol. Evol. 16:330–343.

van Doorn, G. S., P. Edelaar, and F. J. Weissing. 2009. On the origin of species by natural and sexual selection. Science 326:1704–7.

Via, S., A. C. Bouck, and S. Skillman. 2000. Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution. 54:1626–1637.

Whitlock, M. C., P. C. Phillips, and K. Fowler. 2002. Persistence of changes in the genetic covariance matrix after a bottleneck. Evolution. 56:1968–1975.

Whittier, T. S., F. Y. Nam, T. E. Shelly, and K. Y. Kaneshiro. 1994. Male courtship success and female discrimination in the Mediterranean fruit fly (Diptera: Tephritidae). J. Insect Behav. 7:159–170.

Wilson, J. R. U., E. E. Dormontt, P. J. Prentis, A. J. Lowe, and D. M. Richardson. 2009. Something in the way you move: dispersal pathways affect invasion success. Trends Ecol. Evol. 24:136–144.

Yuval, B., and J. Hendrichs. 2000. Behavior of flies in the genus Ceratitis (Dacinae: Ceratitidine). Pp. 429–457 *in* M. Aluja and A. L. Norrbom, eds. Fruit flies (Tephritidae): phylogeny and evolution of behavior. CRC Press LLC, Boca Raton, FL.

Zucoloto, F. S. 1993. Adaptation of a *Ceratitis capitata* population Diptera, Tephritidae to an animal protein-based diet. Entomol. Exp. Appl. 67:119–127.

3.7 Appendices

3.7.1 Appendix 3.1 - Supplementary Tables

Table 3.A1. Sample size (n) and total recorded matings, also broken down by mating pair type (Female/Male: AA, AB, BA, BA), at each generational time point. Raw frequencies shown. Treatments represent: ASG/ASG - Individuals from the ASG regime, tested on the ASG larval diet, ASG/S - Individuals from the ASG regime, tested after two generations of rearing on the Starch larval diet, S/S - Individuals from the Starch regime, tested on the Starch larval diet, S/ASG - Individuals from the Starch regime, tested after two generations of rearing on the ASG larval diet, ASG/G - Individuals from the ASG regime, tested after two generations of rearing on the common garden (Glucose) larval diet, S/G - Individuals from the Starch regime, tested after two generations of rearing on the common garden (Glucose) larval diet.

Generation	Diet A	Diet B	n	Matings	AA	AB	BA	BB
3	ASG/ASG 1	S/S 1	60	58	33	2	23	0
	ASG/ASG 2	S/S 2	60	60	28	1	31	0
	ASG/ASG 3	S/S 3	60	59	32	1	24	2
5	ASG/S 1	ASG/ASG 1	20	18	0	7	0	11
	ASG/S 2	ASG/ASG 2	20	17	0	7	0	10
	ASG/S 3	ASG/ASG 3	20	17	0	8	1	8
	ASG/S 1	S/S 1	20	14	4	4	3	3
	ASG/S 2	S/S 2	20	10	3	3	1	3
	ASG/S 3	S/S 3	20	10	4	3	0	3
	S/ASG 1	ASG/ASG 1	20	20	5	5	3	7
	S/ASG 2	ASG/ASG 2	20	19	5	4	3	7
	S/ASG 3	ASG/ASG 3	20	19	5	3	4	7
	S/ASG 1	S/S 1	20	20	10	0	10	0
	S/ASG 2	S/S 2	20	20	10	0	9	1
	S/ASG 3	S/S 3	20	19	5	3	4	7
30	ASG/ASG 1	S/S 1	70	67	26	2	36	3
	ASG/ASG 2	S/S 2	70	69	26	2	39	2
	ASG/ASG 3	S/S 3	70	63	31	6	23	3
	ASG/S 1	ASG/ASG 1	15	15	0	4	0	11
	ASG/S 2	ASG/ASG 2	15	15	4	4	1	6
	ASG/S 3	ASG/ASG 3	15	11	0	6	0	5
	ASG/S 1	S/S 1	15	12	0	3	2	7
	ASG/S 2	S/S 2	15	11	6	1	2	2
	ASG/S 3	S/S 3	15	11	0	2	4	5
	S/ASG 1	ASG/ASG 1	15	15	5	3	2	5
	S/ASG 2	ASG/ASG 2	15	14	6	1	3	4
	S/ASG 3	ASG/ASG 3	15	15	3	9	3	0
	S/ASG 1	S/S 1	15	14	6	3	2	3
	S/ASG 2	S/S 2	15	13	8	1	4	0
	S/ASG 3	S/S 3	15	15	3	9	3	0
60	ASG/ASG 1	S/S 1	100	100	37	12	26	25
	ASG/ASG 2	S/S 2	100	100	31	11	27	31
	ASG/ASG 3	S/S 3	100	100	34	13	27	26
	ASG/G 1	S/G 1	100	92	26	25	13	28
	ASG/G 2	S/G 2	100	69	22	14	11	22

	ASG/G 3	S/G 3	100	100	13	26	13	51

Pair sexual isolation coefficient (PSI), pair sexual selection coefficient (PSS), and pair total isolation coefficient (PTI), calculated after Rolan-Alvarez and Caballero (2000), for each line pairing, and possible mating pair type. Coefficient values represent mean coefficient values generated after 10,000 bootstrap resamples from the observed (for PTI), or observed and expected frequencies (for PSI and PSS). Stars represent a significant bootstrap probability of rejecting the null hypothesis, that the coefficient is not different from 1 (no preference, or random mating). Lowercase letters represent diet (ASG = a or Starch = s) and replicate information (1-3). Upper case letters represent 'pair types' (homotypic = AA, BB; heterotypic = AB, BA).

Table 3.A2. Quartet style mating tests with all flies reared on their own larval diet, tested at generation 3.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
a1/s1	AA	PSI	0.9972	0.1645	0.9958	
a1/s1	AB	PSI	1.3492	1.1954	0.5782	
a1/s1	BA	PSI	1.0551	0.2593	0.8438	
a1/s1	BB	PSI	0.3803	0.5852	0.946	
a2/s2	AA	PSI	1.0128	0.2042	0.9528	
a2/s2	AB	PSI	0.8552	0.9091	0.508	
a2/s2	BA	PSI	1.0234	0.1873	0.8998	
a2/s2	BB	PSI	0.4032	0.6098	0.7928	
a3/s3	AA	PSI	1.02	0.1774	0.8948	
a3/s3	AB	PSI	0.6293	0.7605	0.9112	
a3/s3	BA	PSI	0.98	0.2263	0.9094	
a3/s3	BB	PSI	1.4053	1.2134	0.5016	
a1/s1	AA	PSS	2.3127	0.2633	0	***
a1/s1	AB	PSS	0.1038	0.084	0	***
a1/s1	BA	PSS	1.5485	0.2587	0.0266	*
a1/s1	BB	PSS	0.0691	0.068	0	***
a2/s2	AA	PSS	1.8825	0.2572	8.00E-04	***
a2/s2	AB	PSS	0.0481	0.0556	0	***
a2/s2	BA	PSS	2.0502	0.2579	0	***
a2/s2	BB	PSS	0.0523	0.0579	0	***
a3/s3	AA	PSS	2.1624	0.2619	0	***
a3/s3	AB	PSS	0.116	0.0872	0	***
a3/s3	BA	PSS	1.699	0.2613	0.004	**
a3/s3	BB	PSS	0.0903	0.0775	0	***
a1/s1	AA	PTI	2.2757	0.2617	0	***
a1/s1	AB	PTI	0.1383	0.0957	0	***
a1/s1	BA	PTI	1.5858	0.2578	0.0172	*
a1/s1	BB	PTI	0.0343	0.0484	0	***
a2/s2	AA	PTI	1.8694	0.2574	8.00E-04	***
a2/s2	AB	PTI	0.0672	0.0664	0	***
a2/s2	BA	PTI	2.0638	0.2582	2.00E-04	***
a2/s2	BB	PTI	0.0327	0.0457	0	***
a3/s3	AA	PTI	2.1727	0.2588	0	***
a3/s3	AB	PTI	0.0677	0.0673	0	***
a3/s3	BA	PTI	1.6238	0.2569	0.0106	*
a3/s3	BB	PTI	0.1359	0.0936	0	***

Table 3.A3. Quartet style mating tests with quartets composed of male and female ASG individuals tested on larval Starch diets versus male and female Starch individuals, at generation 5.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
as1/s1	AA	PSI	1.2461	0.984	0.8412	
as1/s1	AB	PSI	1.2479	0.9852	0.8342	
as1/s1	BA	PSI	1.2681	1.0718	0.8112	
as1/s1	BB	PSI	1.259	1.0729	0.8192	
as2/s2	AA	PSI	1.5345	1.1957	0.5778	
as2/s2	AB	PSI	1.0343	0.8577	0.9616	
as2/s2	BA	PSI	0.6468	0.7317	0.9558	
as2/s2	BB	PSI	1.5285	1.1884	0.5758	
as3/s3	AA	PSI	1.6769	1.2472	0.4698	
as3/s3	AB	PSI	0.9144	0.7249	0.8096	
as3/s3	BA	PSI	0.3375	0.5215	0.696	
as3/s3	BB	PSI	1.7326	1.2801	0.4268	
as1/s1	AA	PSS	1.141	0.4875	0.7982	
as1/s1	AB	PSS	1.1439	0.4855	0.8028	
as1/s1	BA	PSS	0.8561	0.4387	0.6974	
as1/s1	BB	PSS	0.859	0.4444	0.7096	
as2/s2	AA	PSS	0.9582	0.5356	0.8816	
as2/s2	AB	PSS	1.4452	0.6137	0.4784	
as2/s2	BA	PSS	0.637	0.4598	0.4094	
as2/s2	BB	PSS	0.9596	0.5461	0.8924	
as3/s3	AA	PSS	1.2011	0.5636	0.6898	
as3/s3	AB	PSS	1.5918	0.6137	0.2972	
as3/s3	BA	PSS	0.5983	0.4451	0.3986	
as3/s3	BB	PSS	0.7992	0.4922	0.6922	
as1/s1	AA	PTI	1.1397	0.4806	0.8088	
as1/s1	AB	PTI	1.1436	0.4857	0.8006	
as1/s1	BA	PTI	0.8582	0.4377	0.7144	
as1/s1	BB	PTI	0.8585	0.439	0.709	
as2/s2	AA	PTI	1.2008	0.5768	0.7598	
as2/s2	AB	PTI	1.1971	0.5741	0.7638	
as2/s2	BA	PTI	0.3974	0.3784	0.1366	
as2/s2	BB	PTI	1.2047	0.5764	0.7646	
as3/s3	AA	PTI	1.6093	0.6124	0.2852	
as3/s3	AB	PTI	1.1981	0.572	0.699	
as3/s3	BA	PTI	0.1968	0.2673	0.0236	*
as3/s3	BB	PTI	1.1863	0.5681	0.7196	

Table 3.A4. Quartet style mating tests with quartets composed of male and female ASG individuals, tested on larval Starch diet versus male and female ASG individuals, at generation 5.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
as1/a1	AA	PSI	0.4604	0.6537	0.4222	
as1/a1	AB	PSI	1.1033	0.6174	0.895	
as1/a1	BA	PSI	0.433	0.6445	0.6222	
as1/a1	BB	PSI	1.0553	0.3277	0.8466	
as2/a2	AA	PSI	0.4491	0.6472	0.4506	
as2/a2	AB	PSI	1.1003	0.6162	0.89	
as2/a2	BA	PSI	0.4406	0.6555	0.6062	
as2/a2	BB	PSI	1.067	0.3679	0.842	
as3/a3	AA	PSI	0.4326	0.6256	0.7436	
as3/a3	AB	PSI	1.122	0.5199	0.8004	
as3/a3	BA	PSI	0.824	0.8784	0.55	
as3/a3	BB	PSI	1.0502	0.4543	0.9338	
as1/a1	AA	PSS	0.0824	0.1308	0	***
as1/a1	AB	PSS	1.503	0.4467	0.2022	
as1/a1	BA	PSS	0.1249	0.1599	6.00E-04	***
as1/a1	BB	PSS	2.2897	0.456	0.0032	**
as2/a2	AA	PSS	0.0911	0.1401	4.00E-04	***
as2/a2	AB	PSS	1.5758	0.4641	0.211	
as2/a2	BA	PSS	0.1279	0.166	0	***
as2/a2	BB	PSS	2.2052	0.4745	0.0122	*
as3/a3	AA	PSS	0.1718	0.1932	6.00E-04	***
as3/a3	AB	PSS	1.8237	0.4825	0.094	
as3/a3	BA	PSS	0.1813	0.2011	0.0018	**
as3/a3	BB	PSS	1.9374	0.4854	0.0566	
as1/a1	AA	PTI	0.1072	0.1489	2.00E-04	***
as1/a1	AB	PTI	1.4791	0.4392	0.2214	
as1/a1	BA	PTI	0.1041	0.1456	0	***
as1/a1	BB	PTI	2.3095	0.4522	0.002	**
as2/a2	AA	PTI	0.112	0.154	0	***
as2/a2	AB	PTI	1.5498	0.4599	0.2228	
as2/a2	BA	PTI	0.1111	0.1564	2.00E-04	***
as2/a2	BB	PTI	2.227	0.4689	0.0094	**
as3/a3	AA	PTI	0.1187	0.1617	4.00E-04	***
as3/a3	AB	PTI	1.8771	0.4841	0.0718	
as3/a3	BA	PTI	0.2348	0.2241	0.0054	**
as3/a3	BB	PTI	1.8836	0.4802	0.0694	

Table 3.A5. Quartet style mating tests with quartets composed of male and female Starch individuals tested on larval ASG diet versus male and female Starch individuals, tested at generation 5.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
sa1/s1	AA	PSI	1.0624	0.3847	0.8762	
sa1/s1	AB	PSI	0.4262	0.6408	0.5388	
sa1/s1	BA	PSI	1.0618	0.3882	0.8668	
sa1/s1	BB	PSI	0.4249	0.6391	0.5514	
sa2/s2	AA	PSI	1.092	0.4014	0.8058	
sa2/s2	AB	PSI	0.4181	0.6213	0.7858	
sa2/s2	BA	PSI	1.0393	0.4106	0.9488	
sa2/s2	BB	PSI	0.8454	0.8897	0.5224	
sa3/s3	AA	PSI	1.0224	0.5465	0.97	
sa3/s3	AB	PSI	0.8013	0.8677	0.6056	
sa3/s3	BA	PSI	1.0219	0.3579	0.9644	
sa3/s3	BB	PSI	0.7217	0.816	0.8236	
sa1/s1	AA	PSS	1.9066	0.4303	0.0422	*
sa1/s1	AB	PSS	0.0937	0.1327	0	***
sa1/s1	BA	PSS	1.9033	0.4296	0.0434	*
sa1/s1	BB	PSS	0.0964	0.1341	2.00E-04	***
sa2/s2	AA	PSS	1.9471	0.4451	0.0464	*
sa2/s2	AB	PSS	0.1545	0.1701	6.00E-04	***
sa2/s2	BA	PSS	1.8487	0.4424	0.073	
sa2/s2	BB	PSS	0.1473	0.1649	4.00E-04	***
sa3/s3	AA	PSS	1.5902	0.4521	0.1444	
sa3/s3	AB	PSS	0.1832	0.1919	0.002	**
sa3/s3	BA	PSS	2.1807	0.4701	0.0062	**
sa3/s3	BB	PSS	0.2565	0.2251	0.0122	*
sa1/s1	AA	PTI	1.9092	0.4391	0.0442	*
sa1/s1	AB	PTI	0.0929	0.1322	0	***
sa1/s1	BA	PTI	1.9049	0.4371	0.0478	*
sa1/s1	BB	PTI	0.0931	0.1307	0	***
sa2/s2	AA	PTI	1.9996	0.4458	0.0322	*
sa2/s2	AB	PTI	0.0996	0.1375	0	***
sa2/s2	BA	PTI	1.7962	0.4409	0.0902	
sa2/s2	BB	PTI	0.2022	0.1925	8.00E-04	***
sa3/s3	AA	PTI	1.4669	0.4437	0.2362	
sa3/s3	AB	PTI	0.2111	0.2053	0.0054	**
sa3/s3	BA	PTI	2.111	0.4601	0.01	*
sa3/s3	BB	PTI	0.211	0.2066	0.0074	**

Table 3.A6. Quartet style mating tests with quartets composed of male and female Starch individuals tested on larval ASG diet versus male and female ASG individuals, tested at generation 5.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
saa1/a1	AA	PSI	1.5763	1.255	0.5854	
saa1/a1	AB	PSI	0.9726	0.672	0.8606	
saa1/a1	BA	PSI	0.9521	0.8648	0.8352	
saa1/a1	BB	PSI	1.373	0.8689	0.6096	
saa2/a2	AA	PSI	1.6909	1.3254	0.5086	
saa2/a2	AB	PSI	0.9275	0.7353	0.7796	
saa2/a2	BA	PSI	0.8912	0.7976	0.7526	
saa2/a2	BB	PSI	1.4168	0.9009	0.5458	
saa3/a3	AA	PSI	1.6778	1.3003	0.4994	
saa3/a3	AB	PSI	0.8903	0.8044	0.7556	
saa3/a3	BA	PSI	0.923	0.7207	0.7888	
saa3/a3	BB	PSI	1.4235	0.9173	0.5612	
saa1/a1	AA	PSS	0.8074	0.3616	0.763	
saa1/a1	AB	PSS	1.1948	0.4067	0.483	
saa1/a1	BA	PSS	0.8031	0.358	0.7434	
saa1/a1	BB	PSS	1.1947	0.4085	0.484	
saa2/a2	AA	PSS	0.7934	0.3694	0.6474	
saa2/a2	AB	PSS	1.0927	0.4092	0.754	
saa2/a2	BA	PSS	0.8854	0.3797	0.835	
saa2/a2	BB	PSS	1.2285	0.4244	0.5224	
saa3/a3	AA	PSS	0.7977	0.3658	0.646	
saa3/a3	AB	PSS	0.8896	0.3819	0.8442	
saa3/a3	BA	PSS	1.0985	0.4029	0.7432	
saa3/a3	BB	PSS	1.2142	0.4234	0.5412	
saa1/a1	AA	PTI	1	0.3923	0.848	
saa1/a1	AB	PTI	0.9956	0.3894	0.8484	
saa1/a1	BA	PTI	0.6006	0.32	0.3418	
saa1/a1	BB	PTI	1.4038	0.4256	0.225	
saa2/a2	AA	PTI	1.0475	0.4041	0.8466	
saa2/a2	AB	PTI	0.8393	0.3746	0.7328	
saa2/a2	BA	PTI	0.6264	0.3296	0.3314	
saa2/a2	BB	PTI	1.4869	0.443	0.221	
saa3/a3	AA	PTI	1.0515	0.4062	0.8384	
saa3/a3	AB	PTI	0.6301	0.335	0.3332	
saa3/a3	BA	PTI	0.8469	0.377	0.7426	
saa3/a3	BB	PTI	1.4716	0.4454	0.2346	

Table 3.A7. Quartet style mating tests with all flies reared on their own larval diet, tested at generation 30.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
a1/s1	AA	PSI	1.0271	0.2365	0.9308	
a1/s1	AB	PSI	1.121	1.073	0.8364	
a1/s1	BA	PSI	1.0131	0.1682	0.9244	
a1/s1	BB	PSI	1.3148	1.1975	0.8018	
a2/s2	AA	PSI	1.0128	0.2285	0.982	
a2/s2	AB	PSI	1.2656	1.138	0.6432	
a2/s2	BA	PSI	1.021	0.1568	0.8796	
a2/s2	BB	PSI	1.0408	1.0268	0.948	
a3/s3	AA	PSI	0.9998	0.1811	0.9994	
a3/s3	AB	PSI	1.3635	1.0706	0.7608	
a3/s3	BA	PSI	1.0613	0.2862	0.8446	
a3/s3	BB	PSI	1.1026	1.023	0.996	
a1/s1	AA	PSS	1.5447	0.2366	0.0172	*
a1/s1	AB	PSS	0.1252	0.0846	0	***
a1/s1	BA	PSS	2.1555	0.2443	0	***
a1/s1	BB	PSS	0.1746	0.1007	0	***
a2/s2	AA	PSS	1.5253	0.2329	0.027	*
a2/s2	AB	PSS	0.0949	0.0727	0	***
a2/s2	BA	PSS	2.2409	0.2404	0	***
a2/s2	BB	PSS	0.1389	0.0888	0	***
a3/s3	AA	PSS	2.0684	0.2523	0	***
a3/s3	AB	PSS	0.3583	0.1466	4.00E-04	***
a3/s3	BA	PSS	1.3419	0.2395	0.1708	
a3/s3	BB	PSS	0.2314	0.1195	0	***
a1/s1	AA	PTI	1.5471	0.2408	0.0166	*
a1/s1	AB	PTI	0.1196	0.0835	0	***
a1/s1	BA	PTI	2.1547	0.2482	0	***
a1/s1	BB	PTI	0.1786	0.1008	0	***
a2/s2	AA	PTI	1.5078	0.2346	0.0312	*
a2/s2	AB	PTI	0.1147	0.0794	0	***
a2/s2	BA	PTI	2.2611	0.2396	0	***
a2/s2	BB	PTI	0.1164	0.0808	0	***
a3/s3	AA	PTI	2.0357	0.2536	0	***
a3/s3	AB	PTI	0.392	0.1534	2.00E-04	***
a3/s3	BA	PTI	1.3754	0.2434	0.138	
a3/s3	BB	PTI	0.197	0.1114	0	***

Table 3.A8. Quartet style mating tests with quartets composed of male and female ASG individuals tested on larval Starch diets versus male and female Starch individuals, at generation 30.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
as1/s1	AA	PSI	0.4275	0.6195	0.73	
as1/s1	AB	PSI	1.3484	1.1022	0.7288	
as1/s1	BA	PSI	1.2477	1.0748	0.682	
as1/s1	BB	PSI	1.0369	0.4153	0.9168	
as2/s2	AA	PSI	1.347	0.7873	0.5454	
as2/s2	AB	PSI	0.594	0.704	0.798	
as2/s2	BA	PSI	0.8649	0.811	0.838	
as2/s2	BB	PSI	1.5233	1.1948	0.3708	
as3/s3	AA	PSI	0.4125	0.6101	0.9406	
as3/s3	AB	PSI	1.3359	1.1163	0.5838	
as3/s3	BA	PSI	1.4129	1.0748	0.6728	
as3/s3	BB	PSI	1.0375	0.6206	0.9996	
as1/s1	AA	PSS	0.2342	0.2666	0.0084	**
as1/s1	AB	PSS	0.9268	0.4786	0.6488	
as1/s1	BA	PSS	0.5936	0.396	0.1964	
as1/s1	BB	PSS	2.4054	0.5643	0.0208	*
as2/s2	AA	PSS	1.8509	0.5942	0.1042	
as2/s2	AB	PSS	0.7012	0.4632	0.5974	
as2/s2	BA	PSS	1.0559	0.5322	0.8322	
as2/s2	BB	PSS	0.392	0.3596	0.1702	
as3/s3	AA	PSS	0.3508	0.337	0.149	
as3/s3	AB	PSS	0.5565	0.4075	0.4188	
as3/s3	BA	PSS	1.2779	0.5532	0.4752	
as3/s3	BB	PSS	1.9887	0.5999	0.0602	
as1/s1	AA	PTI	0.1662	0.2244	0.0018	**
as1/s1	AB	PTI	0.9978	0.4914	0.7688	
as1/s1	BA	PTI	0.6591	0.422	0.2736	
as1/s1	BB	PTI	2.3369	0.5731	0.0314	*
as2/s2	AA	PTI	2.1731	0.5955	0.031	*
as2/s2	AB	PTI	0.366	0.3496	0.144	
as2/s2	BA	PTI	0.7279	0.4659	0.647	
as2/s2	BB	PTI	0.733	0.4675	0.6532	
as3/s3	AA	PTI	0.184	0.2459	0.0254	*
as3/s3	AB	PTI	0.7174	0.4571	0.6806	
as3/s3	BA	PTI	1.449	0.5778	0.3228	
as3/s3	BB	PTI	1.8235	0.6026	0.1078	

Table 3.A9. Quartet style mating tests with quartets composed of male and female ASG individuals, tested on larval Starch diet versus male and female ASG individuals, at generation 30.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
as1/a1	AA	PSI	0.4515	0.6588	0.3164	
as1/a1	AB	PSI	1.171	0.9405	0.9176	
as1/a1	BA	PSI	0.4107	0.6124	0.7308	
as1/a1	BB	PSI	1.0543	0.2753	0.7806	
as2/a2	AA	PSI	1.8822	1.4394	0.4134	
as2/a2	AB	PSI	0.8877	0.647	0.743	
as2/a2	BA	PSI	0.5243	0.6518	0.6306	
as2/a2	BB	PSI	1.5721	1.1285	0.4912	
as3/a3	AA	PSI	0.4488	0.6356	0.5598	
as3/a3	AB	PSI	1.1282	0.6188	0.8264	
as3/a3	BA	PSI	0.4345	0.6428	0.5052	
as3/a3	BB	PSI	1.1527	0.742	0.8594	
as1/a1	AA	PSS	0.0686	0.1293	0	***
as1/a1	AB	PSS	1.053	0.4378	0.707	
as1/a1	BA	PSS	0.18	0.2075	0.0106	*
as1/a1	BB	PSS	2.6984	0.4671	2.00E-04	***
as2/a2	AA	PSS	0.7103	0.3992	0.5354	
as2/a2	AB	PSS	1.4204	0.5016	0.329	
as2/a2	BA	PSS	0.6281	0.3808	0.407	
as2/a2	BB	PSS	1.2412	0.4798	0.5406	
as3/a3	AA	PSS	0.1776	0.2373	0.0276	*
as3/a3	AB	PSS	1.9847	0.5781	0.0444	*
as3/a3	BA	PSS	0.1536	0.2227	0.0214	*
as3/a3	BB	PSS	1.684	0.568	0.128	
as1/a1	AA	PTI	0.1268	0.1757	0.002	**
as1/a1	AB	PTI	0.9962	0.4328	0.8158	
as1/a1	BA	PTI	0.1246	0.1748	0.0032	**
as1/a1	BB	PTI	2.7524	0.4662	0	***
as2/a2	AA	PTI	1.066	0.4544	0.8112	
as2/a2	AB	PTI	1.0693	0.4496	0.808	
as2/a2	BA	PTI	0.269	0.2606	0.034	*
as2/a2	BB	PTI	1.5957	0.5044	0.1754	
as3/a3	AA	PTI	0.1652	0.2313	0.0248	*
as3/a3	AB	PTI	2.0002	0.5866	0.0372	*
as3/a3	BA	PTI	0.1673	0.2306	0.0238	*
as3/a3	BB	PTI	1.6673	0.5758	0.1382	

Table 3.A10. Quartet style mating tests with quartets composed of male and female Starch individuals tested on larval ASG diet versus male and female Starch individuals, tested at generation 30.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
sa1/s1	AA	PSI	1.37	0.8552	0.5848	
sa1/s1	AB	PSI	0.9766	0.8329	0.885	
sa1/s1	BA	PSI	0.8848	0.8463	0.8534	
sa1/s1	BB	PSI	1.6439	1.3011	0.5068	
sa2/s2	AA	PSI	1.0616	0.4104	0.846	
sa2/s2	AB	PSI	0.8141	0.8669	0.6786	
sa2/s2	BA	PSI	1.2354	0.9909	0.8482	
sa2/s2	BB	PSI	0.4549	0.6692	0.5486	
sa3/s3	AA	PSI	1.1944	0.4599	0.6088	
sa3/s3	AB	PSI	0.8725	0.8421	0.8402	
sa3/s3	BA	PSI	0.8625	0.8375	0.8248	
sa3/s3	BB	PSI	1.5223	1.2094	0.3724	
sa1/s1	AA	PSS	1.4687	0.5089	0.3618	
sa1/s1	AB	PSS	1.0992	0.4767	0.8614	
sa1/s1	BA	PSS	0.8169	0.4293	0.6344	
sa1/s1	BB	PSS	0.6153	0.3884	0.3194	
sa2/s2	AA	PSS	2.4611	0.5481	0.0116	*
sa2/s2	AB	PSS	0.3043	0.2886	0.0314	*
sa2/s2	BA	PSS	1.2311	0.5099	0.7282	
sa2/s2	BB	PSS	0.1517	0.2077	0.002	**
sa3/s3	AA	PSS	2.1546	0.5192	0.0166	*
sa3/s3	AB	PSS	0.7774	0.4059	0.664	
sa3/s3	BA	PSS	0.7831	0.4068	0.6704	
sa3/s3	BB	PSS	0.285	0.2653	0.0388	*
sa1/s1	AA	PTI	1.7191	0.5269	0.171	
sa1/s1	AB	PTI	0.8576	0.4399	0.716	
sa1/s1	BA	PTI	0.5693	0.3726	0.2506	
sa1/s1	BB	PTI	0.8541	0.4416	0.704	
sa2/s2	AA	PTI	2.4562	0.5497	0.0088	**
sa2/s2	AB	PTI	0.3118	0.2919	0.032	*
sa2/s2	BA	PTI	1.2256	0.5138	0.7468	
sa2/s2	BB	PTI	0.1545	0.2134	0.0034	**
sa3/s3	AA	PTI	2.3981	0.506	0.0038	**
sa3/s3	AB	PTI	0.5357	0.3507	0.264	
sa3/s3	BA	PTI	0.5347	0.352	0.2608	
sa3/s3	BB	PTI	0.5314	0.3518	0.2592	

Table 3.A11. Quartet style mating tests with quartets composed of male and female Starch individuals tested on larval ASG diet versus male and female ASG individuals, tested at generation 30.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
sa1/a1	AA	PSI	1.6912	1.2756	0.4744	
sa1/a1	AB	PSI	0.8878	0.7806	0.7448	
sa1/a1	BA	PSI	0.7679	0.7676	0.6964	
sa1/a1	BB	PSI	1.6986	1.2738	0.4728	
sa2/a2	AA	PSI	1.6113	1.0973	0.4314	
sa2/a2	AB	PSI	0.5047	0.6336	0.5744	
sa2/a2	BA	PSI	0.8077	0.675	0.6708	
sa2/a2	BB	PSI	1.9636	1.4606	0.3688	
sa3/a3	AA	PSI	0.7785	0.6418	0.62	
sa3/a3	AB	PSI	1.3355	0.5743	0.4336	
sa3/a3	BA	PSI	2.1315	1.5023	0.2558	
sa3/a3	BB	PSI	0.2795	0.4567	0.4292	
sa1/a1	AA	PSS	0.9971	0.4503	0.936	
sa1/a1	AB	PSS	1.1306	0.4702	0.7018	
sa1/a1	BA	PSS	0.8833	0.4303	0.8606	
sa1/a1	BB	PSS	0.9891	0.4491	0.944	
sa2/a2	AA	PSS	1.2906	0.4981	0.5822	
sa2/a2	AB	PSS	0.7019	0.4059	0.4442	
sa2/a2	BA	PSS	1.2909	0.4993	0.5762	
sa2/a2	BB	PSS	0.7165	0.4105	0.467	
sa3/a3	AA	PSS	1.2348	0.4752	0.5048	
sa3/a3	AB	PSS	1.9613	0.5173	0.0358	*
sa3/a3	BA	PSS	0.3628	0.2916	0.092	
sa3/a3	BB	PSS	0.57	0.3495	0.3262	
sa1/a1	AA	PTI	1.3348	0.487	0.4184	
sa1/a1	AB	PTI	0.803	0.4163	0.7096	
sa1/a1	BA	PTI	0.5308	0.3546	0.2568	
sa1/a1	BB	PTI	1.3314	0.4907	0.4304	
sa2/a2	AA	PTI	1.7141	0.5322	0.1742	
sa2/a2	AB	PTI	0.2844	0.2751	0.03	*
sa2/a2	BA	PTI	0.858	0.4393	0.694	
sa2/a2	BB	PTI	1.1435	0.4847	0.7796	
sa3/a3	AA	PTI	0.7936	0.4035	0.74	
sa3/a3	AB	PTI	2.4018	0.5076	0.003	**
sa3/a3	BA	PTI	0.8011	0.414	0.7588	
sa3/a3	BB	PTI	0.1326	0.1822	0.0024	**

Table 3.A12. Multiple choice mating tests with all flies reared on their own larval diet, tested at generation 60.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
a1/s1	AA	PSI	1.2261	0.2544	0.3244	
a1/s1	AB	PSI	0.8302	0.1939	0.387	
a1/s1	BA	PSI	0.6934	0.2527	0.2542	
a1/s1	BB	PSI	1.3841	0.4039	0.2648	
a2/s2	AA	PSI	1.3132	0.3192	0.2592	
a2/s2	AB	PSI	0.8198	0.1827	0.3426	
a2/s2	BA	PSI	0.6556	0.2516	0.2144	
a2/s2	BB	PSI	1.3185	0.3268	0.2614	
a3/s3	AA	PSI	1.2168	0.2702	0.3746	
a3/s3	AB	PSI	0.8537	0.1932	0.449	
a3/s3	BA	PSI	0.7498	0.2705	0.3602	
a3/s3	BB	PSI	1.3086	0.3669	0.3404	
a1/s1	AA	PSS	1.2346	0.1842	0.1556	
a1/s1	AB	PSS	1.2812	0.1868	0.1062	
a1/s1	BA	PSS	0.7272	0.1526	0.1116	
a1/s1	BB	PSS	0.757	0.1558	0.1666	
a2/s2	AA	PSS	0.9746	0.1718	0.9744	
a2/s2	AB	PSS	1.3488	0.1888	0.0454	*
a2/s2	BA	PSS	0.7051	0.1519	0.0768	.
a2/s2	BB	PSS	0.9714	0.1714	0.958	
a3/s3	AA	PSS	1.1469	0.181	0.3486	
a3/s3	AB	PSS	1.294	0.1846	0.0804	.
a3/s3	BA	PSS	0.7309	0.156	0.1162	
a3/s3	BB	PSS	0.8282	0.1634	0.3466	
a1/s1	AA	PTI	1.4786	0.1936	0.0074	**
a1/s1	AB	PTI	1.0398	0.1765	0.741	
a1/s1	BA	PTI	0.4811	0.1314	6.00E-04	***
a1/s1	BB	PTI	1.0005	0.1729	0.913	
a2/s2	AA	PTI	1.2384	0.1835	0.1518	
a2/s2	AB	PTI	1.0834	0.1786	0.5724	
a2/s2	BA	PTI	0.4396	0.1265	0	***
a2/s2	BB	PTI	1.2386	0.1855	0.156	
a3/s3	AA	PTI	1.359	0.1882	0.04	*
a3/s3	AB	PTI	1.0808	0.1762	0.5712	
a3/s3	BA	PTI	0.5213	0.1345	8.00E-04	**
a3/s3	BB	PTI	1.0389	0.1747	0.7504	

Table 3.A13. Multiple choice mating tests with all flies reared on a common garden glucose based larval diet, tested at generation 60.

Line Pairing	Mate Pair Type	Coefficient	Value	St. dev.	*P* value	Sig
ag1/sg1	AA	PSI	1.2501	0.3338	0.4054	
ag1/sg1	AB	PSI	0.7839	0.2829	0.4168	
ag1/sg1	BA	PSI	0.8729	0.2061	0.536	
ag1/sg1	BB	PSI	1.2265	0.3112	0.422	
ag2/sg2	AA	PSI	1.3401	0.3919	0.3038	
ag2/sg2	AB	PSI	0.735	0.2786	0.3488	
ag2/sg2	BA	PSI	0.7738	0.2522	0.3716	
ag2/sg2	BB	PSI	1.3407	0.3947	0.3142	
ag3/sg3	AA	PSI	1.6498	0.9012	0.298	
ag3/sg3	AB	PSI	0.7563	0.3276	0.4224	
ag3/sg3	BA	PSI	0.8908	0.2086	0.5914	
ag3/sg3	BB	PSI	1.0984	0.1632	0.5276	
ag1/sg1	AA	PSS	0.9398	0.1766	0.8142	
ag1/sg1	AB	PSS	0.7575	0.1621	0.1774	
ag1/sg1	BA	PSS	1.2793	0.1926	0.1128	
ag1/sg1	BB	PSS	1.0234	0.1801	0.8078	
ag2/sg2	AA	PSS	0.9971	0.2077	0.9112	
ag2/sg2	AB	PSS	0.9139	0.2014	0.6092	
ag2/sg2	BA	PSS	1.089	0.2152	0.7374	
ag2/sg2	BB	PSS	1	0.2104	0.9208	
ag3/sg3	AA	PSS	0.3591	0.115	0	***
ag3/sg3	AB	PSS	0.5636	0.1388	0.0058	**
ag3/sg3	BA	PSS	1.1992	0.184	0.2314	
ag3/sg3	BB	PSS	1.8781	0.2014	0	***
ag1/sg1	AA	PTI	1.1308	0.185	0.414	
ag1/sg1	AB	PTI	0.565	0.1465	0.0082	**
ag1/sg1	BA	PTI	1.0902	0.1863	0.5578	
ag1/sg1	BB	PTI	1.2139	0.1928	0.226	
ag2/sg2	AA	PTI	1.2756	0.2237	0.2322	
ag2/sg2	AB	PTI	0.6373	0.1766	0.0422	*
ag2/sg2	BA	PTI	0.8084	0.1932	0.2864	
ag2/sg2	BB	PTI	1.2787	0.2241	0.2466	
ag3/sg3	AA	PTI	0.5215	0.1347	0.002	**
ag3/sg3	AB	PTI	0.3977	0.1194	0	***
ag3/sg3	BA	PTI	1.0423	0.1742	0.7362	
ag3/sg3	BB	PTI	2.0385	0.198	0	***

Table 3.A14. I_{PSI} significance values for generations 3 & 5, calculated using JMATING (Carvajal-Rodriguez and Rolan-Alvarez 2006)**.** Index values shown are mean values generated after 10,000 bootstrap resamples from the observed values of mating pairs. Probability values are the two tailed probability of an index value different from zero (random mating). Lowercase letters represent diet (ASG = a or Starch = s) and replicate information (1-3). Upper case letters represent 'pair types' (homotypic = AA, BB; heterotypic = AB, BA).

Line Pair	I_{PSI} value	St. dev.	*P* value
a1/s1	-0.2278	0.3033	0.394
a2/s2	-0.1422	0.3575	0.4608
a3/s3	0.2158	0.3139	0.486
as1/s1	-0.0067	0.3049	0.8984
as2/s2	0.2861	0.3619	0.5332
as3/s3	0.4688	0.2927	0.1678
as1/a1	0.0432	0.3825	0.716
as2/a2	0.0375	0.3806	0.6834
as3/a3	-0.1412	0.3778	0.4724
sa1/s1	0.0026	0.3732	0.643
sa2/s2	0.1532	0.3757	0.8846
sa3/s3	-0.0603	0.3915	0.7192
sa1/a1	0.2196	0.2402	0.3998
sa2/a2	0.2677	0.2438	0.2728
sa3/a3	0.2748	0.2447	0.2636

Table 3.A15. I_{PSI} Significance values for generations 30. Indices are calculated as described in Table S14. Lowercase letters represent diet (ASG = a or Starch = s) and replicate information (1-3). Upper case letters represent 'pair types' (homotypic = AA, BB; heterotypic = AB, BA).

Line Pair	I_{PSI} value	St. dev.	*P* value
a1/s1	0.0285	0.2639	0.9188
a2/s2	-0.0838	0.298	0.7042
a3/s3	-0.0804	0.1962	0.6928
ass1/s1	-0.1749	0.3756	0.5958
ass2/s2	0.4097	0.3764	0.3172
ass3/s3	-0.2819	0.3447	0.3176
ass1/a1	0.1069	0.412	0.7632
ass2/a2	0.427	0.2589	0.1496
ass3/a3	-0.0248	0.3946	0.5774
saa1/s1	0.281	0.3102	0.4038
saa2/s2	-0.0517	0.4098	0.66
saa3/s3	0.3483	0.3656	0.372
saa1/a1	0.3583	0.2652	0.2146
saa2/a2	0.4916	0.2555	0.1008
saa3/a3	-0.595	0.2458	0.041

4 Behavioural phenotypes of adaptation: male courtship behaviour after experimental evolution in the medfly (*Ceratitis capitata*: Diptera, Tephritidae).

4.1 Introduction

The evolution of reproductive isolation between populations is based upon the establishment of barriers to gene flow between populations (Schluter 2000; Coyne and Orr 2004). Sexual isolation is a key barrier which can develop to reduce gene flow between populations, and arises when individuals from one population are less attracted to individuals of another, or fail to recognise them as potential mates (Dobzhansky and Mayr 1944; Kaneshiro 1980). As mating is the point at which genes can be transferred between populations, mate choice manifests the primary stage at which reproductive isolation can evolve. Due to the complex nature of the traits involved in mate recognition and choice, and the action of sexual selection during mate choice (Lande 1981, 1982), it is hard to ascribe sexual isolation to ecologically driven divergent selection alone (Panhuis et al. 2001; Rundle and Nosil 2005; Nosil 2012). Theoretical models have suggested the combination of natural and sexual selection to be essential to the initiation and completion of speciation (van Doorn et al. 2009). However, recent experimental evidence suggests that where divergence in male and female reproductive optima leads to sexual conflict, sexual selection may impede divergence under natural selection (Chenoweth et al. 2015).

Sexual isolation is based upon the information transferred between a signaller and a signal perceiver (Pillay and Rymer 2012), and the mate choice decision made by these individuals when interacting is one of 'compatibility recognition' (Mendelson and Shaw 2012). The signaller must be able to communicate its compatibility as a mate in terms of individual identity, at the level of species or population. It must also advertise its compatibility in terms of individual quality, in competition with other conspecifics. Coevolution in such mate recognition systems drives behavioural divergence across many well documented sensory modalities (Butlin and Ritchie 1989). These are manifested across a wide range of traits including visual signals mediated by courtship behaviour (e.g. Miller et al. 1998; Boake 2005; Stratton 2005; Arbuthnott and Crespi 2009; Puniamoorthy 2014), or colouration (e.g. Williams and Mendelson 2011); chemosensory signals (e.g. Etges et al. 2009; also reviewed in Smadja and Butlin 2009), and auditory signals (e.g. Ryan and Rand 2003; Honda-Sumi 2005; Snook et al. 2005; Etges et al. 2007).

In some cases, signal traits or their perception fall directly under divergent environmental selection and are referred to as 'magic' (Nagel and Schluter 1998; Jiggins 2008; Maan and Seehausen 2011; Servedio et al. 2011; Chung et al. 2014), or 'multiple effect' (Smadja and Butlin 2011) traits. Such traits provide the most direct link between divergent ecological selection and sexual isolation, but may not be common in nature (Servedio et al. 2011). A key example of a magic trait in the divergence of a wild population is that of the mimetic colour morphs of *Heliconious* butterflies. Divergence between the colour patterns of the sister species *H. melpomene* and *H. cydno*, each following a different pattern of mimicry, has led to the evolution of assortative mating between the two species (Jiggins et al. 2001). Disruptive selection has also been shown to act against hybridisation between the two species (Merrill et al. 2012), as the colour phenotype of the hybrids does not effectively act to deter predators in the same way as either of the parental phenotypes. Hence, natural selection on the signal trait also drives reproductive isolation, which is the characteristic of the so-called 'magic' traits that can facilitate RI.

The action of magic traits is closely paralleled by traits or trait complexes which are condition dependent in their expression (Servedio et al. 2011). As condition dependent traits allow an honest representation of local adaptation, through the maintenance of costly ornamentation (signals) (Rowe and Houle 1996), evolution of the perception of such signals should facilitate population divergence towards the evolution of non random mating (van Doorn et al. 2009; Servedio et al. 2011). Condition dependent traits (signals) communicate the overall functionality of cellular processes (Hill 2011), as well as cognitive ability (Buchanan et al. 2013) relative to successful development in an environment. An example of the expression of cognitive ability expressed through a trait associated with increased fitness is that of the association between greater carotenoid plumage pigmentation in the siskin (*Carduelis spinus*) and elevated problem solving ability in foraging tasks (Mateos-Gonzalez et al. 2011). Carotenoid pigmentation is commonly linked with nutritional condition in birds (e.g. Hill 2000), and is also linked to successful mating (Senar et al. 2004). Its association with problem solving ability has been suggested to allow females to assess the cognitive ability of potential mates (Mateos-Gonzalez et al. 2011). Indeed, such variation in condition dependant traits such as carotenoid pigmentation caused by diffing levels of developmental success in an environment provides the variance in signals that that allows quality to be assessed during compatibility recognition. In

this way, ecological selection pressure can provide the exogenous pathway for the initiation of sexual selection on trait divergence, and thus without acting directly on a magic trait, contribute to the initiation of reproductive isolation (Mendelson and Shaw 2012).

In the previous chapter, the evolution of non-random mating was recorded in an evolution experiment, which altered the larval diet of a base population of the Mediterranean fruit fly (*Ceratitis capitata*, the medfly) to provide a divergent environmental selection pressure. Mate choice was assayed at three time points during the evolution of three replicate populations, of two different dietary treatments. In the 60th generation of experimental evolution, assortative mating by diet was observed consistently across all replicates, during multiple choice mating tests. However, at two previous time points that used limited choice mating tests, no assortative mating was observed. Here, it was proximate effects of larval diet that had the strongest effects, with a significant mating advantage observed for whichever males were reared on the more calorific and nutritious diet (ASG) prior to testing. Multiple choice mating tests have been shown to allow the maximum expression of sexual isolation (Coyne et al. 2005; Jennings and Etges 2009) and it is possible that the limited choice design employed in earlier generations failed to detect divergence in mate choice.

To further examine the mate choice exhibited at the middle time point reported in Chapter 4, the 30th generation of experimental evolution, no choice mating tests were conducted, between all combinations of males and females from both dietary regimes. In the absence of competition, this allowed the compatibility recognition of males and females of both regimes to be assessed. Also this design allowed male courtship behaviour to be recorded to provide a detailed assay of any divergence in signal traits. Further to this, in order to understand the manifestation of the competitive advantage seen by the males reared on the more nutrient rich ASG diet, a single generation diet cross was conducted. This allowed the proximate effects of diet upon male behaviour and resultant mate choice to be assessed.

As described in earlier chapters, the importance of controlling the medfly as a crop pest has lead to a wide range of studies on mate choice and courtship behaviour in this species (Benelli et al. 2014). Medfly court in substrate leks, both on the leaves of host fruit trees, and on the host fruit themselves (Prokopy and

Hendrichs 1979). Leks are convened by males through the release of long range pheromones from a rectal epithelial gland (REG), which is extruded from the tip of the abdomen prior to signalling (Arita and Kaneshiro 1986). Once females are drawn to the lek site, males begin a multimodal courtship display (Feron 1962; Briceño et al. 1996, 2002, 2007; Briceño and Eberhard 1998, 2002a,b; Lux et al. 2002; Benelli et al. 2014). Males begin courtship with continuous wing vibration, initiated in the presence of the female. Males then orient themselves to the female, tracking her movements (Briceño et al. 1996). During continuous wing vibration, the REG remains extruded but is curled below the abdomen, often bearing a droplet of pheromone, suggesting a role for short range pheromonal communication (Briceño et al. 1996). The male then progresses to intermittent wing vibration, or 'buzzing', where it continues to vibrate its wings, moving them back and forth to create an intermittent buzz. This behaviour is often initiated as the female moves closer to the male, and also leads to retraction of the REG (Briceño et al. 1996). Both forms of wing vibration create distinct auditory signals (Briceño et al. 2002). The third component of the male sequence, a visual and potentially mechanical cue (Briceño and Eberhard 2002a), is rapid side-to-side movement of the head, or 'head rocking'. This behaviour often occurs in conjunction with either of the wing vibration behaviours, but is conducted when in direct proximity of the female (Briceño et al. 1996), presumably coinciding with visual range. When the female moves close to the male, and becomes stationary, the male jumps over the female, to a mounting position, and attempts genital intromission (Briceño et al. 1996). As the male passes over the female, several mechanical stimuli are enacted by the male contacting the female with his tarsi (Briceño et al. 1996). If the copulation attempt is unsuccessful, the male may directly re enter the courtship sequence at any point if the female remains in close proximity, or if proximity is lost exit courtship and return to non-courtship behaviour. Successful courtships lead to a copulation which lasts up to two hours (Yuval and Hendrichs 2000). Females are highly selective before accepting copulation, up to 90% of courtships have been shown to be unsuccessful (Whittier et al. 1994).

Detailed recording of this behavioural sequence, as conducted in the study described in this chapter, thus allows the capture of information relating to multiple types of signalling: auditory cues from wing vibration, visual cues from head rocking, and chemosensory cues from REG extrusion. I investigated here whether such information could be used to test the hypothesis that divergent

selection by larval diet results in the expression of different in courtship behaviours associated with non random mating. Therefore, I predicted that males from the two larval diet regimes would display divergent patterns of courtship behaviour. I expected this effect to be present both when males were reared on their regime diet, and on the opposite diet. Also, given the strong proximate effects of larval diet on mating success shown in Chapter 4, and in previous studies (Kaspi et al. 2002; Joachim-Bravo et al. 2009; Anjos-Duarte et al. 2011), detailed analysis of courtship behaviour might also be expected to reveal signatures of variation across courtship signals related to condition dependence. Coupled with the results of no choice mating tests, detailed analysis of courtship behaviour can reveal the role of behavioural divergence in the pattern of non-random mating (at 60 generations, Chapter 3).

The results confirmed the pattern of mating observed in Chapter 3, with ASG males mating at higher frequency. Hence the removal of competition in the no choice tests used here did not alter the outcomes in terms of matings. Diet crossing reversed this effect (as in Chapter 3), but also highlighted potential divergence in male or female mate choice. The results of behavioural analysis showed divergence in courtship behaviour when males were maintained on their own regime diet. However, diet crossing highlighted the strong influence of the proximate diet effects, ultimately determining male attractiveness. Hence, the behavioural profiles seen in the on diet treatments were not retrieved when males were diet crossed. Interestingly, the courtship behaviours showed little relation to copulation success, suggesting that cues affecting female choice were not captured by these analyses.

4.2 Experimental methodology

4.2.1 Origin and maintenance of fly stocks

Flies used in this study were drawn from the 29th and 30th generation of the evolution experiment described in Chapter 4. To examine the proximate effects of 29 generations of experimental evolution on male courtship behaviour, two treatments were created from each of the three replicate ASG and Starch populations. Males were tested when reared on their own regime background diet at generation 29 (on diet) or following one generation of rearing on the opposite diet (diet cross). The 'on diet' versus 'diet cross' treatment comparisons gave insight into the magnitude of evolved versus proximate effects of diet on male behaviour. On diet males were reared on their regime diet until generation

29, and copulation success and behaviour was observed during this generation. Diet crossed males were reared on their regime background diet until generation 29 and then eggs from this generation were seeded onto the opposing diet, creating two diet crossed treatments: 'ASG on Starch' and 'Starch on ASG'. The mating success of males in a no choice scenario, and courtship behaviour of successfully copulating males from the following generation (30), was then recorded. Both on diet and diet cross males were paired with on diet females, which had been reared on their regime background until generation 29 or 30 respectively. This allowed the change in male behaviour associated with diet crossing to be isolated, without undue influence of the female phenotype.

4.2.2 No-choice mating tests

Mating tests were conducted between single pairs of medflies. Flies were reared in single sex cages as described in Chapter 4 until the 7th day post eclosion, when mating tests were conducted. Starting at lights on (09:00), females were aspirated into mating arenas (50mm x 11mm petri dishes) designed for the filming of behaviour, 30 minutes prior to the introduction of a male. Mating arenas had a ~10mm x 30mm strip of paper tape added to the outer lid surface to simulate the underside of a leaf. This ecological component can facilitate normal male courtship behaviour. Mating test observations and filming began with the introduction of the male, and continued for 30 minutes or until a successful copulation occurred. Matings were scored if a male successfully mounted a female, and a resting copulation had been achieved. Matings were recorded independently of filming in five-minute interval visual scans. When a mating was observed, or after 30 minutes had elapsed, filming was ceased, both individuals were discarded, and the mating chamber rinsed with ethanol followed by dH_2O and allowed to air dry before reuse. All mating tests were conducted within four hours after lights on (before 13:00) and set up sequentially to balance the different treatments, in 12 arenas with one camera per arena.

Mating test treatments comprised of single pairs (one male, one female) in factorial combinations as follows: in generation 29: (on diet Female/on diet Male): 'ASG/ASG', 'ASG/Starch', 'Starch/ASG', 'Starch/Starch'; in generation 30 (on diet Female/diet crossed Male): 'ASG/ASG on Starch', 'ASG/Starch on ASG', 'Starch/ASG on Starch', 'Starch/Starch on ASG'.

4.2.3 Behavioural acquisition

Filming was conducted using Sony Handycam CX190 high definition video cameras. An adjustable shelving unit was used to suspend the filming cells 15cm above the cameras, coinciding with their focal depth. Filming was conducted from below as males court females on the underside of leaves in the wild, and courtship was mainly observed on the 'roof' of mating arenas. Filming was conducted using ambient room lighting. All no choice mating tests were filmed, but only replicates in which a male successfully copulated within 30 minutes were used for analysis of courtship behaviour. Replicates in which the males copulated before 120 seconds had elapsed were excluded from the analysis.

4.2.4 Behavioural quantification

Based on preliminary analyses and previous studies (Briceño et al. 1996, 2007,Briceño and Eberhard 2002b), 12 behaviours were selected to quantify courtship. These behaviours are described in Table 4.1. Behaviours were classified as either 'behavioural states' that were mutually exclusive to other behaviours, meaning the occurrence of a bout of that behaviour ended the previous bout of another behaviour, or as 'elementary behaviours' which did not, allowing some behaviours to co-occur. Full detail is given in Appendix 4.1.

Table 4.1. Definitions of pre-copulatory behaviours scored in analysis of medfly pairs. Behaviours in blue represent specific 'courtship' behaviours.

Behaviour	Description
Decamping	Focal male jumps or flies, outside of the courtship sequence
Locomotion	Focal male walks (not associated with female)
Preening	Focal male cleans any body part
Stationary	Focal male stays motionless
Aggression	Focal male strikes female with head
Orientation	Focal male tracks female movement with head
Gland Extrusion	Focal male extrudes anal pheromone gland (not associated with preening)
Continuous Wing Buzzing	Focal male buzzes wings continuously, uninterrupted by forward movement
Head rock	Focal male moves head from side to side rapidly
Intermittent Wing Buzzing	Focal male buzzes wings, but simultaneously moves them rapidly forward and backward
Copulation	Focal male achieves intromission
Copulation attempt	Focal male attempts intromission but is dislodged by female

4.2.5 Video data analysis

Video Data were analysed in real time using VLC media player. Each video was scored for male behaviours using JWatcher ver. 1.0 (Blumstein et al. 2006). All video analyses were conducted double blind, following randomisation and coding of videos by an independent third party. Video files were given a neutral code in order to avoid observer bias. Behaviours were scored sequentially, and then categorised as behavioural states or elementary behaviours using JWatcher's focal 'analysis master file' function. This allowed both bout frequency and bout duration to be recorded simultaneously.

Following sequential scoring of the behavioural sequence for each individual, JWatcher was used to calculate two metrics relating to each behaviour. Bout Frequency (BF) was scored as the count of the number of times a behaviour occurred from the start of filming to the occurrence of copulation. Total time spent in a behaviour (TT) was the sum of the durations of all bouts of a behaviour, in milliseconds, from the start of filming to the occurrence of copulation. To provide a measure of each metric that was comparable between individuals, the raw value of each metric was divided by the duration of the whole behavioural sequence, from the start of filming to the occurrence of copulation, for each individual. This normalised the raw data to the duration of each behavioural sequence, and produced measurements of BF and TT metrics per unit of time. BF was hence measured as 'Bouts per minute of filming', and TT was measured in 'Milliseconds per Second of behaviour'.

The latency of both the initiation of courtship behaviour, and successful copulation were also scored from the JWatcher data. Latency to courtship was the time in milliseconds from the initiation of the mating test to the fist occurrence of one of the four courtship behaviours (Table 4.1). Latency to copulation was the time in milliseconds from the initiation of the mating test to the observation of a settled copulation. Both measurements were converted into minutes for analysis.

All raw data for both BF and TT over all behaviours, as well as courtship and copulation latency were visualised as box an whisker plots with the normalised measure of the behaviour ('Bouts per minute of filming' for BF, 'Milliseconds per Second of behaviour' for TT, 'Minutes from initiation of recording' for latencies) on the Y axis, and the pair types on the X axis. The style of box and whisker plot

used represents the median value (thick bar) and the interquartile range (IQR) of the data (box, coloured by treatment), as well as the tails of the data under 1.5 times the IQR (whiskers). Outliers, points that were more than 1.5 times the IQR, are presented as dots.

4.3 Statistical methodology.

All data analysis was conducted in R ver. 3.2.0 (R Development Core Team 2015).

4.3.1 Copulation success

The success of males in securing copulations during 30 minute no choice mating tests was recorded as success or failure, and analysed using a Chi-Square test for equality of proportions (Wilson 1927). This allowed an overall difference in proportion of successful males between treatments to be tested for.

4.3.2 Principal components analysis

To first assess whether it was possible to reduce the dimensionality of the differential behavioural trait data prior to the implementation of the linear analysis of behaviours described below, principal components analysis (PCA) was implemented using the pr.comp function in R {stats}. Data were scaled within the function so as to have unit variance prior to the analysis, and also zero centred. This PCA analysis was conducted on the total BF and TT datasets for both generations 29 and 30. To evaluate whether the PCA was successful in reducing the number of behavioural variables to be analysed, I assessed the loadings and biplot visualisation (ggbiplot package; Vu 2011) of the data. I found that the dimensionality in the data was not significantly reduced in any of the four analyses. Therefore, the PCA did not offer simplification or additional power to the linear analyses of individual behaviours (an example of the PCA output is presented in Appendix 4.2).

4.3.3 Generalised linear mixed models

Generalised linear mixed models were fitted for the bout frequencies (BF) and total number of milliseconds (TT) for which each behaviour occurred. For the analysis of the generation 29 (on diet) data, fixed effects were the regime diet of the male and the female. For the generation 30 (diet cross) data, fixed effects were the regime diet/rearing diet of the male and the regime diet of the female. A random effect was added to both models to nest the effect of line replicate

within regime diet. Models were offset to the log of the duration until copulation, in order to account for differing lengths of overall courtship time. Mixed models were fitted using the lme4 package (ver. 1.1.12) in R (Bates et al. 2015), and tested using the package lmertest (ver. 2.0; Kuznetsova et al. 2013). A Poisson error structure was fitted and the data tested for overdispersion by comparison of the residual deviance of the maximal model with the corresponding residual degrees of freedom (for fixed effects only). Where there was overdispersion (dispersion factor > 1.2) an observation level variable was added as a random effect. Models that encountered convergence errors were fitted with the 'bobyqa' optimizer (lme4). Model selection was conducted by sequential likelihood ratio testing using lmertest::anova, as was the description of the main effect in the selected models.

4.4 Results

4.4.1 No choice mating tests

The results of the no choice mating tests are presented in Table 4.2. In generation 29, there were significant differences between treatments in the proportions of 'on diet' males that successfully copulated within the experimental period of 30 minutes ($\mathcal{X}^2$ = 38.956, d.f. = 3, P < 0.001). ASG males paired with ASG females secured 74% of copulations, ASG males with Starch females 56%, Starch males with ASG 44% and Starch males with Starch females 23%.

Table 4.2 Mating test results of no choice mating tests conducted during the 29th and 30th generation of the evolution experiment. The total number of copulations recorded for each pair type is presented as well as the total number of these copulations for which courtship behaviour was analysed.

Generation	Male Background	Female Background	Total Mating Tests	Total Copulations	Total analysed for behaviour
29	ASG	ASG	78	58	50
		Starch	75	42	35
	Starch	ASG	59	26	22
		Starch	62	14	12
30	ASG on Starch	ASG	60	12	9
		Starch	67	23	19
	Starch on ASG	ASG	56	21	19
		Starch	58	37	33

In generation 30, when males were reared for one generation on the opposite diets and paired with uncrossed females, there was again a significant difference

between treatments in the proportion of successful copulations achieved within the experimental time period (X^2 =24.838, d.f. = 3, P < 0.001). ASG males reared on Starch secured 20% of matings when paired with ASG females, and 34% of potential copulations when paired with Starch females. Starch males reared on ASG and paired with ASG females secured 38% of available copulations and 64% when paired with starch females.

4.4.2 Courtship and copulation latency

Generation 29 – analysis of behaviour 'on diet'

There was a significant effect of male (glmer, z = 2.53, P = 0.012, Fig 4.1A) and female dietary background (glmer, z = 3.15, P = 0.002, Fig 4.1A) on courtship latency in Generation 29. ASG males were significantly quicker to initiate courtship, and males from both backgrounds initiated courtship if paired with an

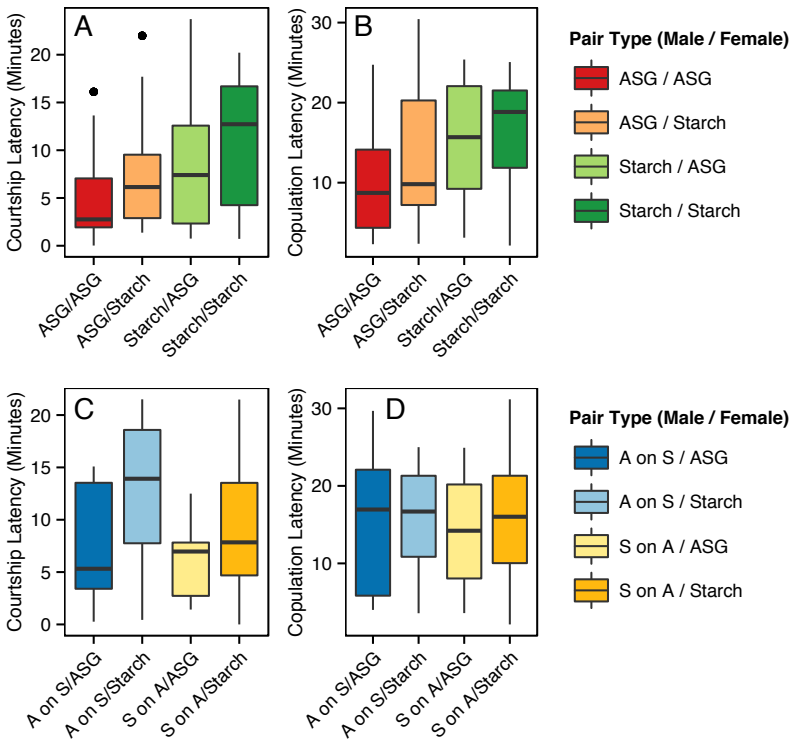

Figure 4.1. Latency to the initiation of courtship and to successful copulation scored in minutes from the start of the mating test. Courtship latency was scored as the time from the start of the mating test to the occurrence of the first courtship behaviour. Copulation latency was the time from the start of the mating test to the successful male genital intromission, and the formation of a settled pairing. Data shown as box plots. A) Generation 29, Courtship latency of on diet males paired with on diet females. B) Generation 29, Copulation latency of on diet males paired with on diet females. C) Generation 30, Courtship latency of diet crossed males paired with on diet females. D) Generation 30, Copulation latency of diet crossed males paired with on diet females.

ASG female (Figure 4.1A). There was also a significant effect of male dietary background on the latency of successful copulation (glmer, z = 3, P = 0.003), with ASG males securing copulation more quickly than did Starch reared males (Figure 4.1B).

Generation 30 – analysis of behaviour on 'cross diets'

There was no significant effect of either male or female dietary background on either courtship or copulation latencies in the 'diet crossed' Generation 30 mating tests (Figure 4.1C & D).

4.4.3 Behavioural analysis - 'on diet' tests (generation 29)

The results of the glmm analysis of the behavioural data recorded from males that were reared on their selection diet are described below. The raw data that formed the basis of these analyses are presented in Figure 4.2 (bout frequency, BF) and Figure 4.3 (total time, TT). Full statistical details of the model selection for each behaviour and metric are presented in Appendix 4.3. Also presented in Appendix 4.3 are full summaries of the final, best fit, statistical models selected in each case.

Courtship behaviour

Male dietary background had a significant effect on the number of bouts of all of the four courtship behaviours recorded (glmer, P < 0.04 in all cases; Appendix 4.3, Section 1). ASG males conducted significantly more bouts of continuous and intermittent wing vibration and head rocking, whilst Starch males conducted significantly more bouts of gland extrusion (Figure 4.2). Female dietary background also had a significant effect on the number of bouts of continuous and intermittent wing vibration and head rocking (glmer, P < 0.02 in all cases; Appendix 4.3, Section 1). However, there was no effect of female diet on gland extrusion bout frequency. Males from both diet backgrounds conducted significantly more bouts of these courtship behaviours when paired with an ASG female (Figure 4.1). There was a marginally significant interaction between the effects of male and female dietary background in the occurrence of bouts of head rocking (glmer, z = 1.74, P = 0.082, see Appendix 4.3, Section 1).

These effects broadly translated to the total time spent in courtship behaviours. Male diet background significantly affected the total time spent in intermittent wing vibration, head rocking, and gland extrusion behaviour (glmer, P < 0.006 in

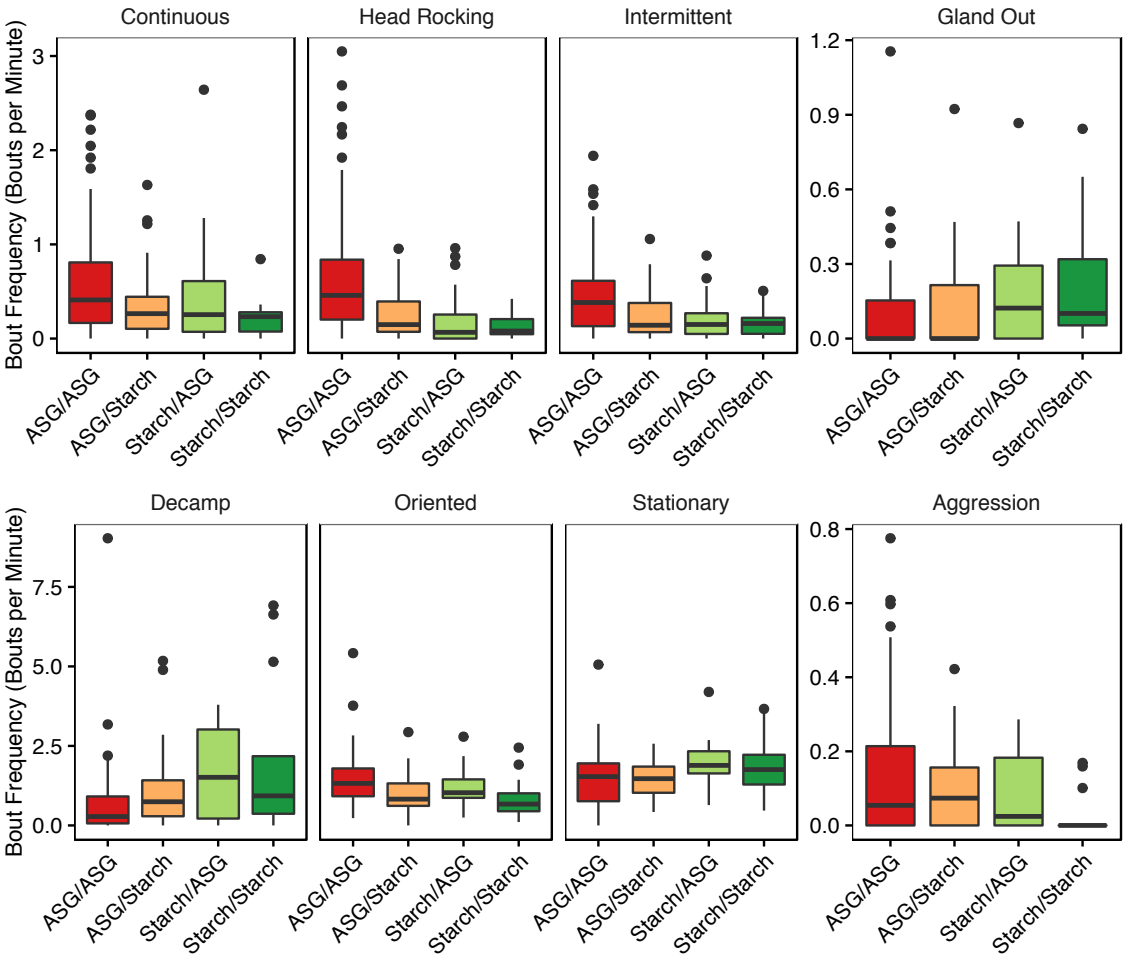

Figure 4.2. Bout frequency of behaviour in on diet males in generation 29 Bout frequency of four courtship behaviours (top row), and four non-courtship behaviours (bottom two rows) that showed significant effects of either male or female diet are presented. Bout frequency was scaled by copulation latency for each individual to give 'bouts per minute of behaviour'. Data shown as box plots.

all cases; Appendix 4.3, Section 1). ASG males spent significantly more total time conducting intermittent wing vibration and head rocking, whilst Starch males spent more time engaged in pheromone gland extrusion. Female dietary background also significantly affected the total time spent in intermittent wing vibration and head rocking behaviours (glmer, $P < 0.03$; Appendix 4.3, Section 1), with ASG females spending more total time in receipt of these behaviours. There was also a marginally non significant effect of female dietary background on the total time spent by males in continuous wing vibration (glmer, z = -0.72, $P = 0.071$; Appendix 4.3, Section 1). There was a significant interaction between the two effects for total time spent in head rocking behaviour (glmer, z = 2.18, $P = 0.029$; Appendix 4.3, Section 1) and a marginally significant interaction for intermittent wing vibration (glmer, z = 1.73, $P = 0.083$; Appendix 4.3, Section 1).

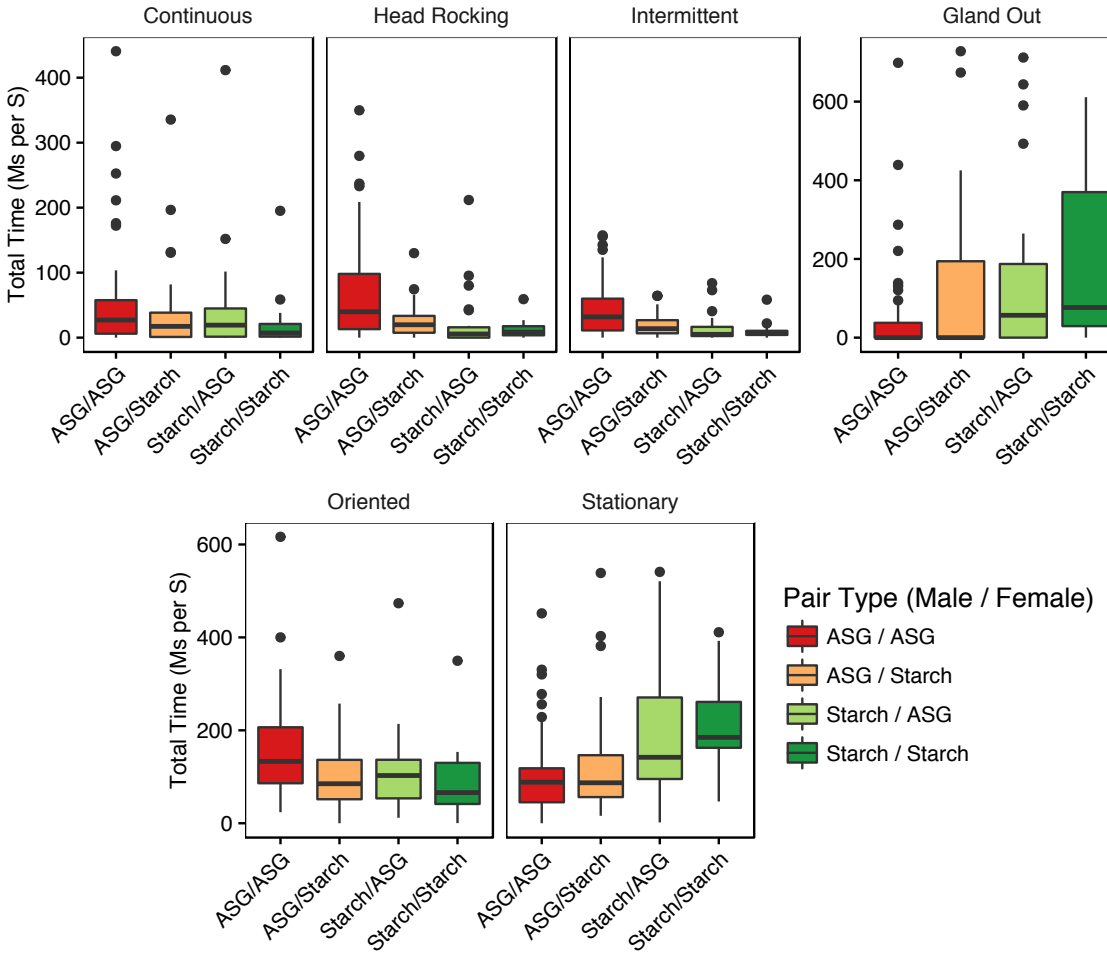

Figure 4.3. Total time spent in courtship and non-courtship behaviours by on diet male flies in generation 29. Four courtship (top row), and two non-courtship (bottom row) behaviours that exhibited significant effects of either male or female dietary treatment are presented. Total durations were scaled by the latency to copulation for each individual to give 'milliseconds per second of behaviour'. Data shown as box plots.

Non courtship behaviour

There was a much less marked effect of the diet treatments on non courtship behaviour. The bout frequency and total time spent engaged in locomotion and preening behaviour was not significantly affected by either male or female dietary background. The number of unsuccessful copulation attempts was also not significantly affected by either male or female dietary background. Male dietary background significantly affected the number of bouts of stationary and decamping behaviour (glmer, $P < 0.04$ in both cases; Appendix 4.3, Section 2), with Starch males exhibiting more frequent bouts of both behaviours. This significant effect of male dietary background was also seen in the total time spent in stationary behaviour (glmer, $z = 2.98$, $P = 0.003$; Appendix 4.3, Section

2), with Starch males spending more time stationary. Female diet background had a significant effect on the number of bouts of orientation behaviour (glmer, z = -3.21, *P* = 0.001; Appendix 4.3, Section 2), with males orienting themselves to ASG females more often. Although marginally affected by male diet background (glmer, z = -1.78, *P* = 0.075; Appendix 4.3, Section 2), there was also a significant effect of diet on the total time spent males spent oriented to females (glmer, z = -2.83, *P* = 0.005; Appendix 4.3, Section 2). Bout frequency of decamping behaviour was also marginally affected by female dietary background (glmer, z = 1.67, *P* = 0.096; Appendix 4.3, Section 2). Finally, the number of bouts of aggressive behaviour was significantly affected by male dietary background (glmer, z = -2.28, *P* = 0.023; Appendix 4.3, Section 2), with ASG males exhibiting more behavioural bouts than for Starch males.

Summary of 'on diet' behavioural analysis results

Following the sequential analysis of courtship behaviour described above, a pattern of activity contingent with the copulatory success described in Table 4.2 was observed. Males selected on the most successful male diet background (at least in terms of copulations secured), namely ASG, initiated courtship faster than Starch males, secured copulations earlier, and also conducted significantly more courtship behaviour. Starch males conducted more bouts of gland extrusion. The background of the female also affected behaviour; ASG females appeared to elicit more courtship than did Starch females, as well as more non-courting 'orientation' behaviour. Other non-courtship behaviours varied little, but 'stationary' and 'decamping' were more common in Starch males. Total time and bout frequencies displayed relatively similar patterns in most behaviours, apart from 'gland extrusion' in which Starch males spent far longer with pheromone gland extruded.

4.4.4 Behavioural analysis – 'diet crossed' tests (generation 30)

The results of the behavioural analysis of courtship behaviour in the no choice mating tests of males crossed onto the opposite selection diet at generation 29 and then tested at generation 30 are given below. In comparison with the tests above, these analyses allowed insight into the proximate and ultimate effects of diet on male courtship behaviour. Female rearing background for all tests here was 'on diet', which allowed the potential proximate effects of female rearing diet on male behaviour to be isolated. All behaviours were analysed using glmms.

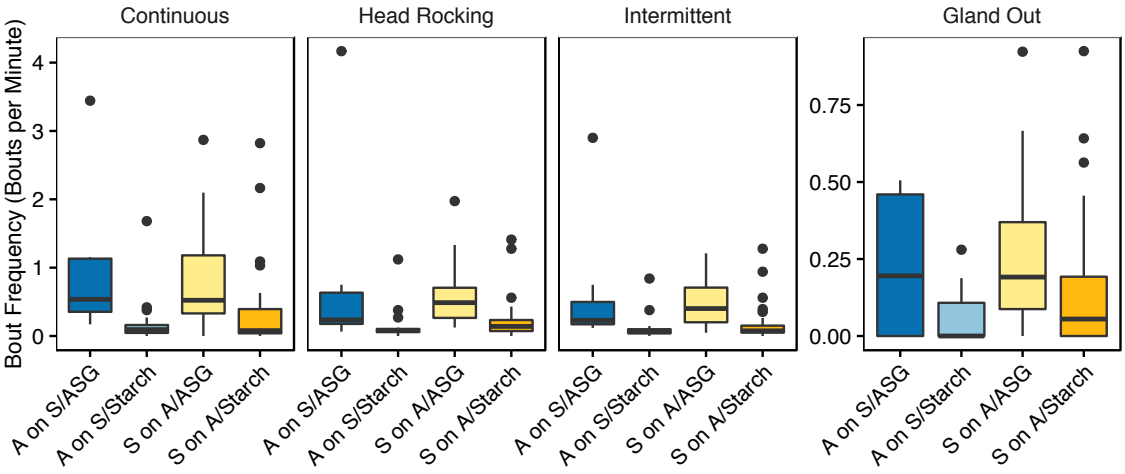

Figure 4.4. Bout frequency of courtship behaviours of diet crossed males in generation 30 Bout frequency is scaled by copulation latency for each individual to give 'bouts per minute of behaviour'. Data shown as box plots.

Results are described below, and the raw data upon which these analyses are based are presented in Figures 4.4 and 4.5 (bout frequency) and Figure 4.6 (total time spent in each behaviour).

Courtship behaviours

There was no significant effect of diet crossed male treatment on courtship behaviours in generation 30. Continuous and intermittent wing vibration, head rocking, and gland extrusion behaviours were significantly affected by female dietary background with respect to both bout frequency and total time spent (glmer, $P < 0.004$ in all cases; Appendix 4.3, Section 3). ASG females elicited more bouts of all courtship behaviours, and males also invested more total time in all courtship behaviours when paired with them.

Non courtship behaviours

Locomotion and decamping behaviour were not significantly affected by either diet crossed male treatment or by female dietary background, in respect to bout frequency or total time spent in these behaviours. There was a significant effect of diet crossed male treatment on preening behaviour (glmer, $z = 3.84$, $P < 0.001$; Appendix 4.3, Section 4), with Starch males reared on ASG conducting significantly more non courtship behavioural bouts. An effect of regime diet was also seen in total time spent preening.

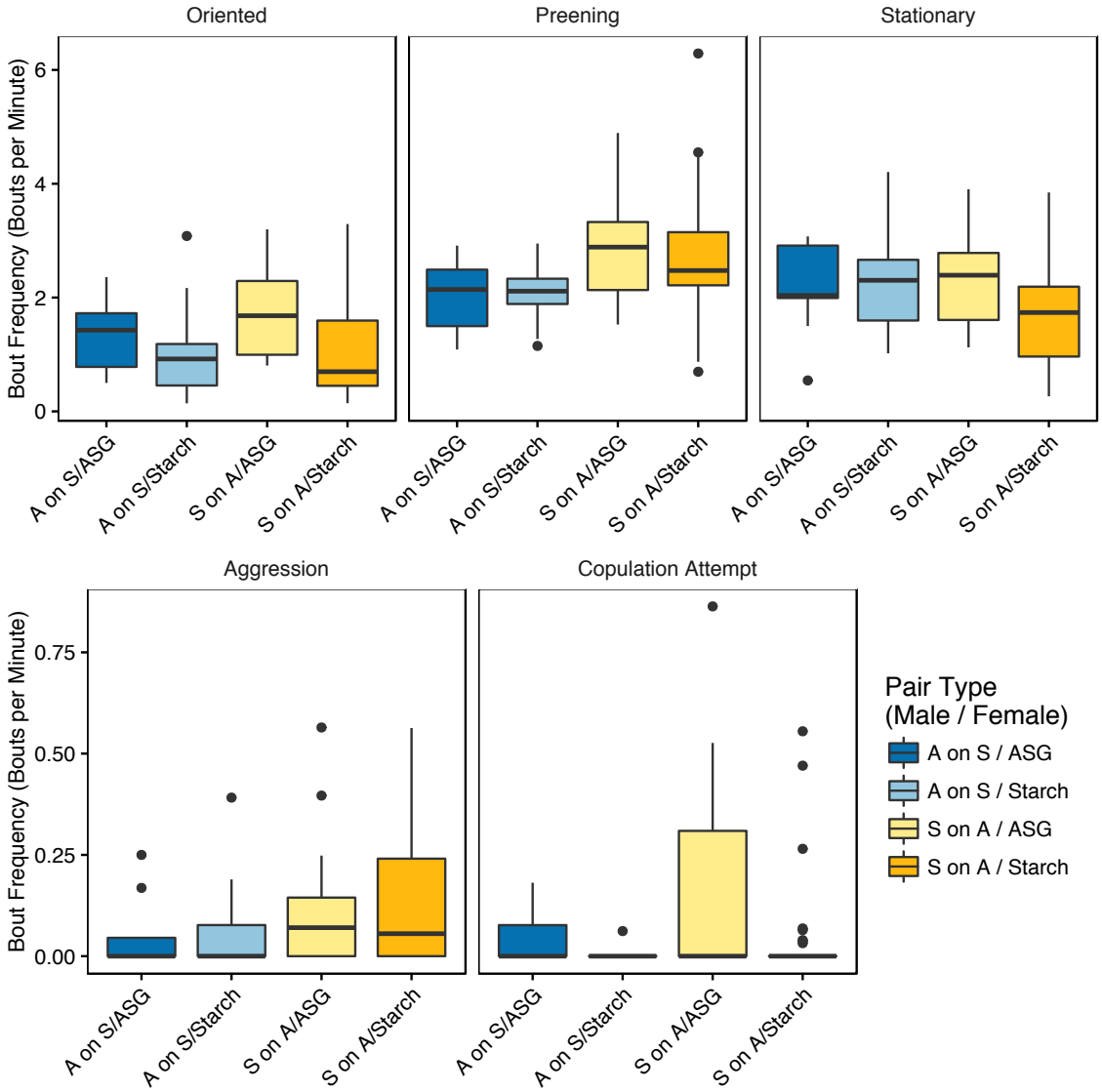

Figure 4.5. Bout frequency of non courtship behaviours of diet crossed males in generation 30 Bout frequency is scaled by copulation latency for each individual to give 'bouts per minute of behaviour'. Data shown as box plots.

The bout frequency of preening and stationary behaviours was significantly affected by diet crossed male treatment (glmer, $P < 0.05$ in both cases; Appendix 4.3, Section 4) with ASG males reared on Starch conducting fewer bouts of preening, and more bouts of stationary behaviour. Stationary behaviour was also affected by female regime background (glmer, z = -2.17, $P = 0.030$; Appendix 4.3, Section 4) with males conducting fewer bouts of stationary behaviour when paired with a Starch female (Fig 4.5). The significant effect of diet crossed male treatment was also seen in the total time spent stationary and in preening behaviour (glmer, z = -2.93, $P < 0.02$ in both cases; Appendix 4.3, Section 4) with Starch males spending less time stationary, but more total time preening. There was also a significant effect of female dietary background on

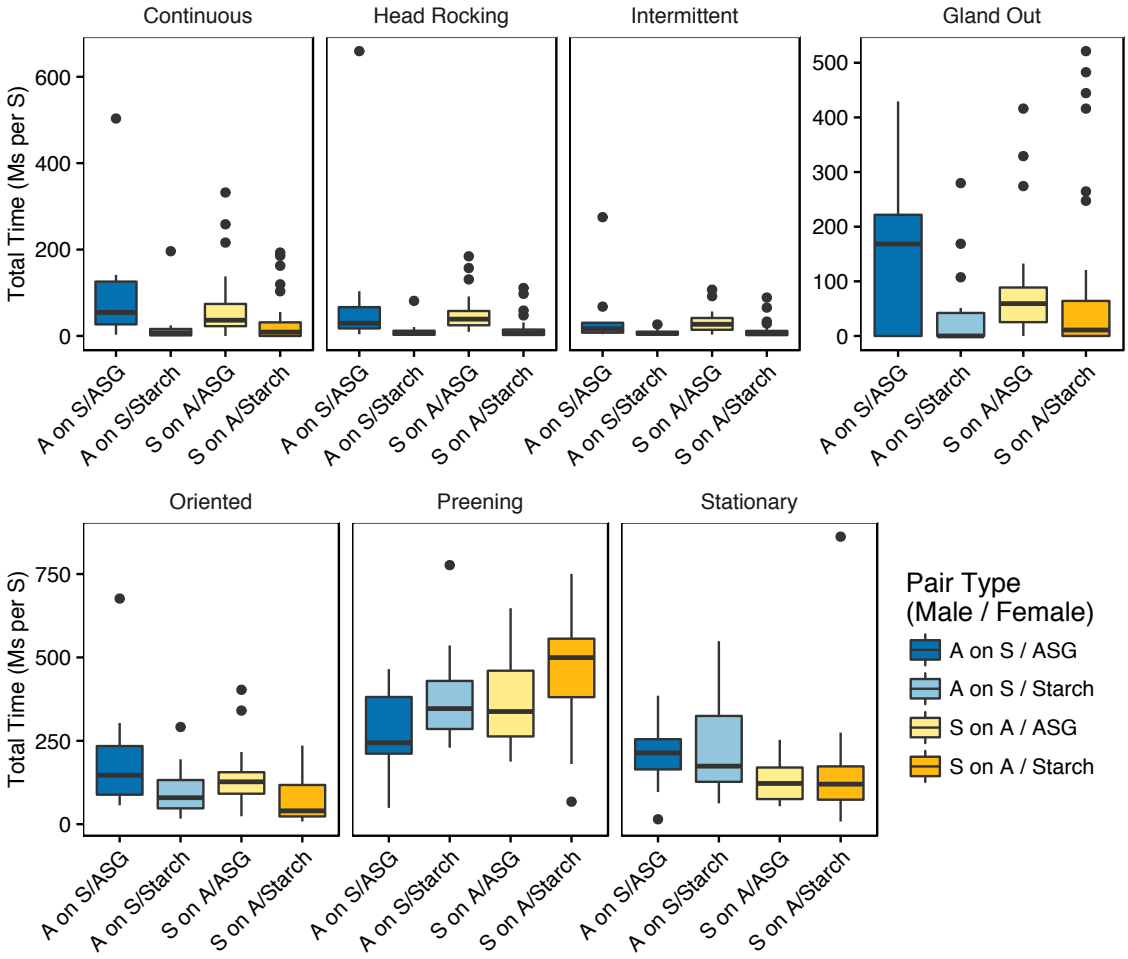

Figure 4.6. Total time spent in behaviours of diet crossed males in Generations 30. Four courtship (top row), and three non-courtship (bottom row) behaviours that showed significant effects of either male or female dietary background are presented. Total durations were scaled by the latency to copulation for each individual to give 'milliseconds per second of behaviour'. Data shown as box plots.

total time spent preening (glmer, z = 2.76, p = 0.006; Appendix 4.3, Section 4), with males spending less time preening when paired with ASG females (Fig 4.6).

Bout frequency of orientation behaviour was significantly affected by female dietary background (glmer, z = -3.52, p < 0.001; Appendix 4.3, Section 4), with males orienting themselves to ASG females more often. This effect was also reflected in the total time males spent oriented to females (glmer, z = -4.11, p < 0.001; Appendix 4.3, Section 4), with males spending significantly longer oriented to ASG females, although this was also marginally affected by diet crossed male treatment (glmer, z = -1.81, p = 0.070; Appendix 4.3, Section 4), with ASG males reared on Starch spending more time in orientation to females.

The frequency of bouts of aggressive behaviours and unsuccessful copulation attempts was significantly affected by diet crossed male treatment (glmer, p <

0.04 in both cases; Appendix 4.3, Section 4), with Starch males reared on ASG conducting more of both behaviours. Attempted copulations were also significantly affected by female dietary background (glmer, $z = -3.66$, $p < 0.001$; Appendix 4.3, Section 4) with males paired with Starch females exhibiting more unsuccessful attempts at copulation.

Summary of 'diet crossed' behavioural analysis results

Following the sequential scoring of behaviours performed by 'diet crossed' males, a behavioural profile emerged that differed markedly from that previously retrieved from males reared on their own regime background diet. The relationship between the increase in courtship behaviour and copulation success suggested by behaviours recorded in the previous generation was not present when the proximate effects of diet were switched between treatments. Here, the effect of female rearing background upon courtship specific behaviours seen in the on diet males tested in the previous generation was also retrieved from diet crossed male behaviour, and to a greater extent. Males of both backgrounds exhibited more bouts of courtship behaviours, and spent more time in them overall, when paired with ASG females. Non-courtship behaviour again showed less variation between treatments, but suggested heightened levels of overall activity in Starch males reared on ASG.

4.5 Discussion

No choice mating tests were used to test for sexual isolation between males and females of the sucrose based 'ASG' and starch based 'Starch' populations of the evolution experiment described in Chapter 3. Three allopatric replicates of each population were tested when reared on their regime diets ('on diet') after 29 generations of selection. Also, in order to reveal the proximate effects of the two diets, eggs from this generation were seeded onto the opposing larval diet to permit a replication of the no choice mating tests in generation 30, where males were 'diet crossed'. During both repeats of the mating tests, the courtship behaviour of males that successfully copulated was analysed for evidence of behavioural divergence between the two populations, in order to gain insight into the potential mechanism of any sexual isolation observed.

The mating tests conducted in both generations confirmed the result seen at generation 30 in Chapter 3: there was no retrievable signal of sexual isolation between the populations at this time point. It was conjectured that the limited

choice mating tests used in Chapter 3 might have obscured any assortative mating. As these tests where conducted on a quartet, where a male and a female of each population were tested simultaneously, the first male to mate may have been favoured by the proximate advantage of development on ASG, the more nutritionally complex diet (Chapter 3). Here, the removal of intrasexual competition showed that this idea was not correct. The competitive advantage which males reared on ASG (either on diet or diet crossed) exhibited remained, as in both generations tested either ASG males, or Starch males reared on ASG, secured significantly higher proportions of matings than either Starch males or ASG males reared on Starch.

Sequential analysis of courtship behaviour leading to successful copulation showed that males reared in their own selective background exhibited different levels of activity prior to copulation. ASG males engaged in more bouts of 'active' courtship behaviours: wing vibration and head rocking. Contrary to this, Starch males exhibited a significantly higher number of bouts of, and spent a higher total time with their rectal epithelial gland (REG) extruded. This behaviour is considered a proxy for pheromonal signalling (Briceño et al. 1996), and may represent less energetically costly 'passive' courtship. Although care must be taken when considering the energetic costs of behaviour (Clark 2012), courtship behaviours are often highly energetic, and have been shown to elevate metabolic rate (e.g. Kotiaho et al. 1998).

The manifestation of a more 'active' courtship profile in males reared on ASG, which is more nutritionally complex and of a higher calorific value (Chapter 3, ASG 684 Kcal/L v. Starch 281 Kcal/L), may represent the fact that this larval diet allows males to store higher levels of nutrients during development, shown to be important to male competitiveness in the medfly (Yuval et al. 1998; Kaspi et al. 2002). Alternatively, this effect could represent some form of developmental 'programming' of metabolism (e.g. Fang et al. 2014). It has been suggested that variation in resting metabolic rate may lead to trade offs between types of behaviour or levels of activity (Biro and Stamps 2010). For example, wolf spiders (*Schizocosa sp.*) exhibit a high metabolic cost of courtship (Kotiaho et al. 1998), and a strong interaction has been shown between developmental diet, adult diet, courtship, and copulation success (Rosenthal and Hebets 2012, 2015). Indeed different *Schizocosa* species, which adopt different 'active' and 'passive' courtship strategies, exhibit large differences in energy expenditure (Cady et al. 2011). To

fully quantify this effect here, detailed calculation of energy budgeting would be required, with inclusion of other behaviour such as food intake. This approach was used by (Trudel et al. 2001) to analyse different (non courtship) activity patterns suggested to drive divergence between morphs of lake whitefish (*Coregonus clupeaformis*).

Despite some evidence that the larval diets used to provide divergent selection between populations may favour different modes of courtship, there was no evidence from these data so far that this difference in courtship behaviour was heritable. When individuals were reared on the opposing larval diet for a single generation, the patterns of behaviour leading to successful copulation did not persist. However, in comparison with the behaviours of the on diet males, diet crossed male behaviour and copulatory success did serve to highlight the potential perception of 'quality' between males and females of the two populations. As diet crossed males were paired with on diet females, females retained the proximate effects of rearing diet present in the experimental evolution populations. Diet crossed males of both backgrounds expressed more of all four courtship behaviours recorded when paired with ASG females. Again, relating to the nutritional quality of larval diet (see above), this shows that females from a more nutritionally rich ASG background were of more interest to males, suggesting higher perceived female 'quality'. This is in line with findings that show female medfly with access to higher levels of protein and sugar during development are more likely to mate, more fecund, and reach sexual maturation faster (Kaspi et al. 2002).

Higher expression of courtship behaviours towards ASG females was also present in the on diet mating pairs, but in these pairs male and female quality was presumably aligned, as proximate effect of regime diet was present in both sexes. This led to the pairs most likely to copulate being 'high quality' ASG males, with 'high quality' ASG females. After diet crossing, 'high quality' ASG females still elicited the most courtship from both crossed male backgrounds, but here females preferentially mated with males of the high quality, Starch males with the transferred proximate advantage of ASG rearing; thus copulatory success followed the proximate effect of the higher quality developmental diet. However, leading from this, it is interesting to note the despite this maintenance of male effort in courtship towards ASG females, the highest proportion of successful

copulations seen in generation 30 was between Starch on ASG males and Starch females.

The fact that diet crossed males from both backgrounds elevated their level of courtship towards higher quality females, regardless of their population, underlines the lack of divergence in mate choice by regime. The frequency and time spent in courtship behaviours performed towards Starch females was significantly lower by both backgrounds of diet crossed males. However, lower quality Starch background females were more likely to form pairs than ASG females. Firstly, this could suggest that low quality females were less able to resist male attempts to copulate. Female resistance to mating has been shown to be important in copulation success in the medfly (Arita and Kaneshiro 1988; Whittier et al. 1994), and large body size, as a product of favourable developmental conditions have been show to lower female ability to resist copulation attempts (Taylor and Yuval 1999). Alternatively, this finding could suggest that the role of female choice is more definitive to the outcome of copulation success in the medfly. In both generations tested here, females could have been expressing choice for high quality males of their own regime, and that the proximate effects of two rearing diets change the availability of high quality males within the population. The high quality ASG diet may have made high quality males are more abundant, thus females of their own mate with them more readily and more ASG/ASG matings occur. When Starch males were reared on ASG the abundance of high quality Starch males was increased (after a generation of high nutrient development), and thus Starch females then mate more readily, and more Starch (on ASG)/Starch matings occur.

In the medfly, female behaviours are important both in initiating and securing successful copulation (Briceño and Eberhard 1998, 2002b). This is in line with other arthropods where female behaviour has been shown to influence male mate choice (e.g. Swierk et al. 2013). It would appear that the choice of both sexes captured here is dominated by proximate quality of individuals, potentially manifested through condition dependence in courtship traits. The pairing Starch females and Starch males on ASG did not match this pattern; despite low occurrence of male courtship behaviour towards Starch females, success copulations were abundant. This could suggest that the behaviours recorded, although selected to proxy multiple modes of signalling, did not capture important information signals such as olfactory or auditory cues, may have

transferred altering compatibility recognition. Although proxies may show the abundance of behaviours associated with these cues; the identity and quality information that they confer may be very different. Auditory cues have been shown to exhibit some divergence between wild populations of medfly (Briceño et al. 2002). Chemosensory cues, mediated by pheromonal communication, are key mechanisms of divergence in many systems (reviewed in Smadja and Butlin 2009), but have been directly associated with speciation driven divergent ecological selection by larval diet both in natural populations (e.g. Etges et al. 2009), and in experimental evolution studies (Rundle et al. 2005). As the medfly has a well characterised pheromonal composition (Baker et al. 1985; Levinson et al. 1987; Jang et al. 1989, 1994; Heath et al. 1991; Light et al. 1999; Gonçalves et al. 2006; Mavraganis et al. 2008; Siciliano et al. 2014), this would be a promising avenue of further study.

To summarise these findings, although sexual isolation was not apparent between the ASG and Starch populations, divergent selection imposed by larval rearing diet may be affecting change on condition dependant signals of quality upon which sexual selection is acting. Many models show that the action of sexual selection can facilitate the rapid divergence of populations (e.g. Lande 1982; Turner and Burrows 1995; Gavrilets and Boake 1998; van Doorn et al. 2009; although see Ryan and Rand 1993). Although heritable differences in male courtship behaviour was not captured here, the manifestation of choice by females may represent the basis for the divergence driven by sexual selection leading to the non random mating observed at a later time point in this evolution experiment (Chapter 3).

Although the findings of this study have highlighted important information regarding the nature of the divergence between populations, several improvements to the experimental design could have been made. Although the design revealed the proximate effects of diet, the diet crossing design could have replaced by a common garden rearing strategy, as used in generation 60 of Chapter 3. This might have facilitated the detection of a genetic basis to the behaviours studied. It is also important to note that in this study, due to logistical considerations, the diet cross treatments were conducted in the generation following the mating tests on the 'on diet' treatments. Hence generation could potentially have confounded the on diet versus diet crossing tests. Future studies could usefully capture both proximate and ultimate effects of diet by employing

common garden rearing on the same generation of flies, if possible. A further extension to the design, which would be essential to fully attributing the changes in behaviour captured here to adaptation to diet, would be the testing between replicate populations within dietary treatments. Such 'same host' mating tests would be the most direct way to account for the possibility that genetic drift between allopatric population pairs was the driver of any behavioural divergence captured. Females used in mating tests at generation 30 were from the relevant experimental regime. Hence proximate effects of diet may have affected choice in these mating tests. Outside of a common garden design, mating tests between diet crossed females an on diet males would be required to fully understand the behaviour and choices reported here. One of the largest improvements to the design would address the bias in copulation success, which led to uneven sampling between lines and across line replicates. Although the analytical method used allowed inference from these data, even sampling between population replicates is essential to assess the action of genetic drift in population divergence, as over representation of a single replicate may hide divergence between replicates. Drift genetic drift is suggested to potentially contribute to population differentiation when working in concert with sexual selection (Lande 1981; Kirkpatrick 1982; Rice 1998). The data from the EE experiment studied here suggests that genetic drift is not a primary driver of divergence. However, this remains to be confirmed by measuring the impact of drift and also using a design that can better account for differences in mating suggested and therefore uniformity of sample size.

The findings suggest great potential for further study of behavioural divergence the medfly. Although condition dependence of behaviours and resulting mate choice is implicated, accurate identification of such condition dependence requires careful recording of both body size and trait values across full range of environmental stresses (Cotton et al. 2004). Further to this, only male behaviour was studied here. Further study of female behaviour could provide insight into compatibility recognition systems in lekking species. If coupled with more in depth quantification of signals such as pheromonal communication, the medfly could act as an ideal experimental model for the study of behavioural divergence in the establishment of reproductive isolation.

4.6 References

Anjos-Duarte, C. S., A. M. Costa, and I. S. Joachim-Bravo. 2011. Sexual

behaviour of the Mediterranean fruit fly (Diptera: Tephritidae): the influence of female size on mate choice. J. Appl. Entomol. 135:367–373.

Arbuthnott, D., and B. J. Crespi. 2009. Courtship and mate discrimination within and between species of *Timema* walking-sticks. Anim. Behav. 78:53–59.

Arita, L. H., and K. Y. Kaneshiro. 1988. Body size and differential mating success between males of two populations of the Mediterranean fruit fly. Pacific Sci. 42:173–177.

Arita, L. H., and K. Y. Kaneshiro. 1986. Structure and function of the rectal epithelium and anal glands during mating behavior in the Mediterranean fruit fly male. Proc. Hawaiian Entomol. Soc. 25:27 – 30.

Baker, R., R. H. Herbert, and G. G. Grant. 1985. Isolation and identification of the sex pheromone of the mediterranean fruit fly, *Ceratitis capitata* (Wied). J. Chem. Soc. Chem. Commun. 824–825.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 67:1–48.

Benelli, G., G. Giunti, A. Canale, and R. H. Messing. 2014. Lek dynamics and cues evoking mating behavior in tephritid flies infesting soft fruits: implications for behavior-based control tools. Appl. Entomol. Zool. 49:363–373.

Biro, P. A., and J. A. Stamps. 2010. Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? Trends Ecol. Evol. 25:653–9.

Blumstein, D. T., C. S. Evans, and J. C. Daniels. 2006. JWatcher 1.0.

Boake, C. R. B. 2005. Sexual selection and speciation in Hawaiian *Drosophila*. Behav. Genet. 35:297–303.

Briceño, R. D., and W. Eberhard. 2002a. Courtship in the medfly, *Ceratitis capitata*, includes tactile stimulation with the male's aristae. Entomol. Exp. Appl. 102:221–228.

Briceño, R. D., and W. G. Eberhard. 2002b. Decisions during courtship by male and female medflies (Diptera, Tephritidae): Correlated changes in male behavior and female acceptance criteria in mass-reared flies. Florida Entomol. 85:14–31.

Briceño, R. D., and W. G. Eberhard. 1998. Medfly courtship duration: a sexually selected reaction norm changed by crowding. Ethol. Ecol. Evol. 10:369–382.

Briceño, R. D., W. G. Eberhard, J. C. Vilardi, P. Liedo, and T. E. Shelly. 2002.

Variation in the intermittent buzzing songs of male medflies (Diptera : Tephritidae) associated with geography, mass-rearing, and courtship success. Florida Entomol. 85:32–40.

Briceño, R. D., W. G. Eberhard, J. Vilardi, J. Cayol, and T. Shelly. 2007. Courtship behavior of different wild strains of *Ceratitis capitata* (Diptera: Tephritidae). Florida Entomol. 90:15–18.

Briceño, R. D., D. Ramos, and W. G. Eberhard. 1996. Courtship behavior of male *Ceratitis capitata* (Diptera: Tephritidae) in captivity. Florida Entomol. 79:130–143.

Buchanan, K. L., J. L. Grindstaff, and V. V. Pravosudov. 2013. Condition dependence, developmental plasticity, and cognition: implications for ecology and evolution. Trends Ecol. Evol. 28:290–296.

Butlin, R. K., and M. G. Ritchie. 1989. Genetic coupling in mate recognition systems: what is the evidence? Biol. J. Linn. Soc. 37:237–246.

Cady, A. B., K. J. Delaney, and G. W. Uetz. 2011. Contrasting energetic costs of courtship signaling in two wolf spiders having divergent courtship behaviors. J. Arachnol. 39:161–165.

Chenoweth, S. F., N. C. Appleton, S. L. Allen, and H. D. Rundle. 2015. Genomic evidence that sexual selection impedes adaptation to a novel environment. Curr. Biol. 25:1860–1866.

Chung, H., D. W. Loehlin, H. D. Dufour, K. Vaccarro, J. G. Millar, and S. B. Carroll. 2014. A single gene affects both ecological divergence and mate choice in *Drosophila*. Science 343:1148–51.

Clark, C. J. 2012. The role of power versus energy in courtship: what is the "energetic cost" of a courtship display? Anim. Behav. 84:269–277.

Cotton, S., K. Fowler, and A. Pomiankowski. 2004. Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? Proc. Biol. Sci. 271:771–83.

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

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Dobzhansky, T., and E. Mayr. 1944. Experiments on Sexual Isolation in *Drosophila*: I. Geographic strains of *Drosophila willistoni*. Proc. Natl. Acad. Sci. U. S. A. 30:238–44.

Etges, W. J., C. C. de Oliveira, E. Gragg, D. Ortíz-Barrientos, M. a F. Noor, and M. G. Ritchie. 2007. Genetics of incipient speciation in *Drosophila mojavensis*. I. Male courtship song, mating success, and genotype X environment interactions. Evolution. 61:1106–1119.

Etges, W. J., C. C. de Oliveira, M. G. Ritchie, and M. A. F. Noor. 2009. Genetics of incipient speciation in *Drosophila mojavensis*: II. Host plants and mating status influence cuticular hydrocarbon QTL expression and G x E interactions. Evolution 63:1712–30.

Fang, L., X.-F. Liang, Y. Zhou, X.-Z. Guo, Y. He, T.-L. Yi, L.-W. Liu, X.-C. Yuan, and Y.-X. Tao. 2014. Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, *Danio rerio*. Br. J. Nutr. 111:808–18.

Feron, M. 1962. L'instinct de reproduction chez la mouche Mediterranéenne des fruits (*Ceratitis capitata*, Wiedemann) (Diptera, Tephritidae). Comportement sexuel. Comportement de ponte. Rev. Pathol. Végétale d'Entomologie Agric. Fr. 41:1–129.

Gavrilets, S., and C. R. Boake. 1998. On the evolution of premating isolation after a founder event. Am. Nat. 152:706–16.

Gonçalves, G. B., C. E. Silva, J. C. G. Dos Santos, E. S. Dos Santos, R. R. Do Nascimento, E. L. Da Silva, A. De Lima Mendonça, M. Do Rosário Tenório De Freitas, and A. E. G. Sant'Ana. 2006. Comparison of the volatile components released by calling males of *Ceratitis capitata* (Diptera: Tephritidae) with those extractable from the salivary glands. Florida Entomol. 89:375–379.

Heath, R. R., P. J. Landolt, J. H. Tumlinson, D. L. Chambers, R. E. Murphy, R. E. Doolittle, B. D. Dueben, J. Sivinski, and C. O. Calkins. 1991. Analysis, synthesis, formulation, and field testing of three major components of male Mediterranean fruit fly pheromone. J. Chem. Ecol. 17:1925–40.

Hill, G. E. 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. Ecol. Lett. 14:625–34.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. J. Avian Biol. 31:559–566.

Honda-Sumi, E. 2005. Difference in calling song of three field crickets of the

genus *Teleogryllus*: the role in premating isolation. Anim. Behav. 69:881–889.

Jang, E. B., D. M. Light, R. G. Binder, R. A. Flath, and L. A. Carvalho. 1994. Attraction of female Mediterranean fruit flies to the five major components of male-produced pheromone in a laboratory flight tunnel. J. Chem. Ecol. 20:9–20.

Jang, E. B., D. M. Light, R. a. Flath, J. T. Nagata, and T. R. Mon. 1989. Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata* to identified volatile constituents from calling males. Entomol. Exp. Appl. 50:7–19.

Jennings, J. H., and W. J. Etges. 2009. Species hybrids in the laboratory but not in nature: a reanalysis of premating isolation between *Drosophila arizonae* and *D. mojavensis*. Evolution 64:587–98.

Jiggins, C. D. 2008. Ecological speciation in mimetic butterflies. Bioscience 58:541–548.

Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. Nature 411:302–5.

Joachim-Bravo, I. S., C. S. Anjos, and A. M. Costa. 2009. The role of protein in the sexual behaviour of males of *Ceratitis capitata* (Diptera: Tephritidae): mating success, copula duration and number of copulations. Zool. (Curitiba, Impresso) 26:407–412.

Kaneshiro, K. Y. 1980. Sexual isolation, speciation and the direction of evolution. Evolution. 34:437–444.

Kaspi, R., S. Mossinson, T. Drezner, B. Kamensky, and B. Yuval. 2002. Effects of larval diet on development rates and reproductive maturation of male and female Mediterranean fruit flies. Physiol. Entomol. 27:29–38.

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

Kotiaho, J. S., R. V. Alatalo, J. Mappes, M. G. Nielsen, S. Parri, and A. Rivero. 1998. Energetic costs of size and sexual signalling in a wolf spider. Proc. R. Soc. B Biol. Sci. 265:2203–2209.

Kuznetsova, A., P. Brockhoff, and R. Christensen. 2013. lmerTest: Tests for random and fixed effects for linear mixed effect models (lmer objects of lme4 package). R Packag. version.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proc. Natl. Acad. Sci. 78:3721–3725.

Lande, R. 1982. Rapid Origin of Sexual Isolation and Character Divergence in a Cline. Evolution. 36:213–223.

Levinson, H. Z., A. R. Levinson, and K. Schäfer. 1987. Pheromone biology of the Mediterranean fruit fly (*Ceratitis capitata*, Wied.) with emphasis on the functional anatomy of the pheromone glands and antennae as well as mating behaviour. J. Appl. Entomol. 104:448–461.

Light, D. M., E. B. Jang, R. G. Binder, R. A. Flath, and S. Kint. 1999. Minor and intermediate components enhance attraction of female Mediterranean fruit flies to natural male odor pheromone and its synthetic major components. J. Chem. Ecol. 25:2757–2777.

Lux, S. A., F. N. Munyiri, J. C. Vilardi, P. Liedo, A. Economopoulos, O. Hasson, and S. Quilici. 2002. Consistency in courtship pattern among populations of Medfly (Diptera: Tephritidae): comparisons among wild strains and strains mass reared for SIT Operations. Florida Entomol. 85:113.

Maan, M. E., and O. Seehausen. 2011. Ecology, sexual selection and speciation. Ecol. Lett. 14:591–602.

Mateos-Gonzalez, F., J. Quesada, and J. C. Senar. 2011. Sexy birds are superior at solving a foraging problem. Biol. Lett. 7:668–9.

Mavraganis, V. G., C. Liaropoulos, N. T. Papadopoulos, N. A. Kouloussis, and T. Broumas. 2008. Whole body extract of Mediterranean fruit fly males elicits high attraction in virgin females. Entomol. Exp. Appl. 127:20–29.

Mendelson, T. C., and K. L. Shaw. 2012. The (mis)concept of species recognition. Trends Ecol. Evol. 27:421–7.

Merrill, R. M., R. W. R. Wallbank, V. Bull, P. C. A. Salazar, J. Mallet, M. Stevens, and C. D. Jiggins. 2012. Disruptive ecological selection on a mating cue. Proc. Biol. Sci. 279:4907–13.

Miller, G., G. Stratton, P. Miller, and E. Hebets. 1998. Geographical variation in male courtship behaviour and sexual isolation in wolf spiders of the genus *Schizocosa*. Anim. Behav. 56:937–951.

Nagel, L., and D. Schluter. 1998. Body Size, Natural Selection, and Speciation in Sticklebacks. Evolution. 52:209–218.

Nosil, P. 2012. Ecological Speciation. Oxford University Press, Oxford.

Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and

speciation. Trends Ecol. Evol. 16:364–371.

Pillay, N., and T. L. Rymer. 2012. Behavioural divergence, interfertility and speciation: a review. Behav. Processes 91:223–35.

Prokopy, R. J., and J. Hendrichs. 1979. Mating behavior of *Ceratitis capitata* on a field-caged host tree. Ann. Entomol. Soc. Am. 72:642–648.

Puniamoorthy, N. 2014. Behavioural barriers to reproduction may evolve faster than sexual morphology among populations of a dung fly (Sepsidae). Anim. Behav. 98:139–148.

R Development Core Team, R. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Rice, W. R. 1998. Intergenomic conflict, interlocus antagonistic coevolution, and the evolution of reproductive isolation. Pp. 261–270 *in* D. J. Howard and S. H. Berlocher, eds. Endless forms: species and speciation. Oxford University Press.

Rosenthal, M. F., and E. A. Hebets. 2012. Resource heterogeneity interacts with courtship rate to influence mating success in the wolf spider *Schizocosa floridana*. Anim. Behav. 84:1341–1346.

Rosenthal, M. F., and E. A. Hebets. 2015. Temporal patterns of nutrition dependence in secondary sexual traits and their varying impacts on male mating success. Anim. Behav. 103:75–82.

Rowe, L., and D. Houle. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc. R. Soc. B 263:1415–1421.

Rundle, H. D., S. F. Chenoweth, P. Doughty, and M. W. Blows. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLoS Biol. 3:e368.

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336–352.

Ryan, M. J., and A. S. Rand. 2003. Sexual selection in female perceptual space: How female Tungara frogs perceive and respond to complex population variation in acoustic mating signals. Evolution. 57:2608–2618.

Ryan, M. J., and A. S. Rand. 1993. Species recognition and sexual selection as a unitary problem in animal communication. Evolution. 47:647–657.

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

Senar, J. C., J. Domenech, and M. Camerino. 2004. Female siskins choose mates

by the size of the yellow wing stripe. Behav. Ecol. Sociobiol. 57:465–469.

Servedio, M. R., G. S. Van Doorn, M. Kopp, A. M. Frame, and P. Nosil. 2011. Magic traits in speciation: "magic" but not rare? Trends Ecol. Evol. 26:389–97.

Siciliano, P., X. L. He, C. Woodcock, J. A. Pickett, L. M. Field, M. A. Birkett, B. Kalinova, L. M. Gomulski, F. Scolari, G. Gasperi, A. R. Malacrida, and J. J. Zhou. 2014. Identification of pheromone components and their binding affinity to the odorant binding protein CcapOBP83a-2 of the Mediterranean fruit fly, *Ceratitis capitata*. Insect Biochem. Mol. Biol. 48:51–62.

Smadja, C., and R. K. Butlin. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity. 102:77–97.

Smadja, C. M., and R. K. Butlin. 2011. A framework for comparing processes of speciation in the presence of gene flow. Mol. Ecol. 20:5123–40.

Snook, R. R., A. Robertson, H. S. Crudgington, and M. G. Ritchie. 2005. Experimental manipulation of sexual selection and the evolution of courtship song in *Drosophila pseudoobscura*. Behav. Genet. 35:245–55.

Stratton, G. E. 2005. Evolution of ornamentation and courtship behaviour in *Schizocosa*: Insights from a phylogeny based on morphology (Araneae, Lycosidae). J. Arachnol. 33:347–376.

Swierk, L., A. Myers, and T. Langkilde. 2013. Male mate preference is influenced by both female behaviour and morphology. Anim. Behav. 85:1451–1457.

Taylor, P. W., and B. Yuval. 1999. Postcopulatory sexual selection in Mediterranean fruit flies: advantages for large and protein-fed males. Anim. Behav. Anim Behav 58:247–254.

Trudel, M., A. Tremblay, R. Schetagne, and J. B. Rasmussen. 2001. Why are dwarf fish so small? An energetic analysis of polymorphism in lake whitefish (*Coregonus clupeaformis*). Can. J. Fish. Aquat. Sci. 58:394–405.

Turner, G. F., and M. T. Burrows. 1995. A model of sympatric speciation by sexual selection. Proc. R. Soc. B Biol. Sci. 260:287–292.

van Doorn, G. S., P. Edelaar, and F. J. Weissing. 2009. On the origin of species by natural and sexual selection. Science 326:1704–7.

Vu, V. 2011. ggbiplot.

Whittier, T. S., F. Y. Nam, T. E. Shelly, and K. Y. Kaneshiro. 1994. Male courtship success and female discrimination in the Mediterranean fruit fly (Diptera:

Tephritidae). J. Insect Behav. 7:159–170.

Williams, T. H., and T. C. Mendelson. 2011. Female preference for male coloration may explain behavioural isolation in sympatric darters. Anim. Behav. 82:683–689.

Wilson, E. B. 1927. Probable inference, the law of succession, and statistical inference. J. Am. Stat. Assoc. 22:209–212.

Yuval, B., and J. Hendrichs. 2000. Behavior of flies in the genus *Ceratitis* (Dacinae: Ceratitidine). Pp. 429–457 *in* M. Aluja and A. L. Norrbom, eds. Fruit flies (Tephritidae): phylogeny and evolution of behavior. CRC Press LLC, Boca Raton, FL.

Yuval, B., R. Kaspi, S. Shloush, and M. S. Warburg. 1998. Nutritional reserves regulate male participation in Mediterranean fruit fly leks. Ecol. Entomol. 23:211–215.

4.7 Appendices

4.7.1 Appendix 4.1 - Supplementary Tables

Table 4.A1. Mutual exclusivity of male behaviours scored during courtship with an individual female. 0 denotes the ability to co-occur, whilst 1 indicates mutual exclusivity. For example: 'Decamping' is mutually exclusive to all other behaviours, when it occurs all other bouts end. 'Locomotion' is mutually exclusive to all behaviours except 'Continuous Wing Vibration' and 'Gland Extrusion', a bout of continuous wing buzzing can continue whilst the male is moving around the filming cell. 'Orientation' is mutually exclusive to itself, other non-courtship traits, and copulation. It can co-occur with other courtship behaviours such as 'Head Rocking', as well as 'aggression', which can occur with out interrupting a male's orientation to the female.

	Gland Extrusion	Continuous Wing Vibration	Orientation	Head Rocking	Intermittent Wing Vibration	Locomotion	Preening	Aggression	Stationary	Decamping	Copulation	Copulation Attempt
Gland Extrusion	1	0	0	0	0	0	0	1	0	1	1	1
Continuous Wing Vibration	0	1	0	0	1	0	0	1	1	1	1	1
Orientation	0	0	1	0	0	1	1	0	1	1	1	1
Head Rocking	0	0	0	1	0	1	1	1	1	1	1	1
Intermittent Wing Vibration	0	1	0	0	1	1	1	1	1	1	1	1
Locomotion	0	0	1	1	1	1	1	1	1	1	1	1
Preening	0	0	1	1	1	1	1	1	1	1	1	1
Aggression	1	1	0	1	1	1	1	1	1	1	1	1
Stationary	0	1	1	1	1	1	1	1	1	1	1	1
Decamping	1	1	1	1	1	1	1	1	1	1	1	1
Copulation	1	1	1	1	1	1	1	1	1	1	1	1
Copulation Attempt	1	1	1	1	1	1	1	1	1	1	1	1

4.7.2 Appendix 4.2 - Principal components analysis exemplar result

Methods

Principal components analysis (PCA) was implemented using the pr.comp function in R {stats} (R Development Core Team 2015). Data were scaled within the function so as to have unit variance prior to the analysis, and also zero centred. This methodology was conducted on the total bout frequency (BF) and total time (TT) datasets for both generations 29 and 30. Success of the analysis was assessed by consultation of the loadings produced in each case, and also by visualisation of the results as biplots, created using the package ggbiplot (Vu 2011).

Results

Presented here are the results of the PCA conducted on the data collected for total time spent in four courtship and four non courtship behaviours from mating tests conducted in generation 29, with males reared 'on diet'. Table 4.A2 shows the proportion of variance each principal component (PC) generated accounts for, as well as the loadings relative to each behaviour scored for TT. The disperse pattern of variance between PCs was seen for all other data analysed. As the majority of the data were described by 6 or more PCs and all PCs carried loadings approaching 1 or -1, it was not seen as advantageous to substitute the raw data for PCs, as dimensionality would not have been reduced.

Figure 4.A1 provides a pairwise comparison the first four PCs described in Table 4.A2. The pattern of crossover seen in the normal ellipses presented in the figure supports the limitations in their descriptive power, again supporting the decision to analyse the raw data.

Table 4.A2. Principal components analysis of total time spent in a suite of 8 behaviours scored sequentially during courtship leading to successful copulation for on diet males in generation 29.

Importance of components:								
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Standard deviation	1.86	1.14	1.06	0.97	0.70	0.63	0.48	0.23
Proportion of variance	0.43	0.16	0.14	0.12	0.06	0.05	0.03	0.01
Cumulative proportion	0.43	0.59	0.74	0.85	0.91	0.96	0.99	1
Loadings:								
Continuous	-0.471	0.196	-0.018	-0.149	-0.016	-0.123	0.820	0.171
Intermittent	-0.485	-0.029	-0.113	0.265	0.123	-0.322	-0.404	0.631
Head Rocking	-0.490	-0.115	-0.083	0.277	0.150	-0.281	-0.090	-0.744
Gland out	-0.153	0.592	-0.102	-0.650	-0.127	-0.189	-0.353	-0.133
Orientation	-0.440	0.121	0.014	0.014	0.129	0.868	-0.144	-0.009
Preening	0.212	0.073	-0.764	-0.045	0.594	0.038	0.094	0.012
Locomotion	-0.120	-0.553	0.314	-0.586	0.481	-0.057	-0.043	0.035
Stationary	0.161	0.521	0.535	0.250	0.588	-0.086	0.025	-0.005

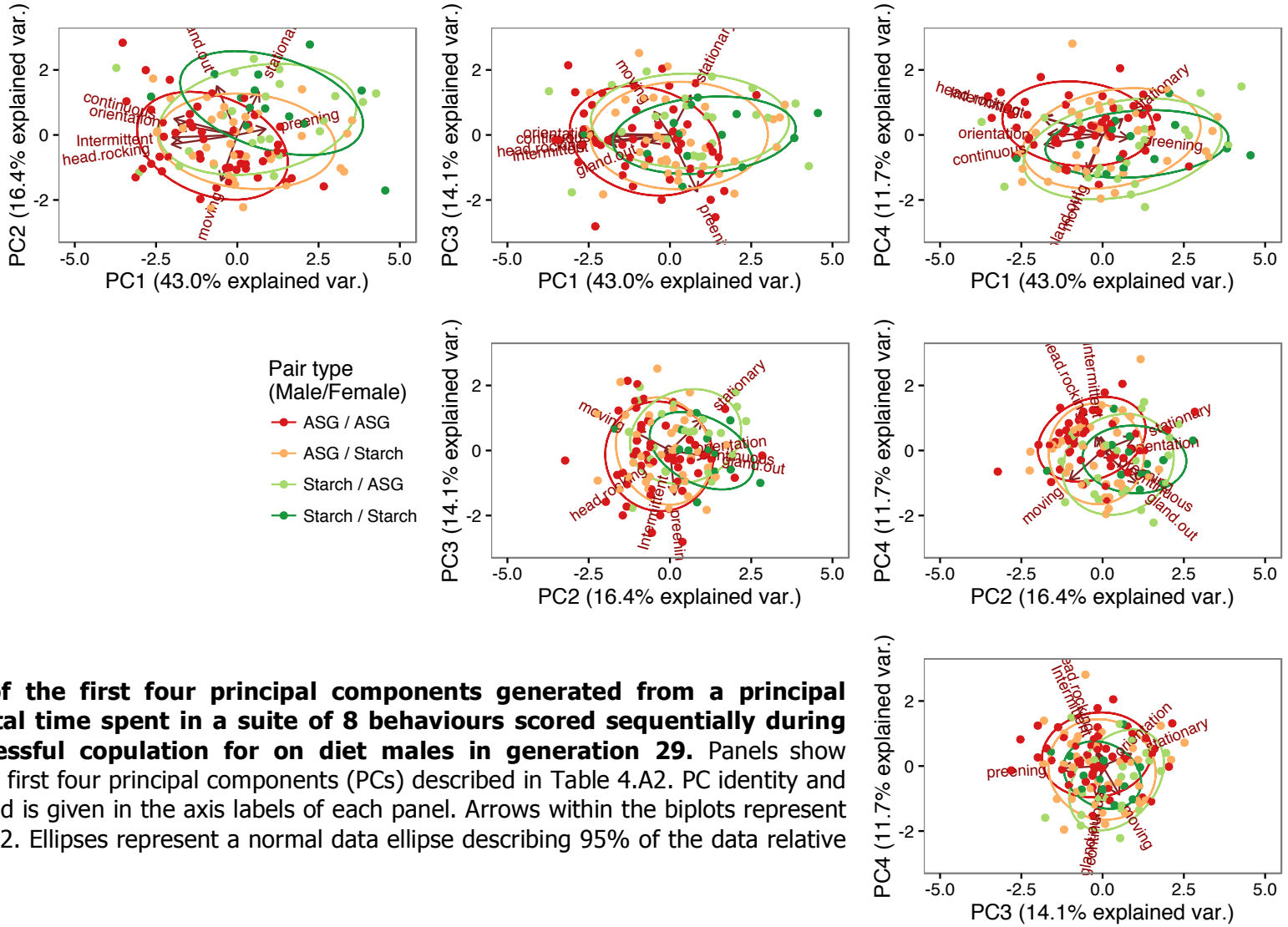

Figure 4.A1 Comparison of the first four principal components generated from a principal components analysis of total time spent in a suite of 8 behaviours scored sequentially during courtship leading to successful copulation for on diet males in generation 29. Panels show covariance biplots between the first four principal components (PCs) described in Table 4.A2. PC identity and proportion of variance explained is given in the axis labels of each panel. Arrows within the biplots represent the loadings given in Table 4.A2. Ellipses represent a normal data ellipse describing 95% of the data relative to each dietary treatment.

4.7.3 Appendix 4.3 - Supplementary statistical information

Methods

Generalised linear mixed models were fitted for discrete counts of bout frequencies and for a discrete measure of the total number of milliseconds for which each behaviour occurred. For the analysis of the generation 29 (on diet) data, fixed effects were the regime diet of the male and the female. For the generation 30 (diet cross) data, fixed effects were the regime diet/rearing diet of the male and the regime diet of the female. A random effect was added into both models to nest the effect of line replicate within regime diet. Models were offset to the log of the duration until copulation, in order to account for differing lengths of overall courtship time. Mixed models were fitted using the lme4 package (ver. 1.1.12) in R (Bates et al. 2015), and tested using the package lmertest (ver. 2.0; Kuznetsova et al. 2013). A Poisson error structure was fitted and the data tested for overdispersion by comparison of the residual deviance of the maximal model with the corresponding residual degrees of freedom (for fixed effects only). Where there was overdispersion (dispersion factor > 1.2) an observation level variable was added as a random effect. Models that encountered convergence errors were fitted with the 'bobyqa' optimizer (lme4). Model selection was conducted by sequential likelihood ratio testing using lmertest::anova, as was description of main effect in selected models.

This appendix contains tables describing the model selection process for each model fitted, and also the parameters of the final model for each behaviour. Models for the bout frequency and total time of each behaviour are presented sequentially. Models which exhibited no significant effect of either fixed effect are not included.

Appendix 4.2 Contents:

Model terms:

n = bout frequency of target behaviour, **tt** = total time spent in target behaviour, **par.diet** = male dietary background, **fem.diet** = female dietary background, **rep** = line replicate, **obs** = observation level variable, **cop** = latency to copulation.

Section 1: Generation 29 Courtship behaviour model selection and summary tables

Bout frequency of continuous wing vibration behaviour

Model selection

m5_b1_cont_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_cont_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_cont_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_cont_n	4	650.53	661.68	-321.26	642.53			
m3_b1_cont_n	6	646.01	662.74	-317.01	634.01	8.5148	2	0.01416
m2_b1_cont_n	7	647.89	667.4	-316.94	633.89	0.1244	1	0.72426

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.8755	0.9357
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-11.9056	0.149	-79.91	<0.001
par.dietstarch	-0.4619	0.2254	-2.05	0.0404
fem.dietS	-0.5093	0.2109	-2.42	0.0157

Total time spent in continuous wing vibration behaviour

Model selection

m5_b1_cont_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_cont_tt: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_cont_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_cont_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_cont_tt	4	2595.90	2607.00	-1293.90	2587.90			
m4_b1_cont_tt	5	2594.70	2608.70	-1292.40	2584.70	3.17	1	0.0752
m3_b1_cont_tt	6	2596.30	2613.00	-1292.10	2584.30	0.45	1	0.5014
m2_b1_cont_tt	7	2597.70	2617.20	-1291.90	2583.70	0.55	1	0.4593

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	13.90	3.73
rep:par.diet	(Intercept)	1.27	1.13
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-4.72	0.65	-7.22	< 0.001
Fem.dietS	-1.29	0.71	-1.81	0.0709

Bout frequency of intermittent wing vibration behaviour

Model selection

m5_b1_ag_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_ag_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_ag_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_ag_n	4	562.84	573.99	-277.42	554.84			
m3_b1_ag_n	6	554.29	571.02	-271.15	542.29	12.54	2	0.0019
m2_b1_ag_n	7	554.61	574.13	-270.31	540.61	1.68	1	0.1949

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.7203	0.8487
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed Effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-12.24	0.15	-84.23	< 0.001
fem.dietS	-0.61	0.22	-2.73	0.0064
fem.dietS	-0.55	0.21	-2.65	0.0081

Total time spent in intermittent wing vibration behaviour

Model selection

m5_b1_int_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_int_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_int_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_int_tt	4	2602.80	2613.90	-1297.40	2594.80			
m3_b1_int_tt	6	2600.50	2617.30	-1294.30	2588.50	6.26	2	0.0436
m2_b1_int_tt	7	2599.60	2619.10	-1292.80	2585.60	2.95	1	0.0858

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	10.28	3.21
rep:par.diet	(Intercept)	0.01	0.11
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed Effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-4.1419	0.4387	-9.442	< 0.001
par.dietstarch	-2.0533	0.6941	-2.958	0.0031
fem.dietS	-0.9574	0.6097	-1.57	0.1164

Bout frequency of head rocking behaviour

Model selection

m5_b1_cont_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_cont_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_cont_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_cont_n	4	607.71	618.86	-299.86	599.71			
m3_b1_cont_n	6	593.60	610.33	-290.80	581.60	18.11	2	0.0001
m2_b1_cont_n	7	592.57	612.08	-289.29	578.57	3.03	1	0.0817

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.9083	0.9531
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-11.83	0.16	-75.10	< 0.001
par.dietstarch	-1.36	0.31	-4.38	< 0.001
fem.dietS	-0.99	0.26	-3.84	< 0.001
par.dietstarch: fem.dietS	0.88	0.51	1.74	0.0818

Total time spent in head rocking behaviour

Model selection

m5_b1_hr_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_hr_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_hr_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_ag_n	4	2635.4	2646.5	-1313.7	2627.4			
m3_b1_ag_n	6	2634.4	2651.1	-1311.2	2622.4	4.98	2	0.0829
m2_b1_ag_n	7	2631.9	2651.4	-1308.9	2617.9	4.55	1	0.0329

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	11.40	3.38
rep:par.diet	(Intercept)	1.39	1.18
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed Effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-3.47	0.84	-4.13	< 0.001
par.dietstarch	-3.74	1.34	-2.80	0.0051
fem.dietS	-1.80	0.77	-2.34	0.0192

Bout frequency of gland extrusion behaviour

Model selection

m5_b1_cont_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_cont_n: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_cont_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_cont_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_go_n	4	414.17	425.32	-203.09	406.17			
m4_b1_go_n	5	411.91	425.85	-200.96	401.91	4.26	1	0.0390
m3_b1_go_n	6	412.94	429.67	-200.47	400.94	0.97	1	0.3250
m2_b1_go_n	7	414.81	434.33	-200.41	400.81	0.13	1	0.7207

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.58	1.26
rep:par.diet	(Intercept)	0.02	0.16
par.diet	(Intercept)	< 0.001	< 0.001

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-13.93	0.26	-53.45	< 0.001
par.dietstarch	0.77	0.37	2.07	0.0382

Total time spent with pheromone gland extruded

Model selection

m5_b1_go_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_go_tt: tt ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_go_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_go_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_go_tt	4	1855.9	1867	-923.95	1847.9			
m4_b1_go_tt	5	1847.7	1861.6	-918.83	1837.7	10.24	1	0.0014
m3_b1_go_tt	6	1849.3	1866	-918.63	1837.3	0.39	1	0.5327
m2_b1_go_tt	7	1850.7	1870.2	-918.35	1836.7	0.58	1	0.4474

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	180.9	13.45
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-19.95	0.91	-22.02	< 0.001
par.dietS	12.00	2.66	4.51	< 0.001

Section 2: Generation 29 Non courtship behaviour model selection and summary tables

Bout frequency of stationary behaviour

Model selection

m5_b1_stat_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_stat_n: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_stat_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_stat_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_stat_n	4	827.33	838.48	-409.67	819.33			
m4_b1_stat_n	5	825.28	839.22	-407.64	815.28	4.05	1	0.0442
m3_b1_stat_n	6	827.19	843.92	-407.6	815.19	0.09	1	0.7645
m2_b1_stat_n	7	828.99	848.5	-407.49	814.99	0.21	1	0.6493

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.12	0.35
rep:par.diet	(Intercept)	0.01	0.10
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-10.66	0.08	-137.27	< 0.001
par.dietstarch	0.31	0.12	2.51	0.0120

Total time spent in stationary behaviour

Model selection

m5_b1_stat_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_stat_tt: tt ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_stat_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_stat_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_stat_tt	4	3020.7	3031.8	-1506.3	3012.7			
m4_b1_stat_tt	5	3017.8	3031.8	-1503.9	3007.8	4.85	1	0.0277
m3_b1_stat_tt	6	3017.4	3034.1	-1502.7	3005.4	2.47	1	0.1163
m2_b1_stat_tt	7	3019.4	3038.9	-1502.7	3005.4	0.01	1	0.9074

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.40	1.18
rep:par.diet	(Intercept)	< 0.001	0.03
par.diet	(Intercept)	< 0.001	< 0.001

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-2.62	0.13	-20.09	< 0.001
par.dietstarch	0.71	0.24	2.99	0.0028

Bout Frequency of orientation behaviour

Model selection

m5_b1_ori_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_ori_n: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_ori_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_ori_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_ori_n	4	810.30	821.45	-401.15	802.30			
m4_b1_ori_n	5	802.39	816.33	-396.20	792.39	9.91	1	0.0016
m3_b1_ori_n	6	802.49	819.21	-395.24	790.49	1.91	1	0.1673
m2_b1_ori_n	7	804.47	823.98	-395.23	790.47	0.02	1	0.8850

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.25	0.50
rep:par.diet	(Intercept)	0.01	0.10
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-10.80	0.08	-130.72	< 0.001
Fem.dietS	-0.36	0.11	-3.21	0.0013

Total time spent orientated to female

Model selection

m5_b1_ori_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b1_ori_tt: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_ori_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_ori_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_ori_tt	4	3020.6	3031.8	-1506.3	3012.6			
m4_b1_ori_tt	5	3015.4	3029.3	-1502.7	3005.4	7.23	1	0.0072
m3_b1_ori_tt	6	3014.4	3031.1	-1501.2	3002.4	3.00	1	0.0831
m2_b1_ori_tt	7	3016.4	3035.9	-1501.2	3002.4	0.00	1	0.9470

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.38	1.17
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed Effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-2.05	0.16	-13.04	< 0.001
par.dietstarch	-0.42	0.24	-1.78	0.0755
fem.dietS	-0.62	0.22	-2.83	0.0046

Bout frequency of decamping behaviour

Model selection

m5_b1_dec_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_dec_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_dec_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_ag_n	4	845.06	856.21	-418.53	837.06			
m3_b1_ag_n	6	843.09	859.81	-415.54	831.09	5.97	2	0.0505
m2_b1_ag_n	7	844.45	863.97	-415.23	830.45	0.64	1	0.4255

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.58	1.26
rep:par.diet	(Intercept)	0.12	0.35
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed Effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-12.03	0.28	-42.91	< 0.001
fem.dietS	0.83	0.40	2.06	0.0391
fem.dietS	0.43	0.26	1.67	0.0957

Bout frequency of aggression behaviour

Model selection

m5_b1_ag_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b1_ag_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b1_ag_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b1_ag_n	4	370.49	381.64	-181.26	362.49			
m3_b1_ag_n	6	366.57	383.30	-177.29	354.57	7.92	2	0.0190
m2_b1_ag_n	7	367.21	386.72	-176.60	353.21	1.36	1	0.2428

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.88	0.94
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 120, groups: obs, 120; rep:par.diet, 6; par.diet, 2

Fixed Effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-13.28	0.21	-63.17	< 0.001
par.dietstarch	-0.75	0.30	-2.49	0.0127
fem.dietS	-0.50	0.27	-1.84	0.0653

Section 3: Generation 30 Courtship behaviour model selection and summary tables

Bout frequency of continuous wing vibration behaviour

Model selection

m5_b2_cont_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_cont_n: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_cont_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_cont_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_cont_n	4	467.65	477.18	-229.82	459.65			
m4_b2_cont_n	5	445.70	457.61	-217.85	435.70	23.95	1	< 0 .001
m3_b2_cont_n	6	447.18	461.48	-217.59	435.18	0.51	1	0.4741
m2_b2_cont_n	7	448.49	465.17	-217.25	434.49	0.69	1	0.4057

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.19	1.09
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-11.56	0.22	-51.77	< 0.001
fem.dietS	-1.51	0.29	-5.14	< 0.001

Total time spent in continuous wing vibration behaviour

Model selection

m5_b2_cont_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_cont_tt: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_cont_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_cont_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_cont_tt	4	1742.4	1751.9	-867.21	1734.4			
m4_b2_cont_tt	5	1732.3	1744.2	-861.14	1722.3	12.13	1	< 0.001
m3_b2_cont_tt	6	1734.2	1748.5	-861.09	1722.2	0.10	1	0.7471
m2_b2_cont_tt	7	1736.1	1752.8	-861.05	1722.1	0.08	1	0.7788

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	16.79	4.10
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-3.44	0.78	-4.43	< 0.001
fem.dietS	-3.50	0.97	-3.62	< 0.001

Bout frequency of intermittent wing vibration behaviour

Model selection

m5_b2_int_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_int_n: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_int_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_int_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_int_n	4	385.6	395.13	-188.8	377.6			
m4_b2_int_n	5	358.32	370.23	-174.16	348.32	29.29	1	< 0.001
m3_b2_int_n	6	359.13	373.43	-173.57	347.13	1.18	1	0.2770
m2_b2_int_n	7	360.78	377.45	-173.39	346.78	0.36	1	0.5504

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.72	0.85
rep:par.diet	(Intercept)	0.01	0.07
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-12.01	0.22	-55.08	< 0.001
fem.dietS	-1.52	0.27	-5.54	< 0.001

Total time spent in intermittent wing vibration behaviour

Model selection

m5_b2_int_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_int_tt: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_int_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_int_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_int_tt	4	1696.9	1706.4	-844.45	1688.9			
m4_b2_int_tt	5	1686.7	1698.6	-838.36	1676.7	12.17	1	< 0.001
m3_b2_int_tt	6	1688.7	1703	-838.34	1676.7	0.04	1	0.8403
m2_b2_int_tt	7	1690.6	1707.3	-838.32	1676.6	0.04	1	0.8469

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	10.00	3.16
rep:par.diet	(Intercept)	0.20	0.45
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-3.94	0.68	-5.76	< 0.001
fem.dietS	-2.79	0.78	-3.57	< 0.001

Bout frequency of head rocking behaviour

Model selection

m5_b2_hr_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_hr_n: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_hr_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_hr_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_hr_n	4	410.02	419.55	-201.01	402.02			
m4_b2_hr_n	5	386.68	398.59	-188.34	376.68	25.34	1	< 0.001
m3_b2_hr_n	6	388.14	402.43	-188.07	376.14	0.54	1	0.4636
m2_b2_hr_n	7	389.65	406.33	-187.83	375.65	0.49	1	0.4840

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.48	0.69
rep:par.diet	(Intercept)	0.13	0.36
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-11.90	0.22	-53.21	< 0.001
fem.dietS	-1.18	0.22	-5.39	< 0.001

Total time spent in head rocking behaviour

Model selection

m5_b2_hr_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_hr_tt: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_hr_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_hr_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_hr_tt	4	1778.5	1788	-885.25	1770.5			
m4_b2_hr_tt	5	1763	1774.9	-876.48	1753	17.55	1	< 0.001
m3_b2_hr_tt	6	1764.9	1779.2	-876.46	1752.9	0.03	1	0.8697
m2_b2_hr_tt	7	1766.5	1783.2	-876.28	1752.5	0.37	1	0.5423

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	4.15	2.04
rep:par.diet	(Intercept)	0.49	0.70
par.diet	(Intercept)	< 0.001	< 0.001

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-3.47	0.50	-6.99	< 0.001
fem.dietS	-2.18	0.49	-4.42	< 0.001

Bout frequency of gland extrusion behaviour

Model selection

m5_b2_go_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_go_n: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_go_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_go_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_go_n	4	331.54	341.07	-161.77	323.54			
m4_b2_go_n	5	325.30	337.21	-157.65	315.30	8.24	1	0.0041
m3_b2_go_n	6	324.39	338.68	-156.19	312.39	2.91	1	0.0879
m2_b2_go_n	7	324.68	341.35	-155.34	310.68	1.71	1	0.1907

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.13	1.07
rep:par.diet	(Intercept)	< 0.001	< 0.001
par.diet	(Intercept)	< 0.001	< 0.001

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-13.17	0.35	-37.58	< 0.001
par.dietstarch	0.58	0.34	1.70	0.0899
fem.dietS	-0.94	0.32	-2.92	0.0035

Total time spent with pheromone gland extruded

Model selection

m5_b2_go_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_go_tt: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_go_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_go_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_go_tt	4	1504.3	1513.8	-748.15	1496.3			
m4_b2_go_tt	5	1502.3	1514.2	-746.17	1492.3	3.95	1	0.04682
m3_b2_go_tt	6	1502.5	1516.8	-745.23	1490.5	1.89	1	0.16951
m2_b2_go_tt	7	1504	1520.7	-745.02	1490	0.41	1	0.52178

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: tt ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Control: glmerControl(optimizer = "bobyqa")

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	4.15	2.04
rep:par.diet	(Intercept)	0.49	0.70
par.diet	(Intercept)	< 0.001	< 0.001

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-8.15	0.63	-12.85	< 0.001
fem.dietS	-3.78	1.03	-3.66	< 0.001

Section 4: Generation 30 non courtship behaviour model selection and summary tables

Bout frequency of stationary behaviour

Model selection

m5_b2_stat_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_stat_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_stat_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_stat_n	4	616.74	626.27	-304.37	608.74			
m3_b2_stat_n	6	613.89	628.18	-300.95	601.89	6.85	2	0.0325
m2_b2_stat_n	7	613.18	629.85	-299.59	599.18	2.71	1	0.0996

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.11	0.33
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-10.10	0.10	-105.98	< 0.001
par.dietstarch	-0.18	0.09	-1.97	0.0485
fem.dietS	-0.20	0.09	-2.17	0.0300

Total time spent stationary

Model selection

m5_b2_stat_tt: tt ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_stat_tt: tt ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_stat_tt: tt ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_stat_tt: tt ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_stat_tt	4	2033.2	2042.7	-1012.6	2025.2			
m4_b2_stat_tt	5	2030.3	2042.2	-1010.2	2020.3	4.83	1	0.0280
m3_b2_stat_tt	6	2032.3	2046.6	-1010.1	2020.3	0.04	1	0.8438
m2_b2_stat_tt	7	2033.5	2050.2	-1009.8	2019.5	0.80	1	0.3720

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: tt ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.52	0.72
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-1.72	0.14	-12.61	< 0.001
par.dietstarch	-0.50	0.17	-2.93	0.0034

Bout frequency of preening behaviour

Model selection

m5_b2_pre_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_pre_n: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_pre_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_pre_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_pre_n	4	613.68	623.21	-302.84	605.68			
m4_b2_pre_n	5	609.54	621.45	-299.77	599.54	6.14	1	0.0132
m3_b2_pre_n	6	611.44	625.73	-299.72	599.44	0.09	1	0.7590
m2_b2_pre_n	7	613.39	630.06	-299.69	599.39	0.06	1	0.8107

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.06	0.25
rep:par.diet	(Intercept)	< 0.001	< 0.001
par.diet	(Intercept)	< 0.001	< 0.001

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-10.30	0.06	-172.97	< 0.001
par.dietstarch	0.28	0.07	3.84	< 0.001

Total time spent preening

Model selection

m4_b2_copat_n: n ~ 1 + (1 | par.diet/rep) + offset(log(cop))

m3_b2_copat_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_copat_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m4_b2_pre_tt	4	2127.1	2136.6	-1059.5	2119.1			
m3_b2_pre_tt	6	2120	2134.3	-1054	2108	11.06	2	0.0040
m2_b2_pre_tt	7	2121.2	2137.8	-1053.6	2107.2	0.84	1	0.3604

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.19	0.44
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-1.35	0.11	-12.40	< 0.001
par.dietstarch	0.25	0.10	2.44	0.0148
fem.dietS	0.28	0.10	2.76	0.0058

Bout frequency of orientation behaviour

Model selection

m5_b2_ori_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_ori_n: n ~ fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_ori_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_ori_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_ori_n	4	591.90	601.42	-291.95	583.90			
m4_b2_ori_n	5	582.11	594.02	-286.06	572.11	11.78	1	< 0.001
m3_b2_ori_n	6	583.15	597.44	-285.57	571.15	0.97	1	0.3252
m2_b2_ori_n	7	585.13	601.80	-285.56	571.13	0.02	1	0.8907

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	0.30	0.54
rep:par.diet	(Intercept)	0.0025	0.05
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-10.63	0.13	-81.51	< 0.001
fem.dietS	-0.52	0.15	-3.52	< 0.001

Bout frequency of aggression behaviour

Model selection

m5_b2_ag_n: n ~ 1 + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m4_b2_ag_n: n ~ par.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m3_b2_ag_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_ag_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m5_b2_ag_n	4	262.45	271.98	-127.22	254.45			
m4_b2_ag_n	5	261.08	272.99	-125.54	251.08	3.37	1	0.0663
m3_b2_ag_n	6	263.08	277.37	-125.54	251.08	< 0.001	1	0.9851
m2_b2_ag_n	7	265.00	281.67	-125.50	251.00	0.08	1	0.7771

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	1.03	1.01
rep:par.diet	(Intercept)	0.02	0.14
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-14.54	0.36	-39.94	< 0.001
par.dietstarch	0.93	0.44	2.13	0.0333

Bout frequency of attempted copulations

Model selection

m4_b2_copat_n: n ~ 1 + (1 | par.diet/rep) + offset(log(cop))

m3_b2_copat_n: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

m2_b2_copat_n: n ~ par.diet * fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
m4_b2_copat_n	3	274.32	281.46	-134.158	268.32			
m3_b2_copat_n	6	173.05	187.34	-80.524	161.05	107.27	3	< 0.001
m2_b2_copat_n	7	174.74	191.41	-80.37	160.74	0.31	1	0.5779

Parameters of selected model

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)

Family: poisson (log)

Formula: n ~ par.diet + fem.diet + (1 | par.diet/rep) + (1 | obs) + offset(log(cop))

Random effects:

Groups	Name	Variance	Std.Dev.
obs	(Intercept)	2.50	1.58
rep:par.diet	(Intercept)	0	0
par.diet	(Intercept)	0	0

Number of obs: 80, groups: obs, 80; rep:par.diet, 6; par.diet, 2

Fixed effects:

	Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-15.12	0.68	-22.11	< 0.001
par.dietstarch	1.50	0.68	2.20	0.0279
fem.dietS	-2.12	0.58	-3.66	< 0.001

5 Transcriptomics of adaptation associated with sexual isolation in the medfly (*Ceratitis capitata*: Diptera, Tephritidae).

5.1 Abstract

Next generation sequencing (NGS) technologies offer new opportunities to describe complex, genome-wide signatures of environmental adaptation in many systems. NGS can also illuminate the importance of the expression of different genes at different life history stages in systems undergoing divergence under ecological selection pressure. One of the key aims of ecological speciation theory is to identify genes that are associated with the development of reproductive isolation in such populations. To investigate the genes associated with ecological divergence, and potentially with sexual isolation (e.g. the significant assortative mating by diet following 60 generations of EE described in Chapter 3), messenger RNA sequencing (RNAseq) was used to describe the transcriptome of male Mediterranean fruit flies (*Ceratitis capitata*, Wiedemann) drawn from two replicated experimental evolution (EE) populations. The populations had been reared on larval diets of differing nutritional complexity and caloric value for 60 generations prior to this study. The transcriptome sequencing was performed on males reared on a common garden diet for two generations prior to sequencing, in order to remove proximate, and possible parental effects, of diet. RNAseq data were analysed using a novel, subsampling-based bootstrap normalisation, which generated high quality and highly comparable datasets for all replicate populations. Differential expression (DE) analysis on the resulting data led to the identification of 109 genes that showed DE above 2 $\log_2$ offset fold change in incident read count between dietary regimes. Functional description of genes showing DE showed an over-representation of many cellular processes including metabolism, oxidative phosphorylation and proteolysis. Three olfactory binding proteins were also found to exhibit DE. These candidates, particularly those involved in oxidative phosphorylation and those that are olfactory receptors, have been shown to be key targets for selection in natural populations.

5.2 Introduction

Next generation sequencing (NGS) technology has revolutionized the study of natural selection (Seehausen et al. 2014), its role in adaptation (Elmer and Meyer 2011; Savolainen et al. 2013) and the instigation of speciation (Nosil 2012). High throughput technologies have allowed the transition from studies of individual genes associated with adaptation and divergence (e.g. Etges et al. 2007, 2009; Matsuo et al. 2007) to those that map the effects of environmental selection pressures on whole genomes (Feder et al. 2012; Nosil 2012). This transition has also allowed the capture of the patterns of gene expression across genomes, and provided insight into the importance of gene expression and it's regulation in the process of species formation (Fay and Wittkopp 2008; Pavey et al. 2010; Bond and Baulcombe 2014).

The study of expression patterns across the genome is referred to as transcriptomics. This branch of NGS is conducted by capturing the whole suite of messenger RNAs (mRNAs) that are transcribed by an organism at any particular time. In it's infancy, transcriptomics was based upon relatively low-throughput sequencing of partial fragments of the 3' and 5' ends of expressed mRNA, reverse transcribed into copy DNA (cDNA), otherwise known as expressed sequence tag (EST) sequencing (Adams et al. 1991). This technique was key to early gene discovery studies (e.g. Verdun et al. 1998) and allowed transcriptomics to form an essential bridge between model and non-model systems (Ekblom and Galindo 2011). From this basis, expansion in sequencing technologies, and the reduction in their cost, has allowed the study of transcriptomics to move wholly into the realm of high-throughput sequencing of cDNA. The increasing availability of reference genomes for many model systems (e.g. the 12 Drosophila genomes; Drosophila 12 Genomes Consortium (2007)) has facilitated the study of gene expression using DNA microarrays (Ranz and Machado 2006). This technique relies upon the hybridization of tagged cDNA from the test sample, with cDNA probes of all known mRNAs in a genome that have been rendered onto a solid support. It therefore relies on the utilisation of existing information about all mRNAs in a genome in order to capture the expression levels of known candidate genes, through the quantification of fluorescence levels generated by the extent of hybridization (Ranz and Machado 2006). Microarrays cannot therefore detect unknown transcripts nor variation due to alternative splicing events.

Microarrays have continuing utility in the study of in transcriptomics – however, transcriptome profiling through RNA sequencing (RNAseq) has become increasingly

popular (Graveley 2008; Shendure 2008). In RNAseq, cDNA is sequenced on a high throughput NGS platform, providing huge numbers of 'reads', i.e. small fragments of cDNA (typically 50 nucleotides), typically well over 100M per sequencing lane, which can be identified to provide a highly sensitive picture of gene expression (Wang et al. 2009). RNAseq avoids drawbacks of hybridisation based microarray methods, such as requirement for detailed prior knowledge of the transcriptome, and complicated analytical processing of expression levels (Wang et al. 2009). Indeed, RNAseq has served to allow the study of transcriptomics to develop rapidly and move away from model species or their close relatives. It allows the rapid *de novo* assembly of the transcriptome of non model genomes (e.g. Vera et al. 2008), insight into novel processes involved in gene expression such as alternative splicing of exons (Matlin et al. 2005) as well as highlighting the huge importance, and underappreciated sophistication of the regulation to expression in generating evolutionary novelty (Fay and Wittkopp 2008; Wang et al. 2009). Furthermore, recent advances in the study of gene expression offer 'spatially resolved transcriptomics', able to capture the gene expression within individual cells or tissues types (Crosetto et al. 2014).

In the past decade this powerful technology has been utilised to great effect, and transcriptomics has allowed new levels of insight into the genome wide expression profiles associated with development (e.g. Street et al. 2008; Vesterlund et al. 2011; Jiménez-Guri et al. 2013; Tan et al. 2013) and different life history stages (e.g. Daines et al. 2011; Etges et al. 2015). Whole genome expression studies have also elucidated the signatures of gene expression associated with specific behavioural events such as copulation (e.g. Gioti et al. 2012; Gerrard et al. 2013; Manfredini et al. 2015). This sensitivity has also made transcriptomics an essential tool for the study of physiological response to perturbation in environmental factors (Evans 2015). The transcriptome has been used to profile genomic response to stresses such as hypoxia (e.g. Gracey 2007), salinity (e.g. Whitehead et al. 2013), pollutants (e.g. Chapman et al. 2011; Whitehead et al. 2012) and temperature (reviewed by Porcelli et al. 2015). The ability to capture changes in gene expression in different environments, and at different stages of life history has opened new avenues for study relating to the long term evolutionary consequences of differences in gene expression, their role in adaptive genetic divergence, and ultimately speciation (Pavey et al. 2010).

As the study of speciation has become tractable using NGS, and the technology capable of identifying candidate genes which differentiate under divergent selection pressure has been applied, there has been an increased focus on finding genes that

link genetic divergence to the evolution of RI (Nosil and Schluter 2011; Nosil 2012). Gene expression studies have furthered the search for "speciation genes" (Nosil and Schluter 2011) in two main ways. Firstly, due to the power of mRNA based methods in facilitating exploration of the genomes of non-model species (Ekblom and Galindo 2011; Riesgo et al. 2012), transcriptomic studies have allowed many important species pairs, incipient species, and host races/ecotypes to be studied at the level of the expressed coding sequence. This has facilitated the search for candidate genes involved in well described examples of adaptive divergence (e.g. *Rhagoletis pomonella*: Schwarz et al. 2009; *Littorina saxitalis*: Galindo et al. 2010; *Timema cristinae*: Comeault et al. 2012; *Cichlid fishes*: reviewed by Henning and Meyer 2014).

Secondly, in systems that have already have well studied reference genomes, or that are closely related to species that have, it has been possible to finesse knowledge of genomic divergence by isolating specific patterns of expression associated with divergent phenotypes, allowing candidate genes, or gene groups associated with adaptation to be isolated, with far greater precision. A prime example of a system which is characterised by repeated parallel evolution of distinct phenotypic morphs exhibiting RI, which have evolved in response to ecological selection, is the lake whitefish species complex (*Coregonus sp.*) (Landry et al. 2007; Bernatchez et al. 2010). Studies of whitefish utilised their close relatedness with Atlantic salmon to employ cDNA microarrays to describe a pattern of several hundred genes exhibiting differential expression (DE) in adult fish of each morph (Derome and Bernatchez 2006; St-Cyr et al. 2008). The specificity of expression level study allowed the pattern of this DE to be isolated to metabolic genes expressed only in adult fish (Nolte et al. 2009), correlating well with divergent energy budgets required between niches (Trudel et al. 2001). These patterns have more recently been validated by studies using RNAseq (Jeukens et al. 2010; Renaut et al. 2010), also allowing the additional capture of sequence polymorphisms associated with this metabolic divergence.

Patterns of gene expression generated by ecological selection pressures have also been studied in a range of *Drosophila* species, facilitated by the wealth of sequenced genomes in this genus (Drosophila 12 Genomes Consortium 2007). Microarrays and RNAseq have been employed to study divergence within incipient species, and also between species pairs (e.g. Mezey et al. 2008; Frentiu et al. 2009; Graze et al. 2009; McManus et al. 2010; Wurmser et al. 2011; Matzkin 2012; Guillén et al. 2015). A particularly well-characterised species that exhibits incipient RI based on host choice is *Drosophila mojavensis* (Etges 2014). Populations from different regions of the Sonoran

desert in Baja California exhibit premating isolation, mediated by cuticular hydrocarbon profiles (CHCs) and courtship song (Etges et al. 2007, 2009), based on the cactus host they utilise for reproduction. Transcriptomic studies have identified a small suite of genes specifically affected by the host food plant at different points in the life history of *D. mojavensis* (Matzkin 2012; Rajpurohit et al. 2013). Some of these candidates have also directly been linked to host-dependent mate choice between allopatric populations (Smith et al. 2013; Etges 2014). Recent studies have combined these two findings, showing life history stage specific changes in gene expression, influenced by rearing host, which underlie RI in this species (Etges et al. 2015), with genes involved in metabolic function being an important area of divergence during developmental life history and those associated with olfactory and behavioural traits divergently expressed in adult life history, where mate choice is made.

Despite the increasing well-understood role of gene expression in adaptation, population divergence and RI in wild systems, relatively few studies have built upon the findings to study gene expression in response to experimental selection. Experimental evolution has yielded useful information into the speciation process (Rice and Hostert 1993; Kawecki et al. 2012). Recent innovations based on the increasingly low cost of NGS study have ushered in '*evolve & resequence'* techniques (Schlötterer et al. 2014). By imposing selection on a particular trait or whole suite of traits, experimental evolution studies are able to ask questions about the adaptive response to this selection in real time. Using this power Remolina et al. (2012) were able to identify a suite of 38 genes which evolved DE between a control population, and a population selected for longevity and late life fecundity for 50 generations, suggesting strong candidates for roles in the regulation of aging. The retrieval of such a strong signal of DE over a relatively small number of generations highlights the suitability of the combination between evolution experiments and transcriptomics. Despite this fact, no laboratory study has yet captured the effects of selection mediated by environment on gene expression under an experimental evolution framework. As previous studies have shown the possibility for the evolution of assortative mating in response to dietary adaptation during experimental evolution (Dodd 1989; Rundle et al. 2005, Chapter 3), transcriptomic study of such adaptation has the potential to elucidate candidate genes involved in this process.

In this chapter, this omission is addressed through the utilisation of RNAseq to compare gene expression between sexually mature males derived from the 62nd generation of the evolution experiment described in Chapter 3. In this evolution

experiment, divergent selection is provided by two differing larval diets. This chapter uses the emergent nature of *Ceratitis capitata* (medfly) as a model organism (organism with sequenced genome) and high throughput RNAseq to isolate the signature of differential expression associated with assortative mating. This is described previously (Chapter 3) or under the underlying associated behavioural phenotypes (Chapter 4).

Medfly provide an ideal candidate for such a study, as alongside the wealth of ecological, behavioural and population scale information available, described in previous chapters, the species has a genome project in progress, and thus a reference genome is now available (https://www.hgsc.bcm.edu/arthropods/mediterranean-fruit-fly-genome-project). It's great importance as a crop pest, infesting >350 hosts of commercial importance (Liquido et al. 1991) has led to great interest in medfly genetics, to facilitate the development of genetically-mediated control measures (Leftwich et al. 2015) and optimisation of contemporary control efforts (Calla et al. 2014; Scolari et al. 2014). Studies of gene expression in the medfly originated in tissue-specific (e.g. Davies and Chapman 2006), and then genome-wide (Gomulski et al. 2008; Scolari et al. 2012; San Andrés et al. 2013) EST sequencing. From this basis, microarrays were employed to identify the transcriptional profile of head tissue derived from immature, sexually mature virgin and non virgin adult medfly (Gomulski et al. 2012). RNAseq has been used to create a *de novo* assembly of the medfly embryonic transcriptome (Salvemini et al. 2014) and, coupled with the recent advent of the medfly reference genome, has been used to capture genome wide expression patterns of divergence between wild and long established laboratory populations (Calla et al. 2014).

In this chapter the medfly is used for the first time to study adaptation and the evolution of reproductive isolation. RNAseq is used to retrieve body part specific (Head/Thorax and Abdomen) gene expression profiles in sexually mature males. Males were sourced from the 62nd generation of the evolution experiment described in Chapters 3 and 4, which divided an ancestral population onto divergent novel larval rearing diets (sucrose-based 'ASG' vs. starch-based 'Starch'). In the 60th generation, significant, male-driven, assortative mating by larval diet was observed across three replicate population pairs within this experiment (Chapter 3). Here, mRNAseq was conducted on males reared for 2 generations in a common garden glucose diet, to reveal evolved differences in gene expression at adult male sexual maturity. A highly uniform expression pattern was retrieved across the three replicate line pairs, and 1214 transcripts were identified as DE between dietary treatments. This allowed the

functional annotation of 109 genes (51 Head/Thorax, 58 Abdomen), showing DE. This DE was in a suite of genes involved in nutrient metabolism, oxidative phosphorylation (OXPHOS), proteolysis, and in some olfactory binding proteins.

5.3 Experimental methodology

5.3.1 Origin and maintenance of fly stocks

For origin of the flies used in this study and the rearing methods, please see Nash & Chapman (2014, Chapter 2). Eggs used here were taken from the experimental evolution lines described by Nash et al. (2015, in revision, Chapter 3) in the same generation as the mating tests were conducted to assay for RI, following 60 generations of experimental evolution. Flies from both experimental evolution dietary treatments, 'ASG' (A) and 'Starch' (S), described above, were reared on the glucose common garden (CG) diet (Chapter 3), under standard conditions and density. Adults emerging from daily collected cohorts of pupae were sex-sorted at birth and reared in single sex cages, under standard adult rearing conditions (Nash & Chapman 2014, Chapter 2) until seven days post eclosion. 17 - 30 male flies were then flash frozen in liquid N_2 30 minutes after lights on (09:30), in Eppendorf tubes, in groups of 10 - 15. These samples were then stored at -80°C until RNA extraction.

5.3.2 RNA extraction

Total RNA was extracted from samples of 17 - 22 flies pooled within each replicate of each dietary treatment. Each pool of individuals was split into two tissue types, Head/Thorax (HT), and Abdomen (Ab), prior to extraction. Flies were transferred from -80°C storage, and placed directly onto dry ice. A scalpel was then used to gently divide the two body parts. Thus, 12 extractions per sex were conducted in total. Total RNA was extracted using the *mir*Vana kit (Ambion), used according to the manufacturers instructions. 5µg (>200ng/µl) of Total RNA from male flies was then submitted for mRNA sequencing.

5.3.3 RNAseq

Messenger RNA (mRNA) sequencing was conducted by BaseClear (Netherlands). Libraries were prepared using the Illumina Truseq protocol following polyA enrichment for mRNA. Sequencing was single end, and conducted on the Illumina HiSeq 2500 platform (Rapid Mode), at SR50 cycles.

5.4 Bioinformatic methodology

5.4.1 Quality control

Initial quality control was performed on FASTQ files delivered by the provider. FASTQ files were converted into FASTA format, and the accuracy of the conversion calculated. Reads containing Ns (< 1%) were discarded. Files were then transformed to non-redundant format, with each sequence occurring once (sequence abundance, number of times it was found in a sample, was coded in the sequence identifier), and the complexity (ratio of non-redundant reads to redundant reads) calculated, creating the original non-redundant read dataset.

5.4.2 Subsampling normalisation

In order to initially reduce inter-sample variability, all samples were checked for internal consistency using incremental subsampling (95:50% of data). A fixed total, based on the sample with the lowest number of reads (AG3MHT, 34M reads), was then selected. All samples were then subsampled to a fixed total of 34M reads using a bootstrapping approach. This was to check if the selected subset was representative to the original sample and similar to any other subset of the same total. One of the 34M read subsamples created for each sample was then accepted as biologically informative and used in the fixed total bootstrap subsampled (bstrp) dataset. Information further justifying the bootstrap normalisation approach, and comparing it to alternative methodologies for normalisation can be found in Appendix 5.1.

The patMaN software (Prüfer et al. 2008) was used to map the reads from both the original and bstrp datasets to the Cc01172013 version of the *Ceratitis capitata* genome, full length, with 0 or 1 mis-match (1mm). A full mismatch approach was used, i.e. a mismatch was counted as a mismatch between any nucleotide pair (e.g. A != T, A != C, A != G etc.), with no half scoring (e.g. G = U).

Due to the low quality of the available reference transcriptome, and to create a reference set relevant to the genes expressed in the experimental structure, a new set of reference transcripts were generated, at exon level, from the pooled data. Three reference transcriptomes were created; one from pooled HT samples only, one from pooled Ab samples only, and a third generated from a pool of all transcripts. The patMaN mapping was conducted in triplicate, using all three sets of transcripts as the reference. Here, only the expression levels derived from the 'all transcripts' set are analysed further, as the expression matrix generated was comprehensive due to a

more uniform coverage of all exons. This is achieved as the 'all transcripts' reference set compensates for the slight variation in start and stop codons between HT and Ab samples, as it contains all variants of both.

Using the 1mm matching reads from the original and bstrp datasets, transcribed regions were identified which were fully covered with reads (no gaps are permitted within a transcript). This generated two expression matrices for further analysis following normalisation.

5.4.3 Further normalisation

Normalisation is essential to the analysis of RNAseq data, as it serves to remove unwanted between sample distributional technical differences, and allows accurate analysis of gene expression (Dillies et al. 2013; Risso et al. 2014). Alongside the subsampling methodology described above, two further methods of normalisation were utilised in this study:

- Reads per million (RPM) normalisation (Mortazavi et al. 2008) was applied to the expression levels calculated from the original dataset. First, a normalisation factor was calculated as the sum of the redundant reads per transcript divided by the total number of reads per sample (with a normalization constant of the sum of the redundant read totals divided by the absolute value of the sample redundant reads), for each sample, and applied to the matrix containing the expression levels.
- Quantile (Q) normalisation, described by (Bolstad et al. 2003), matches the rank distribution of read abundances between samples. It was conducted on the expression levels resulting from the bstrp dataset, in order to further minimize remaining between-sample variation.

5.4.4 Differential expression analysis

Firstly, in order to describe differential expression (DE) between replicates, transcript DE between replicates was identified using the $\log_2$ offset of the fold change, with an empirically determined offset of 20 (Mohorianu et al. 2011). This offset allowed compensation for noise at low levels of expression (<20, See Appendix 5.1 for justification). The $\log_2$ offset fold ratio (OFR) was then calculated pairwise between replicates. This DE was then examined at 3 thresholds of $\log_2$(OFR) (>2, >3, >4) with transcripts occurring at a total abundance of <100 removed for the >2 and >3 $\log_2$(OFR) comparisons, and with transcripts occurring at a total abundance of <20 for the >4 $\log_2$(OFR) comparison.

Secondly, of main relevance to the further analysis conducted below, DE between tissues and treatments was done using a hierarchical approach with two levels, first the body part (tissue) (HT/Ab), followed the dietary treatment (A/S). As far more genes were expected to exhibit DE between HT and Ab tissue then between dietary treatments, this allowed the set of transcripts analysed for DE between treatments to be drawn from a larger set of transcripts based on tissue specific expression. The DE call between treatment types was made on maximal intervals [min replicate expression, max replicate expression] (MIs). The DE call was based on the amplitude between the proximal ends of the MIs using a $\log_2$ offset fold change, again with an offset of 20. Genes with a $\log_2$(OFC) greater than 1 (corresponding to a 2 fold change difference) were called as exhibiting DE. Further information on the advantages of the MI approach, and comparison with other methods of DE calling are presented in Appendix 5.1.

5.4.5 Annotation and functional description

The annotation of DE transcripts at the A/S level was conducted using BLASTN (on the nr database) and BLASTX (on the UniprotKB Swiss databases). As the BLASTN search is more conservative because it is conducted at the nucleotide level, the annotations derived from this search were then manually curated; the GO terms were assigned using similarity search on the UniprotKB (SwissProt & TrEMBL) public database.

Large proportions of the annotation set did not yield meaningful annotations following the BLASTN similarity search and were removed from the functional description set. Also, several large groups of transcripts were found to match the same annotation, despite being spread across wide stretches of the reference genome. These 'non-adjacent duplicates' were also excluded from the functional description set, as it was not possible to distinguish between sequencing errors and true repeat sequences. Transcripts that matched *Ceratitis capitata* annotations, but that were either uncharacterised loci or hypothetical proteins were also excluded from the functional description set, as no further information would be gained from such annotations.

In most cases exact matches were found for *Ceratitis capitata*, but where this was not possible homologues were identified in either the closely related Tephritid fruit flies *Bactrocera dorsalis* or *B. cucurbritae*, or in *Drosophila melanogaster*. In cases where *D. melanogaster* homologues were used, the suffix '-like' was added to gene names assigned to annotations. Annotations that could not be identified to genes within this

context, or genes that were not associated with any GO terms were discarded at this point.

Following functional description, genes were grouped by manually assigned 'keywords' linking GO terms. These specific keywords referenced GO terms from the 'Biological Process' (BP) domain, and an additional single keyword was drawn from the 'Cellular component' (CC) domain. Secondary keywords that linked genes already within a keyword group by more specific GO commonalities were also assigned. The use of these manually assigned keywords was advantageous because it facilitated the curation of genes within keyword categories into tight functional groupings. The nine specific manually assigned keyword groupings were: 'Metabolism', 'Oxidative Phosphorylation', 'Proteolysis', 'DNA', 'Signalling', 'Transport', 'Biosynthesis', 'Response', & 'Membrane'.

5.4.6 Candidates for qRT-PCR validation

Although it was not possible to conduct validation during this chapter, candidates for qRT-PCR validation were selected in order to enable future work by allowing the confirmation of patterns of DE in both tissue types. Candidates were selected on several criteria. Firstly, candidates were selected to validate patterns of DE within GO groupings. Where two genes that were exhibited DE in different directions within a grouping were present, they were selected. Secondly, candidates were required to exhibit a universally high level of expression in all replicates of both treatments, above 200. Finally, candidates had to show high query coverage in the BLASTN search result, with a minimum set at 30%. Following selection on these criteria, candidates were further examined by the plotting of the algebraic sum of abundances of incident reads at every position of the reference transcript, or expression profile, of each gene. A gene that passed the three selection criteria, and had an expression profile suitable for primer design, was considered as a candidate for qRT-PCR.

5.5 Results

5.5.1 Quality control

Raw FASTQ files contained between 34,385,212 and 53,240,596 reads (Table 5.1). Conversion to FASTA format left 34,348,097 to 53,183,504 reads remaining, with the acceptance over 99% for every sample (Table 5.1). Next, the files were transformed from redundant to non-redundant format, yielding between 7,625,995 & 10,214,330 unique (non-redundant) reads, with a complexity of between 0.192 and 0.229 across samples (Table 5.1). Following matching to the *Ceratitis capitata* genome with no

mismatches, the proportion of reads matching to the genome was: redundant between 56 & 62%, non-redundant between 50 & 54%, with complexity of between 0.172 and 0.207. When one mismatch was allowed, the proportion of reads matching the reference genome rose to: redundant between 14 & 21%, and non-redundant between 63 & 91%, with complexity between 0.201 and 0.232 (Table 5.1).

5.5.2 Subsampling normalisation

The fixed total (34M) was found by subsampling between 95 and 60% of reads. When all samples were resampled at the fixed total (34M), the non-redundant read count was between 7,055,508 and 8,382,537 with complexity between 0.208 and 0.231 (which was equivalent across replicates and tissue types) (Table 5.1). When the resulting bstrp dataset was mapped to the *Ceratitis capitata* genome with 0 mismatches, the proportion of reads matching the genome was: redundant between 56 & 62%, non-redundant between 51 & 54%, with complexity ranging from 0.187 to 0.218. When one mismatch was permitted, the proportion of reads matching the reference genome was: redundant between 72 & 79%, and non-redundant between 76 & 77%, with complexity between 0.22 and 0.249.

5.5.3 Expression analyses

Using the 1 mis-match annotations of the original, and bstrp datasets described above, gene/transcript expression level was calculated as the algebraic sum of the abundances of all incident reads with each transcript. Further to this RPM normalisation was applied to the resulting original expression matrix, and Q normalisation was applied to the bstrp expression matrix. This provided four potential expression matrices for further analysis.

5.5.4 Differential expression - between replicates

As exemplified by Figure 5.1, the four expression matrices created showed varying levels of inter-replicate distributional difference. The Q normalised bstrp expression matrix was used in all analyses as this combination of normalisation techniques provided a uniform distribution of expression, with all replicate pairs centred on zero, for all replicates of both treatments (Figure 5.1). The use of the Q normalised bstrp expression matrix allowed all three replicates to be used in the DE analysis.

Table 5.1. Quality control and genome matching of RNAseq data used in this study. 'fastq2fasta' describes the conversion of sequence data FASTQ format to FASTA format, '%Acc' describes the accuracy of this conversion. 'R2NR' denotes the conversion of the FASTA files into non-redundant format (see text), 'NR' = non redundant, 'R' = redundant, 'C' = complexity (see text). 'Bstrp' describes the 34M fixed total subsampling of the data, conducted as a normalisation step (see text).

	fastq2fasta				R2NR		Genome matching					Bstrp			Bstrp Genome Matching				
Sequence tag	total	accepted	rejected	%Acc	NR	C	R	NR	C	%R	%NR	R	NR	C	R	NR	C	%R	%NR
AG1MHT	38,396,086	38,264,651	131,435.00	0.9966	7,625,995	0.1993	22,215,703	4,031,344	0.1815	0.5806	0.5286	34,000,000	7,781,859	0.2289	20,917,722	4,138,080	0.1978	0.6152	0.5318
AG1MAb	38,312,455	38,184,106	128,349.00	0.9966	8,388,873	0.2197	23,490,912	4,412,477	0.1878	0.6152	0.5260	34,000,000	7,055,508	0.2075	19,739,977	3,769,477	0.1910	0.5806	0.5343
SG1MHT	43,633,436	43,485,426	148,010.00	0.9966	9,873,856	0.2271	26,331,708	5,165,733	0.1962	0.6055	0.5232	34,000,000	7,898,462	0.2323	20,637,113	4,117,740	0.1995	0.6070	0.5213
SG1MAb	35,937,875	35,815,136	122,739.00	0.9966	8,172,070	0.2282	21,739,637	4,240,382	0.1951	0.6070	0.5189	34,000,000	8,382,537	0.2465	20,588,820	4,482,220	0.2177	0.6056	0.5347
AG2MHT	39,812,520	39,699,610	112,910.00	0.9972	8,184,858	0.2062	23,215,704	4,370,199	0.1882	0.5848	0.5339	34,000,000	7,820,598	0.2300	20,773,135	4,103,579	0.1975	0.6110	0.5247
AG2MAb	36,359,555	36,257,468	102,087.00	0.9972	8,151,440	0.2248	22,152,788	4,252,887	0.1920	0.6110	0.5217	34,000,000	7,389,313	0.2173	19,885,130	3,997,797	0.2010	0.5849	0.5410
SG2MHT	39,710,643	39,598,821	111,822.00	0.9972	8,269,615	0.2088	23,153,157	4,305,920	0.1860	0.5847	0.5207	34,000,000	7,562,735	0.2224	20,836,043	3,901,004	0.1872	0.6128	0.5158
SG2MAb	37,370,799	37,265,802	104,997.00	0.9972	8,026,054	0.2154	22,836,556	4,106,364	0.1798	0.6128	0.5116	34,000,000	7,480,731	0.2200	19,879,025	3,949,171	0.1987	0.5847	0.5279
AG3MHT	34,385,212	34,348,097	37,115.00	0.9989	7,899,535	0.2300	20,485,515	4,229,627	0.2065	0.5964	0.5354	34,000,000	7,677,981	0.2258	20,898,243	4,050,120	0.1938	0.6147	0.5275
AG3MAb	43,671,582	43,625,453	46,129.00	0.9989	9,018,415	0.2067	26,812,963	4,654,093	0.1736	0.6146	0.5161	34,000,000	7,845,869	0.2308	20,277,634	4,204,598	0.2074	0.5964	0.5359
SG3MHT	43,889,931	43,842,538	47,393.00	0.9989	8,481,398	0.1935	24,601,967	4,236,816	0.1722	0.5611	0.4995	34,000,000	7,697,240	0.2264	20,755,046	4,044,996	0.1949	0.6104	0.5255
SG3MAb	53,240,596	53,183,504	57,092.00	0.9989	10,214,330	0.1921	32,467,648	5,154,109	0.1587	0.6105	0.5046	34,000,000	7,177,756	0.2111	19,079,164	3,671,020	0.1924	0.5612	0.5114
							with 1 mis-match								with 1 mis-match				
AG1MHT							29,704,593	6,443,110	0.2169	0.1684	0.8449				26,450,812	5,993,956	0.2266	0.7780	0.7702
AG1MAb							28,336,614	5,846,187	0.2063	0.1531	0.6969				25,178,737	5,423,767	0.2154	0.7406	0.7687
SG1MHT							27,287,494	6,208,718	0.2275	0.1428	0.6288				25,904,164	6,008,660	0.2320	0.7619	0.7607
SG1MAb							32,957,133	7,518,721	0.2281	0.2099	0.9201				25,768,840	6,421,626	0.2492	0.7579	0.7661
AG2MHT							28,245,127	6,254,547	0.2214	0.1575	0.7642				26,486,797	6,010,010	0.2269	0.7790	0.7685
AG2MAb							29,709,878	6,284,518	0.2115	0.1733	0.7710				25,446,416	5,694,754	0.2238	0.7484	0.7707
SG2MHT							28,755,484	6,111,166	0.2125	0.1543	0.7390				26,236,926	5,771,695	0.2200	0.7717	0.7632
SG2MAb							29,127,386	6,281,231	0.2156	0.1686	0.7826				25,009,970	5,703,960	0.2281	0.7356	0.7625
AG3MHT							34,356,624	6,887,754	0.2005	0.2005	0.8719				26,776,387	5,900,300	0.2204	0.7875	0.7685
AG3MAb							26,139,226	6,061,222	0.2319	0.1389	0.6721				25,874,160	6,021,569	0.2327	0.7610	0.7675
SG3MHT							40,761,022	7,696,949	0.1888	0.1756	0.9075				26,057,594	5,870,392	0.2253	0.7664	0.7627
SG3MAb							31,707,793	6,380,437	0.2012	0.1200	0.6247				24,590,981	5,434,344	0.2210	0.7233	0.7571

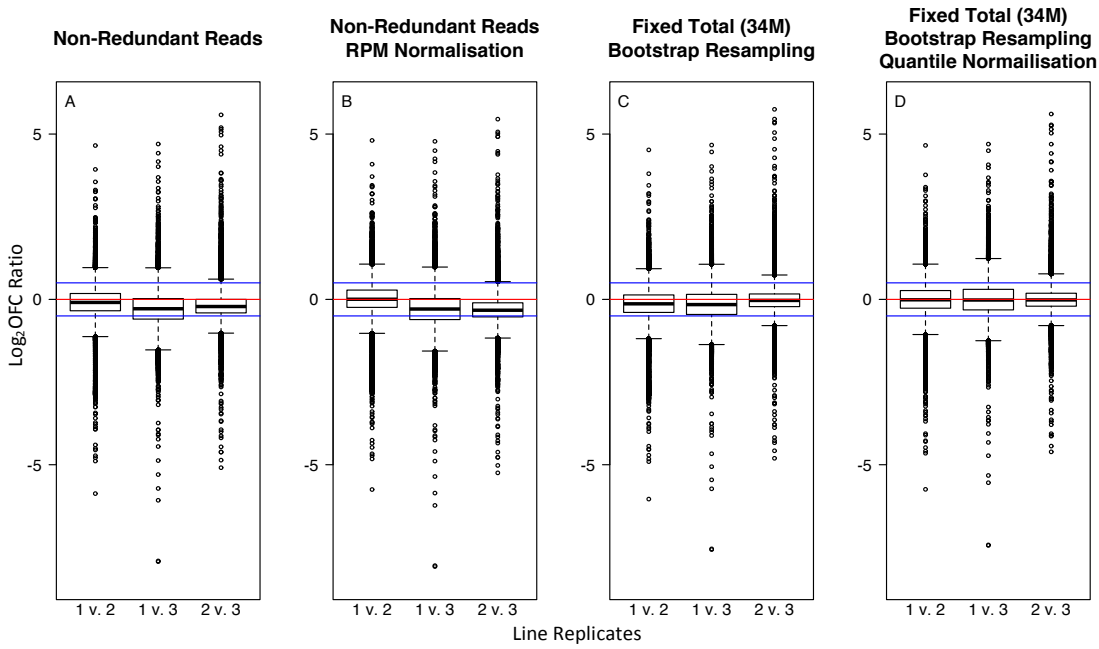

Figure 5.1 Plots showing pairwise log_2(OFR) of differential expression between replicates of abdominal (Ab) tissue from ASG reared male *Ceratitis capitata*, demonstrating three types of normalisation. A) The original expression matrix with no normalisation. B) The original expression matrix with RPM normalisation. C) The bstrp expression matrix with no normalisation. D) The bstrp expression matrix with Q normalisation. Blue lines represent +/- 0.5 for log_2(OFR) this is the detection limit for low throughput validation such as qRT-PCR or northern blot.

All replicates showed strong correlation in expression pattern. Figure 5.2 shows the pairwise correlation between ASG Ab replicates. The whole data set showed strong correlation between expression patterns (Figure 5.3). As the correlations were high across all replicates in both tissue types of both treatments, only ASG Ab samples and their correlations are presented here, as an example of this pattern.

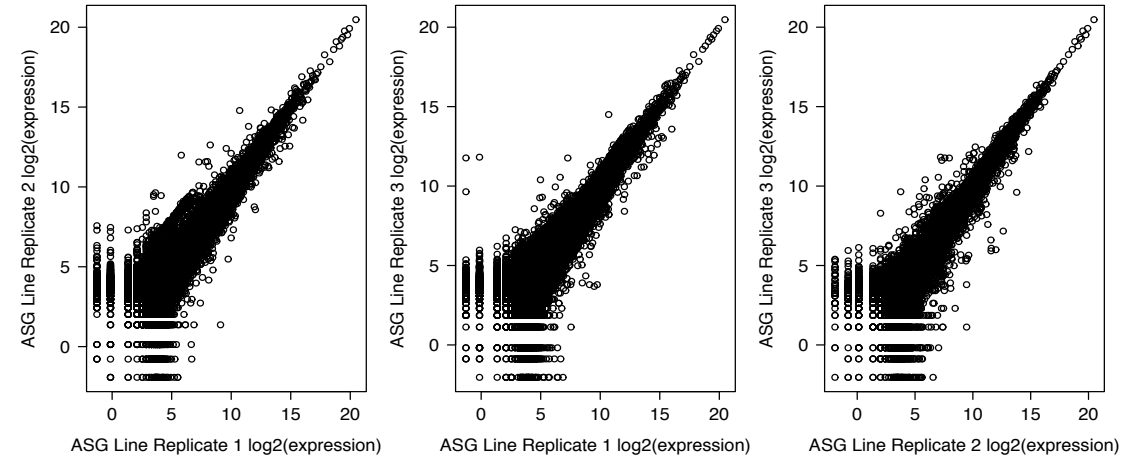

Figure 5.2. Pairwise log_2 expression scatter plot between replicates of abdominal (Ab) tissue from ASG reared male *Ceratitis capitata*. Correlation between all replicates was high (See Figure 5.3).

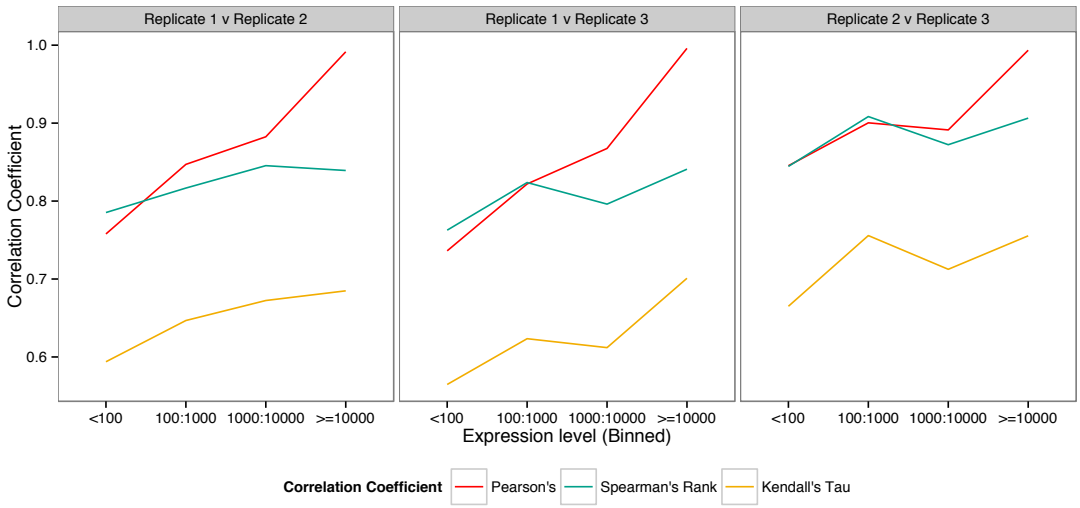

Figure 5.3. Correlation between expression levels of three line replicates of ASG Abdomen tissue, over four bins of expression (<100; 100:1,000; 1,000:10,000; >=10,000). Three correlation coefficients are presented; Pearson's (red), Spearman's Rank (green), Kendall's Tau (yellow), all are highly significant (P < 0.001).

Table 5.2. Numbers of transcripts DE between replicates at >4, >3, and >2 $\log_2$(OFR), and numbers of transcripts which were annotated to *Ceratitis capitata* genes. For transcripts DE at >3 and >2 $\log_2$(OFR), transcripts of total expression across replicates <100 were not counted, and for transcripts DE at >4 $\log_2$(OFR), transcripts of total expression across replicates <100 were not counted.

Diet	Tissue	Replicate	Transcript log2(OFR) DE			Annotations		
			> 4	> 3	> 2	*Ceratitis*	Unrelated	None
ASG	Ab	1 v. 2	9	18	530	51	41	438
		1 v. 3	10	24	75	39	20	16
		2 v. 3	14	13	104	25	40	39
	HT	1 v. 2	3	15	53	23	22	8
		1 v. 3	9	10	47	26	8	13
		2 v. 3	9	19	78	24	29	25
Starch	Ab	1 v. 2	7	16	190	45	16	129
		1 v. 3	6	4	90	22	8	60
		2 v. 3	0	10	66	12	9	45
	HT	1 v. 2	9	20	111	47	26	38
		1 v. 3	3	12	108	46	31	31
		2 v. 3	1	14	46	15	6	25

Transcripts that showed DE between replicates at each threshold were functionally described using BLASTN similarity search, Table 5.2 describes the results of the between replicate DE analysis. The resulting set of enriched DE genes represented a subset of DE trend described between treatments below. There was also a much more marked influence of GO categories representing random patterns between groups of individuals, for example genes related to immune response (GO:0006955).

5.5.5 Hierarchical differential expression

The predicted pattern of hierarchical DE, with more transcripts exhibiting DE between tissues (HT/Ab) than between treatments (A/S) was confirmed (Figure 5.4). The frequency of transcripts showing DE when replicate and treatment levels were summed to show tissue level DE (black line, Figure 5.4A), was higher than when tissue and replicate expression levels were summed to show treatment level DE (red line, Figure 5.4). This allowed transcripts to be separated by tissue type, and then analysed for DE at treatment level (Figure 5.4B).

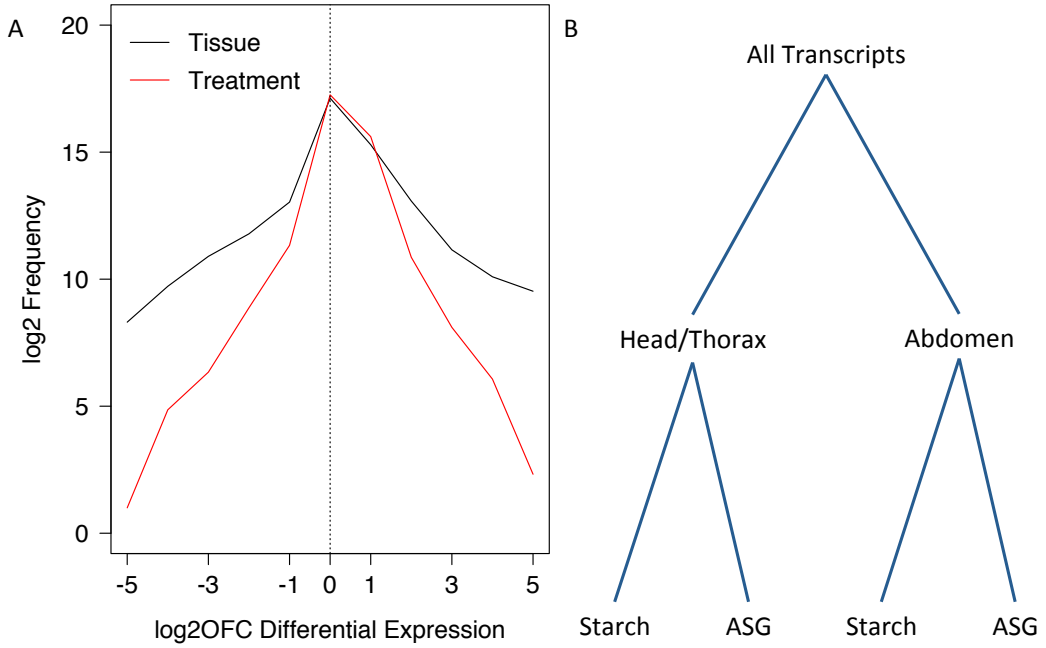

Figure 5.4. Hierarchical DE analysis. A) Frequency distribution of log_2OFC DE between sum expression in tissue types (HT/Ab), and in treatment (A/S). The distribution of DE between treatment types, in red, falls below the distribution of DE between tissue types, in black. B) Design of hierarchical DE analysis, with treatment level DE called on tissue specific transcripts.

5.5.6 Differential expression between treatments

The number of transcripts exhibiting DE between treatments is described in Table 5.3. A large proportion of transcripts were discarded during the annotation process, particularly due to lack of possible annotation, and also due to the presence of 'non-adjacent duplicates'. It is interesting to note that, despite an unbiased lane design during sequencing, the Starch samples exhibited a large number of non-adjacent duplicate groups, in comparison to very few seen in the ASG samples. A total of 109 annotated genes were fully functionally described across both treatments and tissue types. When the GO of these genes was divided between keywords, 9 main clusters emerged. Figures 5.5 & 5.6 display the major clusters found in the functionally

described DE genes and their expression level, Figure 5.7 shows the expression level rescaled to highlight DE patterns between treatments at each gene.

5.5.7 Head/Thorax tissue differential expression

34 genes with meaningful functional descriptions exhibited DE in HT tissue between of sexually mature males reared on ASG and Starch larval diets (Figure 5.5 & 5.7). These genes represented 7 of the 9 main keyword groups. No keyword groups showed a completely unidirectional difference in expression pattern, with each containing genes expressed at a minimum of 2 $\log_2$(OFC) above the opposing treatment, observed for both treatments. 6 of 7 genes grouped under the keyword 'metabolism', associated with nutrient metabolism (e.g. BPs: Catabolic process GO:0009056; Glucose import, GO:0046323; protein targeting to Golgi, GO:0000042; MFs: hydrolase activity, GO:0016787; ligase activity, GO:0016874) were more highly expressed in Starch flies than in ASG. Opposite to this, 4 of 5 genes grouped under the keyword 'Oxidative Phosphorylation' (OXPHOS), associated with oxidation and ATP synthesis (e.g. BPs: oxidation-reduction process, GO:0055114; protein ADP-ribosylation, GO:0006471; MFs: oxireductase activity, GO:0016491; CCs: mitochondrion, GO:0005739; respiratory chain, GO:0070469), were more highly expressed in ASG HT tissue than in Starch. The keyword 'proteolysis', (e.g. BPs: proteolysis, GO:0006508; MFs:peptidase activity, GO:0008233) contained 10 genes, 6 of which were expressed at higher levels in Starch than ASG, the other 4 were more highly expressed in ASG than Starch. The keyword 'DNA' grouped a range of BPs relating to DNA (e.g. transcription, DNA-templated; GO:0006351, DNA replication, GO:0006260; DNA repair, GO:0006281). 4 of the 5 genes grouped within this keyword were expressed at higher levels in Starch over ASG. The other three keywords ('Signalling', 'Transport', 'Response' showed a mixture of expression patterns, predominantly with higher expression in seen in Starch. Interestingly, within the keyword cluster 'Response'; two odorant binding (GO:0005549) genes were differentially expressed in different directions, between different treatments.

Table 5.3. Differentially expressed (DE) transcripts used in the annotation and functional description steps, prior to visualisation in Figures 5.5, 5.6, & 5.7. Annotation Step: Un-annotated transcripts did not match any known genes following BLASTN similarity search, and were discarded. Non-adjacent duplicates were transcripts which all matched to the same BLASTN identification following similarity search. These were discarded, as it was not possible to distinguish assembly error in these groups. Unidentified transcripts were associated with *Ceratitis capitata*, but either as unidentified loci, or as hypothetical proteins, these were also discarded. Functional Description Step: of the total transcripts successfully annotated, transcripts not matching a gene known in *C. capitata* (or homologs, see methods), or that did not have gene ontology (GO) terms associated with them were discarded. Prior to visualisation, genes which represented a GO keyword with less than 2 gene associated with it were also excluded.

	Tissue			
	Head/**T**horax		**Ab**domen	
Starch	**Total DE transcripts**	**286**	**Total DE transcripts**	**653**
	Un-annotated	53	Un-annotated	359
	Non-adjacent duplicates	163	Non-adjacent duplicates	190
	Unidentified	27	Unidentified	49
	Ceratitis annotations	40	*Ceratitis* annotations	50
	Unrelated annotations	3	Unrelated annotations	7
	Adjacent duplicates	3	Adjacent duplicates	6
	Total used	34	Total used	35
	No Gene/GO	3	No Gene/GO	0
	≤2 in GO	6	≤2 in GO	9
	Used in heat map	**25**	Used in heat map	**26**
ASG				
	Total DE	**95**	**Total DE**	**180**
	Un-annotated	30	Un-annotated	114
	Non-adjacent duplicates	12	Non-adjacent duplicates	3
	Unidentified	18	Unidentified	23
	Ceratitis annotations	30	*Ceratitis* annotations	32
	Unrelated annotations	5	Unrelated annotations	7
	Adjacent duplicates	8	Adjacent duplicates	6
	Total used	17	Total used	23
	No Gene/GO	5	No Gene/GO	0
	≤2 in GO	3	≤2 in GO	8
	Used in heat map	**9**	Used in heat map	**12**

5.5.8 Abdomen tissue differential expression

41 genes with meaningful functional descriptions showed DE in Ab tissue between of sexually mature males reared on ASG and Starch larval diets (Figure 5.6 & 5.7). More genes related to the keywords 'metabolism' and OXPHOS were present in the abdomen samples. These were mostly expressed at higher levels in Starch line males, with the division between ASG and Starch expression patterns seen in the HT not being

apparent. The divide between genes associated with proteolysis remained, with 3 of 5 being more highly expressed in ASG over Starch. The keyword cluster 'DNA' showed 2 genes expressed above 2 $\log_2$(OFC) difference in each treatment. Two keyword clusters were observed in Ab tissues that were not seen in HT tissue. 'Biosynthesis' (e.g. BPs: biosynthetic process, GO:0009058; steroid biosynthetic process, GO:0006694), which had 2 genes expressed at higher levels in ASG, and one gene expressed at higher levels in Starch. 'Membrane' was based in the cellular component domain of GO (GO:0016020), although it offered limited descriptive information, associated BPs (cell adhesion, GO:0007155), were DE at higher levels in Starch. The remaining groups, 'Transport' and 'Response' again show mixed DE patterns, Ion transport (GO:0006811) associated genes being expressed higher in both treatments. However, transporter activity (GO:0005215) was only expressed at a higher level in Starch. This was also true of all three 'Response' related genes: one odorant binding (GO:0005549) associated gene, and two related to behavioural BPs.

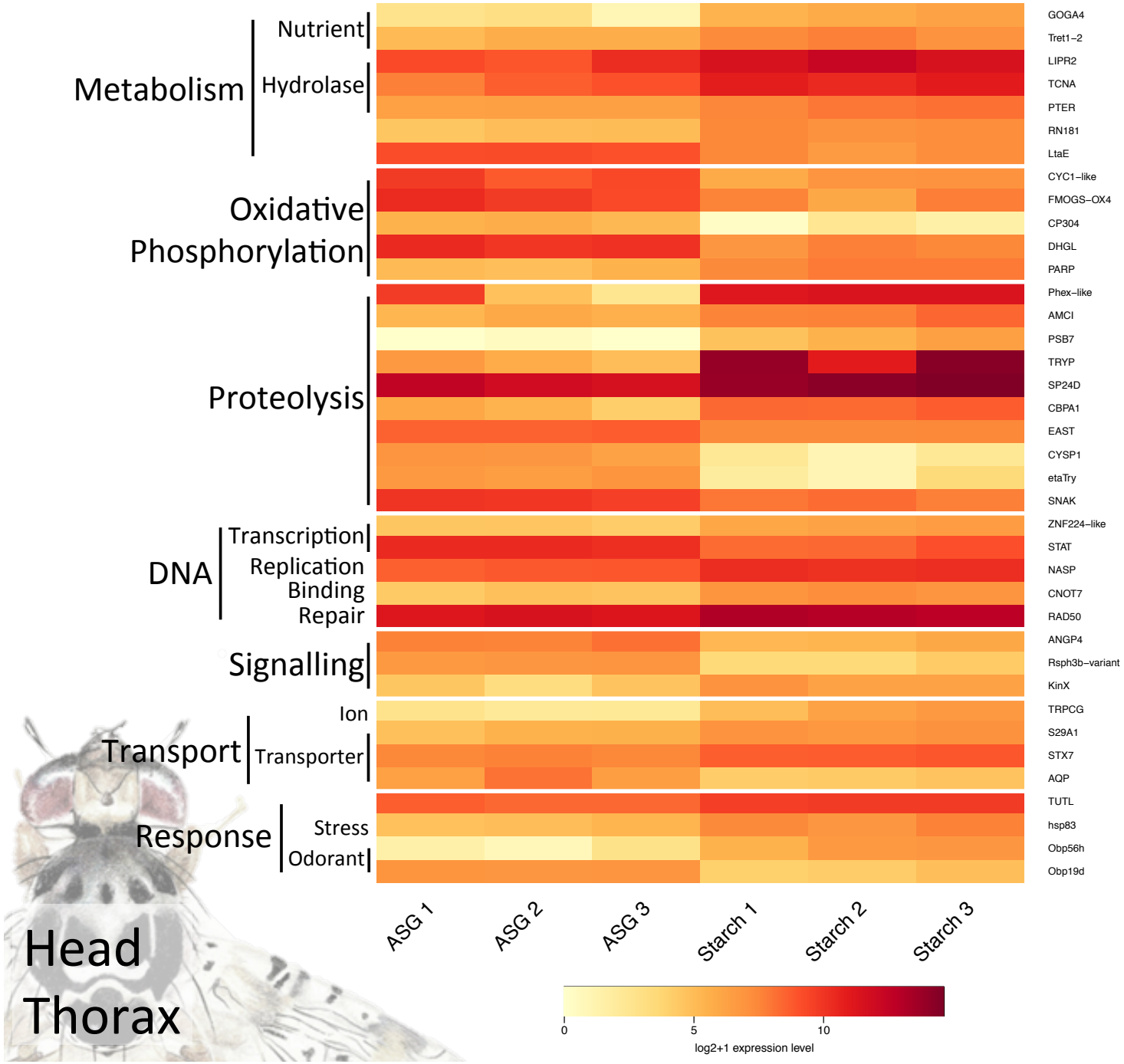

Figure 5.5. Heat map representing $\log_2 + 1$ expression levels for genes exhibiting DE in HT tissue. 34 genes showing DE in HT tissue, genes selected represent groups of GO terms that contained more than 2 genes. Major key words are represented in large text on the left, those in smaller text represent secondary groupings. Gene names represented on the right hand side. Darker colours represent higher expression level.

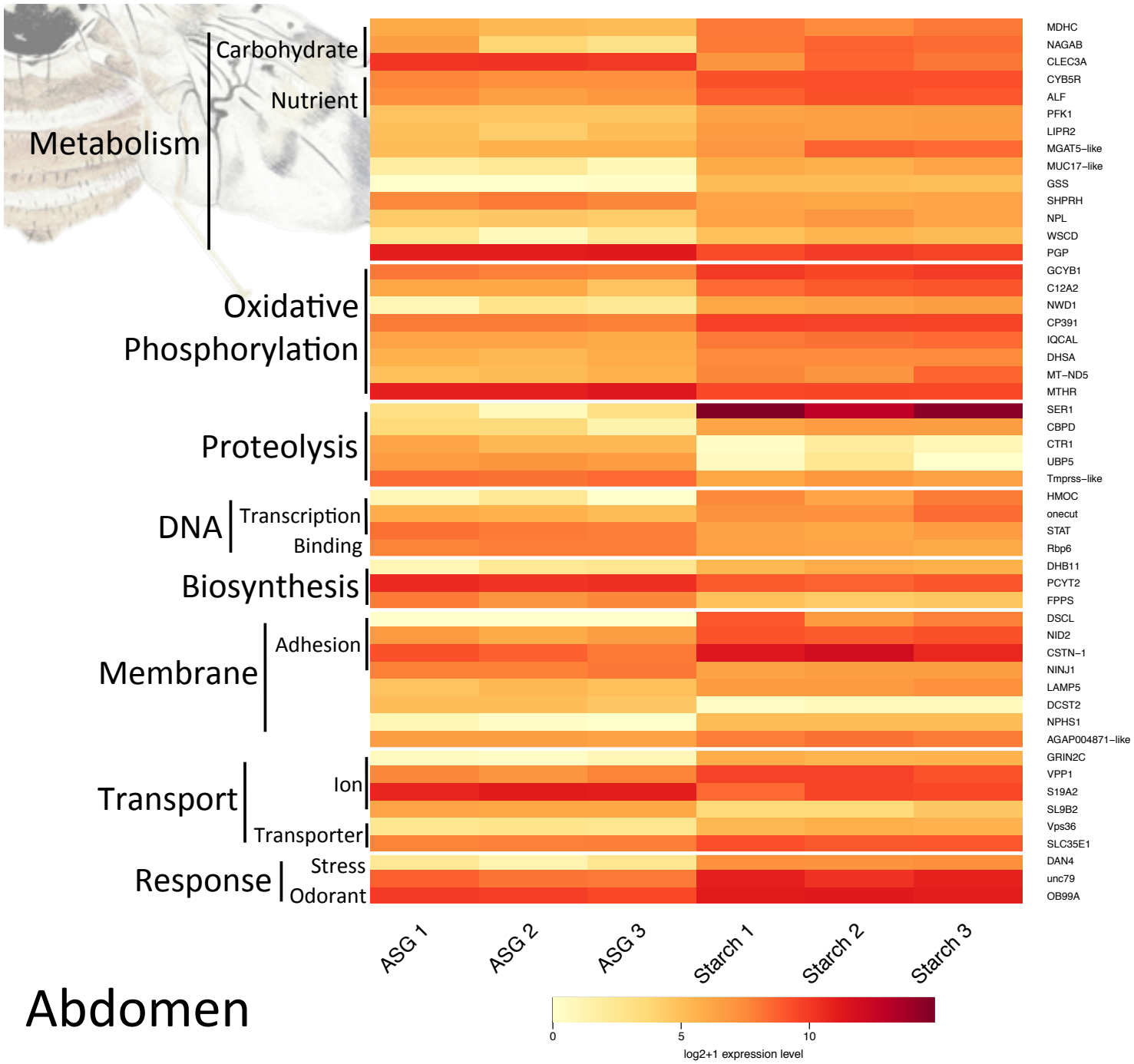

Figure 5.6. Heat map representing $\log_2$ + 1 expression levels for genes exhibiting DE in Ab tissue. 38 genes showing DE in Ab tissue. Format follows that of Figure 5.5.

Abdomen

5.5.9 Candidates for low throughput validation

Figure 5.8 shows four examples of the expression profile plots that were used to assess the suitability of candidates for low throughput validation via qRT-PCR. 16 candidates were chosen from the 109 annotated genes exhibiting DE, 9 from HT tissue, and 7 from the Ab tissue. These candidates are highlighted in Figure 5.7. Plotting of the expression profiles of these candidates revealed three patterns of DE within the study.

- Up or down regulation (Figure 5.8A), where the gene was expressed in both treatments, but at higher levels in one. The most common signal, and presenting a good candidate for validation
- Presence/Absence DE (Figure 5.8B), where a gene was expressed in only one treatment. An excellent candidate for validation.
- Up or down regulation, but with both transcripts are expressed within the noise level (around 50 abundance) (Figure 5.8C), a questionable candidate for validation, as it could provide no signal in the qRT-PCR.
- Small fragment based DE (Figure 5.8D), not helpful for qRT-PCR candidacy in this context, but potentially informative about the presence of non-coding RNA (ncRNA).

Following the visual assessment of expression profiles, 7 genes from the Head/Thorax tissue were retained as candidates for qRT-PCR validation. These genes were: from the metabolism grouping, TCNA, which showed up regulation in Starch, and LtaE, which showed up regulation in ASG. From the proteolysis grouping, TRYP which showed presence in Starch and absence in ASG, and SNAK which showed up regulation in ASG. From the OXPHOS grouping, DGHL, which showed up regulation in ASG. From the DNA grouping, RAD50, which showed up regulation in Starch, and STAT, which showed up regulation in ASG.

From the Response grouping, both Obp19d and Obp56h were assessed as candidates, but rejected due to low levels of expression in Opb19d, and small fragment size in the case of Obp56h. However, when the secondary structure of this small fragment was analysed (inset, Figure 5.8D) using the RNAfold in the ViennaRNA package 2.0 (Lorenz et al. 2011), it presented the classic hairpin structure characteristic of microRNAs (Bartel 2009).

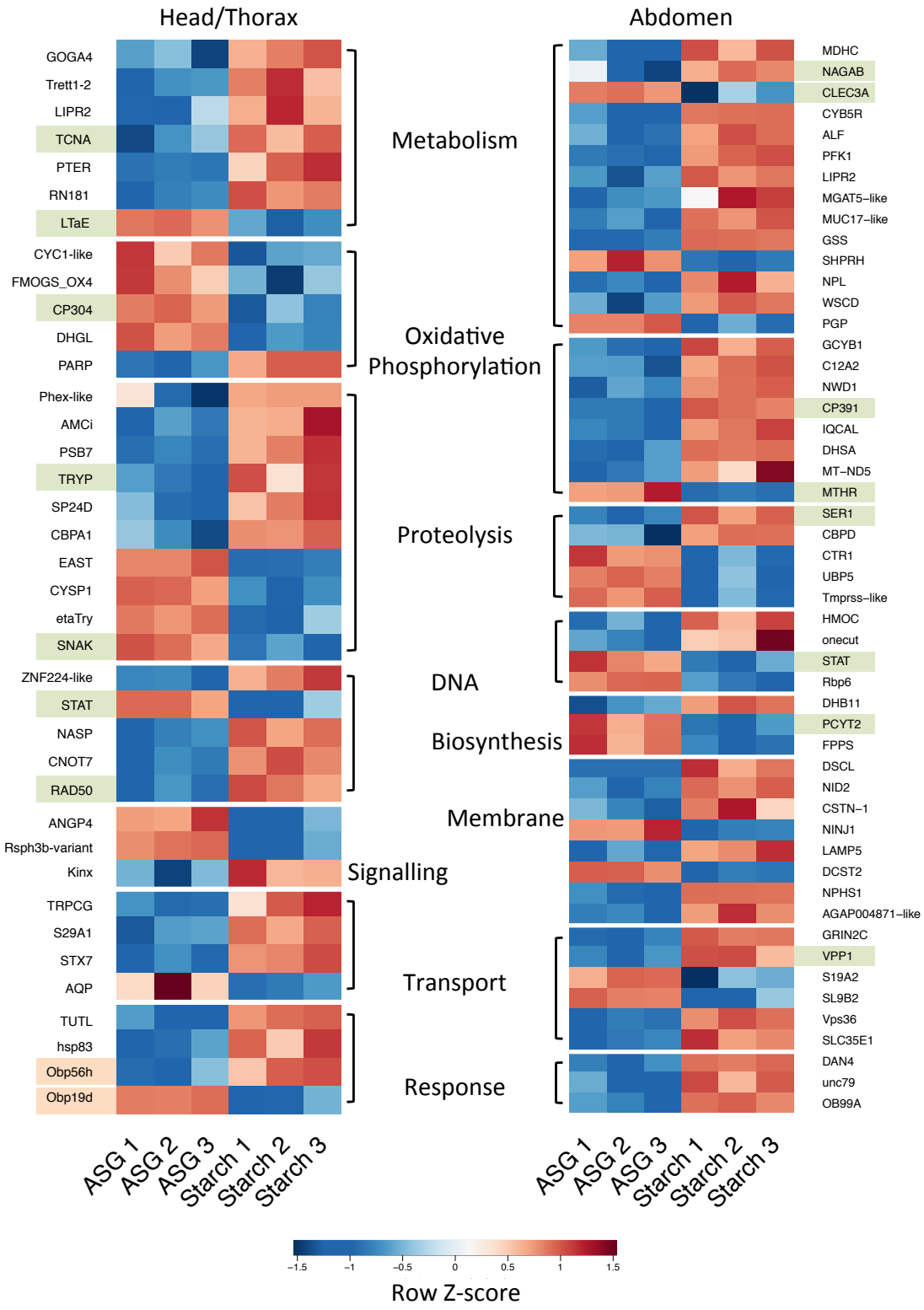

Figure 5.7. Heat maps of differential expression. Heat maps presented in Figures 5 and 6, recolored to highlight DE patterns. Colour represents row wise Z-score of expression levels normalised by row mean and standard deviation, Red represents comparative up regulation, blue represents comparative down regulation Left hand heat map represents genes exhibiting DE in the HT tissues, Right hand heat map represents genes exhibiting DE in the Ab tissue. Groupings based on GO terms are displayed between the heat maps. Genes from both tissues that were successfully tested as candidates for qRT-PCR validation of expression highlighted in orange.

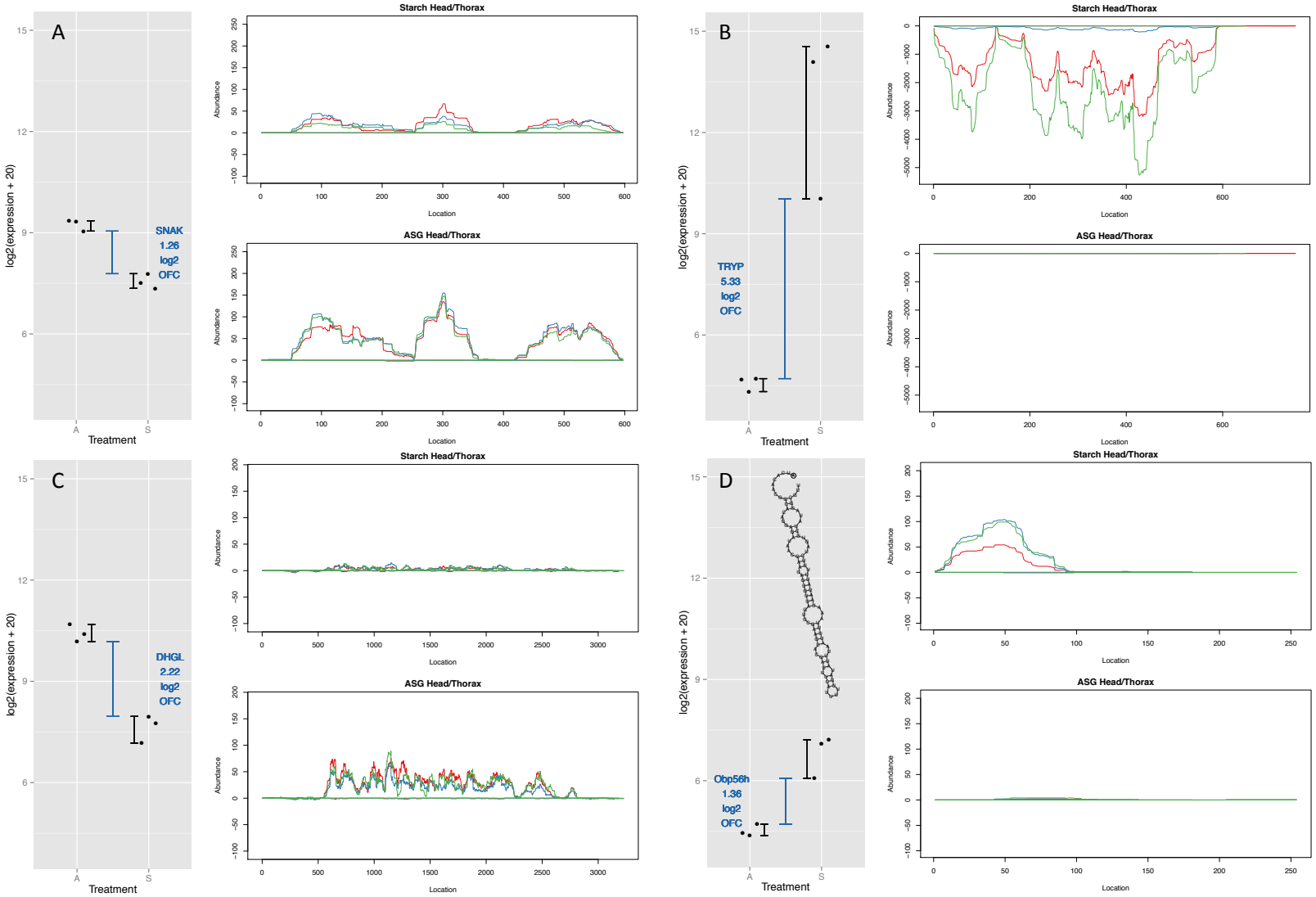

Figure 5.8. Expression profiles of four examples of putative candidate genes for low throughput validation. Plots show the expression level, maximal intervals, and DE of the three line replicates of each treatment (A = ASG, S = Starch, left hand plot), and the algebraic sum of abundances of incident reads at every position of the reference transcript, for each treatment (Starch top, ASG bottom, red = replicate 1, blue = replicate 2, green = replicate 3). Genes shown are selected from the Head/Thorax tissue. **A)** SNAK, DE by up or down regulation. **B)** TRYP, Presence/Absence DE. **C)** DHGL, DE by up or down regulation, but within the noise level. **D)** Obp56h, Small fragment based DE, with secondary structure of 100 bp that is DE inset

All 7 candidates for validation chosen from the Abdomen tissue were retained after there expression profiles were examined, they were: From the metabolism grouping, NAGAB, which showed up regulation in Starch, and CLEC3A, which showed up regulation in ASG. From the proteolysis grouping, SER1, which showed presence in starch and absence in ASG. From the OXPHOS grouping, CP391 which, showed up regulation in Starch, and MTHR, which showed up regulation in ASG. The remaining two candidates; PCYT2 from Biosynthesis, and VPP1 from Transport, exhibited up or down regulation within the noise level of expression, so may not prove useful as candidates, but will be retained for further testing.

5.6 Discussion

RNAseq was used to capture a portrait of gene expression in sexually mature males resulting from the 62nd generation of an evolution experiment based upon divergent larval rearing diet (Chapter 3). The base population was divided between two novel larval diets, sucrose based 'ASG', and starch based 'Starch', with three allopatric replicates of each. During the 30th generation of this experiment, males were observed to adopt different courtship behavioural phenotypes when the proximate effects of diet were intact (Chapter 4). In the 60th generation of this experiment, significant assortative mating by diet was observed, and maintained to marginal significance when individuals were reared on a common garden diet. Here, eggs from the 60th generation of the evolution experiment were reared for two generations on the same common garden diet, and then males were maintained to sexual maturity in identical conditions. Thus, the gene expression captured was not influenced by the nutritional differences in the selection diets, by any potential parental effects, or by differences in adult conditions. The results should therefore reflect genetic and evolved differences in gene expression in response to the two experimental evolution diets.

The use of a novel, subsampling based bootstrap normalisation (Mohorianu et al. in revision) to a fixed total of 34M reads, yielded 3 high quality independent biological replicates for each regime and body part. Using a maximal interval based approach, the DE call utilised these 3 replicates to show transcripts that exhibited DE between treatments across all three replicates. Based upon this method, a set of 1,214 transcripts that were DE above 2 $\log_2$OFC between treatments was isolated. The uniformity of the replicates indicates both the power of the normalisation method, an essential analytical step following RNAseq (Dillies et al. 2013), and the repeatability of the adaptive response to divergent larval rearing diet in this study. This uniformity was also seen in the mate choice exhibited in the 60th generation mating tests (Chapter 3).

This is an interesting result as the repeatability of evolutionary patterns has been widely debated (Boake 1989; Arendt and Reznick 2008; Stern and Orgogozo 2008; Hughes 2010; Rosenblum et al. 2010; Lachapelle et al. 2015), and this study, in line with other recent evolution experiments (Remolina et al. 2012; Burke et al. 2014), suggests that such designs can retrieve repeatable signals of adaptation. Further, these results highlight the medfly as an extremely suitable organism for further experimental evolution studies.

Within the set of DE transcripts, 109 transcripts yielded meaningful annotations (9%). This small subset represents the emergent nature of the medfly as a model species, and also the conservative nature of the annotation techniques used in the analysis (e.g. BLASTN search only). Further study on this data set should incorporate medfly-specific annotation resources which are not available through the BLAST network, such as the MEET database (Salvemini et al. 2014), in order to extend possibilities of successful annotation. Despite this, the relatively small number of meaningful annotations retrieved, the functional description of these genes suggested several interesting patterns of divergence in gene expression patterns at sexual maturity between ASG and Starch line males.

Starch diet males exhibited higher expression of genes associated with metabolism and nutrient processing, across both head thorax and abdomen tissue. In the HT tissue, this is manifested by genes involved in the breakdown & transport of nutrients, such those associated with lipases (e.g. LIPR2), hydrolases (e.g. PTER), ligases (e.g. RN181), lyases (e.g. ALF), and trelahose transport (Tret1-2). In the Ab tissue, a DE in a similar suite of genes was observed. Particularly associated with carbohydrate metabolism, cytoplasmic malate (MDHC) and alpha-n-acetylgalactosaminidase (NAGAB) were up regulated in Starch males, where as the c-type lectin associated gene CLEC3A was up regulated in ASG. As the major difference between the divergent larval diets was the complexity and diversity of carbohydrate content, DE in nutrient metabolism was expected. Several Drosophilid systems that exhibit divergence driven by host specialisation show similar patterns of DE in nutrient related metabolic genes by host (e.g. Dworkin and Jones 2009; Wurmser et al. 2011; Etges 2014; Etges et al. 2015; Guillén et al. 2015). This effect is to be expected, as the nutrients available in the larval diet can have large effects on survivorship (Chapter 2), and have the potential to drive selection for optimal ability to utilise novel host nutrients. Within the medfly, the impact of larval rearing diet on male adult life history is substantial (e.g. Arita and Kaneshiro 1988; Kaspi et al. 2002). DE in metabolic genes could be

associated with phenotypes manifested through effects on body size and nutrient reserves (Davidowitz and Nijhout 2004; Edgar 2006), but also through the potential for metabolic programming of adult expression patterns by larval conditions, as seen in zebrafish (e.g. Fang et al. 2014).

An interesting pattern of divergent DE was observed in genes associated with ATP and OXPHOS. In both HT and Ab tissue, genes expressed in the mitochondria expressed strong DE between treatments. In the HT tissue, ASG males showed higher expression in two cytochrome genes (CYC1, CP304), a flavin containing monooxygenase (FMOGS-OX4), and a glucose dehydrogenase (DHGL), all genes associated with ATP synthesis. In the Ab tissue, the majority of OXPHOS genes were expressed at higher levels in Starch males. These genes included two cytochrome p450 genes (C12A2, CP391), a guanylate cylase associated gene (GYC1B), and a succinate dehydrodgenase gene (DHSA). These genes are involved in ATP synthesis through the oxidation-reduction process, heme binding, or ATP binding and transport. Genes involved in OXPHOS are expressed in, or interact with the mitochondria which, due to their role as the centres of cellular energy production and rapidly evolving independent genome (mtDNA), are thought to be essential genes in adaptation and speciation (Gershoni et al. 2009; Ballard and Melvin 2010). An important example of this is the lake whitefish species complex (*Coregonus spp.*), in which divergence in OXPHOS gene expression in adult fish shows a tight relationship to the energetic phenotypes represented by two reproductively isolated morphs (Trudel et al. 2001; Derome et al. 2006; St-Cyr et al. 2008; Nolte et al. 2009; Jeukens et al. 2010; Renaut et al. 2010; Evans and Bernatchez 2012).

The tissue specific DE seen in OXPHOS genes between males from ASG and Starch backgrounds may have a correlation with the behavioural phenotypes described in Chapter 4, which were based on different levels of activity during courtship. ASG males were observed to conduct a higher frequency of bouts of wing vibration behaviour during courtship, than starch males, and also more locomotion. Starch males were significantly less mobile, and exhibited less 'active' courtship behaviours, favouring instead more bouts and a higher total time spent in pheromone gland extrusion. The up regulation of genes associated with OXPHOS related energy production genes in ASG male HT tissue could indicate the initiation of this behaviour, or priming for it, as the thorax is where the musculature associated with locomotion and wing movement is located in insects (Gullan and Cranston 2009). As Starch males were seen to engage in behaviour related to the expression of pheromone (Chapter 4), the up regulation of

genes related to energy creation in the Starch male abdomen could be associated with energetically costly process of pheromone biosynthesis (Dicke and Sabelis 1992; Jurenka 2004).

The DE seen between genes associated with proteolysis, the process of protein degradation, which is active in digestion, but also in driving many facets of the cell cycle (King et al. 1996) did not show a mainly unidirectional change in expression. In both tissues, different genes associated with proteases and peptidases, the enzymes that conduct proteolysis, were more highly expressed within each treatment. Five different serine proteases (SP24D, SNAK, EAST, SER1, Tmprss) exhibited DE between treatments, as well as other digestive enzymes such as trypsin (TRYP) and proteasome associated genes (e.g. PSB7). The pattern of DE seen in genes associated with proteolysis also carried the largest fold changes in expression seen in this analysis. Starch males expressed some genes within this grouping at very high levels that were absent in ASG males (e.g. HT: SER1, Ab: TRYP). The different sets of genes relating to proteolysis that were up regulated in sexually mature males of each treatment, coupled with the fact that Starch males were expressing genes which were not expressed in ASG males, may represent divergent adaptation in proteolytic strategy between the Starch and ASG lines. As the two diets differed in the total availability of protein, with ASG containing both yeast and corn meal as protein sources, and Starch containing only yeast, the need for different strategies of proteolysis may have been selected for during development, creating a different 'programme' for the adult proteolytic phenotype.

Other patterns in DE were less well defined with smaller numbers of genes associated with particular domains of GO present. However, it is possible to define a pattern in these genes in the Head/Thorax tissue, with higher levels of regulation in Starch males, across DNA related genes, such as zinc finger protein 224 (ZNF224) which regulates transcription, and *nasp* (NASP) which is involved in DNA replication. This could suggest, in association with the elevated level of metabolism, more cellular processes were occurring. This pattern was less clearly defined in the abdomen tissue, with all subsequent GO groupings showing up regulation in both treatments. However, coupled with the overall pattern of divergence in the expression of genes associated with nutrient metabolism and processing, OXPHOS, and proteolysis, these genes could represent other members in larger gene regulatory networks. Such networks are essential to the phenotypic plasticity that can facilitate adaptive evolution (Espinosa-Soto et al. 2011), and network divergence is increasingly understood as a driver of

adaptation and speciation (e.g. Chapman et al. 2011; Filteau et al. 2013; Pfennig and Ehrenreich 2014). As it is possible to identify 'hub genes' within networks, those likely to be centres for pleiotropy (Evans 2015), such analyses can offer great insight in genomic response to environmental selection, and should be considered in future analysis of the data presented here.

Where ecological speciation is driven by host specialisation, although genes relating to metabolism and energy production often form the main body of changes in expression levels between populations, genes that confer specific host-related functions have also been reported as important in transcriptomic divergence. These functions can be jointly conferred by metabolic genes, for example the role of certain OXPHOS genes in detoxification and host related hormone regulation (Dworkin and Jones 2009; Wurmser et al. 2011). Genes that show divergence outside of dual functions are equally important, and another gene set associated with population divergence by host specialisation is olfactory and gustatory receptor (OGR) genes (McBride et al. 2007; Wurmser et al. 2011; Etges 2014). Divergence in chemosensory traits associated with OGR genes is commonly associated with the establishment of prezygoitc barriers during speciation (reviewed by Smadja and Butlin 2009). These genes mediate chemosensory responses and are associated with host recognition, but also perception of pheromonal communication (Galindo and Smith 2001). Three OGR genes exhibit DE in this study; all are olfactory binding proteins (OBPs) (Obp19d, Obp56h, Obp99a). Of these, Obp56h is of particular interest, as it is seen to show DE between allopatric populations of *Drosophila mojavensis*, adapted to different host cacti, and is differentially expressed in conjunction with other OBPs and behavioural genes (Etges 2014). In *D. mojavensis*, Obp56h is expressed in the antenna, and is associated with sensory perception of smell, and also response to pheromones (Etges 2014).

In this study, Obp56h was expressed at higher levels in the Starch HT tissue, a pattern that is mirrored by another OBP, Obp19d, which is expressed at higher levels in ASG HT tissue. Obp19d has also been associated with chemosensory response and nutrient sensing (Arya et al. 2010). As the experimental design removed the opportunity for active host selection, pheromonal signalling could drive this divergence in expression. Both genes have previously identified as expressed in medfly adult head, antennae and palps (Siciliano et al. 2014), further supporting a sensory role. The role of OGR genes and particularly OBPs are well established and Obp57d & Obp57e in *Drosophila sechillia* (Matsuo et al. 2007) are considered candidates for 'ecological speciation genes' (Nosil 2012). DE of OBPs in this study is interesting, and they are definite

candidates for further study. It is also interesting to note the putative miRNA structure of the fragment of Obp56h exhibiting DE in this study (inset, Figure 5.8D). Such regulatory molecules may represent an underappreciated source for facilitating rapid evolutionary change via the creation of new variation (Bond and Baulcombe 2014; Konczal et al. 2015).

The results described here suggest that the medfly may be a prime candidate to study adaptation and population divergence using evolution experiments, as the repeatability of the adaptive response in the transcriptomic data was high. Even with a conservative annotation protocol, it was possible to retrieve a signal of adaptation to novel environments that aligned with several examples of divergence under environmental selection pressure in the wild. Divergence in the expression of metabolic, OXPHOS and proteolysis genes was seen in both treatments, and several candidates (e.g. Obp56h) associated with behaviour showed DE. Further designs could be extended to incorporate sequencing of larval medfly to investigate the relationship between developmental gene expression and adult gene expression. The difference in expression seen in species such as *Drosophila mojavensis* have shown this to be an important division in adaptive gene expression (Nolte et al. 2009). The renewed interest in experimental evolution heralded by 'evolve & re-sequence' designs, which couple evolution experiments with repeated sequencing of whole genomes or expression profiles throughout their progress (Schlötterer et al. 2014), offer vast potential to answer questions concerning adaptation and speciation. The findings described here could inform such experiments.

It is interesting to consider whether evolution would come to the same answer using the same genes or expression patterns if different base populations had been used to initiate selection. Given that the timescale of this study was short, it is unlikely that the appearance of new mutations in the adapting populations would have effected the evolved changes captured here. For this reason the standing variation represented by the founding population is essential to the resulting adaptation, and also in separating the action of adaptive evolution from genetic drift. Although it was not quantified here, the repeatability of the changes in gene expression, and significant and repeatable evolved changes in traits presented in earlier Chapters, do not suggest the founding population used to initiate the replicated populations studied here were lacking in genetic variation. However, it will be essential for future studies to quantify standing variation in their based populations, as it is increasingly understood to have great bearing on the power and repeatability of experimental evolution designs (e.g. Kessner

and Novembre 2015). The data presented here also have great potential to yield far more information about the exact pattern of divergence in expression patterns. Firstly, analysis of between replicate DE highlighted the presence of small quantities of transfer RNA (tRNA) and ribosomal RNA (rRNA) within the analysed data set. As tRNA/rRNA should be removed during the sequencing process, this will distort the picture of DE when coupled with the fixed total subsampling normalisation methodology employed here. Although its effects are expected to be minor, the annotation and removal of these transcripts, and consequent recalculation of abundance distributions and subsampling could refine the picture of DE between treatments. It will also be possible to utilise the data described here to isolate sequence polymorphism present between replicates and treatments (e.g. Renaut et al. 2010), and to investigate patterns of alternate splicing (e.g. Smith et al. 2013) between treatments or replicates. Evidence of DE in several transposable elements was also observed between treatments. Such elements have been implicated as important in rapid responses to ecological divergence (Crespi and Nosil 2013; Kim et al. 2014), and would be interesting candidates to explore further.

The first stage in further work is to validate the DE using the candidates suggested by this analysis (Figure 5.8). Using low throughput methodologies such as qRT-PCR for validation is essential in NGS studies of gene expression (Pavey et al. 2010), and is expected to confirm the expression patterns described here. This would be the first stage in fully connecting the functional elements highlighted by RNAseq data to the phenotypes observed (Chapter 3, 4). Candidate genes from this study could be used in gene knock down studies (e.g. Marshall et al. 2009; Streisfeld et al. 2013; Li 2014), or manipulated through transgenic alteration of expression (Kobayashi et al. 2013), including using gene editing methods such as CRISPRi (Larson et al. 2013).

5.7 References

Adams, M., J. Kelley, J. Gocayne, M. Dubnick, M. Polymeropoulos, H. Xiao, C. Merril, A. Wu, B. Olde, R. Moreno, and a. et. 1991. Complementary DNA sequencing: expressed sequence tags and human genome project. Science (80-.). 252:1651–1656.

Arendt, J., and D. Reznick. 2008. Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? Trends Ecol. Evol. 23:26–32.

Arita, L. H., and K. Y. Kaneshiro. 1988. Body size and differential mating success between males of two populations of the Mediterranean fruit fly. Pacific Sci. 42:173–177.

Arya, G. H., A. L. Weber, P. Wang, M. M. Magwire, Y. L. S. Negron, T. F. C. Mackay, and R. R. H. Anholt. 2010. Natural variation, functional pleiotropy and transcriptional contexts of odorant binding protein genes in *Drosophila melanogaster*. Genetics 186:1475–85.

Ballard, J. W. O., and R. G. Melvin. 2010. Linking the mitochondrial genotype to the organismal phenotype. Mol. Ecol. 19:1523–39.

Bartel, D. P. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136:215–33.

Bernatchez, L., S. Renaut, A. R. Whiteley, N. Derome, J. Jeukens, L. Landry, G. Lu, A. W. Nolte, K. Ostbye, S. M. Rogers, and J. St-Cyr. 2010. On the origin of species: insights from the ecological genomics of lake whitefish. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365:1783–800.

Boake, C. R. B. 1989. Repeatability: Its role in evolutionary studies of mating behavior. Evol. Ecol. 3:173–182.

Bolstad, B. M., R. . Irizarry, M. Astrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185–193.

Bond, D. M., and D. C. Baulcombe. 2014. Small RNAs and heritable epigenetic variation in plants. Trends Cell Biol. 24:100–7.

Burke, M. K., G. Liti, and A. D. Long. 2014. Standing genetic variation drives repeatable experimental evolution in outcrossing populations of *Saccharomyces cerevisiae*. Mol. Biol. Evol. 31:3228–39.

Calla, B., B. Hall, S. Hou, and S. M. Geib. 2014. A genomic perspective to assessing quality of mass-reared SIT flies used in Mediterranean fruit fly (*Ceratitis capitata*) eradication in California. BMC Genomics 15:98.

Chapman, R. W., A. Mancia, M. Beal, A. Veloso, C. Rathburn, A. Blair, A. F. Holland, G. W. Warr, G. Didinato, I. M. Sokolova, E. F. Wirth, E. Duffy, and D. Sanger. 2011. The transcriptomic responses of the eastern oyster, *Crassostrea virginica*, to environmental conditions. Mol. Ecol. 20:1431–49.

Comeault, A. A., M. Sommers, T. Schwander, C. A. Buerkle, T. E. Farkas, P. Nosil, and T. L. Parchman. 2012. De novo characterization of the *Timema cristinae* transcriptome facilitates marker discovery and inference of genetic divergence. Mol. Ecol. Resour. 12:549–61.

Crespi, B., and P. Nosil. 2013. Conflictual speciation: species formation via genomic

conflict. Trends Ecol. Evol. 28:48–57.

Crosetto, N., M. Bienko, and A. van Oudenaarden. 2014. Spatially resolved transcriptomics and beyond. Nat. Rev. Genet. 16:57–66.

Daines, B., H. Wang, L. Wang, Y. Li, Y. Han, D. Emmert, W. Gelbart, X. Wang, W. Li, R. Gibbs, and R. Chen. 2011. The *Drosophila melanogaster* transcriptome by paired-end RNA sequencing. Genome Res. 21:315–24.

Davidowitz, G., and H. F. Nijhout. 2004. The physiological basis of reaction norms: the interaction among growth rate, the duration of growth and body size. Integr. Comp. Biol. 44:443–449.

Davies, S. J., and T. Chapman. 2006. Identification of genes expressed in the accessory glands of male Mediterranean Fruit Flies (*Ceratitis capitata*). Insect Biochem. Mol. Biol. 36:846–56.

Derome, N., and L. Bernatchez. 2006. The transcriptomics of ecological convergence between 2 limnetic coregonine fishes (Salmonidae). Mol. Biol. Evol. 23:2370–8.

Derome, N., P. Duchesne, and L. Bernatchez. 2006. Parallelism in gene transcription among sympatric lake whitefish (*Coregonus clupeaformis*, Mitchill) ecotypes. Mol. Ecol. 15:1239–49.

Dicke, M., and M. W. Sabelis. 1992. Costs and benefits of chemival information conveyance: proximate and ultimate factors. Pp. 122–155 *in* Insect chemical ecology : an evolutionary approach. Chapman & Hall, New York.

Dillies, M.-A., A. Rau, J. Aubert, C. Hennequet-Antier, M. Jeanmougin, N. Servant, C. Keime, G. Marot, D. Castel, J. Estelle, G. Guernec, B. Jagla, L. Jouneau, D. Laloë, C. Le Gall, B. Schaëffer, S. Le Crom, M. Guedj, and F. Jaffrézic. 2013. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief. Bioinform. 14:671–83.

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

Drosophila 12 Genomes Consortium. 2007. Evolution of genes and genomes on the *Drosophila* phylogeny. Nature 450:203–18.

Dworkin, I., and C. D. Jones. 2009. Genetic changes accompanying the evolution of host specialization in *Drosophila sechellia*. Genetics 181:721–36.

Edgar, B. A. 2006. How flies get their size: genetics meets physiology. Nat. Rev. Genet. 7:907–916.

Ekblom, R., and J. Galindo. 2011. Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity. 107:1–15.

Elmer, K. R., and A. Meyer. 2011. Adaptation in the age of ecological genomics: insights from parallelism and convergence. Trends Ecol. Evol. 26:298–306.

Espinosa-Soto, C., O. C. Martin, and A. Wagner. 2011. Phenotypic plasticity can facilitate adaptive evolution in gene regulatory circuits. BMC Evol. Biol. 11:5.

Etges, W. J. 2014. No boundaries: genomes, organisms, and ecological interactions responsible for divergence and reproductive isolation. J. Hered. 105 Suppl :756–70.

Etges, W. J., C. C. de Oliveira, E. Gragg, D. Ortíz-Barrientos, M. a F. Noor, and M. G. Ritchie. 2007. Genetics of incipient speciation in *Drosophila mojavensis*. I. Male courtship song, mating success, and genotype X environment interactions. Evolution. 61:1106–1119.

Etges, W. J., C. C. de Oliveira, M. G. Ritchie, and M. A. F. Noor. 2009. Genetics of incipient speciation in *Drosophila mojavensis*: II. Host plants and mating status influence cuticular hydrocarbon QTL expression and G x E interactions. Evolution 63:1712–30.

Etges, W. J., M. V Trotter, C. C. de Oliveira, S. Rajpurohit, A. G. Gibbs, and S. Tuljapurkar. 2015. Deciphering life history transcriptomes in different environments. Mol. Ecol. 24:151–79.

Evans, M. L., and L. Bernatchez. 2012. Oxidative phosphorylation gene transcription in whitefish species pairs reveals patterns of parallel and nonparallel physiological divergence. J. Evol. Biol. 25:1823–34.

Evans, T. G. 2015. Considerations for the use of transcriptomics in identifying the "genes that matter" for environmental adaptation. J. Exp. Biol. 218:1925–1935.

Fang, L., X.-F. Liang, Y. Zhou, X.-Z. Guo, Y. He, T.-L. Yi, L.-W. Liu, X.-C. Yuan, and Y.-X. Tao. 2014. Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, *Danio rerio*. Br. J. Nutr. 111:808–18.

Fay, J. C., and P. J. Wittkopp. 2008. Evaluating the role of natural selection in the evolution of gene regulation. Heredity. 100:191–9.

Feder, J. L., S. P. Egan, and P. Nosil. 2012. The genomics of speciation-with-gene-flow. Trends Genet. 28:342–50.

Filteau, M., S. A. Pavey, J. St-Cyr, and L. Bernatchez. 2013. Gene coexpression networks reveal key drivers of phenotypic divergence in lake whitefish. Mol. Biol. Evol.

30:1384–96.

Frentiu, F. D., M. Adamski, E. A. McGraw, M. W. Blows, and S. F. Chenoweth. 2009. An expressed sequence tag (EST) library for *Drosophila serrata*, a model system for sexual selection and climatic adaptation studies. BMC Genomics 10:40.

Galindo, J., J. W. Grahame, and R. K. Butlin. 2010. An EST-based genome scan using 454 sequencing in the marine snail *Littorina saxatilis*. J. Evol. Biol. 23:2004–16.

Galindo, K., and D. P. Smith. 2001. A large family of divergent Drosophila odorant-binding proteins expressed in gustatory and olfactory sensilla. Genetics 159:1059–72.

Gerrard, D. T., C. Fricke, D. A. Edward, D. R. Edwards, and T. Chapman. 2013. Genome-wide responses of female fruit flies subjected to divergent mating regimes. PLoS One 8:e68136.

Gershoni, M., A. R. Templeton, and D. Mishmar. 2009. Mitochondrial bioenergetics as a major motive force of speciation. Bioessays 31:642–50.

Gioti, A., S. Wigby, B. Wertheim, E. Schuster, P. Martinez, C. J. Pennington, L. Partridge, and T. Chapman. 2012. Sex peptide of *Drosophila melanogaster* males is a global regulator of reproductive processes in females. Proc. R. Soc. B 279:4423–32.

Gomulski, L. M., G. Dimopoulos, Z. Xi, F. Scolari, P. Gabrieli, P. Siciliano, A. R. Clarke, A. R. Malacrida, and G. Gasperi. 2012. Transcriptome profiling of sexual maturation and mating in the Mediterranean fruit fly, *Ceratitis capitata*. PLoS One 7:e30857.

Gomulski, L. M., G. Dimopoulos, Z. Xi, M. B. Soares, M. F. Bonaldo, A. R. Malacrida, and G. Gasperi. 2008. Gene discovery in an invasive tephritid model pest species, the Mediterranean fruit fly, *Ceratitis capitata*. BMC Genomics 9:243.

Gracey, A. Y. 2007. Interpreting physiological responses to environmental change through gene expression profiling. J. Exp. Biol. 210:1584–92.

Graveley, B. R. 2008. Molecular biology: power sequencing. Nature 453:1197–8.

Graze, R. M., L. M. McIntyre, B. J. Main, M. L. Wayne, and S. V Nuzhdin. 2009. Regulatory divergence in *Drosophila melanogaster* and *D. simulans*, a genomewide analysis of allele-specific expression. Genetics 183:547–61, 1SI–21SI.

Guillén, Y., N. Rius, A. Delprat, A. Williford, F. Muyas, M. Puig, S. Casillas, M. Ràmia, R. Egea, B. Negre, G. Mir, J. Camps, V. Moncunill, F. J. Ruiz-Ruano, J. Cabrero, L. G. de Lima, G. B. Dias, J. C. Ruiz, A. Kapusta, J. Garcia-Mas, M. Gut, I. G. Gut, D. Torrents, J. P. Camacho, G. C. S. Kuhn, C. Feschotte, A. G. Clark, E. Betrán, A. Barbadilla, and A. Ruiz. 2015. Genomics of ecological adaptation in cactophilic *Drosophila*. Genome Biol.

Evol. 7:349–66.

Gullan, P. J., and P. S. Cranston. 2009. The Insects: An Outline of Entomology. John Wiley & Sons.

Henning, F., and A. Meyer. 2014. The evolutionary genomics of cichlid fishes: explosive speciation and adaptation in the postgenomic era. Annu. Rev. Genomics Hum. Genet. 15:417–41.

Hughes, K. A. 2010. More than one way to blanch a lizard. Proc. Natl. Acad. Sci. U. S. A. 107:1815–6.

Jeukens, J., S. Renaut, J. St-Cyr, A. W. Nolte, and L. Bernatchez. 2010. The transcriptomics of sympatric dwarf and normal lake whitefish (*Coregonus clupeaformis spp.*, Salmonidae) divergence as revealed by next-generation sequencing. Mol. Ecol. 19:5389–403.

Jiménez-Guri, E., J. Huerta-Cepas, L. Cozzuto, K. R. Wotton, H. Kang, H. Himmelbauer, G. Roma, T. Gabaldón, and J. Jaeger. 2013. Comparative transcriptomics of early Dipteran development. BMC Genomics 14:123.

Jurenka, R. 2004. Insect pheromone biosynthesis. Top. Curr. Chem. 239:97–132.

Kaspi, R., S. Mossinson, T. Drezner, B. Kamensky, and B. Yuval. 2002. Effects of larval diet on development rates and reproductive maturation of male and female Mediterranean fruit flies. Physiol. Entomol. 27:29–38.

Kawecki, T. J., R. E. Lenski, D. Ebert, B. Hollis, I. Olivieri, and M. C. Whitlock. 2012. Experimental evolution. Trends Ecol. Evol. 27:547–60.

Kessner, D., and J. Novembre. 2015. Power analysis of artificial selection experiments using efficient whole genome simulation of quantitative traits. Genetics 199:991–1005.

Kim, Y. B., J. H. Oh, L. J. McIver, E. Rashkovetsky, K. Michalak, H. R. Garner, L. Kang, E. Nevo, A. B. Korol, and P. Michalak. 2014. Divergence of *Drosophila melanogaster* repeatomes in response to a sharp microclimate contrast in Evolution Canyon, Israel. Proc. Natl. Acad. Sci. U. S. A. 111:10630–5.

King, R. W., R. J. Deshaies, J.-M. Peters, and M. W. Kirschner. 1996. How proteolysis drives the cell cycle. Science (80-.). 274:1652–1659.

Kobayashi, M. J., Y. Takeuchi, T. Kenta, T. Kume, B. Diway, and K. K. Shimizu. 2013. Mass flowering of the tropical tree *Shorea beccariana* was preceded by expression changes in flowering and drought-responsive genes. Mol. Ecol. 22:4767–82.

Konczal, M., W. Babik, J. Radwan, E. T. Sadowska, and P. Koteja. 2015. Initial

molecular-level response to artificial selection for increased aerobic metabolism occurs primarily through changes in gene expression. Mol. Biol. Evol. 32:1461–73.

Lachapelle, J., J. Reid, and N. Colegrave. 2015. Repeatability of adaptation in experimental populations of different sizes. Proc. Biol. Sci. 282:20143033–.

Landry, L., W. F. Vincent, and L. Bernatchez. 2007. Parallel evolution of lake whitefish dwarf ecotypes in association with limnological features of their adaptive landscape. J. Evol. Biol. 20:971–84.

Larson, M. H., L. A. Gilbert, X. Wang, W. A. Lim, J. S. Weissman, and L. S. Qi. 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat. Protoc. 8:2180–96.

Leftwich, P. T., M. Bolton, and T. Chapman. 2015. Evolutionary biology and genetic techniques for insect control. Evol. Appl. 9:212-230.

Li, Z. 2014. An investigation of novel genes involved in female decision-making in *Drosophila melanogaster*. Discussions 10.

Liquido, N. J., L. A. Shinoda, and R. T. Cunningham. 1991. Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): an annotated world review. Entomological Society of America, Lanham.

Lorenz, R., S. H. Bernhart, C. Höner Zu Siederdissen, H. Tafer, C. Flamm, P. F. Stadler, and I. L. Hofacker. 2011. ViennaRNA Package 2.0. Algorithms Mol. Biol. 6:26.

Manfredini, F., M. J. F. Brown, V. Vergoz, and B. P. Oldroyd. 2015. RNA-sequencing elucidates the regulation of behavioural transitions associated with the mating process in honey bee queens. BMC Genomics 16:563.

Marshall, J. L., D. L. Huestis, Y. Hiromasa, S. Wheeler, C. Oppert, S. A. Marshall, J. M. Tomich, and B. Oppert. 2009. Identification, RNAi knockdown, and functional analysis of an ejaculate protein that mediates a postmating, prezygotic phenotype in a cricket. PLoS One 4:e7537.

Matlin, A. J., F. Clark, and C. W. J. Smith. 2005. Understanding alternative splicing: towards a cellular code. Nat. Rev. Mol. Cell Biol. 6:386–98.

Matsuo, T., S. Sugaya, J. Yasukawa, T. Aigaki, and Y. Fuyama. 2007. Odorant-binding proteins OBP57d and OBP57e affect taste perception and host-plant preference in *Drosophila sechellia*. PLoS Biol. 5:e118.

Matzkin, L. M. 2012. Population transcriptomics of cactus host shifts in *Drosophila mojavensis*. Mol. Ecol. 21:2428–39.

McBride, C. S., J. R. Arguello, and B. C. O'Meara. 2007. Five *Drosophila* genomes reveal nonneutral evolution and the signature of host specialization in the chemoreceptor superfamily. Genetics 177:1395–416.

McManus, C. J., J. D. Coolon, M. O. Duff, J. Eipper-Mains, B. R. Graveley, and P. J. Wittkopp. 2010. Regulatory divergence in *Drosophila* revealed by mRNA-seq. Genome Res. 20:816–825.

Mezey, J. G., S. V Nuzhdin, F. Ye, and C. D. Jones. 2008. Coordinated evolution of co-expressed gene clusters in the *Drosophila* transcriptome. BMC Evol. Biol. 8:2.

Mohorianu, I., F. Schwach, R. Jing, S. Lopez-Gomollon, S. Moxon, G. Szittya, K. Sorefan, V. Moulton, and T. Dalmay. 2011. Profiling of short RNAs during fleshy fruit development reveals stage-specific sRNAome expression patterns. Plant J. 67:232–46.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5:621–8.

Nash, W. J., and T. Chapman. 2014. Effect of dietary components on larval life history characteristics in the medfly (*Ceratitis capitata*: Diptera, Tephritidae). PLoS One 9:e86029.

Nolte, A. W., S. Renaut, and L. Bernatchez. 2009. Divergence in gene regulation at young life history stages of whitefish (*Coregonus sp.*) and the emergence of genomic isolation. BMC Evol. Biol. 9:59.

Nosil, P. 2012. Ecological Speciation. Oxford University Press, Oxford.

Nosil, P., and D. Schluter. 2011. The genes underlying the process of speciation. Trends Ecol. Evol. 26:160–7.

Pavey, S. A., H. Collin, P. Nosil, and S. M. Rogers. 2010. The role of gene expression in ecological speciation. Ann. N. Y. Acad. Sci. 1206:110–29.

Pfennig, D. W., and I. M. Ehrenreich. 2014. Towards a gene regulatory network perspective on phenotypic plasticity, genetic accommodation and genetic assimilation. Mol. Ecol. 23:4438–40.

Porcelli, D., R. K. Butlin, K. J. Gaston, D. Joly, and R. R. Snook. 2015. The environmental genomics of metazoan thermal adaptation. Heredity. 114:502–514.

Rajpurohit, S., C. C. Oliveira, W. J. Etges, and A. G. Gibbs. 2013. Functional genomic and phenotypic responses to desiccation in natural populations of a desert Drosophilid. Mol. Ecol. 22:2698–715.

Ranz, J. M., and C. A. Machado. 2006. Uncovering evolutionary patterns of gene

expression using microarrays. Trends Ecol. Evol. 21:29–37.

Remolina, S. C., P. L. Chang, J. Leips, S. V Nuzhdin, and K. A. Hughes. 2012. Genomic basis of aging and life-history evolution in *Drosophila melanogaster*. Evolution 66:3390–403.

Renaut, S., A. W. Nolte, and L. Bernatchez. 2010. Mining transcriptome sequences towards identifying adaptive single nucleotide polymorphisms in lake whitefish species pairs (*Coregonus spp*. Salmonidae). Mol. Ecol. 19 Suppl 1:115–31.

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

Riesgo, A., S. C. S. Andrade, P. P. Sharma, M. Novo, A. R. Pérez-Porro, V. Vahtera, V. L. González, G. Y. Kawauchi, and G. Giribet. 2012. Comparative description of ten transcriptomes of newly sequenced invertebrates and efficiency estimation of genomic sampling in non-model taxa. Front. Zool. 9:33.

Risso, D., J. Ngai, T. P. Speed, and S. Dudoit. 2014. Normalization of RNA-seq data using factor analysis of control genes or samples. Nat. Biotechnol. 32:896–902.

Rosenblum, E. B., H. Römpler, T. Schöneberg, and H. E. Hoekstra. 2010. Molecular and functional basis of phenotypic convergence in white lizards at White Sands. Proc. Natl. Acad. Sci. U. S. A. 107:2113–7.

Rundle, H. D., S. F. Chenoweth, P. Doughty, and M. W. Blows. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLoS Biol. 3:e368.

Salvemini, M., K. P. Arunkumar, J. Nagaraju, R. Sanges, V. Petrella, A. Tomar, H. Zhang, W. Zheng, and G. Saccone. 2014. De novo assembly and transcriptome analysis of the Mediterranean fruit fly *Ceratitis capitata* early embryos. PLoS One 9:e114191.

San Andrés, V., P. Castañera, and B. Sabater Muñoz. 2013. Transcriptome analysis in *Ceratitis capitata* to unveil genes involved in ageing-maturation process. Spanish J. Agric. Res. 11:842.

Savolainen, O., M. Lascoux, and J. Merilä. 2013. Ecological genomics of local adaptation. Nat. Rev. Genet. 14:807–20.

Schlötterer, C., R. Kofler, E. Versace, R. Tobler, and S. U. Franssen. 2014. Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation. Heredity. 114:431–440.

Schwarz, D., H. M. Robertson, J. L. Feder, K. Varala, M. E. Hudson, G. J. Ragland, D.

A. Hahn, and S. H. Berlocher. 2009. Sympatric ecological speciation meets pyrosequencing: sampling the transcriptome of the apple maggot *Rhagoletis pomonella*. BMC Genomics 10:633.

Scolari, F., L. M. Gomulski, P. Gabrieli, M. Manni, G. Savini, G. Gasperi, and A. R. Malacrida. 2014. How functional genomics will impact fruit fly pest control: the example of the Mediterranean fruit fly, *Ceratitis capitata*. BMC Genet. 15 Suppl 2:S11.

Scolari, F., L. M. Gomulski, J. M. C. Ribeiro, P. Siciliano, A. Meraldi, M. Falchetto, A. Bonomi, M. Manni, P. Gabrieli, A. Malovini, R. Bellazzi, S. Aksoy, G. Gasperi, and A. R. Malacrida. 2012. Transcriptional profiles of mating-responsive genes from testes and male accessory glands of the Mediterranean fruit fly, *Ceratitis capitata*. PLoS One 7:e46812.

Seehausen, O., R. K. Butlin, I. Keller, C. E. Wagner, J. W. Boughman, P. A. Hohenlohe, C. L. Peichel, G.-P. Saetre, C. Bank, A. Brännström, A. Brelsford, C. S. Clarkson, F. Eroukhmanoff, J. L. Feder, M. C. Fischer, A. D. Foote, P. Franchini, C. D. Jiggins, F. C. Jones, A. K. Lindholm, K. Lucek, M. E. Maan, D. A. Marques, S. H. Martin, B. Matthews, J. I. Meier, M. Möst, M. W. Nachman, E. Nonaka, D. J. Rennison, J. Schwarzer, E. T. Watson, A. M. Westram, and A. Widmer. 2014. Genomics and the origin of species. Nat. Rev. Genet. 15:176–92.

Shendure, J. 2008. The beginning of the end for microarrays? Nat. Methods 5:585–7.

Siciliano, P., F. Scolari, L. M. Gomulski, M. Falchetto, M. Manni, P. Gabrieli, L. M. Field, J.-J. Zhou, G. Gasperi, and A. R. Malacrida. 2014. Sniffing out chemosensory genes from the Mediterranean fruit fly, Ceratitis capitata. PLoS One 9:e85523.

Smadja, C., and R. K. Butlin. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity. 102:77–97.

Smith, G., Y. Fang, X. Liu, J. Kenny, A. R. Cossins, C. C. de Oliveira, W. J. Etges, and M. G. Ritchie. 2013. Transcriptome-wide expression variation associated with environmental plasticity and mating success in cactophilic Drosophila mojavensis. Evolution 67:1950–63.

St-Cyr, J., N. Derome, and L. Bernatchez. 2008. The transcriptomics of life-history trade-offs in whitefish species pairs (*Coregonus sp.*). Mol. Ecol. 17:1850–70.

Stern, D. L., and V. Orgogozo. 2008. The loci of evolution: how predictable is genetic evolution? Evolution 62:2155–77.

Street, N. R., A. Sjödin, M. Bylesjö, P. Gustafsson, J. Trygg, and S. Jansson. 2008. A cross-species transcriptomics approach to identify genes involved in leaf development.

BMC Genomics 9:589.

Streisfeld, M. A., W. N. Young, and J. M. Sobel. 2013. Divergent selection drives genetic differentiation in an R2R3-MYB transcription factor that contributes to incipient speciation in *Mimulus aurantiacus*. PLoS Genet. 9:e1003385.

Tan, M. H., K. F. Au, A. L. Yablonovitch, A. E. Wills, J. Chuang, J. C. Baker, W. H. Wong, and J. B. Li. 2013. RNA sequencing reveals a diverse and dynamic repertoire of the *Xenopus tropicalis* transcriptome over development. Genome Res. 23:201–16.

Trudel, M., A. Tremblay, R. Schetagne, and J. B. Rasmussen. 2001. Why are dwarf fish so small? An energetic analysis of polymorphism in lake whitefish (*Coregonus clupeaformis*). Can. J. Fish. Aquat. Sci. 58:394–405.

Vera, J. C., C. W. Wheat, H. W. Fescemyer, M. J. Frilander, D. L. Crawford, I. Hanski, and J. H. Marden. 2008. Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Mol. Ecol. 17:1636–47.

Verdun, R. E., N. Di Paolo, T. P. Urmenyi, E. Rondinelli, A. C. C. Frasch, and D. O. Sanchez. 1998. Gene discovery through expressed sequence tag sequencing in *Trypanosoma cruzi*. Infect. Immun. 66:5393–5398.

Vesterlund, L., H. Jiao, P. Unneberg, O. Hovatta, and J. Kere. 2011. The zebrafish transcriptome during early development. BMC Dev. Biol. 11:30.

Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 10:57–63.

Whitehead, A., B. Dubansky, C. Bodinier, T. I. Garcia, S. Miles, C. Pilley, V. Raghunathan, J. L. Roach, N. Walker, R. B. Walter, C. D. Rice, and F. Galvez. 2012. Genomic and physiological footprint of the Deepwater Horizon oil spill on resident marsh fishes. Proc. Natl. Acad. Sci. U. S. A. 109:20298–302.

Whitehead, A., S. Zhang, J. L. Roach, and F. Galvez. 2013. Common functional targets of adaptive micro- and macro-evolutionary divergence in killifish. Mol. Ecol. 22:3780–96.

Wurmser, F., D. Ogereau, T. Mary-Huard, B. Loriod, D. Joly, and C. Montchamp-Moreau. 2011. Population transcriptomics: insights from *Drosophila simulans*, *Drosophila sechellia* and their hybrids. Genetica 139:465–77.

5.8 Appendix 5.1 - Comparative analysis of existing and novel approaches for data normalization and differential expression call

5.8.9 Scaling approaches

The initial methods proposed for RNA-seq data analysis were based on the identification of a scaling factor, which was applied indiscriminately, on the abundances of all genes. Newer approaches such as edgeR (Robinson and Oshlack 2010; Zhou et al. 2014) and DESeq (Love et al. 2014) have subsequently refined the detection of such scaling factors. However, it can be argued that a scaling approach, i.e. the use of a unique scaling factor for all genes across the abundance range, is not appropriate. To illustrate this, I calculated the ratio of abundances (the "real" scaling factors), for each gene, between biological replicates (Figure 1). It is observed that, although the distributions of ratios are narrow towards higher abundances, they are never reduced to a single value. Moreover, for the smaller abundances (e.g. $> 2^6 = 64$) the distributions are wide and include numerous outliers.

A normalization using a single scaling factor would introduce differential expression for the low abundance transcripts (as exemplified for the edgeR analysis, see below) and would distort the available sequencing space for the high abundance transcripts, distortion. This may lead to artificial DE (especially when the expected DE is subtle).

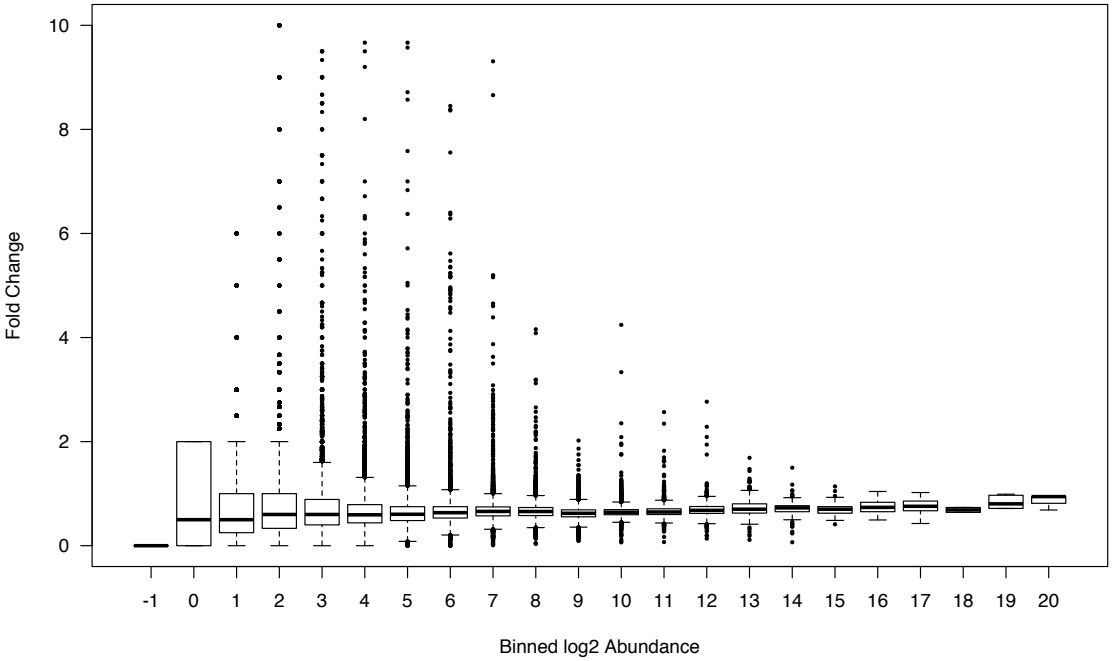

Figure 1. Distribution of scaling factors, calculated as fold changes between biological replicates, over abundance. The samples chosen as examples for this analyses were S3Ab and S2Ab with 32,467,648 and 22,836,556 genome matching reads. On the y axis is shown the fold change in linear scale (the abundances in sample S3Ab were divided to the abundances in sample S2Ab). The x-axis shows abundance, in $\log_2$ scale. For all abundances a distribution of scaling factors can be seen, proving that a scaling normalization using a single factor would be appropriate only for a subset of genes and would lead to artefacts in the differential expression call, especially for low abundance genes.

5.8.10 Subsampling normalization coupled with hierarchical differential expression performs better than DESeq2 or EdgeR

The efficiency of the subsampling normalization coupled with the hierarchical DE in comparison to other methods is supported by several lines of argument, as outlined below:

Comparability of the samples in terms of distributions of expression levels.

The distributions of expression levels produced by different normalisation methods used in this study are presented in Figure 2. A necessary, but not sufficient, condition of an appropriate normalization is to produce comparable distributions of expression levels. This condition is necessary as wholesale shifts in the distribution of expression levels imply that there is a very large proportion of DE transcripts. This goes against the assumption that the majority of the transcripts are not expected to be DE between treatments (even more so between biological replicates). However, comparable distributions can be generated by DE transcripts. To exclude this scenario replicate-to-replicate MA plots were generated (Figure 3).

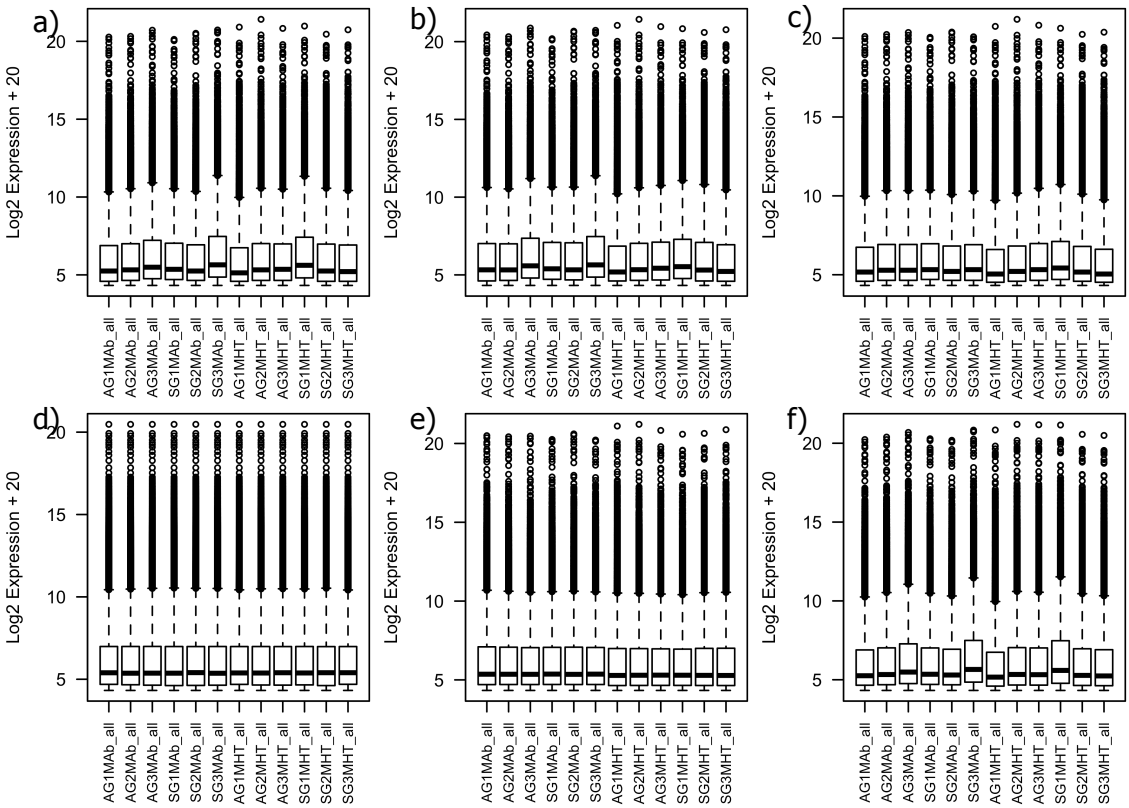

Figure 2. Distributions of expression levels generated by different normalisations. Panel a) represents the raw data, b) RPM normalisation, c) Subsampling normalisation followed by quantile correction, e) normalisation conducted within DESeq2, f) normalisation conducted within EdgeR. On the X axis are the samples and on the Y the normalised gene expression level according to the different normalization methods.

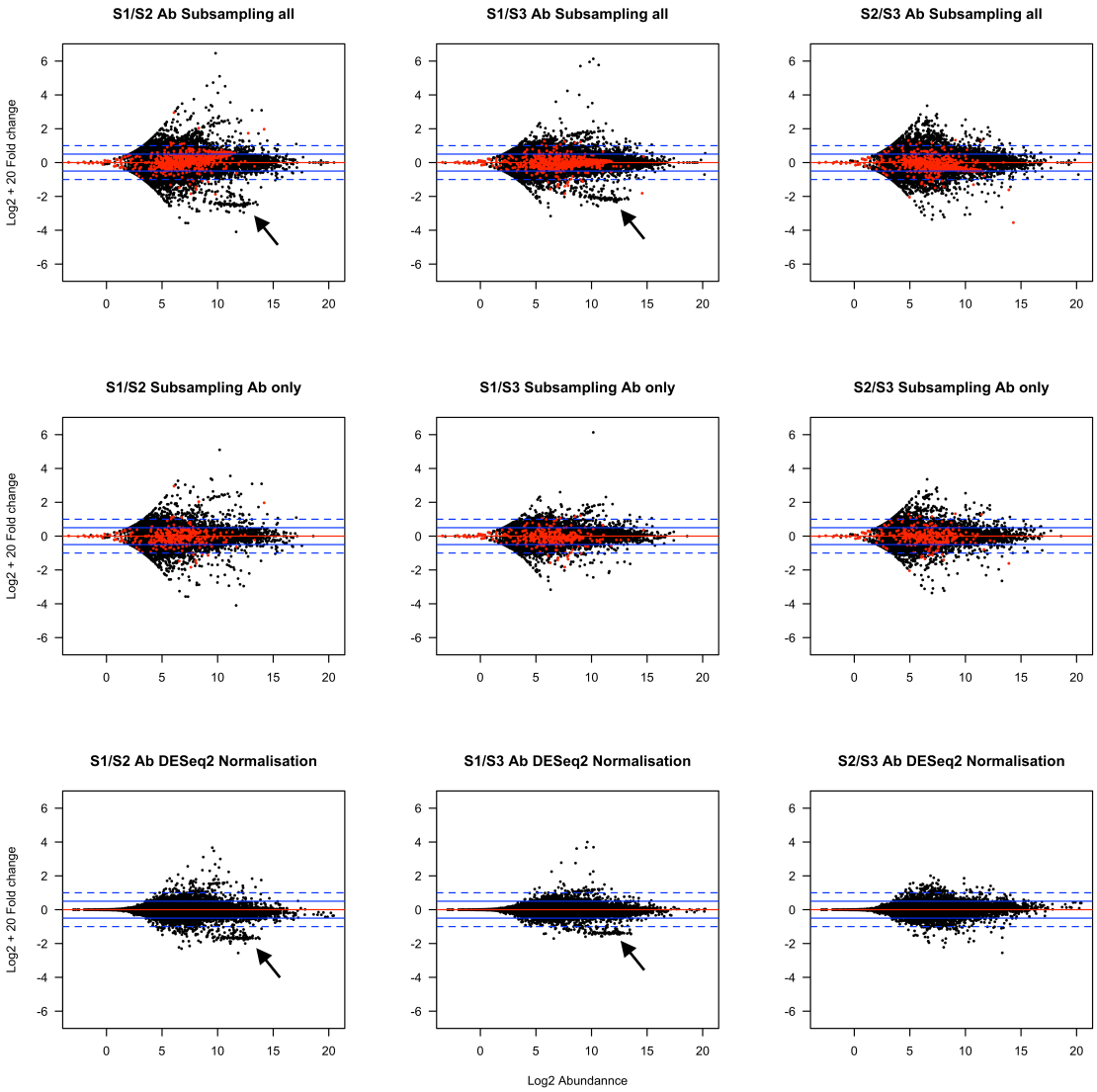

Figure 3. Replicate-replicate Brand-Altman (MA) plots. The top row represents replicate-replicate pairwise comparisons conducted on the subsampling normalisation prior to the HDE call, the middle row represents the subsampling normalisation after the HDE call had removed transcripts that were expressed at significantly higher levels in the head/thorax tissue, the bottom row represents the normalisation conducted within the DESeq2 package. To avoid averaging effects, all pairwise comparisons were conducted between Abdomen replicates for the S treatment. On the y axis is shown the $\log_2$(OFC), with an offset of 20, on the x axis the average abundance of the difference between the samples being compared. The red line corresponds to the 0 $\log_2$(OFC). The blue lines to ± 0.5 and ±1 $\log_2$(OFC) (continuous and dashed lines, respectively) are accepted DE thresholds for biological validation corresponding to technical limitations for low throughput (e.g. qRT-PCR) validations. In red are shown the genes that were called DE between treatments by the subsampling analysis. The HDE step removed 'leaky' genes (indicated with the arrow). The treatment DE genes were consistent between replicates. In terms of spread, the replicate-to-replicate MA plot for the subsampling has a similar spread as the DESeq2 approach. However, it is more stringent at the DE call step (see Figure 4).

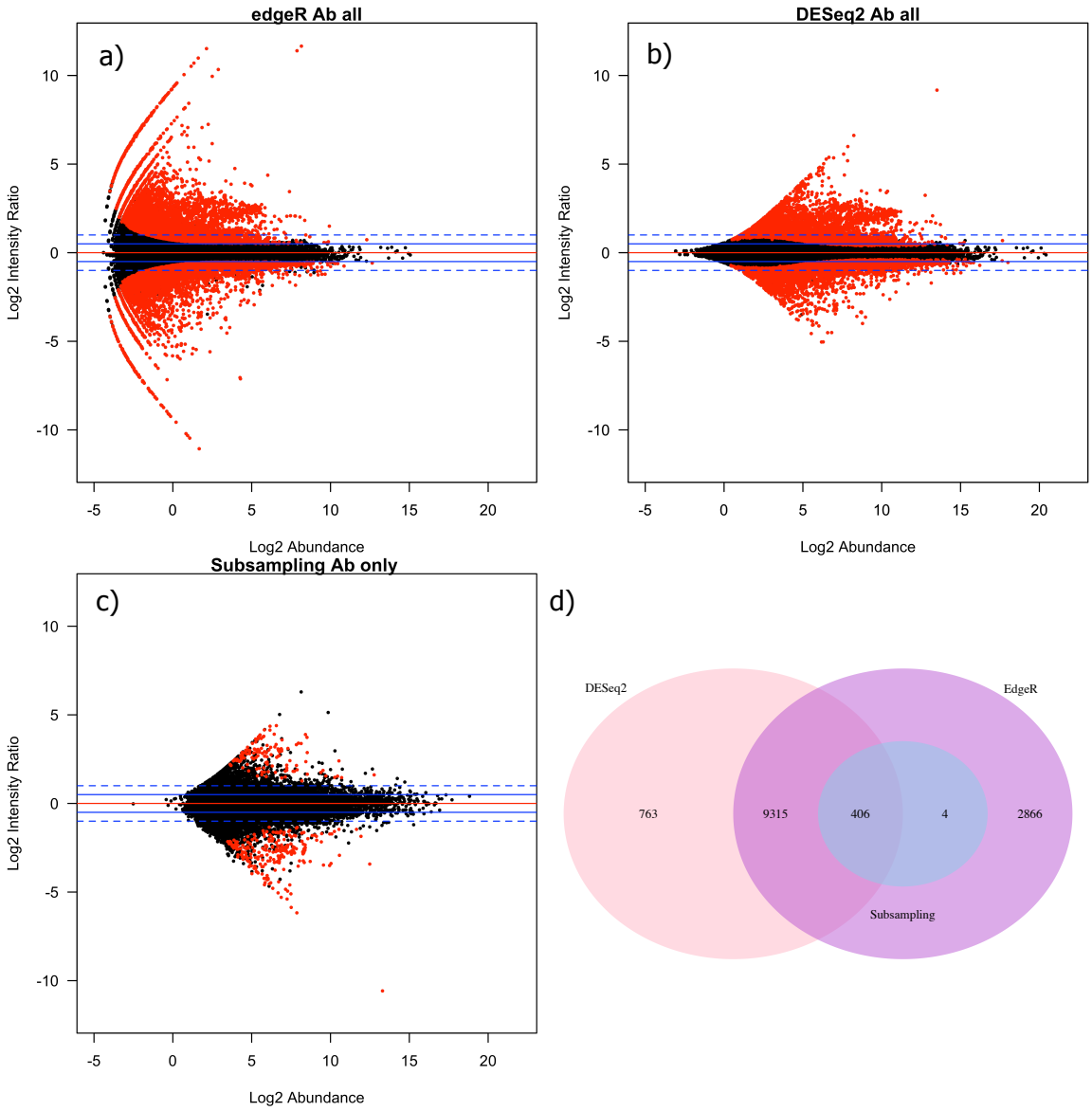

Figure 4. Comparison of DE call obtained using the subsampling normalization plus Heirarchical Differential Expression (HDE), DESeq2 and edgeR. MA plots, with x-axis showing $\log_2$ average abundances against FC (Panels A and B) and OFC with an offset of 20 (Panel C). The example shown is for the abdominal tissue DE comparison (A vs S treatment). The red line indicates 0 $\log_2$ FC/OFC and the blue lines ±0.5 (solid) and ±1 (dashed) $\log_2$ FC/OFC. Red data points represent the genes 'called' differentially expressed by each of the methods. Panel A shows the results for edgeR, Panel B for DEseq2 and Panel C for subsampling normalization with DE calculated using the hierarchical approach. Panel D shows a Venn diagram with the number of differentially expressed genes identified by two or more methods versus uniquely by each.

All genes called DE using the subsampling and the HDE were also called DE using either edgeR or DESeq2 methods (Figure 4d). However, the number of DE genes was inflated (10,078 more genes in DESeq2, and 12,181 in edgeR) and the number and identity of these genes will have an effect on the biological interpretation of the results. In addition, using the edgeR approach numerous low abundance genes were called DE. Using the DESeq approach, genes with FC less than 0.5 on $\log_2$ scale are proposed as DE. Common "mistakes" for both approaches are the averaging on the replicate expression levels and the distortion of the DE distribution derived from the presence of leaky genes. I illustrate these sources of bias, in turn in (Figure 5).

5.8.11 Justification of the offset

The offset indicates the "noise threshold" since a transcript with an abundance at the noise level in one sample and 0 in the other sample would be classified as DE (e.g. log2((offset+offset)/(0+offset))=1). The practical justification of the offset is that it excludes reads with high fold change but low expression levels e.g. a variation between 2 and 10 has a fold change of 5; a variation between 200 and 1000 has a fold change of 5. For former lies within the inherent sequencing noise, whereas the latter could be validated using low throughput methods and can have a biological relevance.

The noise threshold is given based on a reproducibility of the expression profile. A characteristic of transcripts with low expression is the inconsistency of hits along the transcripts. It is generally accepted that a Pearson Correlation Coefficient above 0.5 – 0.7 indicates a good reproducibility. To evaluate the consistency of the sequencing pattern across transcripts, the point-to-point Pearson correlation coefficient was calculated on the expression profile and represented against the variation of abundance (Figure 6). I observed that the distribution of point-to-point PCC was above the 0.5 line for reads with abundance above $2^3 – 2^4$, corresponding to an abundance of 8 to 16. Since the offset must be consistent for all samples, I conservatively chose an offset of 20.

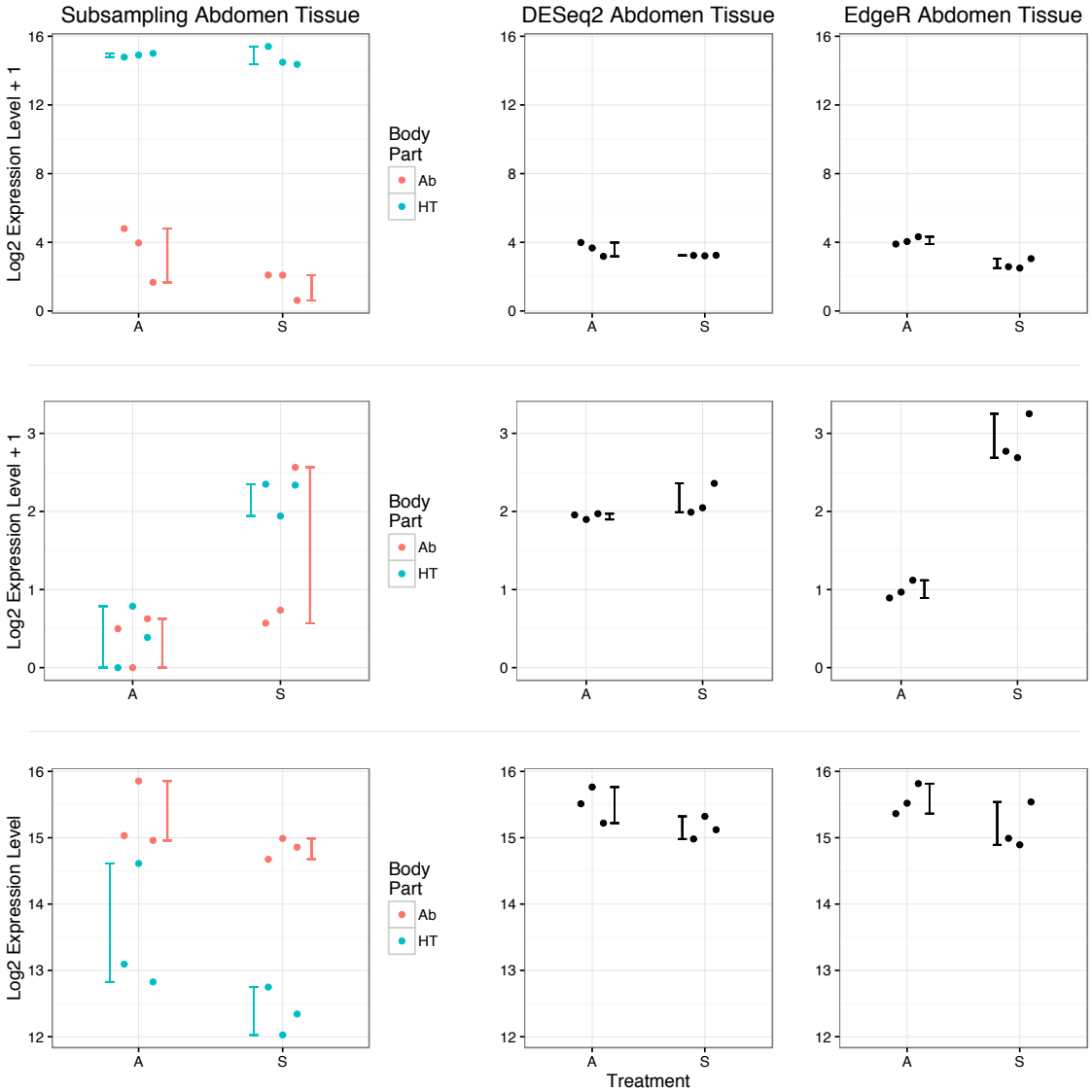

Figure 5. Examples of expression levels for genes called DE using the DESeq2 and EdgeR but not called DE by the subsampling and HDE approach. The DE call here was from the abdominal tissue samples. The points in each panel represent expression levels of each of the Abdomen tissue replicates. In the subsampling panel HT expression levels are also represented so as to illustrate whether transcripts are 'leaky' or not. Panels show the $\log_2$ expression level of the same transcript normalised by the three different methodologies. Each row represents a transcript called as DE by DESeq2 and EdgeR but not by the subsampling methodology. The top row shows 'leaky genes' i.e. genes present at high abundance in the other body part – HT and which retain an expression signature in the AB body part. The middle row represents inflation of low abundance variation called DE by DESeq2 and EdgeR approaches due to the large (artificial) fold change. The bottom row represents false inference inferred by averaging across wide maximal intervals of expression (in the DESeq2 and EdgeR methodologies).

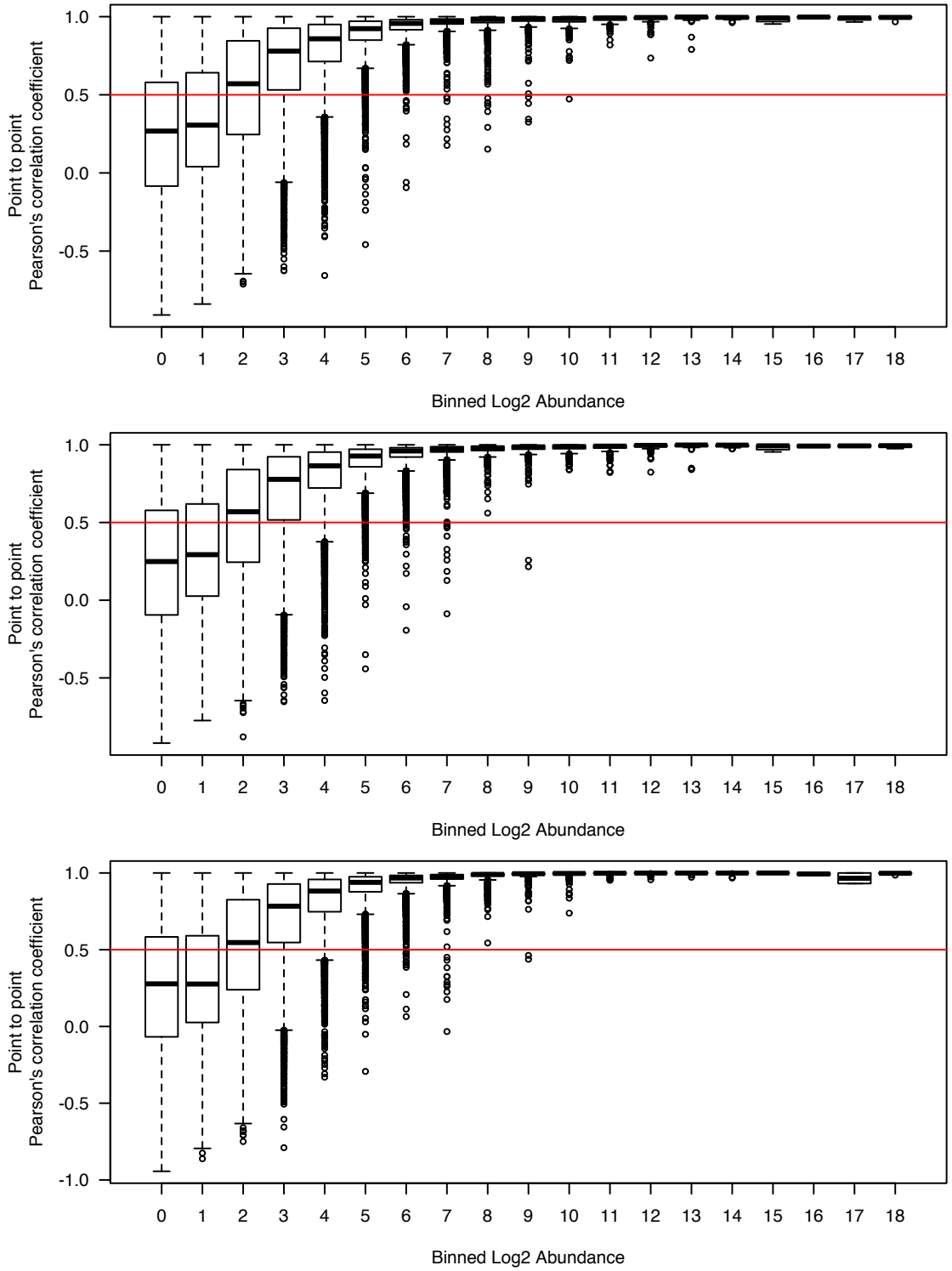

Figure 6. Distribution of point-to-point PCC between gene expression profiles against gene expression levels (log_2 scale) for pairwise comparisons between the 3 replicates of ASG Abdomen tissue. Panel A shows replicate 1 vs 2, B replicate 1 vs 3 and C replicate 2 vs 3. Shown are the raw data, prior to normalization. For all replicate comparisons, more variability was consistently observed at lower abundances. The red line is the noise ratio of 0.5 - where the boxes stop interacting with the line is where an good offset lies - here it is at ~log2 4, which justifies the offset used in the HDE of 20.

5.8.12 Evaluation of normalization efficiency using replicate-to-replicate differential expression

Commonly, the efficiency of normalization is determined according to the number and abundances of DE transcripts identified when replicates are compared. The null hypothesis is that there should be no DE genes between replicates, against the alternative (H1) that DE genes are present. Under this, the 'true positives' (TP) consist of the genes that do not show DE between replicates and where the DE call is 'not DE'. The 'false positives' (FP) are the genes that show DE and are called 'not DE'. The 'false negatives' (FN) are the genes that do not show DE between replicates, yet are called DE. The 'true negatives' (TN) are the genes that do show DE between replicates and are called as such.

The analysis which is summarised in Figure 7 shows that when p-value criteria are used, the maximum number of replicate-to-replicate DE genes was less than 150 for DESeq2 (Panel a) and edgeR (Panel b). Using the subsampling normalization and the hierarchical DE, the number of replicate-to-replicate DE genes was less than 600 (Panel C). I conclude that the proposed method falls within the comparable range of existing approaches.

To assess the efficiency of the DE calls is problematic in general, as it cannot usually be known a priori which genes should show DE, either between treatments or even replicates. However, bearing this problem in mind it is still possible to make an estimate of the false discovery rate, e.g. assuming that true biological replicates should not in general contain genes showing DE. Based on the FDR our proposed method is comparable to the existing methods such as edgeR and DEseq2. I conclude that the subsampling normalization produces comparable results in terms of FDR in comparison with the edgeR and DESeq2 (on the basis that for edgeR and DESeq2, I consider as differentially expressed the genes with a p-value of less than 0.05).

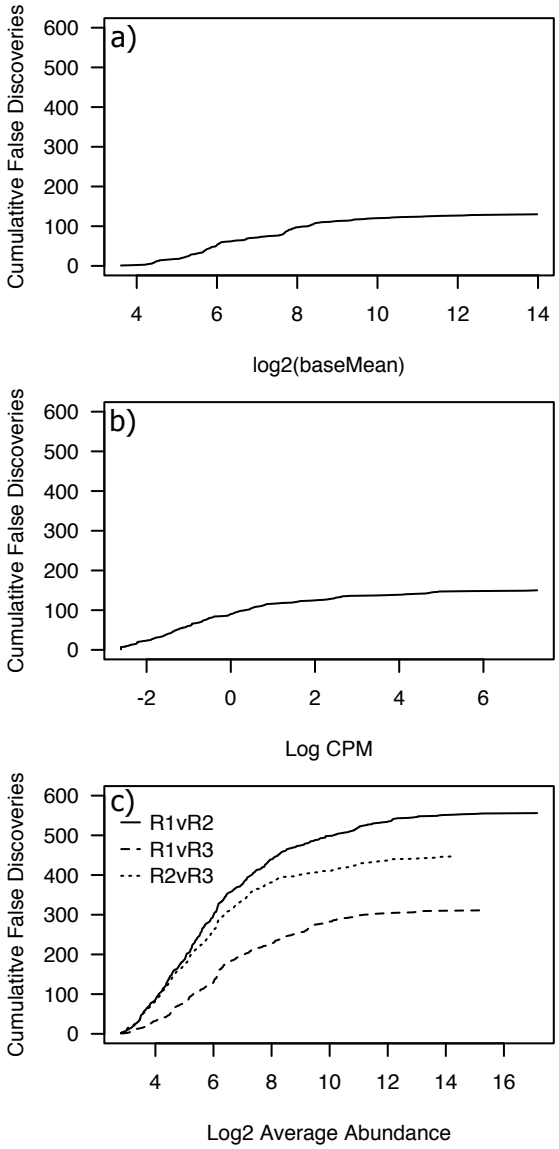

Figure 7. ROC curves for the number of false discoveries using the data from Figs. 2 & 3 for a) DESeq2, with p-value DE criteria b) edgeR, with p-value DE criteria, c) subsampling and hierarchical DE with FC >1 DE criteria. DESeq2 (a) and EdgeR (b) analyses show false discovery rate between the SAb replicate 1 & 2 and SAb replicate 1 & 3 comparisons, The subsampling false discovery rate (c) is show for all SAb rep-rep comparisons. This analysis shows that the number of false discoveries of the proposed method fell within a comparable range in comparison to existing methods.

References

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550.

Robinson, M. D., and A. Oshlack. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 11:R25.

Zhou, X., H. Lindsay, and M. D. Robinson. 2014. Robustly detecting differential expression in RNA sequencing data using observation weights. Nucleic Acids Res. 42:e91.

6 General Discussion

6.6 Summary of key findings

The unifying aim of this thesis research has been to quantify the response of the Mediterranean fruit fly (*Ceratitis capitata*, Wiedemann) (medfly) to changes in developmental nutrition, and to test if divergent selection between different developmental diets can cause populations to rapidly evolve incipient reproductive isolation (RI), potentially as a by product of ecological adaptation. To address this goal multiple experimental methodologies were combined, but centred around an experimental evolution (EE) study. Using these populations as a focus the specific objectives of this thesis were addressed and presented in four data chapters. In the first, a range of specific nutritional manipulations was made to a standard larval diet and the proximate developmental responses of medfly larvae analysed (Chapter 2). These data showed that the selective pressures resulting from larval diet manipulations were potentially strong and divergent. To test the effects of longer-term divergent selection imposed by rearing on different larval diets on the evolution of assortative mating, an experimental evolution experiment was conducted and mating tests were performed at three time points on populations undergoing EE on two larval diets of different composition and calorific value (Chapter 3). To confirm the results of these mating tests, and to investigate the changes in the behavioural mechanisms underlying differences in mate choice observed, single choice mating tests and video analysis of behaviour were conducted on flies from the EE populations (Chapter 4). Finally, in order to retrieve evidence of differential gene expression in sexually mature males drawn from the EE populations, messenger RNA sequencing (RNAseq) was conducted and a hierarchical differential expression (DE) analysis employed (Chapter 5). The key findings of these approaches in relation to the specific objectives of the thesis are summarised here, followed by a synthesis of these findings and suggestions for future work.

1. *Developmental response of a laboratory population to manipulation specific nutritional components of larval diet.* In Chapter 2, alteration of the quantity or type of protein, and type of carbohydrate available in larval diet showed that a laboratory strain of medfly, domesticated for over 20 years (Morrison et al. 2009), retained the capacity to manifest significant phenotypic plasticity in developmental life history in response to nutritional challenge. The effects of diet alteration were apparent at different life history stages. Reduction of protein quantity or quality caused higher mortality in, and increased the duration of, the larval stage of development. Alteration of carbohydrate source

caused higher mortality during the pupal stage. Neither protein or carbohydrate alteration effected pupal weight, when the protein or carbohydrate source lay within the host range of the medfly. The inclusion of 'alien' sources of protein revealed the extent of the adaptive plasticity that can be manifested by the medfly.

2. *Evolution of RI between replicate EE populations developing on divergent larval diets.* Chapter 3 introduced a set of replicated EE populations that formed the basis of the research presented in the following chapters. The EE design divided a single outbred stock population between two larval diets that differed in nutritional complexity and caloric content. Over the course of 60 generations, assortative mating indicative of RI evolved within these populations. Assortative mating was observed in all replicate populations when they were tested with the proximate effects of diet intact, and was also present when proximate and parental effects of diet were removed in a common garden experiment. The assortative mating, suggesting sexual isolation between the dietary regimes, was not observed at two earlier testing points. However, the combination of two mating test methodologies; limited, and multiple choice mating tests, prevented a complete description of the evolution of assortative mating across time. At 3, 5 and 30 generations of EE, mating preferences between populations were tested using limited choice quartet mating tests. This design revealed a competitive advantage for males reared on the more complex and calorically rich 'ASG' diet. Calculation of descriptive coefficients based upon mating frequencies suggested that a decay in this advantage co-occurred with the observation of assortative mating.

3. *Behavioural mechanisms associated with patterns of mating observed in the EE populations.* The behavioural mechanisms of mate choice between the 'ASG' and 'Starch' diet EE populations were further examined in Chapter 4. Using files from the 29th & 30th generation of EE, the middle time point tested in Chapter 3, 'no choice' mating tests were conducted. The aim of these tests was validate whether the pattern mate choice observed in limited choice mating tests (Chapter 3) would be replicated with the effects intrasexual competition removed. Four combinations of homo- and heterotypic mating pairs were tested. Mating tests were conducted with the proximate effects of diet intact, and with them switched by reciprocal crossing of males for one generation. No

choice tests confirmed that no sexual isolation was present at 29 & 30 generations of EE. Both sets of no choice tests ('on diet' and 'diet cross') were filmed and male courtship behaviour leading to successful copulation analysed. The frequency of courtship and non-courtship behaviours was recorded, as well as the total time exhibited in each behaviour prior to copulation. This detailed analysis aimed to retrieve divergence of male courtship behaviour between dietary regimes. Although different aspects of courtship behaviour were exaggerated when the proximate effects of the different diets remained, this effect did not persist when they were switched. This result suggested no divergence in the genetic basis of male courtship phenotype. However, the combination of the mating test results, and the behavioural profiles associated with each pair combination revealed a mismatch between courtship vigour in males, and number of copulations secured. This highlighted the role of female choice, in line with the lek-based mating system exhibited by the medfly.

4. *Genes associated with adaptation to divergent developmental environment or differences in mate choice between the EE populations.* The assortative mating observed in Chapter 3 still remained even when flies from both dietary regimes of the EE populations were reared on a common garden diet. This suggested that the assortative mating observed was associated with genetic divergence between populations. In order to assay this divergence, the gene expression profiles of sexually mature males from both ASG and Starch populations were captured using RNAseq (Chapter 5). Males from which total RNA was extracted were reared from eggs taken from the 60th generation of EE, that in which assortative mating was observed, which had been reared on a common garden diet for two generations to remove possible proximate and parental effects of the regime diets. The bioinfomatics methodology described in Chapter 5 imposed rigorous quality control and normalisation steps on the raw sequence data in order to produce three high quality independent samples for each treatment, one representing each population replicate. These samples were then mapped to the medfly reference genome, and the incident read count used to quantify gene expression levels across the genome. DE between treatments was then calculated based on the expression matrix produced, with transcripts that were DE above two $\log_2$ offset fold change (OFC) considered for annotation and functional enrichment.

1,214 transcripts exhibited DE above two log_2OFC between the two dietary regimes, and of this set, 109 yielded meaningful annotations. Key patterns of DE between regimes involved genes that were associated with GO keywords 'metabolism', 'oxidative phosphorylation' (OXPHOS), and 'proteolysis'. DE was also observed in lesser abundance in genes associated with a wide range of cellular functions. A small number of genes associated with behavioural GO terms, as well as three olfactory binding proteins also exhibited DE. The DE analysis revealed tissue specific expression patterns, with a range of OXPHOS related genes expressed at higher levels in the head/thorax tissue of ASG males, and a separate set of OXPHOS related genes expressed at higher levels in Starch male abdominal tissue. Starch males also expressed higher expression of metabolism-associated genes, in both tissue types. Different sets of genes associated with proteolysis were expressed at higher levels in males drawn from either regime.

6.7 Synthesis of findings and future work

The findings described above represent the implementation of a range of techniques that have not previously been applied to the medfly. In conducting one of the first EE studies in the medfly (e.g. Diamantidis et al. 2011) this research has added valuable information to the understanding of the species, and also shown that it is a responsive model for the study of adaptation and it's implications in population divergence under ecological selection pressure in laboratory populations. In conducting a transcriptomic analysis of the gene expression associated with dietary adaptation this study joins a small number of studies that have considered the transcriptomic response to EE, and is the first to report gene expression changes that are a product of EE on divergent developmental diets. The association of this divergent expression with putative RI allows novel parallels to be drawn with studies of natural populations, and opens the field to much future study. Here, the findings of each chapter are synthesised to draw the main conclusions of the work, identify limitations, and outline avenues of study within the medfly system, and in wider biology.

6.7.9 Developmental diet nutrients as a source of selection

The role of nutritional resources as a basis of natural selection is a well-established concept within evolutionary biology (e.g. Mayr 1963; Ehrlich and Raven 1964; Roughgarden 1972; Stearns 1992; Schluter 2000; Coyne and Orr 2004). Within herbivorous insects, several key systems have provided important examples of the way adaptation to divergent selection associated with the acquisition of dietary nutrients

can act to cause population divergence and species formation (e.g. Futuyma and Moreno 1988; Funk et al. 2002; Nosil 2012). These examples often centre on host specialisation, and the way in which host shifting can impose barriers to gene flow between populations through factors such as temporal isolation (e.g. Feder et al. 1994), or immigrant inviability (e.g. Nosil et al. 2005). Adaptation to specific host qualities such as toxicity have also been shown to be an important driver of population divergence (e.g. R'Kha et al. 1991; Legal et al. 1992; Moreteau et al. 1994; Farine et al. 1996). However, only a small number of studies have experimentally tested how such specialisation might begin, and how rapidly it can lead to barriers forming between species (e.g. Dodd 1989; Rundle 2003; Rundle et al. 2005; Dettman et al. 2008). The work described in this thesis adds an important example to this body of research, and confirms the role that relatively simple changes to nutritional environment can facilitate population divergence.

Chapter 2 represents a robust test of the responses of medfly to alteration of larval dietary nutrients. The motivation of the research in this chapter was to quantify the potential for selection imposed by the diets used in the EE populations. The strong effects on the proportion of surviving individuals in both larval and pupal stages highlight the potential for selection mediated by alteration in both protein and carbohydrate constituents of diet. Also, the adaptive plasticity exhibited during larval development may have become important in consecutive generations due to potential for genetic accommodation of this plasticity (West-Eberhard 2003). Although the diets used as a basis for EE in this thesis where based on changes in nutritional composition of a similar magnitude to those tested in Chapter 2, direct comparisons to the selective conditions that the EE populations experienced are not possible. Future EE studies that consider adaptation to developmental diets should impose methodologies similar to that used in Chapter 2 to fully quantify effects on survival and chart the adaptive response to diets over the course of EE. Also, a fundamental extension to this approach is to quantify the effects of divergent developmental environments on the demographic response exhibited by the adult population. This would allow the effect of selection during development on key traits such as longevity, reproductive potential, and the timing of sexual maturity to be included in interpretation of adaptation to novel developmental environments.

Although the nature of the selection experienced during EE can only be inferred from the results of this research, firm evidence is presented that divergence in mate choice and also gene expression patterns had evolved between populations after 60

generations of EE. A key finding of this study was the similarity in gene expression differences seen between EE populations and those observed in several natural populations evolving under divergent selection (discussed in detail in Chapter 5). The role of genes involved in key functions such as OXPHOS, and also in chemosensory function, are already well established as key targets for selection during population divergence (Gershoni et al. 2009; Smadja and Butlin 2009). The observation of differential expression in a suite of OXPHOS related genes and also some olfactory binding protein genes in response to a simple environmental manipulation over a short time scale is a novel result. It provides a valuable insight into the potentially fundamental nature of such genes in the action of adaptive divergence and the instigation of pre-mating barriers over short timescales.

6.7.10 The evolution of reproductive isolation

The key result of this work was the observation of assortative mating between EE populations reared on divergent larval diets after 60 generations. This finding represents the first observation of assortative mating as a product of EE under divergent natural selection in a herbivorous insect outside of the *Drosophila.* However, considerable extensions to this finding are needed to fully attribute this finding of to the action of divergent natural selection based on diet. Although parallel divergence between the experimental populations was suggested by the uniformity of the patterns of assortative mating described in Chapter 3, as well as the uniformity of the gene expression profiles seen in males across replicate populations in Chapter 5, the data presented here cannot rule out the effects of other drivers of differentiation such as random genetic drift (Coyne and Orr 2004). The most prescient suggestion for future work is to conduct 'same host' mating tests on the EE populations studied here. The comparison of isolation of 'same host verses different host' pairs is essential in ascribing the role in ecologically mediated natural selection in their divergence (Funk and Nosil 2008). It is conceivable that founder effects and result drift may have caused all six populations within the EE study to diverge independently. Although no experimental support has been found for such effects (e.g. Rundle 2003), further experimentation is required to rule out this possibility.

The sexual isolation between EE populations described in Chapter 3 was observed in multiple-choice mating tests. This finding is in line with previous studies within several *Drosophila* systems that show mating test design is important to the retrieval of signatures of adaptation in mate choice (Coyne et al. 2005; Jennings and Etges 2009). The three mate choice designs implemented within Chapters 3 and 4 (limited choice,

multiple choice, and no choice) highlight the role of different designs in generating different information regarding the nature of choice within speciation experiments. Future studies aiming to quantify the evolution of sexual isolation should take care to consider this, and implement a uniform mating test design at regular time point during the progress of EE.

The alteration of mating test design between generations 30 and 60 (Chapter 3), although contributing to the description of sexual isolation, removes the ability to fully interpret its evolution. Across the three time points at which mating tests were conducted, sexual isolation seems to have evolved through either a decline in a fitness advantage which was originally seen in males form the ASG populations, or by an increase in the ability of Starch males to secure matings. As both mating test and the method of removing proximate effects of diet were altered in generation 60, the evidence of this advantage, seen under the 'crossing' design coupled with limited choice mating tests, was not retrieved. Further testing of these populations, which are now beyond 100 generations of EE, should incorporate both the both mating test designs to resolve the evolutionary trajectory of the apparent completive advantage, and also to further document the evolution of sexual isolation and how 'complete' it becomes (Nosil et al. 2009).

6.7.11 Mechanisms of reproductive isolation

The behavioural manifestation of the competitive advantage exhibited by ASG males in the 30th generation mating tests (Chapter 3) was described in Chapter 4. ASG males were much more active in both courtship and non-courtship behaviour than males from the Starch populations. However, this effect did not persist to any significant level when proximate effects were crossed. Although a heritable divergence in courtship behaviours was not observed, the results of behavioural analysis suggested the existence of different energetic optima present in the selection lines. Although this did not describe the mechanism of the mate choice observed, when compared to the number of copulations secured it highlighted the role of female choice within the system, and thus the action of sexual selection. As the medfly is a lekking species (Prokopy and Hendrichs 1979; Arita and Kaneshiro 1985), the action of female choice is expected as it is essential to lek dynamics (Höglund and Alatalo 1995).

The interaction between natural and sexual selection in speciation is complex (Safran et al. 2013), but theoretical models have suggested that, through the advertisement of local adaptation, sexual selection can facilitate population divergence under natural

selection (van Doorn et al. 2009). Understanding the phenotypic basis of RI on a trait by trait basis when natural and sexual selection combine has been suggested to be important (Safran et al. 2013). To fully understand the traits that govern mate choice in the EE populations studied here, more work is needed. The visual analysis of courtship employed in Chapter 3 showed that the courtship behaviours recorded were not correlated with mating success, suggesting the importance of other cues in mate choice in the medfly. Key candidates for further study are the pheromones males use to convene leks, and also to signal to the female (e.g. Prokopy and Hendrichs 1979; Briceño et al. 1996). DE in olfactory binding protein genes between the EE populations, as well as several genes involved in biosynthesis (Chapter 5), further support this suggestion. Adaptive divergence of chemosensory traits in response to factors such as host switches is commonly seen to drive the evolution of premating barriers to gene flow (*reviewed in* Smadja and Butlin 2009), hence this would be a promising avenue for further study. Also, the pheromone chemistry of the medfly is well documented (e.g. Jang et al. 1989, 1994; Light et al. 1999; Gonçalves et al. 2006), and would provide a good basis for the study of divergence.

6.7.12 Genetic basis of reproductive isolation

As the assortative mating observed after 60 generations of EE (Chapter 3) remained when proximate effects of diet were removed, suggesting sexual isolation (SI) between populations has a heritable genetic basis. The patterns of mating observed at earlier time points (3 & 5, 30) did not persist, and even reversed when proximate effects were removed. This suggests a gradual build up of SI between populations caused by pleiotropic association with traits that were under selection during adaptation to larval diet, or by tight linkage between loci associated with SI and those under selection during adaptation and sexual isolation. Although it is beyond the scope of this thesis to identify such mechanisms, the transcriptomic dataset generated in Chapter 5 provides a comprehensive staring point for genomic studies of adaptation in the medfly. This data will contribute to a growing body of work around the recently developed medfly genome, work which is expected to have great applied significance (Scolari et al. 2014). Several key extensions to the findings of Chapter 5 are necessary before the gene expression differences between males of the two EE populations can be confidently described. Of key importance is the validation of the DE patterns using qRT-PCR. Once validated, this dataset has the potential to inform future studies on the on going EE populations, i.e. regarding corresponding female expression patterns, or the variation in expression of these candidates over life history. However, to fully

understand any correspondence of such candidates with the sexual isolation described in Chapter 3, specific targets related to the mate choice underlying this isolation would have to be determined.

Without the possibility of assessing the gene expression patterns of the ancestral population that was used to seed the ASG and Starch EE populations, direct confirmation of the role of any of the candidates suggested in adaptation or sexual isolation would not be possible. Consideration of the ancestral population is a key component of studies which adopt recently popularised 'evolve and resequence' (E&R) designs (Kofler and Schlötterer 2014; Schlötterer et al. 2014a), which are proving to be a powerful tool in understanding the molecular architecture of adaptation (*reviewed in* Long et al. 2015). As such studies utilise a sequencing approach that is based on the of pooling large numbers of individuals, costs are significantly reduced and accuracy of base calling increased (Poolseq, (Schlötterer et al. 2014b)). This allows repeat sampling of the whole genome (e.g. Tobler et al. 2014), and the pattern of its expression (e.g. Konczal et al. 2015) over the course of EE. This technique is already proving to be a powerful tool in identifying genome-wide patterns of selection response when used in combination with EE designs (e.g. Turner and Miller 2012; Turner et al. 2013; Tobler et al. 2014), and would present an attractive option for future study of the genetic architecture of reproductive isolation in the medfly.

6.8 Conclusions and wider perspectives

The research presented in this thesis goes some way to developing the medfly as a model species for the study of adaptation and the evolution of sexual isolation in experimental populations. The findings of this research also enable exciting possibilities for future study. As the experimental populations studied here are still extant, and are now over 100 generations into experimental evolution, their continued study has the potential to provide insight into maintenance and evolutionary trajectory of the sexual isolation shown in this thesis. Further work guided by the findings of this thesis could also provide more insights into the mechanisms by which isolation has evolved between these populations. Given the evidence described here that the medfly can manifest an adaptive response to environmental selection over a small evolutionary time scale, the potential of the medfly as a laboratory model is highlighted. In light of the relatively close phylogenetic relationship between the medfly and *Drosophila*, the medfly may also prove a useful tool in testing hypotheses that have solely developed in *Drosophila*.

This work also has the potential to contribute to fields that aim to understand and control the medfly as a crop pest. The rapid global radiation of the medfly has led it to interact with a huge range of host environments (Liquido et al. 1991). Although global populations appear to exhibit divergence at a genotypic (e.g. Gasperi et al. 2002) and phenotypic level (e.g. Briceño et al. 2002, 2007), no reproductive isolation has been recorded between global populations (Cayol 2000; Cayol et al. 2002). Although this radiation is young, global populations may have the potential for specialisation resulting in speciation. As gene flow between global populations is suggested to be becoming limited (Karsten et al. 2015), this potential maybe increased. Having shown the medfly to be able to develop sexual isolation based on divergent selection, this thesis supports the medfly's capacity to exhibit host specialisation. As intraspecific divergence is increasingly understood to be of importance in understanding the invasive potential of *Tephritid* flies (Godefroid et al. 2015), the work presented here or its implications may inform population level study in the medfly.

Overall, this thesis provides support for the role of divergent natural selection in creating barriers to gene flow, or ecological speciation (ES). In doing so, it joins a growing body of evidence that suggests that this process maybe central to the generation of biological diversity. This work conforms to several of the 'major unanswered questions' regarding (ES Table 9.1, Nosil 2012), specifically the 'commonality' of ES, it's 'rapidity', and the role gene expression may play in ES. The studies this work enables should use it as a foundation to fully address these, and further unanswered questions towards the theory of ES.

6.9 References

Arita, L. H., and K. Y. Kaneshiro. 1985. The dynamics of the lek system and mating success in males of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Proc. Hawaiian Entomol. Soc. 25:39–48.

Briceño, R. D., D. Ramos, and W. G. Eberhard. 1996. Courtship behavior of male *Ceratitis capitata* (Diptera: Tephritidae) in Captivity. Florida Entomol. 79:130–143.

Briceño, R. D., W. G. Eberhard, J. C. Vilardi, P. Liedo, and T. E. Shelly. 2002. Variation in the intermittent buzzing songs of male medflies (Diptera : Tephritidae) associated with geography, mass-rearing, and courtship success. Florida Entomol. 85:32–40.

Briceño, R. D., W. G. Eberhard, J. Vilardi, J. Cayol, and T. Shelly. 2007. Courtship behavior of different wild strains of *Ceratitis capitata* (Diptera: Tephritidae). Florida Entomol. 90:15–18.

Cayol, J. P. 2000. Changes in sexual behavior and life history traits of tephritid species caused by mass-rearing processes. Fruit Flies Phylogeny Evol. Behav. 843–860.

Cayol, J. P., P. Coronado, M. Taher, and Cayol. 2002. Sexual compatibility in medfly (Diptera : Tephritidae) from different origins. Florida Entomol. 85:51–57.

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

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

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

Diamantidis, A. D., J. R. Carey, C. T. Nakas, and N. T. Papadopoulos. 2011. Ancestral populations perform better in a novel environment: Domestication of Mediterranean fruit fly populations from five global regions. Biol. J. Linn. Soc. 102:334–345.

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

Ehrlich, P. R., and P. H. Raven. 1964. Butterflies and plants: a study in coevolution. Evolution. 18:586–608.

Farine, J.-P., L. Legal, B. Moreteau, and J.-L. Le Quere. 1996. Volatile components of ripe fruits of *Morinda citrifolia* and their effects on *Drosophila*. Phytochemistry 41:433–438.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. U. S. A. 91:7990–4.

Funk, D. J., K. E. Filchak, and J. L. Feder. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. Pp. 251–267 *in* Genetics of Mate Choice: From Sexual Selection to Sexual Isolation. Springer, Netherlands.

Funk, D. J., and P. Nosil. 2008. Comparative analyses of ecological speciation. Pp. 117–135 *in* K. Tilmon, ed. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Oakland, CA.

Futuyma, D. J., and G. Moreno. 1988. The evolution of ecological specialization. Annu. Rev. Ecol. Syst. 19:207–233.

Gasperi, G., M. Bonizzoni, L. M. Gomulski, V. Murelli, C. Torti, A. R. Malacrida, and C. R. Guglielmino. 2002. Genetic differentiation, gene flow and the origin of infestations of the medfly, *Ceratitis capitata*. Genetica 116:125–135.

Gershoni, M., A. R. Templeton, and D. Mishmar. 2009. Mitochondrial bioenergetics as a major motive force of speciation. Bioessays 31:642–50.

Godefroid, M., A. Cruaud, J.-P. Rossi, and J.-Y. Rasplus. 2015. Assessing the risk of invasion by Tephritid fruit flies: Intraspecific divergence matters. PLoS One 10:e0135209.

Gonçalves, G. B., C. E. Silva, J. C. G. Dos Santos, E. S. Dos Santos, R. R. Do Nascimento, E. L. Da Silva, A. De Lima Mendonça, M. Do Rosário Tenório De Freitas, and A. E. G. Sant'Ana. 2006. Comparison of the volatile components released by calling males of *Ceratitis capitata* (Diptera: Tephritidae) with those extractable from the salivary glands. Florida Entomol. 89:375–379.

Höglund, J., and R. V. Alatalo. 1995. Leks. Princeton University Press.

Jang, E. B., D. M. Light, R. G. Binder, R. A. Flath, and L. A. Carvalho. 1994. Attraction of female mediterranean fruit flies to the five major components of male-produced pheromone in a laboratory flight tunnel. J. Chem. Ecol. 20:9–20.

Jang, E. B., D. M. Light, R. a. Flath, J. T. Nagata, and T. R. Mon. 1989. Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata* to identified volatile constituents from calling males. Entomol. Exp. Appl. 50:7–19.

Jennings, J. H., and W. J. Etges. 2009. Species hybrids in the laboratory but not in nature: a reanalysis of premating isolation between *Drosophila arizonae* and *D. mojavensis*. Evolution 64:587–98.

Karsten, M., B. Jansen van Vuuren, P. Addison, and J. S. Terblanche. 2015. Deconstructing intercontinental invasion pathway hypotheses of the Mediterranean fruit fly (*Ceratitis capitata*) using a Bayesian inference approach: are port interceptions and quarantine protocols successfully preventing new invasions? Divers. Distrib. 1–13.

Kofler, R., and C. Schlötterer. 2014. A guide for the design of evolve and resequencing studies. Mol. Biol. Evol. 31:474–83.

Konczal, M., W. Babik, J. Radwan, E. T. Sadowska, and P. Koteja. 2015. Initial molecular-level response to artificial selection for increased aerobic metabolism occurs primarily through changes in gene expression. Mol. Biol. Evol. 32:1461–73.

Legal, L., J. R. David, and J. M. Jallon. 1992. Toxicity and attraction effects produced by *Morinda citrifolia* fruits on the *Drosophila melanogaster* complex of species. Chemoecology 3:125–129.

Light, D. M., E. B. Jang, R. G. Binder, R. A. Flath, and S. Kint. 1999. Minor and intermediate components enhance attraction of female Mediterranean fruit flies to natural male odor pheromone and its synthetic major components. J. Chem. Ecol. 25:2757–2777.

Liquido, N. J., L. A. Shinoda, and R. T. Cunningham. 1991. Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): an annotated world review. Entomological Society of America, Lanham.

Long, A., G. Liti, A. Luptak, and O. Tenaillon. 2015. Elucidating the molecular architecture of adaptation via evolve and resequence experiments. Nat. Rev. Genet. 16:567–582.

Mayr, E. 1963. Animal Species and Evolution. Harvard University Press, Cambridge, MA.

Moreteau, B., S. R'Kha, and J. R. David. 1994. Genetics of a nonoptimal behavior: Oviposition preference of *Drosophila mauritiana* for a toxic resource. Behav. Genet. 24:433–441.

Morrison, N. I., D. F. Segura, K. C. Stainton, G. Fu, C. A. Donnelly, and L. S. Alphey. 2009. Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*. Entomol. Exp. Appl. 133:146–153.

Nosil, P. 2012. Ecological Speciation. Oxford University Press, Oxford.

Nosil, P., L. J. Harmon, and O. Seehausen. 2009. Ecological explanations for (incomplete) speciation. Trends Ecol. Evol. 24:145–156.

Nosil, P., T. H. Vines, and D. J. Funk. 2005. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution. 59:705–19.

Prokopy, R. J., and J. Hendrichs. 1979. Mating behavior of *Ceratitis capitata* on a field-caged host tree. Ann. Entomol. Soc. Am. 72:642–648.

R'Kha, S., P. Capy, and J. R. David. 1991. Host-plant specialization in the *Drosophila melanogaster* species complex: a physiological, behavioral, and genetical analysis. Proc. Natl. Acad. Sci. 88:1835–1839.

Roughgarden, J. 1972. Evolution of niche width. Am. Nat. 106:683–718.

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

Rundle, H. D., S. F. Chenoweth, P. Doughty, and M. W. Blows. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLoS Biol. 3:e368.

Safran, R. J., E. S. C. Scordato, L. B. Symes, R. L. Rodríguez, and T. C. Mendelson. 2013. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: A research agenda. Trends Ecol. Evol. 28:643–650.

Schlötterer, C., R. Kofler, E. Versace, R. Tobler, and S. U. Franssen. 2014a. Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation. Heredity. 114:431–440.

Schlötterer, C., R. Tobler, R. Kofler, and V. Nolte. 2014b. Sequencing pools of individuals — mining genome-wide polymorphism data without big funding. Nat. Rev. Genet. 15:749–763.

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

Scolari, F., L. M. Gomulski, P. Gabrieli, M. Manni, G. Savini, G. Gasperi, and A. R. Malacrida. 2014. How functional genomics will impact fruit fly pest control: the example of the Mediterranean fruit fly, *Ceratitis capitata*. BMC Genet. 15 Suppl 2:S11.

Smadja, C., and R. K. Butlin. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity. 102:77–97.

Stearns, S. C. 1992. The Evolution of Life Histories. Oxford University Press, Oxford.

Tobler, R., S. U. Franssen, R. Kofler, P. Orozco-Terwengel, V. Nolte, J. Hermisson, and C. Schlötterer. 2014. Massive habitat-specific genomic response in *D. melanogaster* populations during experimental evolution in hot and cold environments. Mol. Biol. Evol. 31:364–75.

Turner, T. L., and P. M. Miller. 2012. Investigating natural variation in *Drosophila* courtship song by the evolve and resequence approach. Genetics 191:633–642.

Turner, T. L., P. M. Miller, and V. A. Cochrane. 2013. Combining genome-wide methods to investigate the genetic complexity of courtship song variation in *Drosophila melanogaster*. Mol. Biol. Evol. 30:2113–20.

Van Doorn, G. S., P. Edelaar, and F. J. Weissing. 2009. On the origin of species by natural and sexual selection. Science 326:1704–7.

West-Eberhard, M. J. 2003. Developmental Plasticity and Evolution. Oxford University Press, USA.