FOOD, SEX AND DEATH: COSTS OF REPRODUCTION AND THE MECHANISTIC BASIS OF AGEING.

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

PHD THESIS

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C. RUTH ARCHER

Submitted by Catharine Ruth Archer, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, April 2012.

ABSTRACT

Ageing is the progressive decline in physiological performance with age, which is almost universal amongst multicellular organisms. While understanding ageing is an important aim in biological research, our current understanding of how and why we age is incomplete. In this thesis, I examine how sexual selection affects the evolution and mechanistic basis of ageing. I then explore how diet affects lifespan and reproduction in either sex. Finally, I test the hypotheses that oxidative stress, which occurs when cellular levels of Reactive Oxygen Species exceed circulating antioxidant defences causes ageing (i.e. the free radical theory of ageing) and/or constrains life-history strategies.

To ask these questions, I employ quantitative genetics in decorated crickets *Gryllodes sigillatus* to examine the genetic co(variance) of ageing, lifespan, reproductive effort, oxidative damage and antioxidant protection. In the Australian field cricket, *Teleogryllus commodus,* I apply the geometric framework of nutrition to examine how lifespan, reproductive effort, oxidative damage and antioxidant capacity respond to dietary manipulation.

In *G. sigillatus*, I found that sexual selection caused divergent strategies of age-dependent reproductive effort across the sexes and that this, in turn, promoted different rates of ageing in males and females. I found a trade-off between early reproductive effort and ageing rate in both sexes, although this trade-off was more pronounced in females (Chapter 3). I then explored the mechanistic basis of these sex-specific life-history strategies and, in support of the free radical theory of ageing, I found that oxidative damage was greatest in the shortest lived sex (females) and was negatively genetically correlated with lifespan. Additionally, oxidative damage was a cost of female reproductive effort that accelerated ageing, showing that oxidative stress may mediate sex-specific life-history strategies in decorated crickets (Chapter 4).

If sexual selection affects reproduction and lifespan it should promote sex-specific life-history responses to dietary manipulation. In Australian black field crickets *Teleogryllus commodus,* I found that males and females have distinct dietary optima for lifespan and reproductive effort and that diet mediated a trade-off between these

traits. I found that mating affected responses to dietary manipulation and caused sexual dimorphism in dietary intake under choice (Chapter 5). However, oxidative stress did not explain these life-history responses to dietary manipulation across the sexes (Chapter 6): although oxidative damage was greatest in the shortest lived sex (i.e. females), diets that extended lifespan did not reduce oxidative damage.

My thesis illustrates the importance of considering sexual selection when considering the evolution and mechanistic basis of ageing. It offers equivocal support for the free radical theory of ageing but shows that oxidative stress may help underpin sex-specific life-history strategies. However, my results highlight that unravelling the relationship between oxidative stress and life-history strategies across the sexes will be a very difficult task.

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AUTHOR'S DECLARATION

During the research contributing to this thesis Catharine Ruth Archer (CRA) was supported by a studentship from NERC. All of the chapters presented in this thesis were written by CRA with comments and editing from John Hunt (JH) and Nick Royle (NR), however, others contributed to each chapter as detailed below.

CHAPTER ONE

JH, NR, Sandra South (SS) and Colin Selman (CS) helped comment on the geometric framework section of the general introduction, which is adapted from a review written by CRA, published in 2009 in The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **64**: 956-959.

CHAPTER THREE

Scott Sakaluk (SK): established the inbred lines used in this chapter and commented on draft manuscript. Felix Zajitschek (FZ): help train CRA in conducing survival analyses and commented on draft manuscript. Accepted for publication in Evolution.

CHAPTER FOUR

SK: established the inbred lines used in this chapter. CS: provided training in biochemical protocols.

CHAPTER FIVE

CRA: data collection, analyses. SS: provided protocols for making diets according to the geometric framework. JH: trained CRA in conducting response surface analyses. Kim Jensen: useful discussion

CHAPTER SIX

SS: as above. CS: support via email optimizing biochemical protocols. Sheridan Willis measured vitamin E and was trained to do so by Christopher Mitchell.

DEFINITIONS

Additive genetic effects	Gene action whereby alleles at a locus do not affect the expression of other alleles at either the same, or different loci. In other words, heritable genetic variation without dominance or epistasis.
Ampulla	The sperm containing sack transferred to females at mating by male crickets. In most cricket species, this is the sole component of the **spermatophore**, but in *Gryllodes sigillatus* the ampulla is attached to a nuptial gift called a **spermatophylax**.
Antagonistic pleiotropy	A special case of pleiotropy, whereby a locus has a positive effects on early life fitness but a negative effect on fitness expressed later in life.
Antioxidants	A compound that inhibits oxidation and therefore helps regulate ROS levels to maintain redox homeostasis. May be enzymatic (e.g. catalase, superoxide dismutase) or non-enzymatic (e.g. vitamin E).
Dominance	Interactions between alleles at a single locus.
Epistasis	Interactions between two or more alleles at different loci.
Extrinsic mortality	Environmental sources of mortality such as predation or drought. Assumed to be condition independent.
Free radicals	Highly reactive chemical species that have an unpaired electron in their outermost shell.
Geometric framework	A state-space modelling approach that enables us to examine the association between traits of interest and two or more nutrients simultaneously.
Heritability	Broad sense heritability estimates are the proportion of phenotypic variance explained by genes. Narrow sense heritability estimates are the proportion of

	phenotypic variance explained by additive genetic variation.
Inbreeding depression	Decrease in fitness associated with inbreeding.
Intake array	The amount of nutrients (e.g. protein and carbohydrate) eaten in a range of nutritional imbalanced diets. The shape of the intake array reveals how animals resolve trade-offs between over- and under- ingesting nutrients when they are unable to reach their **intake target**.
Intake target	The amount of particular nutrients that an animal needs to ingest to reach its **nutritional target**.
Mutation	A permanent alternation to a DNA molecule.
Non-additive genetic effects	The sum of **Epistatic** and **Dominance** genetic effects.
Nutritional rail	A rail is used to represent the nutritional composition of food, it starts at the origin in nutritional space (0) and its gradient represents the ratio of two nutrients in a particular diet.
Nutritional space	The area that is defined by nutritional rails, usually two-dimensional.
Nutritional surface	A three-dimensional representation of the relationship between a phenotypic traits and intake of two or more nutrients. Also called a **nutritional landscape.**
Nutrient target	The intake of two or more nutrients that optimises individual fitness, which may be dynamic changing over an organism's lifetime. Regulated by a suite of behavioural (e.g. dietary intake) and physiological mechanisms (e.g. post-ingestive regulation).
Ovipositor	The appendage through female crickets lay eggs into damp substrate.
Oxidative damage	Damage arising due to oxidation of biological molecules, for example, lipid peroxidation or protein carbonyl group formation.

Oxidative stress	A pro-oxidant state experienced in cells when production of ROS overwhelms circulating antioxidant defences.
Pleiotropy	When one locus affects the expression of more than one trait, for example, body mass and height, causing a genetic correlation.
Prooxidants	Compound formed on the incomplete reduction of oxygen that oxidise cellular molecules.
Reactive Oxygen Species	Oxygen based chemical species, formed on the incomplete reduction of oxygen. May be extremely unstable (e.g. hydroxyl radicals) or comparatively long lived (e.g. hydrogen peroxide).
Senescence	Deleterious age-associated changes, causing the progressive decline in physiological function with age and increased risk of death over time.
Spermatophore	Sperm containing sack transferred to females at mating in crickets.
Spermatophylax	The large gelatinous proportion of the **spermatophore**, which does not contain any sperm and is eaten by females post mating in decorated crickets, *Gryllodes sigillatus.* Not present in *Teleogryllus commodus.*

1. GENERAL INTRODUCTION

1.1 AN INTRODUCTION TO AGEING

Ageing (or **senescence**) is the irreversible decline in physiological performance with age, which progressively increases an individual's risk of dying (Finch 1990). Ageing is almost universal amongst multicellular organisms and understanding its evolution and mechanistic basis is a longstanding and important aim in biology (reviewed in Hughes and Reynolds 2005). However, unravelling the process(es) that underpin senescence is challenging and, despite decades of research, we still do not fully understand either how or why we age (Kenyon 2010; Partridge 2010).

Our incomplete understanding of senescence is most likely due to the complexity of the ageing process. Rates of ageing vary tremendously both within and between species (Kirkwood and Finch 2002; Nussey et al. 2008a). For example, fruit flies experience a rapid decline in physiological function soon after reaching maturity (Finch 1990) whilst bristlecone pines age imperceptibly slowly over thousands of years (Finch and Austad 2001; Finch 2009). Age-associated changes may even occur at different rates within an individual, for example, in nematodes neurons retain their function throughout old age whilst muscles progressively deteriorate (e.g. Herndon et al. 2002). Despite a considerable body of theory explaining the evolution of ageing (Medawar 1946, 1957; Williams 1957; Kirkwood 1977), evolutionary models cannot provide a wholly satisfactory explanation for this variation in ageing rates seen across and within species (Nussey et al. 2008a). Likewise, while hundreds of theories have been proposed to explain the proximate causes of senescence (Medvedev 1990), none of these can explain fine-scale differences in age-associated pathologies (Sohal et al. 2002).

Over the last decade, leading researchers in the field of gerontology have suggested that to better understand the evolution and mechanistic basis of ageing we need to adopt a more integrated approach to ageing research (e.g. Partridge and Barton 1996; Promislow 2003; Williams and Day 2003; Williams et al. 2006; Bourke 2007; Graves 2007; Bonduriansky et al. 2008). However, what do these calls for integration mean? Integration of what and at what level of analysis? In this

introduction I will summarise our current understanding of senescence. In particular, I will highlight where our understanding of ageing may be improved by integrating ageing research with other areas of biology and I summarise how I have attempted to take this approach during my doctoral research.

1.2 THE EVOLUTION OF AGEING

Explaining the evolution of ageing once posed one of the greatest challenges to evolutionary theory (Rose 1991). In fact, for a long time senescence was neglected by evolutionary biologists and considered an inevitable bi-product of somatic wear and tear (Williams 1957). However, wearing out is the accumulation of injuries seen in systems where there is no intrinsic repair over time, for instance, the erosion of engine parts. In contrast, an organism is capable of repairing physiological damage and so, while wear and tear may cause functional decline, ageing reflects an increasing inability to repair it (Williams 1957).

August Weismann was amongst the first to consider how ageing evolved (reviewed in Kirkwood and Cremer 1982) and, in a series of lectures published in 1891, proposed that death is an adaptive trait that removes decrepit individuals to limit overcrowding and competition within a species. However, this hypothesis was fundamentally flawed. Firstly, because Weismann predicted that ageing removes worn out members of a population, his theory relied on that which it attempted to explain (i.e. aged, decrepit animals) (Kirkwood and Holliday 1979). Moreover, because old animals are very seldom seen in nature (Austad 1997) aged animals are rarely exposed to natural selection (Williams 1957; Kirkwood and Austad 2000; Kirkwood 2002). Consequently, even if ageing confers an adaptive benefit, without being subject to natural selection, a gene for ageing could not evolve (Kirkwood 2002). This criticism applies to any theory that posits the existence of an "ageing gene" and a direct, adaptive benefit to senescence.

Our understanding of the evolution of ageing was advanced by the insight that because very few individuals survive environmental hazards such as predation and disease to reach old age, the strength of natural selection diminishes with age (Haldane 1942; Medawar 1946; 1952; Hamilton 1966). Medawar (1946; 1952) realised that this weak natural selection late in life would allow the accumulation of late acting

deleterious mutations that could cause the functional decline characteristic of senescence. Additionally, because the impact on fitness of mutations (and therefore, the strength of selection against them) would depend on both when they were expressed and their magnitude, Medawar proposed that ageing is a result of numerous, late acting, mildly deleterious mutations. Crucially, Medawar's (1952) **mutation** accumulation theory of ageing predicts that senescence is not directly adaptive but instead a consequence of weak natural selection late in life allowing the accumulation of alleles with age-specific, deleterious effects.

The decline in strength of natural selection is also central to the **antagonistic pleiotropy** theory of ageing (Williams 1957). The theory predicts that selection favours alleles with positive effects on early life fitness even if they have deleterious late acting, **pleiotropic** effects. As a result, a trade-off between traits that affect fitness early and late in life is predicted, such that an increase in reproductive investment early in life should accelerate ageing, reduce lifespan or reproductive output later on (Williams 1957). In contrast to the mutation accumulation theory, antagonistic pleiotropy assumes that while ageing is not directly adaptive, it is a consequence of the evolution of an optimal life-history strategy (Partridge and Barton 1993).

A trade-off between fitness early and late in life is also predicted by the disposable soma theory of ageing (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Rose 1991), which is often considered a derivative of the antagonistic pleiotropy theory (Hughes and Reynolds 2005). The disposable soma theory predicts that the high risk of **extrinsic mortality** in natural populations should lead to selection for organisms to invest resources in current reproduction rather than in long-term somatic maintenance, as they are unlikely to reach old age. Therefore, there is an optimal level of somatic repair: if investment is too low the soma may deteriorate before an organism is able to reproduce, however, if resources are diverted to somatic maintenance when extrinsic mortality is high, this investment will probably be wasted (Kirkwood 1977; 2002; Kirkwood and Holliday 1979). The disposable soma and antagonistic pleiotropy theories are conceptually very similar (Hughes and Reynolds 2005) and very difficult to tease apart empirically (but see Carranza et al. 2004) so the disposable soma theory is not discussed further here.

The mutation accumulation (Medawar 1946; 1952) and antagonistic pleiotropy theories of ageing (Williams 1957) assume that weak natural selection amongst greater age classes allows the accumulation of late acting, costly alleles. Therefore, each predicts that greater levels of extrinsic mortality should cause the strength of natural selection to decline more rapidly and, in turn, promote the evolution of shorter lives and more rapid senescence (discussed in Hughes and Reynolds 2005). This prediction has received substantial support from comparative studies in mammals (Austad 1993), and insects (Keller and Genoud 1997) and from studies of experimental evolution, whereby lines subjected to different rates of adult mortality evolve divergent rates of ageing across selection lines (Stearns et al. 2000).

Whilst these results offer broad support to evolutionary theories of ageing, they cannot determine whether mutation accumulation (Medawar 1946; 1952) or antagonistic pleiotropy (Williams 1957) causes senescence. While both processes could contribute to ageing across and within populations, determining their relative contribution is important (Hughes 2010). The antagonistic pleiotropy theory is an optimality model, which predicts that the evolution of longer lives must be associated with reduced early reproductive effort (Williams 1957). Conversely, the mutation accumulation theory assumes that alleles with costly late acting effects on fitness have neutral effects early in life and therefore, an increase in survival need not be associated with any changes in early life fitness (Hughes 2010). Therefore, determining the contribution of mutation accumulation and antagonistic pleiotropy informs us whether we should expect correlated changes in other life-history traits, (e.g. early reproductive effort) in response to the evolution of longer life. Moreover, this question is relevant to general evolutionary problems, for example, understanding the relationship between the strength of selection and adaptation (discussed by Partridge and Barton 1993).

Studies that have attempted to determine the relative contributions of mutation accumulation and antagonistic pleiotropy to the evolution of ageing largely rely on quantitative genetics. Quantitative genetics allows us to partition variation in trait expression across individuals of known relatedness (e.g. siblings), as being due to the environment or due to genes. Genetic variation in trait expression may then be further divided as being **additive** or **non-additive** (i.e. **epistasis** or **dominance**) genetic

effects. Quantitative genetics allows us to test evolutionary theories of ageing because mutation accumulation (Medawar 1946; 1952) and antagonistic pleiotropy (Williams 1957) should produce different patterns of age-associated changes in genetic variance (Charlesworth and Hughes 1996; Hughes et al. 2002; Hughes 2010). If mutation accumulation contributes to senescence, traits that affect fitness will show age-associated increases in additive and non-additive genetic variance and a parallel increase in **inbreeding depression** (Charlesworth and Hughes 1996; Hughes et al. 2002; Hughes 2010). This is because these variance components and the inbreeding load are proportional to the frequency of alleles with deleterious effects at equilibrium. Under the mutation accumulation theory (Medawar 1946; 1952), the frequency of such deleterious alleles is expected to increase over time. These predictions have been met in a broad range of species including fruit flies (*Drosophila sp)* (Lesser et al. 2006; Swindell and Bouzat 2006; Reynolds et al. 2007), snails (*Phyta acuta)* (Escobar et al. 2008) and song sparrows (*Melospiza melodia*) (Keller et al. 2008). However, this support for mutation accumulation is by no means universal. For example, in seed beetles (*Callobruschus maculatus)* inbreeding load for loci affecting mortality did not increase over time (Fox et al. 2006).

Tests for antagonistic pleiotropy have largely looked for negative genetic correlations that indicate trade-offs between traits that affect early- and late- life fitness. Such trade-offs have been found in the field between early reproductive success and ageing rate in antler flies (*Protopiophila litigata*) (Bonduriansky and Brassil 2005), early and late reproductive effort in mute swans (*Cygnus olor)* (Charmantier et al. 2006) and early life fecundity and offspring weight in red deer (*Cervus elaphus*) (Nussey et al. 2008b). Laboratory studies using *Drosophila* species have also found evidence for trade-offs between life-history traits expressed early and late in life (e.g. Luckinbill et al. 1984; Rose 1984a; Partridge and Fowler 1992), however, these trade-offs are not evident in all *Drosophila* species (Schnebel and Grossfield 1988).

Collectively, these studies show that both mutation accumulation and antagonistic pleiotropy are likely to contribute to the evolution of ageing (Partridge and Barton 1993; Hughes 2010). These evolutionary theories of ageing form the foundation of our understanding of why we age. However, as we collect data on rates of senescence across different natural populations, patterns of ageing are emerging

that these theories alone cannot explain. For example, a population of guppies (*Poecilia reticulata*) that evolved under high predation regimes, aged more slowly and lived longer than populations evolved under low levels of predation (Reznick et al. 2004). This directly contradicts a central prediction of evolutionary theories of ageing, namely, that higher levels of extrinsic mortality should cause the evolution of shorter lives and more rapid ageing. However, this prediction assumes that mortality is a random, condition independent event (Williams 1957) and in nature, this is often not the case (Bronikowski and Promislow 2005). In these guppies, animals in poor condition were most vulnerable to predation and therefore, high predation selected for greater net physiological performance and genes that have a positive, pleiotropic effect on longevity (Williams and Day 2003). This example highlights the importance of better understanding the association between ageing and ecology (Abrams 1993; Williams and Day 2003; Reznick et al. 2004; Bonduriansky and Brassil 2005; Sparkman et al. 2007) to resolve apparent mismatches between the predictions of evolutionary theories of ageing and data.

However, ecology is not the only field of biology that has been poorly integrated with evolutionary theories of ageing. Recently, there is a growing appreciation that sexual selection and sexual conflict could influence the evolution of ageing and lifespan (Partridge and Barton 1996; Promislow 2003; Graves 2007; Bonduriansky et al. 2008). Understanding how sexual selection affects lifespan and ageing may explain sex differences in survival, ageing rates and the sex-specific resolution of the trade-off between ageing and reproductive effort predicted by the antagonistic pleiotropy theory of ageing (Williams 1957). Despite this, very few studies have examined how sexual selection and sexual conflict affect the evolution of senescence (Bonduriansky et al. 2008).

1.3 SEXUAL SELECTION, SEXUAL CONFLICT AND THE EVOLUTION OF SENESCENCE

Evolutionary theories of ageing are based on the assumption that the strength of natural selection on survival is constant until the age of first reproduction, at which point it begins to decline (Haldane 1942). Changing the scheduling of reproductive effort over time should therefore affect the rate at which natural selection weakens

and in turn, ageing rates (discussed in Hughes and Reynolds 2005). However, research examining the evolution of ageing has neglected a strong form of selection on age-dependent reproductive effort; sexual selection (Bonduriansky et al. 2008).

Sexual selection occurs because one sex (typically females) invests more resources in reproduction than the other (males) (Trivers 1972; Andersson 1994). Most fundamentally, females produce a few, large eggs while males produce numerous, tiny sperm. This means that males have more resources free to allocate in competing for access to females (Trivers 1972) and therefore, fitness is typically more variable in males than females. As a result, both the opportunity for and intensity of sexual selection is generally greater in males (Bateman 1948; Trivers 1972). This difference in the intensity of sexual selection across the sexes is predicted to have repercussions for how males and females invest in reproductive effort over their lifetime (Bonduriansky et al. 2008).

In females, natural selection is expected to promote low-risk, low-wear-and-tear strategies of reproductive effort that yield moderate rates of return over extended time periods (Bonduriansky et al. 2008). This is because female reproductive success relies on having time to amass resources to produce and rear offspring. In contrast, males may invest intensively in reproductive early in life, a strategy that may yield high fitness returns over a short time frame (Vinogradov 1998; Bonduriansky et al. 2008). This high investment in early reproductive effort may be achieved by sacrificing longevity (Kokko 1997; 2001). Theory predicts that when males adopt this "live fast and die young" strategy of reproductive effort (e.g. Hunt et al. 2004; Bonduriansky and Brassil 2005; Preston et al. 2011), selection should favour the evolution of shorter lives and more rapid ageing in males than females. In keeping with this prediction, male mortality is higher than female mortality in a number of taxa (Promislow 1992) and differences in lifespan and ageing across the sexes positive covary with the intensity of sexual selection in both comparative studies (Promislow 1992; Clutton-Brock and Isvaran 2007) and in lines subject to experimental evolution (Maklakov et al. 2007).

However, males may live as long, or even longer than females (Williams et al. 2006; Bonduriansky et al. 2008) if males increase their reproductive effort with age (e.g. Mysterud et al. 2004). An age-dependent increase in reproductive investment

may occur because reproductive success relies on a trait, such as mass, which takes time to attain (e.g. Le Boeuf 1974; Clutton-Brock 1982; Botero et al. 2009). Alternatively, because the probability of surviving to mate again decreases with advanced age, a male may improve his fitness by increasing his investment in reproduction over time (Kokko 1997). Where a male improves his fitness by investing increasingly in reproductive effort, sexual selection may favour the evolution of a longer life in males than females (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). Sexual selection may therefore shape divergent strategies of age-dependent reproductive effort across the sexes and, in turn, the evolution of ageing and lifespan in either sex.

Where sexual selection favours sex-specific optima for life-history traits the genetic interests of either sex may diverge, a process known as sexual conflict (Parker 1979). Sexual conflict occurs when there is antagonistic selection on alleles either at one locus (*intralocus conflict*) or at different loci (*interlocus conflict*) (Arnqvist and Rowe 2005). Either form of sexual conflict may influence the evolution of ageing and lifespan (Promislow 2003; Bonduriansky et al. 2008). A great deal of research has explored how interlocus sexual conflict and antagonistic co-evolution may drive an evolutionary “arms race” between the sexes where adaptations in one sex are harmful for the other (Arnqvist and Rowe 2005). Indeed, it is well documented that males can influence the lifespan of their mate, either by causing direct physical damage during mating (e.g. Crudgington and Siva-Jothy 2000; Stutt and Siva-Jothy 2001) or by manipulating the female’s reproductive schedule to increase short-term reproduction at the expense of lifespan (Chapman et al. 1995).

Much less work has asked how intralocus sexual conflict can affect the evolution of lifespan and ageing (Bonduriansky et al. 2008). Intralocus sexual conflict occurs when traits that are expressed in either sex have a common genetic basis but are subject to contrasting selection (Bonduriansky and Chenoweth 2009). This differential selection means that these alleles are under an evolutionary "tug-of-war" (Rice and Chippindale 2001), which may restrict the independent evolution of the traits in either sex and may prevent one, or both, of the sexes from reaching their phenotypic optimum (Bonduriansky and Chenoweth 2009). Recently, it has been suggested that sex differences in the optimal timing and relative costliness of

reproductive effort can mediate intralocus sexual conflict over lifespan and rates of ageing (Zajitschek et al. 2007; Bonduriansky et al. 2008). In particular, intralocus sexual conflict over lifespan and ageing may result from differences in the way that males and females trade early-life reproductive effort against lifespan and ageing, or early versus late-life reproductive effort (Zajitschek et al. 2007; Bonduriansky et al. 2008). According to the antagonistic pleiotropy theory of ageing (Williams 1957) this should manifest itself as differences in the direction or strength of genetic correlations between lifespan, ageing and reproductive effort across the sexes (Zajitschek et al. 2007).

Despite the potential importance of sexual selection and sexual conflict in the evolution of ageing and lifespan, very few studies have asked how strategies of age-dependent reproductive effort, shaped by sexual selection, affect the evolution of ageing in either sex (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). I examine how sexual selection affects the evolution of age-dependent reproductive effort across the sexes and ask how this, in turn, affects the evolution of ageing and lifespan. In Chapter 3, I quantify the genetic (co)variance between lifespan, ageing parameters, and age-dependent reproductive effort in both male and female decorated crickets, *Gryllodes sigillatus.* I provide evidence demonstrating an important role for sexual selection in the evolution of lifespan and ageing in this species and find the potential for intralocus sexual conflict over the evolution of ageing and lifespan in the decorated cricket. The remaining parts of my thesis examine the proximate basis of sex-specific rates of ageing and schedules of reproductive effort. The first step in which, is asking if males and females manage their life-history strategies via behavioural regulation of their diet.

1.4 FOOD, SEX AND DEATH: WHAT IS THE CONNECTION?

If sexual selection affects the evolution of reproductive effort, ageing and lifespan, it should also affect how these life-history traits respond to dietary manipulation. In a broad range of species, diet affects lifespan and reproductive effort. Specifically, when animals eat a restricted diet, but not so restricted as to cause malnutrition, they live longer, age more slowly and experience fewer age associated diseases than fully fed animals. Increased lifespan and/ or improved health under

Dietary Restriction (DR) has been seen in species including yeast (Jiang et al. 2000), fruit flies (Mair et al. 2005), spiders (Austad 1989), rotifers (Verdone-Smith and Enesco 1982), mice (Weindruch et al. 1986) and primates (Colman et al. 2009). However, this increase in lifespan is often associated with reduced reproductive effort (e.g. Klass 1977; Chippindale et al. 1993; Bishop and Guarente 2007). Understanding the relationship between ageing, lifespan, reproductive effort and diet is a central aim of gerontology (Mair and Dillin 2008), which may also offer insight into the proximate basis of ageing, age-associated diseases and ultimately highlight therapeutic interventions that may improve human health and survival (Mair and Dillin 2008). Moreover, sex-specific responses to dietary manipulation and regulation of dietary intake may help us understand the proximate mechanisms by which either sex manages their life-history strategies (Maklakov et al. 2008). However, despite decades of research, we still do not fully understand why animals live longer when they eat less (Flatt 2009; Tatar 2010)

Our poor understanding of lifespan extension under DR can be attributed to a number of technical challenges. One of the main issues concerns whether the effects of DR on longevity are primarily artefacts of laboratory environments, for example, given that there is little data available on food consumption patterns of animals in the wild, the beneficial effects of DR may simply reflect the adverse effects of overfeeding in the lab (Mockett et al. 2006; Lee et al. 2008). Methodological limitations also pose problems. Empirically uncoupling the effects of calories from those of the nutrients from which they are derived remains a major challenge. Perhaps as a consequence of this, DR studies often apply too few dietary treatments (Lee et al. 2008). In addition, it is difficult to accurately quantify food consumption, especially where liquid diets are used and restriction is accomplished via nutrient dilution. Without precise measurements of dietary consumption it may not be possible to detect compensatory feeding (Mockett et al. 2006; Piper and Partridge 2007; Tatar 2010); a process whereby animals consume greater quantities of diluted nutrients so as to regulate intake and, in doing so, mitigate the effects of DR. Studies seldom address assimilation efficiency, which may not be constant across an organism's lifetime. Finally, animals have often been housed in mixed sex groups and therefore, sexual activity and reproductive investment have not been controlled (Piper and Partridge 2007; Tatar 2010). Even in

studies where reproductive effort has been measured, it is often measured with greater precision (and more frequently) in females than in males (e.g. Rose and Charlesworth 1981; Rose 1984a; Partridge and Fowler 1992; Carey et al. 2002). This is possibly due to the relative ease with which female reproductive effort can be quantified in laboratory model organisms (counting eggs) compared with measuring lifetime investment by males in sexual signalling.

Two studies on insects, however, have overcome many of these limitations via the application of the geometric framework of nutrition. The progress made with this technique surpasses that of the last decade (Archer et al. 2009), not only in advancing our understanding of the link between DR and ageing but also in providing a practical framework that promises to further progress research on this topic.

1.5 THE GEOMETRIC FRAMEWORK OF NUTRITION

The central premise behind the **geometric framework** is that growing animals, or those that consume a nutritionally heterogeneous diet, strive to optimise their intake of specific nutrients to maximise fitness (Simpson and Raubenheimer 1993; 1995; Raubenheimer and Simpson 1997). This **intake target** is the multidimensional nutritional point towards which animals feed. This can be identified by providing several diets and determining the intake optimal for fitness or by identifying the dietary intake animals actively defend when provided with choice (Simpson and Raubenheimer 1995). Around this target, it is possible to construct a **nutrient surface** by feeding animals diets that vary in their nutrient ratios (along **nutrient rails**) and concentration (Figure 1-1). This surface is like a geographical map, where different nutrients replace latitude and longitude, on which various traits (including longevity and reproductive effort) may be regressed (Figure 1-2). The effects of specific nutrients on that trait can be determined by forcing animals to eat suboptimal diets and analysing nutritional landscapes using powerful response surface methodologies (Lande and Arnold 1983). Compensatory feeding can be detected if animals maintain their intake target across a range of nutrient dilutions (Lee et al. 2008). The geometric framework has been used to great effect to investigate the relationship between nutrition, longevity and reproductive effort (e.g. Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Cotter et al. 2011; Jensen et al. 2011; South et al. 2011).

Lee et al. (2008) employed nutritional geometry in an elegant study of *D. melanogaster*. Liquid diet of varying protein and carbohydrate ratio and concentration was provided in microcapillary tubes and consumption measured according to a scale bar. Lee et al. (2008) found that longevity was greatest at a Protein:Carbohydrate (P:C) ratio of 1:16. The fecundity of mated females was optimized under higher protein: the rate of egg production peaked at a P:C of 1:2 and lifetime fecundity at a P:C of 1:4. The difference in optimal P:C for longevity and fecundity suggests a trade-off. However, it appears that the interplay between reproduction and lifespan is not integral to determining how the latter responds to DR. Since the P:C contour lines (i.e. those lines showing the effect of protein and carbohydrate intake on trait expression) ran almost perpendicular to the isocaloric line (i.e. the line linking diets of equal caloric value) this shows that calories *per se* did not have a significant influence on any of the investigated life history attributes. Flies attempted to mitigate the effect of nutrient dilution by eating more and could regulate their intake to consume a P:C ratio corresponding to that which maximises lifetime reproductive success across a range of nutrient concentrations.

Maklakov et al. (2008) used similar methods to investigate senescence in the Australian field cricket, *Teleogryllus commodus*. Male *T. commodus* call to attract females and this energetic sexual advertisement is associated with a four-fold increase in metabolic rate (Kavanagh 1987) and is under strong selection through female choice (Bentsen et al. 2006). In a no-choice test, 24 powdered diets (Figure 1-1) were provided in excess and intake measured by weighing the dried food every 3 days throughout the cricket's lifetimes. Male calling effort was monitored using an acoustic chamber and the fecundity of mated females quantified by counting the number of eggs that females produced. Two choice experiments were also conducted to examine how either sex regulated their nutritional intake. Maklakov et al. (2008) first provided isocaloric diets that differed in their P:C ratio to determine which ratio of protein to carbohydrate the crickets preferentially consumed (i.e. to identify the intake target) and then, in a separate study, provided a choice between three pairs of foods that varied in both the ratio of nutrients (P:C) and energetic content (P+C) to determine how well crickets could defend their intake target.

As was the case for *D. melanogaster*, lifespan was maximised on a low P:C ratio, in this case a 1:3 ratio of proteins to carbohydrate. However, in *T. commodus* male longevity declined with high carbohydrate intake, while in either sex, as the P:C intake ratio increased, longevity decreased. This study provided the first, clear evidence that there is potential for sexual conflict over optimal diet (but see Chapter 5). Male calling effort was greatest at the P:C ratio that maximised lifespan, while in females, greater dietary protein increased fecundity but decreased longevity. This trade-off between female reproductive fitness and long life did not manifest itself in a female's choice of diet: whilst there was evidence of active regulation of diet influenced in both sexes, both sexes consumed a 1:3 ratio of P:C and there was little sexual dimorphism in nutrient regulation. While neither sex apparently chose the diet that maximised their lifetime reproductive success in the laboratory paradigm in which they were tested, female fecundity was highly compromised by their choice of diet.

These studies advanced our understanding of how diet affects reproductive effort and lifespan across the sexes. However, the geometric framework has a number of potential applications to ageing research that have not yet been fully utilised. For example, the geometric framework allows us to examine how either sex manages their life-history strategy via behavioural regulation of dietary intake. Maklakov et al. (2008) aimed to explore such intersexual differences, but this study compared nutritional landscapes for mated females and virgin males. This approach is problematic for two main reasons. Foremost, the availability of mates changes the expression of a suite of reproductive behaviours in crickets (Loher 1981; Souroukis 1992; Hill 1998; Dolwing and Simmons 2012), each of which may have particular nutritional requirements. Mating could therefore shift the nutritional optimum for fitness. Secondly, the rationale behind expecting sex-specific strategies of nutrient regulation is that either sex may improve their fitness by optimising different life-history traits (e.g. reproductive effort in males but lifespan in females). In the absence of mates, we might predict that virgins eat to maximise their lifespan until mates become available. Mating could therefore change the optimal strategy of nutrient regulation across the sexes. In Chapter 5, I construct nutritional landscapes for mated males and mated females and ask how mating changes responses to dietary manipulation (by comparing my nutritional landscapes to those produced by Maklakov et al. 2008) and then

examine how mating status affects intake targets across the sexes (by collecting intake data for virgin and mated animals). I use this data to create an **intake array** to determine how either sex manages the costs of over- and under- ingesting different nutrients when they are restricted to a single, nutritionally imbalanced diet.

The geometric framework may also be used to examine the mechanistic basis of ageing. In Chapter 6, I use diets constructed according to the geometric framework of nutrition to examine the role of **oxidative stress** in causing senescence and by doing so, test the free radical theory of ageing (Harman 1956).

1.6 THE FREE RADICAL THEORY: INTEGRATING ULTIMATE AND PROXIMATE APPROACHES TO AGEING RESEARCH

In the 1950s Denis Harman proposed that **free radicals** generated within cells caused the functional decline characteristic of ageing. At the time, the free radical theory of ageing (Harman 1956) was controversial, but when the endogenously produced **antioxidant** superoxide dismutase was discovered (McCord and Fridovich 1969), an enzyme that functions solely to scavenge free radicals, Harman's hypothesis gained its first empirical support (Finkel and Holbrook 2000). Today, we know that oxidative damage may be caused by a diverse group of pro-oxidants, collectively referred to as **Reactive Oxygen Species** (ROS). ROS are formed on the incomplete reduction of oxygen both in response to environmental stimuli (e.g. ionizing radiation) and intracellularly, primarily in the mitochondria during aerobic metabolism (Boveris and Chance 1973).

ROS are highly reactive, which makes them excellent signalling molecules that are used to regulate a number of vital physiological processes, including cellular growth, apoptosis and differentiation (Apel and Hirt 2004; Veal et al. 2007). However, this reactivity also carries costs and means that ROS readily react with cellular molecules such as lipids, DNA and proteins and, by doing so, impair their function (e.g. Halliwell and Chirico 1993; Berlett and Stadtman 1997; Aitken and Krausz 2001). For example, protein oxidation may inactivate enzyme complexes (Dalle-Donne et al. 2003) while lipid oxidation reduces membrane integrity (Brigelius-Flohe and Traber 1999). Optimal ROS homeostasis is therefore a compromise between meeting the functional demands for these **pro-oxidants** (e.g. as signalling molecules) and mitigating

the cellular damage that they cause when in excess (Finkel and Holbrook 2000). **Antioxidants** are central to maintaining this homeostatic balance. These dietary derived molecules (e.g. vitamin E) or endogenously produced enzymes (e.g. catalase) react with ROS and render them inert (Finkel and Holbrook 2000). However, when ROS production is perturbed such that it overwhelms the capacity of antioxidant defences, cells enter a pro-oxidant state called oxidative stress and **oxidative damage** to cellular molecules accumulates (Finkel and Holbrook 2000). The free radical theory of ageing predicts that the accumulation of this oxidative damage, under prolonged or severe oxidative stress, causes ageing (Harman 1956).

The free radical theory (Harman 1956) makes a number of readily testable predictions. Foremost, it predicts that variation in lifespan across species should be due to variation in oxidative stress, either because of different rates of ROS production, variation in antioxidant defences or rates of repair of oxidative damage. Secondly, oxidative damage should accumulate over time. Thirdly, manipulations that affect the rate at which oxidative damage accumulates should, in turn, affect lifespan. Numerous studies have tested each of these predictions but the results are largely equivocal or contradictory (Beckman and Ames 1998).

Studies which have compared oxidative damage and antioxidant protection across species, strains or the sexes that differ in the lifespan or rates of ageing, have often produced evidence in support of the free radical theory (Beckman and Ames 1998; Barja 2002). These comparative studies have found that oxidative damage or production of ROS may negatively correlate with lifespan (Sohal et al. 1993a,b; 1995). For example, longer lived species of snakes generate fewer ROS than shorter lived species (Robert et al. 2007). In mammals, long lived white footed mice produce fewer ROS and have more active antioxidant defences than shorter lived house mice (Sohal et al. 1993b). However, recent studies have shown that very long lived species often have high levels of oxidative damage when compared to other shorter lived, closely related species (reviewed by Speakman and Selman 2011) challenging a key prediction of the free radical theory (Harman 1956). Comparisons within species have shown that oxidative damage may be greater in the shorter lived sex (e.g. Ide et al. 2002; Ali et al. 2006), however, the direction of intersexual differences in oxidative damage is inconsistent across studies (Satori-Valinotti et al. 2007). Research that has quantified

age-associated changes in oxidative damage or antioxidant protection have found that damage frequently accrues over time (e. g. Forster et al. 1996; Hudson et al. 1998; Mecocci et al. 1999; Head et al. 2002). However, this is not always the case, for example in wild kestrels oxidative damage is greatest in the youngest animals (Costantini et al. 2006).

Correlative studies such as these can only offer, at best, weak support for the free radical theory because they cannot show that oxidative damage is the cause, rather than a symptom of, senescence. A more robust test of the free radical theory is to manipulate oxidative stress and determine its effects on ageing (Beckman and Ames 1998). Research that has attempted to do this has also found mixed support for the free radical theory. For example, studies have shown that antioxidant supplementation increased longevity in nematodes (Harrington and Harley 1988), rotifers (Enesco and Verdone-Smith 1980; Sawada and Enesco 1984), fruit flies (Driver and Georgeou 2003) and mice (Navarro et al. 2005) but increased the risk of disease or death in humans with diabetes (Lonn et al. 2005). Where studies have found positive effects on lifespan of antioxidant supplementation, these findings are not always repeatable (Morley and Trainor 2001) and depend heavily on the timing and dose of supplementation (Driver and Georgeou 2003).

Manipulations of the expression of antioxidant enzymes using transgenic or knockout model organisms have also challenged the free radical theory (reviewed in Jang and Remmen 2009). Over-expression of genes that encode antioxidants may increase longevity (Spencer et al. 2003; Schriner et al. 2005), but of eighteen such manipulations in mice, only one (deletion of a gene encoding the antioxidant enzyme superoxide dismutase) affected longevity in the direction predicted by the free radical theory (Pérez et al. 2009). The failure of antioxidant manipulation to consistently and predictably influence lifespan has been declared a decisive and absolute blow against the free radical theory of ageing (e.g. Howes 2006; Jang and Remmen 2009; Pérez et al. 2009).

However, there is an increasing awareness that we should not expect the relationship between antioxidants and lifespan to be quite this simple. Several recent studies have highlighted that oxidative stress could mediate life-history trade-offs (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al.

2011) and if this hypothesis is correct, we should not always expect elevated antioxidant capacity to be related to increased lifespan. For example, if antioxidants are used in reproduction (e.g. pigment based sexual display - Blount et al. 2003; Pike et al. 2007), antioxidants could drive a resource based trade-off between reproductive effort and somatic maintenance. If increasing reproductive effort improves fitness more than a long life does (e.g. Hunt et al. 2004), then supplementary antioxidants could be allocated to reproduction rather than to the soma, which would increase reproductive effort but not lifespan. Oxidative stress could also mediate the association between reproductive effort and lifespan if the increased metabolic rate often associated with reproduction (e.g. Kavanagh 1987), increases ROS production from the electron transport chain, pushing cells into oxidative stress and accelerating senescence. Testing the role of oxidative stress in life-history evolution may improve our understanding of the proximate basis of life-history trade-offs (Stearns et al. 2000; Zera and Harshman 2001; Barnes and Partridge 2003; Lessells 2008; Nijhout et al. 2010), individual variation in life-history strategies and trade-offs between traits that are separated in time, e.g. between development rates and lifespan (Isaksson et al. 2011). In principle, oxidative stress could explain sex-specific life-history strategies. For example, sexual selection may promote a live fast die young strategy of reproductive effort in males (Hunt et al. 2004; Punzalan et al. 2008) and therefore, favour the allocation of antioxidants to sexual signals rather than somatic maintenance. However, despite the potential importance for oxidative stress as a proximate constraint on life-history evolution, very little work has explored the role of oxidative stress in life-history trade-offs (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011).

In Chapter 4, I use *G. sigillatus* to ask how sex specific schedules of reproductive effort, ageing and lifespan, shaped by sexual selection, correspond to age-dependent oxidative damage and protection across the sexes and calculate the genetic (co)variance between these traits to explore the genetics of oxidative stress. In doing so, I aim to test the free radical theory of ageing, the role of oxidative stress in mediating life-history strategies and ask how sexual selection affects optimal investment in antioxidant defences relative to oxidative damage.

If oxidative stress does mediate life-history strategies, we should expect levels of damage or investment in antioxidant protection to respond to dietary manipulation. Whilst numerous studies have manipulated a single nutrient and asked how DR affects oxidative stress (e.g. Ayala et al. 2007; Sanz et al. 2006), none of these have used the geometric framework of nutrition to explore responses to manipulation of more than one nutrient simultaneously. Furthermore, these studies have not measured reproductive effort across the sexes to see if oxidative damage is a cost of reproductive effort. In Chapter 6, I quantified oxidative damage to proteins and antioxidant capacity in crickets fed one of four diets that affect lifespan and reproductive effort in either sex. I also supplemented half of these diets with the antioxidant vitamin E, to quantify how antioxidant supplementation affects the expression of these life-history traits.

1.7 AIMS AND OBJECTIVES OF MY DOCTORAL RESEARCH

Recently, experts in the field have highlighted that to further develop our understanding of the evolution of ageing, we need to better integrate different avenues of ageing research (Partridge 2010). In this thesis, I hope to integrate sexual selection with evolutionary and mechanistic theories of senescence (e.g. Promislow 2003; Graves 2007; Bonduriansky et al. 2008). Using the geometric framework of nutrition I determine if males and females can regulate their life-history strategies via behavioural regulation of their nutritional intake. Finally, I use diet to manipulate lifespan and reproductive effort to see how this affects oxidative damage and antioxidant protection across the sexes. My aims are therefore threefold: (1) elucidate the role of sexual selection on the evolution and mechanistic basis of ageing, (2) examine the role of oxidative stress in causing senescence and as a proximate constraint on life-history evolution and (3) examine how nutrition affects oxidative stress to better understand the mechanisms by which diet affects ageing and lifespan.

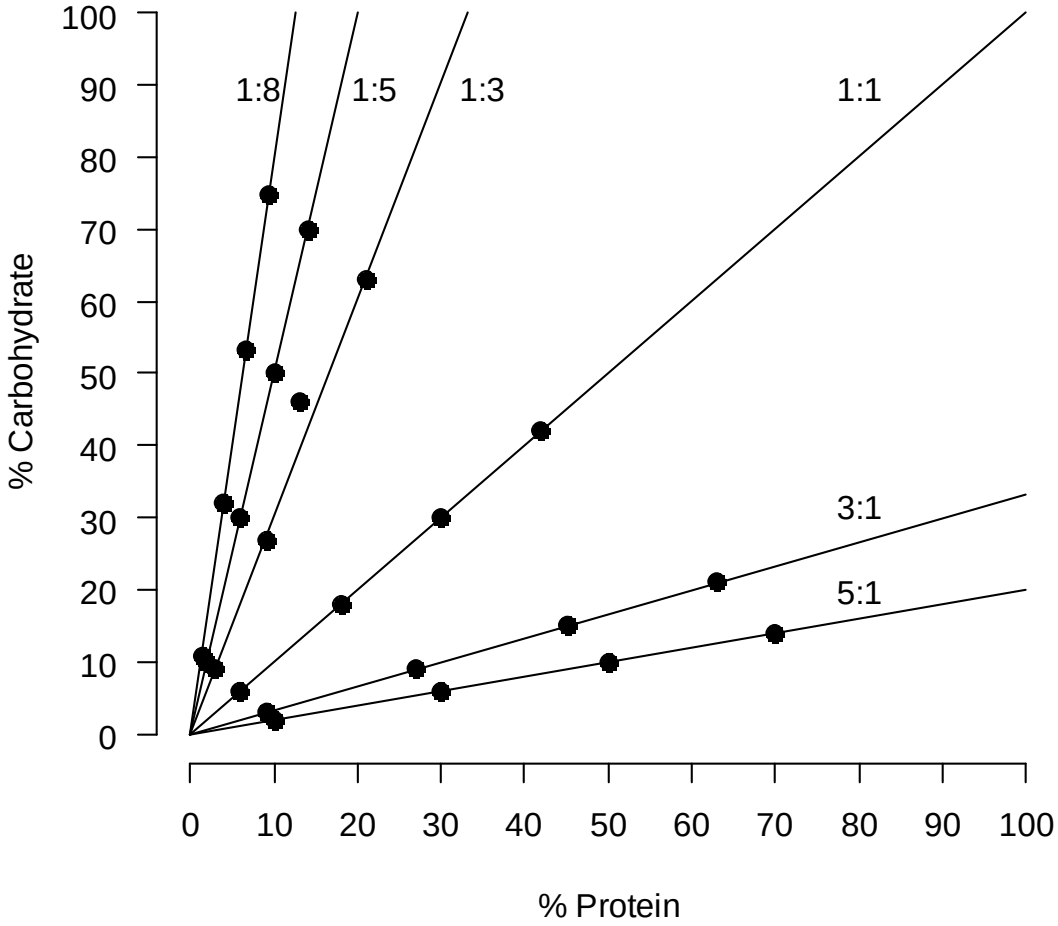

Figure 1-1. Location of the 24 diets that define the **nutritional space**.

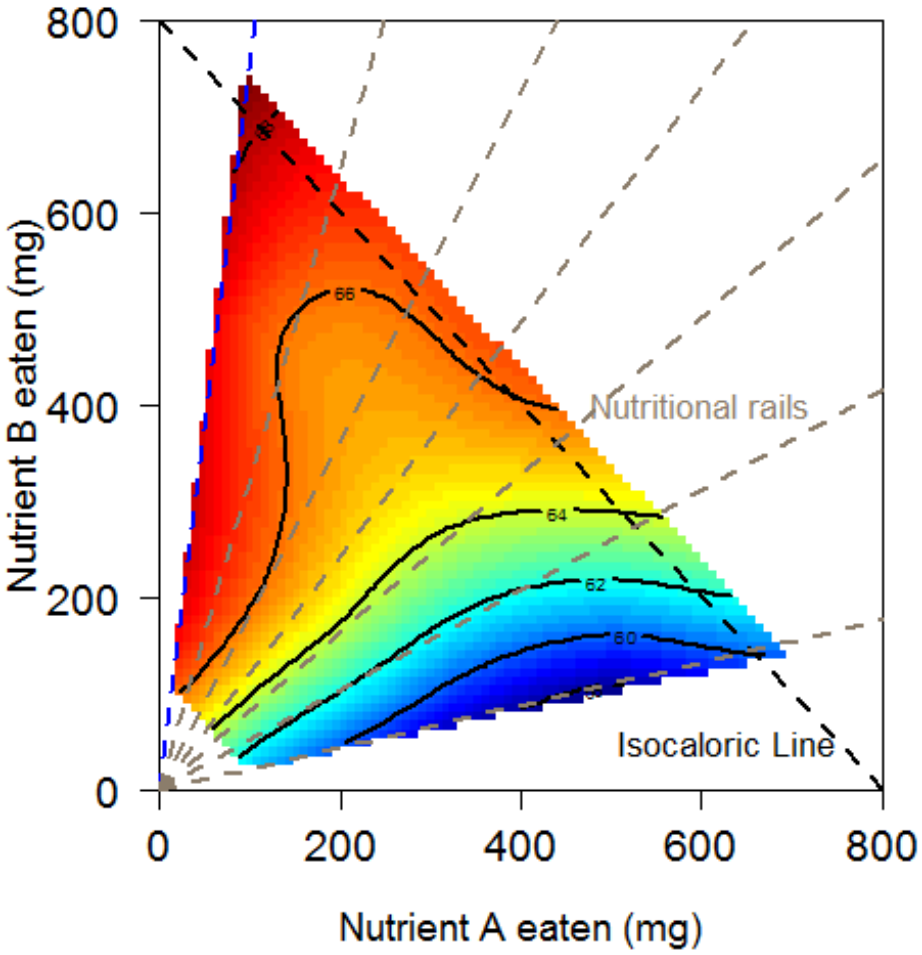

Figure 1-2. Example of a nutritional landscape. Animals may feed along nutritional rails (beige dotted lines) and a trait of interest is mapped onto the intake data for nutrients A and B. Red regions of the surface correspond to optimal expression of the trait of interest, whilst blue regions show low values for expression of that trait. Dotted black lines are isocaloric lines which connect diets of equal caloric value and the nutritional rail highlighted blue, corresponds to the diet ratio optimal for the trait of interest.

2. GENERAL METHODS

2.1 DECORATED HOUSE CRICKETS AND AGEING: THE POTENTIAL FOR (AND LIMITATIONS OF) USING INBRED LINES

For half of my empirical research, I use inbred lines of the decorated house cricket, *Gryllodes sigillatus* (Walker 1869) (Figures 2-1 to 2-3). *G. sigillatus* (previously *Gryllodes supplicans)* are probably native to south-western Asia (Williams 2011) but now occur in tropical regions across the globe and are often associated with human habitation (Thomas 1985). This species shows homodynamic development whereby all developmental stages are present throughout the year (Masaki and Walker 1987), often in very high density populations (Sakaluk 1987). This means that in *G. sigillatus*, competition for mates is high and therefore there is the opportunity for intense sexual selection and sexual conflict. This has promoted the evolution of a number of adaptations in males (and counter-adaptations in females) to maximise sex-specific reproductive success.

Foremost, male *G. sigillatus* produce advertisement songs to attract females from burrows in gravel, cracks in cement or from under temporary refugia such as fallen leaves (Figure 2-1) (Sakaluk 1987). Females move above ground towards potential mates (Sakaluk 1987) and exert pre-copulatory mate choice amongst competing males (Ivy and Sakaluk 2007). Female mate choice appears to be influenced, in part, by how long and how frequently a male calls and so calling effort is the primary determinant of a male's mating success (Ketola et al. 2007).

Once a female has chosen a mate, she mounts him and the male then transfers a large, sperm containing **spermatophore** to the female, attaching it immediately beneath the base of her **ovipositor** (Figure 2-2) (Sakaluk 1984). This spermatophore consists of a large **spermatophylax**, which does not contain any sperm and is eaten by females (Sakaluk 1984) and a sperm containing **ampulla** (Figure 2-2). Seconds after mating ends, females dismount their mates and remove and begin to consume the spermatophore, leaving the ampulla attached. This allows sperm transfer from the ampulla to the female's sperm storage organ (Figure 2-3). Once the spermatophore has been consumed, the female then removes the ampulla and sperm transfer

terminates. This means that investment in a spermatophore affects a male's reproductive success: the larger the spermatophore the longer the ampulla remains attached to the female (Sakaluk 1985) and, therefore, the more sperm is transferred (Sakaluk 1984). This may help determine a male's fertilization success (Sakaluk 1986). This means that investing in large spermatophores may improve male fertilization success, however, producing a spermatophore is extremely costly. For example, males that invest more heavily in spermatophores tend to have lower immune defences (Ker et al. 2010). How much males invest in their spermatophores is influenced, in part, by the risk of competition from other males (Mallard and Barnard 2003).

The reason for this is that even after mating, males are in competition for fertilization success because female decorated crickets mate multiply. Over 20 days, a female decorated cricket mates on average 22 times, whilst males mate on average 10.9 times across their lifetime (Sakaluk 1987). Females that mate with several different males have a fitness advantage over virgins or monogamous females because they produce more offspring that survive to maturity (Ivy and Sakaluk 2005). Multiple mating even appears to improve female lifespan, perhaps because spermatophylax consumption improves female condition (Burpee and Sakaluk 1993). Whilst polyandry improves female fitness it reduces each individual male's fertilization success (Sakaluk 1986). Therefore, there is selection for multiple mating in females but selection for males to reduce female mating frequency.

To this end, males have evolved two adaptations to affect female mating frequency. Firstly, males invest more heavily in a spermatophore in the presence of competitors (Mallard and Barnard 2003) to increase the time that the ampulla is attached to a female post mating and increase the number of sperm that are transferred. In turn, females exert post-mating mate choice and can behaviourally regulate how much sperm is transferred by a particular male. Studies have shown that females leave the ampulla attached for longer and therefore, accept more sperm from, an attractive male than an unattractive male (Ivy and Sakaluk 2007). To help prevent a female prematurely terminating sperm transfer and to exclude rival males, males actively guard females post mating (Bateman and MacFayden 1999). In response, females attempt to evade their guards by jumping and kicking to gain access to any new males (Bateman and MacFadyen 1999).

In summary, in *G. sigillatus* either sex has evolved adaptations to affect how much sperm is transferred during mating (i.e. spermatophylax production by males and removal of the ampulla by females) as well as mechanisms to affect the frequency of female re-mating (i.e. producing large spermatophores and mate guarding versus evading guards). In this species, that has evolved under high male-male competition, there is intense sexual selection and sexual conflict over mating behaviours. This means that decorated crickets are a good model organism to examine how sexual selection affects the evolution of ageing and lifespan. Moreover, in this species, we can readily assay investment in reproductive effort across the sexes; in females, fecundity can be measured by counting eggs (e.g. Head et al. 2005; Hunt et al. 2006; Zajitschek et al. 2007), while male calling effort, an important determinant of male reproductive success in decorated crickets (Ketola et al. 2007) can be measured (e.g. Hunt et al. 2004, 2006; Zajitschek et al. 2007; Judge et al. 2008).

Our stocks of *G sigillatus* are descended from 500 adult crickets collected in Las Cruces, New Mexico in 2001. These animals were used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictically (hereafter, referred to as the outbred population). Nine inbred lines were created by subjecting a random subset of crickets from this population to 23 generations of full sib mating followed by 12 generations of panmixis within each line (Ivy and Sakaluk 2005). Crickets were imported to Tremough Campus at this point, where panmixis has been maintained within all lines since.

Crickets were housed in 15L plastic containers in an environmental chamber (Percival I-66VL) maintained at 32°C on a 14h:10h light/dark cycle. Crickets were provided with cat food pellets (Friskies Go-Cat Senior) and water in 60ml glass test tubes plugged with cotton wool ad libitum, as well as an abundance of egg cartons to provide shelter. As soon as adults were detected, moistened cotton wool was provided in a petrie-dish (10 cm diameter) as an oviposition substrate. Each inbred line was maintained in 2 containers and our outbred population in 8 containers. Each generation, crickets were randomly mixed between containers and were maintained at a density of approximately 300 crickets per container.

I use these inbred lines to calculate quantitative genetic parameters. In particular, I calculate the genetic (co)variance between ageing parameters, lifespan

and age-dependent reproductive effort (Chapter 3) and oxidative damage and antioxidant protection (Chapter 4) between and within the sexes. It is important to highlight that using inbred lines in quantitative genetics has limitations. One such frequently cited limitation was proposed by Rose (1984b), who suggests that inbred lines commonly show positive genetic correlations between traits when at equilibrium and hence, are a poor tool for quantifying trade-offs. This commonly held view is far from being unequivocally established in the empirical literature. For example, a direct test of this argument in the cricket *Gryllus firmus* by Roff & DeRose (2001) showed that inbreeding influenced the intercept of relationship between body size and fecundity (equivalent to mean fecundity) but not the slope (equivalent to genetic correlation between these traits). Studies from other laboratories using the same inbred lines of *G. sigillatus* have documented trade-offs, for example, Gershman et al. (2010) found a negative genetic correlation between different measures of immune function. Therefore, I am confident that trade-offs can be (and have been) detected using these inbred lines. Despite the limitations of using inbred lines in evolutionary research, I believe, as many other researchers do, that they are a powerful tool for understanding the genetic architecture of complex traits (e.g. David et al. 2004; Rohlfs 2006; Gershman et al. 2010). This is particularly true for my research because I estimate the genetic architecture of ageing parameters (Chapter 3), which is best achieved using genetically identical animals (Finch 1990) and requires large sample sizes.

In all studies where I use these inbred animals (Chapters 3 and 4) I estimate all traits of interest in a large sample of the outbred crickets that were used to propagate our inbred lines. This allows me to add these trait estimates for the outbred crickets to all figures, providing the opportunity for direct comparison with the equivalent values in the inbred lines. This provides some insight into the effect that inbreeding has had on mean trait expression but does not enable us to determine if or how inbreeding has influenced our quantitative genetic estimates.

2.2 AUSTRALIAN BLACK FIELD CRICKETS (*TELEOGRYLLUS COMMODUS*) AS A MODEL SPECIES FOR STUDYING SENESCENCE

I conduct the second half of my PhD research using the Australian black field cricket, *Teleogryllus commodus* (Walker) (Orthoptera: Gryllidae) (Figure 2-4). *T.*

commodus is widely distributed across southern Australia (Figure 2-5) (Hill et al. 1972; Fontana and Hogan 1969). In the wild, these crickets enter diapause over winter (Hogan 1965) and emerge in spring, when males begin producing advertisement calls to attract females from simple burrows and crevices in the earth (Evans 1983). However, diapause is not obligate and crickets may be reared throughout the year in the laboratory, making *T. commodus* amenable to laboratory research.

In particular, *T. commodus* is an excellent model species for exploring the relationship between ageing, lifespan and reproduction. Importantly, as in *G. sigillatus,* reproductive effort can be accurately quantified in both sexes; once more female fecundity can be measured by counting eggs (e.g. Head et al. 2005; Hunt et al. 2006; Zajitschek et al. 2007) and male reproductive effort can be measured as the time males spend calling (e.g. Hunt et al. 2004, 2006; Zajitschek et al. 2007; Judge et al. 2008), which is an important determinant of male reproductive success in this species (Bentsen et al. 2006). Moreover, there is a large body of data examining ageing, lifespan and reproductive effort in *T. commodus.* For example, Zajitschek et al. (2009a,b) measured sex-specific ageing rates in the field and in the laboratory studies have examined how diet affects lifespan and reproductive effort (Hunt et al. 2004; Maklakov et al. 2008) and characterised sex-specific life-history strategies (Hunt et al. 2006; Maklakov et al. 2008). These studies exploring the evolution of ageing, lifespan and reproductive effort provide an excellent foundation for research exploring the mechanistic basis of ageing in *T. commodus*.

2.3 COLLECTION AND MAINTENANCE OF STOCK *T. COMMODUS*

Between February and April of 2009, I collected *T. commodus* from a number of locations across Australia (Figure 2-5). Crickets were collected between 10pm and 3am, when males were actively calling to attract females and females were moving above ground towards potential mates. Crickets were caught by hand and stored in a bucket filled with lengths of cardboard and cat food (Friskies GoCat Senior). At the end of each night, crickets were transferred into large plastic boxes (c. 80L), which contained stacks of egg carton for shelter, commercially available cat food and damp cotton wool in which females could lay eggs. I collected *ca*. 200 gravid females and 200 males from each location and allowed these to mate and lay eggs *ad libitum.*

These crickets were then transferred by plane to a laboratory at the Australian National University (ANU) in Canberra. In my research I only use crickets from one of these populations (Smith's Lake, Figure 2-5), which is the most widely studied of the six (i.e. this population was used in all studies discussed in section 2-2). The other populations are being used in the laboratory at Tremough Campus for a NERC funded research project examining the genetics of call structure.

At ANU, population containers were maintained at 28°C, under a 13:11 light dark cycle. Crickets were provided with stacks of egg boxes for shelter and were cleaned and fed Friskies GoCat Senior cat food daily. Water was provided in an upturned plastic beaker lodged in a petri-dish lid containing moist cotton wool. Egg pads consisted of damp cotton wool provided in the bases of take-away containers, cut to a depth of *ca*. 0.5 inches, which were sprayed with water daily to ensure they did not dry out. Once a week, egg pads were shipped back to the laboratory at Tremough Campus where the nymphs hatched (Figure 2-4B).

At Tremough Campus, all crickets were housed under the same temperature and light-dark conditions as at ANU. Nymphs were maintained in small boxes (17 x 12 x 6 cm) for the first month post hatching, and water was provided in 2.5ml test tubes plugged with cotton wool. Nymphs were fed whole cat food pellets in a small piece of egg carton and, in addition, cat food was provided ground up in the lid of an 1.5ml eppendorf. After a month these nymphs were transferred to larger boxes (10L) but were otherwise maintained in the same manner. When nymphs reached their final developmental instar, they were transferred into 100L boxes and maintained exactly as in ANU. Egg pads were changed weekly and nymphs collected from these pads over six weeks after being removed from the colony.

2.4 MAKING DIETS ACCORDING TO THE GEOMETRIC FRAMEWORK

In Chapter 5, I made twenty-four, dry granular diets that varied in both their ratio of protein to carbohydrate (P:C) and absolute amount of protein and carbohydrate (P+C) according to the established protocol detailed in Simpson and Abisgold (1985). In each diet, proteins consisted of casein, albumen and peptone in a 3:1:1 ratio. Carbohydrates consisted of a 1:1 mix of sucrose and dextrin. Wesson's salts (2.5%), ascorbic acid (0.28%), cholesterol (0.55%) and vitamin mix (0.18%) were

present in all diets in equal amounts. To this mixture of protein, carbohydrate and micronutrients, the diet was diluted to the required amount with crystalline cellulose, which is indigestible to the majority of insects (Martin et al. 1991). The diets used in each experiment, and their composition are provided in Table 2-1 and Figure 1-1 shows the location of these diets in nutritional space.

To make these diets, individual vitamins were weighed into a small weighing boat using a clean microspatula and microbalance and then added to a mortar. This mixture was ground and then stored at -20°C in a sealed container until it was needed. To make the main body of the diets, I added cellulose and casein sequentially to a large glass beaker. Equal amounts of cholesterol were then weighed into smaller glass beakers and linoleic acid was added to the cholesterol using a pipette. Where appropriate (Chapter 6) vitamin E was added at this stage. This cholesterol / linoleic acid mixture was dissolved thoroughly in chloroform and then added to the dry casein-cellulose mix. The diets were left in a fume hood and stirred regularly over the next 24 hours while the chloroform evaporated. Once all chloroform had evaporated, the appropriate amounts of Wesson salts, sucrose, dextrin, peptone, albumen and vitamin C were added. Gloves, spatulas and weighing boats were changed each time a new ingredient was added and the diets were stirred thoroughly on addition of each new ingredient. Vitamin mix was then dissolved in 20% pure ethanol and added to the powdered diets.

Diets were then mixed in a kitchen food processor for *ca.* 2 minutes before being put into a pyrex baking tray and dried in the oven at 30°C for 24 hours. The next day, diets were removed from the oven and blended briefly in the food processor and then returned to the oven. Twenty four hours later, dry diets were ground using a grain mill and then, if necessary, a mortar and pestle to produce a homogenous, fine powder. Finished diets were stored at -18°C in sealed containers until they were needed (Chapter 5) or at -80°C under inert gas (N_2) so that the vitamin E would not degrade (Chapter 6). In Chapter 6, diet was re-made once a month to mitigate vitamin E degradation.

Figure 2-1. A male decorated cricket, *Gryllodes sigillatus*, calling to attract a mate. Photo courtesy of Scott Sakaluk.

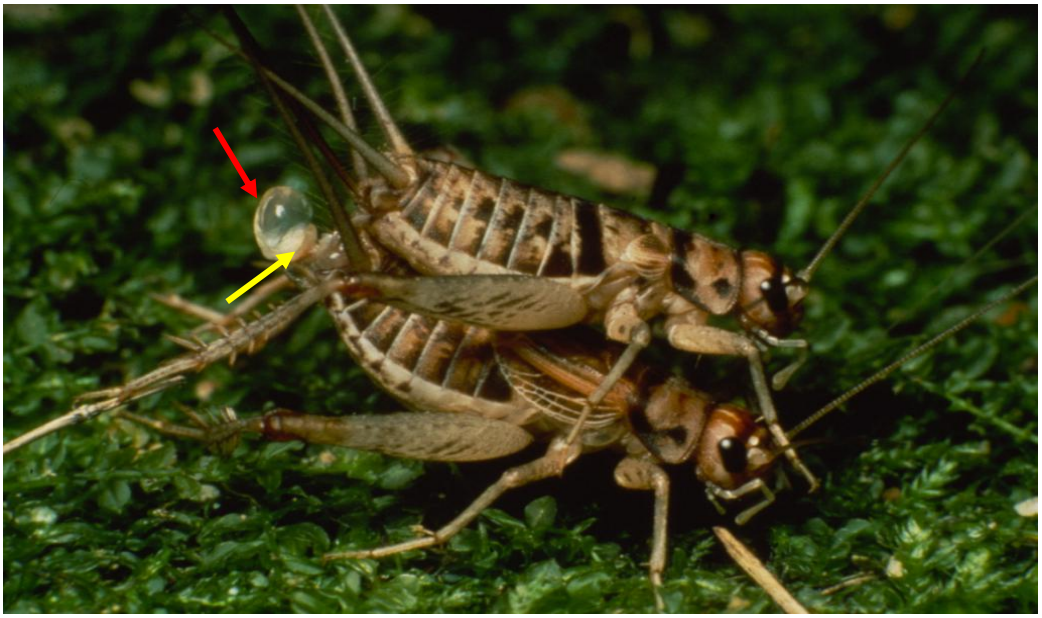

Figure 2-2. A mating pair of decorated crickets, *Gryllodes sigillatus*. During mating, males transfer a complex spermatophore to the female that consists of a sperm containing package (ampulla, yellow arrow) and a large gelatinous gift (spermatophylax, red arrow) that the female feeds on during sperm transfer. Photo courtesy of Scott Sakaluk.

Figure 2-3. A female decorated cricket, *Gryllodes sigillatus,* consuming a spermatophylax (red arrow), whilst the ampulla (yellow arrow) remains attached transferring sperm. After females consume the spermatophylax, she will then remove the ampulla and sperm transfer will stop. Photo courtesy of Scott Sakaluk.

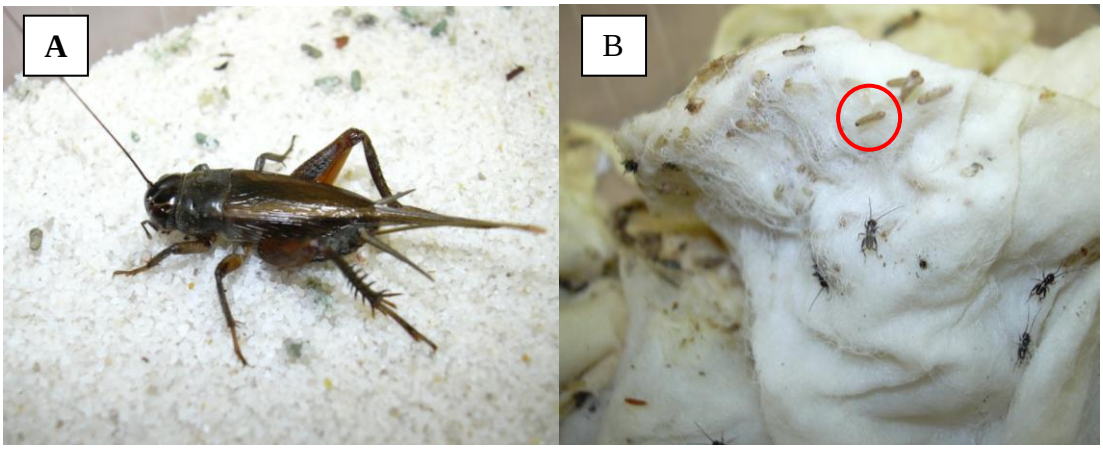

Figure 2-4. Australian field crickets, *Teleogryllus commodus* **(A)** A female black field cricket on damp cotton wool **(B)** Nymphs hatching onto damp cotton wool alongside some un-hatched eggs (circled in red).

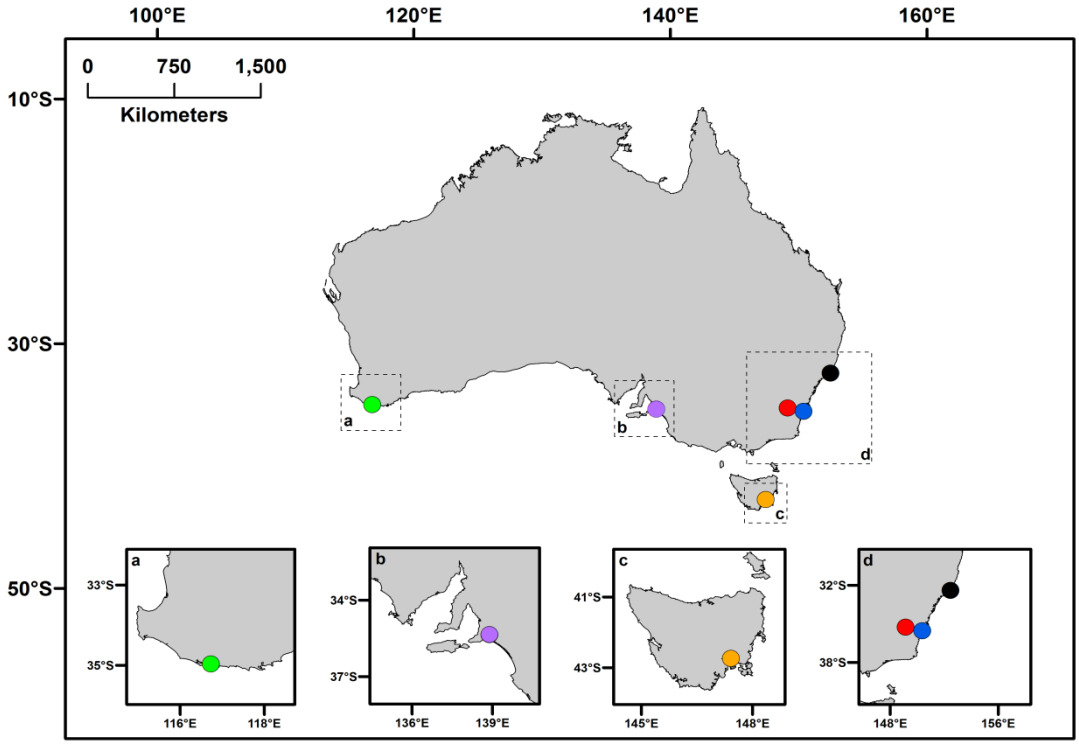

Figure 2-5. Sites where crickets were collected from across Australia. Green circle = West Australia, pink = South Australia, orange = Tasmania, blue = Kioloa, red = Australian National Territories and black = Smith's Lake (SL).

Table 2-1. Protein (P) and carbohydrate (C) composition of the 24 artificial diets used in my feeding experiments. The total nutrients present in each diet is given as the sum of the percentage protein and percentage carbohydrate, with the remaining percentage consisting of indigestible crystalline cellulose.

Protein (P)	Carbohydrate (C)	P + C	P : C	Diet Number
10	2	12	5:1	1
30	6	36	5:1	2
50	10	60	5:1	3
70	14	84	5:1	4
9	3	12	3:1	5
27	9	36	3:1	6
45	15	60	3:1	7
63	21	84	3:1	8
6	6	12	1:1	9
18	18	36	1:1	10
30	30	60	1:1	11
42	42	84	1:1	12
3	9	12	1:3	13
9	27	36	1:3	14
15	45	60	1:3	15
21	63	84	1:3	16
2	10	12	1:5	17
6	30	36	1:5	18
10	50	60	1:5	19
14	70	84	1:5	20
1.33	10.66	12	1:8	21
4	32	36	1:8	22
6.66	53.55	60	1:8	23
9.33	74.66	84	1:8	24

3. SEXUAL SELECTION AFFECTS THE EVOLUTION OF LIFESPAN AND AGIENG IN THE DECORATED CRICKET, *GRYLLODES SIGILLATUS*

3.1 ABSTRACT

Recent work suggests that sexual selection can influence the evolution of ageing and lifespan by shaping the optimal timing and relative costliness of reproductive effort in the sexes. I use inbred lines of the decorated cricket, *Gryllodes sigillatus*, to estimate the genetic (co)variance between age-dependent reproductive effort, lifespan and ageing within and between the sexes. Sexual selection theory predicts that males should die sooner and age more rapidly than females. However, a reversal of this pattern may be favoured if reproductive effort increases with age in males but not in females. I found that male calling effort increased with age, whereas female fecundity decreased, and that males lived longer and aged more slowly than females. These divergent life-history strategies were underpinned by a positive genetic correlation between early-life reproductive effort and ageing rate in both sexes, although this relationship was stronger in females. Despite these sex differences in life-history schedules, age-dependent reproductive effort, lifespan and ageing exhibited strong positive intersexual genetic correlations. This should, in theory, constrain the independent evolution of these traits in the sexes and may promote intralocus sexual conflict. My study highlights the importance of sexual selection to the evolution of sex differences in ageing and lifespan in *G. sigillatus*.

Keywords; ageing, life-history theory, quantitative genetics, sexual selection

3.2 INTRODUCTION

Senescence (or ageing) is the general decline in organismal fitness and performance with age and is an almost universal feature of multicellular organisms (reviewed in Hughes and Reynolds 2005). Evolutionary theory suggests that senescence evolves because extrinsic mortality means that few individuals reach old age in a population, which reduces the intensity of natural selection with age

(Medawar 1946; 1952; Williams 1957). This weakening of natural selection with age may result in the accumulation of alleles with deleterious effects that are only expressed late in life (*mutation accumulation,* Medawar 1952) or that enhance fitness early in life at the expense of fitness late in life (*antagonistic pleiotropy,* Williams 1957). Under both scenarios, higher extrinsic mortality should result in the evolution of accelerated senescence (Medawar 1952; Williams 1957; Hamilton 1966; Rose 1991). Whilst this prediction has received support from experimental studies (e.g. Service et al. 1988; Stearns et al. 2000) and comparative analyses (e.g. Keller and Genoud 1997; Bryant and Reznick 2004), recent theory suggests that this outcome may not be realised under all conditions (Williams et al. 2006). For example, if high extrinsic mortality selects for increased investment in somatic maintenance, it is theoretically possible for a decelerated rate of senescence to evolve (Abrams 1993; Williams and Day 2003). This appears to occur in natural populations of guppies (*Poecilia reticulata*) subject to different predation regimes (Reznick et al. 2004).

More recently, there has been a growing appreciation that sexual selection also plays an important role in the evolution of senescence (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). In most sexually-reproducing species one sex (male) produces numerous, small gametes that compete for access to smaller numbers of large gametes (female). This dichotomy in reproductive investment between the sexes means that males typically allocate more resources to competing for additional matings than females (Trivers 1972). As a result, male fitness is generally more variable than female fitness and this increases both the opportunity for and intensity of sexual selection in males (Bateman 1948; Trivers 1972). In general, female fitness is limited by the time investment and resource acquisition demands of offspring production and viability selection acting on females is expected to promote low-risk, low-wear-and-tear strategies with moderate rates of return over extended time periods (Bonduriansky et al. 2008). In contrast, sexual selection means that males are expected to pursue high-risk, “live fast, die young” life-history strategies that have the potential to yield high fitness returns over a short time frame (Vinogradov 1998; Hunt et al. 2004; Bonduriansky et al. 2008; Punzalan et al. 2008) and theoretical models show that this may be achieved by trading lifespan for enhanced mating success (Kokko 1997; 2001). In support of this prediction, male mortality is higher than female mortality

across a range of taxa (Finch 1990) and has been shown to covary with the intensity of sexual selection in both comparative studies (e.g. Promislow 1992; Clutton-Brock and Isvaran 2007) and experimental evolution (e.g. Maklakov et al. 2007). However, empirical support for this general prediction is far from universal (Williams et al. 2006; Bonduriansky et al. 2008) and this prediction may, in fact, be incorrect in species that exhibit age-dependent changes in reproductive effort (Partridge and Barton 1996; Graves 2007; Bonduriansky et al. 2008). In species where male reproductive effort increases with age (e.g. Mysterud et al. 2004), sexual selection may favour the evolution of a slower rate of ageing in males than in females if the increase in reproductive effort with age enhances male mating success (Partridge and Barton 1996; Graves 2007; Bonduriansky et al. 2008). In fact, theory suggests that an increase in male reproductive effort with age represents an evolutionary stable strategy (ESS) over a wide range of conditions and may even drive the evolution of female mating preferences for older males in the population (Kokko 1997).

Despite the potential importance of sexual selection to the evolution of lifespan and ageing, there are relatively few direct empirical studies that quantify the role of sexual selection in the evolution of lifespan and ageing (reviewed in Bonduriansky et al. 2008). One method that has been used is experimental evolution, where replicate populations are permitted to evolve with differing opportunities for sexual selection (i.e. monogamy or polygamy) (e.g. Promislow et al. 1998; Maklakov et al. 2007; 2010; Maklakov and Fricke 2009). Although this approach has provided valuable support for a role of sexual selection in the evolution of lifespan and ageing in the sexes (e.g. Promislow et al. 1998; Maklakov et al. 2007 but see Maklakov and Fricke 2009), a recent study suggests that this pattern may actually represent differential selection on life-history regimes rather than the effects of sexual selection *per se* (Maklakov et al. 2010). Furthermore, none of these studies have examined whether differences in the opportunity for sexual selection also leads to an evolutionary divergence in age-dependent reproductive effort in the sexes (Bonduriansky et al. 2008). This is, in part, due to the difficulties with accurately quantifying male reproductive effort in most species. In this regard, field crickets have proven to be an important model for ageing research as both male and female reproductive effort can be easily quantified: male reproductive effort can be

measured as the time spent calling to attract a mate (Hunt et al. 2004; 2006; Zajitschek et al. 2007; Judge et al. 2008) and female reproductive effort can be measured as the number of eggs produced (Head et al. 2005; Hunt et al. 2006; Zajitschek et al. 2007). In a number of cricket species, the number of females attracted to a calling male on a given night is positively correlated with his calling effort (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muňoz et al. 2010) and is therefore, an important determinant of male reproductive success. Moreover, as calling is metabolically costly (Kavanagh 1987), nightly calling effort also constitutes an important form of male reproductive effort. Despite this, the majority of studies examining the relationship between age-dependent reproductive effort and ageing in crickets have focussed exclusively on lifespan (Hunt et al. 2004; 2006; Zajitschek et al. 2007; Judge et al. 2008), which may not always provide an accurate measure of the rate of senescence (Pletcher 1999; Monaghan et al. 2008).

Here I examine the role that sexual selection plays in the evolution of lifespan and ageing in the decorated cricket, *Gryllodes sigillatus*. Using eight inbred lines, I first quantify differences between the sexes in lifespan, measures of ageing (Gompertz baseline mortality and rate of ageing), and how reproductive effort changes with age and use this to estimate the genetic basis of these traits in males and females. If sexual selection is important to the evolution of lifespan or ageing, I predict that these traits will be heritable and differ across the sexes, the direction of which should reflect how males and females alter their reproductive effort with age. I then estimate the genetic correlations between these traits, as well as between early and late-life reproductive effort, to test the hypothesis that the relationship between these traits differs between the sexes. Finally, I estimate the genetic correlations between male and female lifespan, measures of ageing and age-dependent reproductive effort. A positive genetic correlation for these traits across the sexes would indicate that these traits are unable to evolve independently in the sexes and may provide a signature of intralocus conflict if coupled with sex-specific differences in selection operating on these traits (Bonduriansky and Chenoweth 2009). To my knowledge, this is the first study to quantify genetic (co)variance between lifespan, ageing parameters, and age-dependent reproductive effort in both males and females and therefore provides

compelling evidence demonstrating an important role for sexual selection in the evolution of lifespan and ageing in this species.

3.3 MATERIALS AND METHODS

Inbreeding protocol and cricket maintenance

G. sigillatus used in this study descended from 500 adult crickets collected in Las Cruces, New Mexico in 2001, and used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictically (hereafter, referred to as the outbred population). Nine inbred lines were created by subjecting a random subset of crickets from this population to 23 generations of full-sib mating followed by 12 generations of panmixis within each line (Ivy and Sakaluk 2005). Of the 9 original lines, 8 were used in my experiment, referred to as lines A, B, D-I, respectively.

Crickets were housed in 15L plastic containers in an environmental chamber (Percival I-66VL) maintained at 32 ± 1ºC on a 14h:10h light/dark cycle. Crickets were provided with cat food pellets (Friskies Go-Cat Senior®) and water in 60ml glass test tubes plugged with cotton wool *ad libitum*, as well as an abundance of egg cartons to provide shelter. As soon as adults were detected, moistened cotton wool was provided in a petri-dish (10 cm diameter) as an oviposition substrate. Each inbred line was maintained in 2 containers and the outbred population in 8 containers.
Each generation, crickets were randomly mixed between containers and were maintained at a density of approximately 300 crickets per container.

Experimental design

On the day of hatching, 270 nymphs from each of the 8 inbred populations were isolated in individual plastic containers (5cm x 5cm x 5cm). Each nymph was provided a piece of cardboard egg box for shelter and water in a 2.5ml test tube plugged with cotton wool. Crickets were fed cat food pellets (Friskies GoCat Senior) and their enclosure cleaned once a week. For the first three feedings, food was provided in powder form in the lid of a 2.5ml eppendorf. For subsequent feedings, two cat food pellets were provided every week to each cricket. As soon as nymphs reached final instar they were checked daily for eclosion to adulthood to adult age. Adult survival was checked daily from eclosion until death.

I measured the reproductive effort of male and female *G. sigillatus* on days 10, 20 and 30 post-eclosion and each cricket was measured on only one of these days, being allocated to these sampling periods at random prior to adult eclosion. Table 3-1 presents the number of crickets alive in each of these three sampling periods, plus at 40 days post-eclosion. My main rationale for choosing these sampling periods was to ensure that sufficient crickets were alive at each sampling period to ensure that an accurate measure of reproductive effort could be attained. As can be seen in Table 3-1, insufficient males from one of the inbred lines (line A) were alive to assess reproductive effort at 40 days post-eclosion. This sampling regime has been used in other ageing studies on field crickets (Hunt et al. 2004; 2006; Zajitschek et al. 2007), thereby making my work directly comparable. For simplicity, I refer to these sampling periods as reproductive effort measured "early", "mid" and "late" in life, although I appreciate that this classification is not perfect for all lines or for both sexes (Table 3.1).

Prior to day 8 post eclosion, all animals were reared in isolation and were therefore virgins. On each of days 8, 18 and 28 post-eclosion, every experimental cricket was paired with a cricket of the opposite sex taken at random from the outbred population and allowed 48 hours to mate. All animals were paired, irrespective of whether their reproductive effort was assayed or not, to incorporate the effects of mating on reproductive effort and survival. Pairs were then separated and all experimental females were provided with a small petri-dish (5cm diameter) full of moist sand for oviposition. While all experimental females were given access to this substrate to oviposit across their lifetime, I measured female reproductive effort in a subset of females (see below) as the number of eggs produced in the 48 hours immediately following their allotted sampling period. The content of each petri-dish was emptied into a container of water, swirled and the eggs removed with fine forceps and counted.

In male crickets, the amount of time spent calling is a good measure of mating success because females strongly prefer males that call more in both the laboratory (Hunt et al. 2006) and field (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muňoz et al. 2010). It is also a good measure of reproductive effort because calls are metabolically expensive to produce (e.g. Kavanagh 1987). Consequently, I measured

the amount of time each male spent calling on a given night (hereafter referred to as calling effort) using a custom-built electronic monitoring device. Each male was placed in an individual recording chamber (5 x 5 x 5 cm), with a condenser microphone (c1163, Dick Smith®) embedded in the lid, on his night of sampling. Each recording chamber was, in turn, placed inside a larger foam container (15 x 15 x 15 cm) to ensure each male was acoustically isolated. Each microphone was connected via leads to a data acquisition unit (DaqBook 120, IO-Tect, Cleveland) and computer (Dell™ OptiPlex™ 580). The data acquisition unit activates a single microphone at a time, which then relays the sound level to the PC board, where it is compared to the background noise. If the received signal is ≥10 dB louder, this is recorded as a call. The microphone is then deactivated and the next one in the series is activated, with each recording chamber being sampled and recorded 10 times per second. For the current analyses, I used the number of seconds a male called each night between 18:00 to 09:00. I expect that male calling effort is under strong sexual selection, while female reproductive schedules are shaped by natural selection. When I refer to sexual selection promoting divergent strategies of reproductive effort across the sexes, this is because it is predicted to affect male, rather than female, schedules of reproductive investment over time.

The use of inbred organisms in evolutionary research can be problematic, particularly if inbreeding depression results in a large reduction in the fitness (or fitness-related traits) of individuals or if the inbred lines do not represent a true random sample of genotypes from the outbred population from which they were derived (Hoffmann and Parsons 1988). Moreover, there is the potential for inbreeding to bias quantitative genetic parameters, particularly the sign and strength of genetic correlations between traits (for discussion see Rose 1984b). Therefore, in addition to the inbred lines, I also established 270 nymphs from the outbred population and measured lifespan, rates of ageing and age-dependent reproductive as outlined above. It is important to note, however, that my aim was not to provide an explicit test of how inbreeding has influenced these traits, as this would require a vastly different experimental design, but rather to simply provide a baseline for comparison. Consequently, these outbred crickets are not included in any of my statistical analyses but are provided in my figures only for visual comparison. Importantly, trait means for

the inbred lines were within the natural range documented in the outbred population, suggesting that inbreeding *per se* has not influenced trait expression (see Results). My design, however, does not allow us to determine if inbreeding has influenced quantitative genetic estimates. Therefore, as with all studies employing inbred lines, my quantitative genetic estimates should be interpreted with a degree of caution.

With the exception of males from lines A and E, I measured the reproductive effort of 20 crickets of each sex at each sampling period (i.e. days 10, 20 and 30 post-eclosion) per line. Due to higher nymph mortality in lines A and E, and the fact that I assigned animals at random to sampling periods prior to adult eclosion, calling effort was only measured in 15 males per sampling period. My estimates of survival and ageing parameters come from a total of 1791 crickets (878 females and 913 males) and the samples sizes for each sex and line are provided in Table 3-1. Using simulation models, Pletcher (1999) provided a number of general guidelines on sample sizes needed to accurately estimate mortality parameters: (*i*) more than 50 individuals are needed to detect changes in the rate of ageing and identification of the correct mortality function, (*ii*) 75 – 100 individuals are required to detect levelling off in ageing late in life and (*iii*) 100 individuals are needed to detect a change in baseline mortality. It is important to note that these guidelines are based on the use of outbred individuals and that the statistical power of these estimates is dramatically increased when inbred individuals are used (Pletcher 1999). Based on the sample sizes presented in Table 3-1, I am therefore confident in my estimated mortality parameters.

Statistical analyses

Analysis of lifespan and survival

I examined differences in lifespan across the lines and sexes using a generalized linear mixed model (GLMM), including line as a random effect and sex as a fixed effect in the model, implemented in the "nlme" package (Pinheiro et al. 2011) in R (version R. 2.11.1; R_Development_Core_Team 2010). Lifespan could not be transformed to meet a normal distribution in either sex nor did it adhere to any other well defined statistical distribution. I therefore tested the significance of the terms in my model using a randomization test where I compared the *F* values from the above GLMM (F_{real}) to those produced when the same GLMM was executed but lifespan was shuffled at

random (without replacement) across lines and the sexes (F_{random})(Manly 2007). This shuffling procedure was iterated 10,000 times using a Monte Carlo simulation and the proportion of times (p) that F_{random} exceeded F_{real} was calculated for each term in the model. Two tailed significance values were calculated for each term in the model as $2p$ if $p < 0.5$ or as $2(1-p)$ if $p > 0.5$ (Manly 2007). All randomization tests were executed in R using a modified version of the "shuffle" function. I analysed differences in survival between lines and sexes using a Cox proportional-hazards regression, implemented using the "coxph" function of the "survival" package (Therneau and Lumley 2011) in R. Line, sex and their interaction were included as explanatory variables in this model.

Estimating and comparing rates of ageing

I used a maximum likelihood approach implemented in the "bbmle" package of R (Bolker 2009) to compare five statistical different models that describe the demographic rate of change in mortality with age: Gompertz, Gompertz-Makeham, Logistic, Logistic-Makeham and Weibull. I compared these models separately for each sex per line and the best fitting model was taken as the one with the lowest Akaike's Information Criterion (AIC). In each instance, the Gompertz model always provided the best fit to the data with a ΔAIC always exceeding 10. I therefore used the Gompertz model to compare age-specific mortality across lines and between the sexes. The Gompertz model (Gompertz 1825) is defined as:

$$\mu_x = \alpha e^{\beta x}$$

where x is the age at death and μ_x is an estimate of the mortality hazard rate. Parameter α can be interpreted as the baseline mortality rate and β as the ageing parameter that affects the exponential increase of mortality rate late in life.

I compared Gompertz models that had either sex-specific α, sex-specific β or sex-specific α and β, with models that included line-specific effects on these two parameters, respectively. To test for an ageing signature in mortality rate increase ($\beta > 0$), I constrained β to be zero in the Gompertz models fitted to each line, separately for males and females, and compared them to their unconstrained versions. For fitted Gompertz parameters, I estimated variance by randomly re-sampling 95% of the occurred ages at death in a sample and repeating this step 1000 times. For each re-

sampled subsample, I then estimated the Gompertz parameters and calculated the variance of these 1000 values. For model comparison, I used AIC values and weights (Burnham and Anderson 2002).

Age-dependent reproductive effort

I examined differences in age-dependent reproductive effort across the lines and sampling periods using a GLMM, including line as a random effect and sampling period as a fixed effect in the model, implemented in the "nlme" package in R (Pinheiro et al. 2011). Female fecundity and male calling effort were not normally distributed, so I determined the significance of terms in my models using the randomization procedure outlined above for the analysis of adult lifespan. As my fixed effect had multiple levels (day 10, 20 and 30 post-eclosion), I also used this randomization procedure to conduct post-hoc tests.

Quantitative genetic analysis

I estimated heritabilities from my inbred lines by calculating the coefficient of intraclass correlation (t) as:

$$t = \frac{nV_b - V_w}{nV_b + (n-1)V_w}$$

where n is the number of lines (in this case 8) and V_b and V_w are the between-line and within-line variance components, respectively, estimated directly from an ANOVA including line as the main effect. The standard error of the intraclass correlation (SE(t)) was calculated according to Becker (1984) as:

$$SE(t) = \sqrt{\frac{2(1-t)^2[1+(k-1)t]^2}{k(k-1)(n-1)}}$$

where k is the number of individuals sampled within each line. The heritability (h^2) of each phenotypic trait was then estimated according to Hoffmann & Parsons (1988) as:

$$h^2 = \frac{2}{\left(\frac{1}{t} - 0.5\right)}$$

The standard error of this estimate, (SE(h^2)), was calculated according to Hoffmann & Parsons (1988) as:

$$SE(h^2) = \frac{2}{\left(1 - \frac{t}{2}\right)^2} SE(t)$$

Gompertz parameters are population based estimates and therefore the above analysis based on within and between line variance cannot be used to estimate the heritability of these parameter estimates. To estimate the heritabilities of Gompertz parameter (α and β) for males and females, I generated 1000 pseudo-Gompertz parameter estimates for each inbred line (separately for each sex) by sampling at random from a normal distribution where the mean was the actual Gompertz parameter estimate for the line and the standard deviation was calculated from the standard error of this actual estimate. I therefore assume that the standard error associated with each Gompertz parameter is not only a measure of the accuracy of the estimation routine, but can also be used to infer the variance of this parameter within each line. I then used these pseudo-estimates for each line to extract the within and between line variance components for the Gompertz parameters using a linear mixed effects model implemented in R using the "lme4" package (Bates and Maechler 2009).

I estimated genetic correlations (and their standard errors) between traits using the jackknife method of Roff and Preziosi (1994). This procedure first estimates the genetic correlation between two traits using inbred line means. Next, a sequence of N (in this case 8) pseudo-values is computed by dropping, sequentially, each of the lines, estimating the resulting correlations using the formula:

$$S_{N,i} = Nr_N - (N-1)r_{N-1,i}$$

where $S_{N,i}$ is the ith pseudo-value, r_N is the genetic correlation estimated using the means of all N inbred lines, and $r_{N-1,i}$ is the genetic correlation obtained by dropping the ith inbred line alone (Roff and Preziosi 1994). The jacknife estimate of the genetic correlation (r_j) is the mean of the pseudo-values, and an estimate of the standard error (SE) is given by:

$$SE = \frac{\sum_{i=1}^{i=N} (S_{N,i} - r_j)^2}{N(N-1)}$$

Simulation models have shown that this jacknife approach provides more accurate genetic estimates than those based on conventional inbred line means when

fewer than 20 inbred lines are used in the analysis (Roff and Preziosi 1994). These estimates of genetic (co)variance from inbred lines contain variance due to dominance or epistasis and therefore should be considered broad-sense estimates (Falconer and Mackay 1996). As crickets were reared individually, I reduced the variance between lines due to common environment effects. Heritability estimates and genetic correlations were considered statistically significant if the estimates divided by their standard errors were greater than 1.96, rejecting the null hypothesis of no correlation based on a two tailed *t*-distribution with infinite degrees of freedom.

3.4 RESULTS

Lifespan and rates of ageing

There was significant variation in adult lifespan across lines (p =1.00, P < 0.0001) and also across the sexes (p = 1.00, P < 0.0001), with males living an average of 15 days longer than females (male lifespan: 58.45 ± 0.94 days, female lifespan: 43.70 ± 0.60 days). However, the magnitude of this sexual asymmetry in lifespan differed significantly across lines (line x sex: p = 1.00, P < 0.0001) being small in some lines (e.g. lines A and H) and larger in others (e.g. lines G and I) (Figure 3-1, Table 3-1). Qualitatively similar results were found when I analysed the probability of survival using a Cox proportional hazards regression (line: χ^2 = 273.12, df = 7, P = 0.0001; sex: χ^2 = 214.94, df = 1, P = 0.0001; line x sex: χ^2 = 54.22, df = 7, P = 0.0001) (Figure 3-1).

I compared ageing parameters derived from the Gompertz model and found that the best-fit model included sex-specific baseline mortality (α) and ageing rate (β) demonstrating that the sexes exhibit different patterns of ageing (Table 3-2). Indeed, females always aged faster than males (Figure 3-2). Consequently, I compared ageing parameters across lines separately for each sex (Table 3-3). For males, the best-fit model included line-specific baseline mortality and ageing rate (Table 3-3) indicating that both ageing parameters (α and β) differ across lines (Figure 3-2B). In contrast, the best-fit model for females included a line-specific ageing rate only (Table 3-3), demonstrating that females from the same lines differed in their rate of ageing but not their baseline mortality (Figure 3-2A).

Age-dependent reproductive effort

Females showed clear signs of reproductive ageing, with fecundity significantly decreasing with age (p = 1.00, P = 0.0001)(Figure 3-3). Post-hoc tests revealed that female fecundity was significantly greater at 10 days of age than at 20 (p = 0.00, P= 0.0001) or 30 (p = 0.00, P = 0.0001) days of age but that fecundity did not change between 20 and 30 days of age (p = 0.44, P = 0.87)(Figure 3-3). Although female fecundity varied significantly across lines (p = 1.00, P = 0.0001), females from the different lines showed a similar decline in reproductive effort with age (line x age: p = 0.90, P = 0.21)(Figure 3-3).

In contrast to females, male reproductive effort significantly increased with age (p = 1.00, P = 0.0001)(Figure 3-4). Post-hoc tests revealed that males spent more time calling per night at 30 days of age than at 10 (p = 0.00, P = 0.0001) and 20 (p = 0.002, P = 0.006) days of age, and more time calling at 20 than at 10 days of age, although this difference was not significant (p = 0.03, P = 0.057)(Figure 3-4). As observed in females, male nightly calling effort differed significantly across lines (p = 0.00, P = 0.0001), but the increase in reproductive effort with age did not significantly differ across lines (line x age: p = 0.93, P = 0.133)(Figure 3-4).

The genetics of lifespan, ageing and age-dependent reproductive effort

Consistent with the large differences I observed between lines, I found significant heritability estimates for lifespan, ageing parameters and age-dependent reproductive effort in both males and females (Table 3-4). In both sexes, there were consistent positive genetic correlations between reproductive effort measured at the different ages (Table 3-4). There were positive genetic correlations between lifespan and reproductive effort in males and in females but the strengths of these associations were typically much weaker in females (Table 3-4). Lifespan and baseline mortality were negatively genetically correlated in both sexes and between lifespan and the rate of ageing in males but not in females (Table 3-4).

Importantly, in both males and females there was a significant positive genetic correlation between early-life reproductive effort and the rate of ageing (Table 3-4). This genetic association was strongest in females and did not extend to mid or late-life reproductive effort in either sex (Table 3-4). Moreover, there were consistent negative

genetic correlations between baseline mortality and reproductive effort measured at the different ages for both sexes (Table 3-4). This suggests that genes promoting elevated reproductive effort early in life were associated with those causing more rapid ageing but a reduced baseline mortality.

All measures of age-dependent reproductive effort, ageing and lifespan exhibited positive genetic correlations between the sexes, although this association was not significant for early-life reproductive effort (Table 3-5). Likewise, the majority of intersexual genetic correlations between measures of age-dependent reproductive effort were positive, as were the intersexual genetic correlations between the rate of ageing and age-dependent reproductive effort (Table 3-5). In contrast, the intersexual genetic correlations between baseline mortality and measures of age-dependent reproductive effort were consistently negative (Table 3-5).

The intersexual genetic correlations involving lifespan were far less consistent in their pattern (Table 3-5). While there were consistent negative genetic correlations between baseline mortality and lifespan across the sexes, the intersexual genetic correlations between ageing rate and lifespan differed in sign and magnitude (Table 3-5). Likewise, the intersexual genetic correlations between age-dependent reproductive effort and lifespan were largely positive, although the genetic correlation between early-life reproductive effort in females and lifespan in males was negative.

3.5 DISCUSSION

Ageing is almost ubiquitous across multicellular organisms and understanding how this process evolves remains a central question in evolutionary biology (Hughes and Reynolds 2005; Williams et al. 2006; Bonduriansky et al. 2008; Monaghan et al. 2008). The role that natural selection plays in the evolution of lifespan and ageing is already well established (Medawar 1952; Williams 1957; Hamilton 1966; Rose 1991), but more recently there has been a growing appreciation that sexual selection may also play a significant role in shaping this process (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). However, surprisingly little empirical evidence currently exists on how sexual selection contributes to the evolution of lifespan and ageing in the sexes (Bonduriansky et al. 2008). Studies that have attempted to quantify the influence of sexual selection on the evolution of lifespan and ageing have used

experimental evolution (e.g. Promislow et al. 1998; Maklakov et al. 2007; 2010; Maklakov and Fricke 2009), but have not examined whether differences in the opportunity for sexual selection also leads to an evolutionary divergence in age-dependent reproductive effort in the sexes (Bonduriansky et al. 2008). This information is critical because differences in how the sexes alter their reproductive effort with age, and the effect that this has on sex-specific fitness, is predicted to determine the way that sexual selection will influence the evolution of lifespan and ageing in the sexes (Partridge and Barton 1996; Graves 2007; Bonduriansky et al. 2008).

Here I show that sexual selection has facilitated divergence in the way that male and female decorated crickets (*Gryllodes sigillatus*) alter their reproductive effort across their lifetime; while males increase their reproductive effort with age, females show a decline in fecundity over time. These contrasting schedules of reproductive effort were associated with differences in lifespan and ageing between the sexes, with males living longer and ageing more slowly than females. As my measures of reproductive effort are important determinants of fitness in this species, these findings suggest that the selective value of lifespan and ageing differ considerably for the sexes. Mediating this sexual divergence, I found a positive genetic correlation between early-life reproductive effort and ageing rate in both sexes indicative of antagonistic pleiotropy, although this relationship was much stronger in females than in males. Despite clear sex differences in lifespan, ageing and age-dependent reproductive effort, I found strong positive genetic correlations for these traits across the sexes suggesting that these traits are unlikely to evolve independently in males and females. My results therefore highlight a clear and important role for sexual selection in the evolution of lifespan and ageing in the sexes of *G. sigillatus*.

Classic sexual selection theory predicts that males should maximise reproductive success by investing in sexual advertisement at the expense of lifespan (Kokko 1997; 1998; Kokko et al. 2002; Getty 1998), whereas females should adopt a low risk strategy allowing time to accrue the resources needed to meet the relatively higher demands of reproduction (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). This general prediction has received considerable empirical support, with males having higher mortality rates than females across a range of taxa (Comfort 1979; Finch

1990; Promislow and Harvey 1990), as well as a positive relationship between the intensity of sexual selection and male-biased mortality being documented across a variety of vertebrate species (Promislow 1992; Clutton-Brock and Isvaran 2007). However, this support is far from conclusive with many counter-examples existing where mortality rates are female biased (Promislow and Haselkorn 2002; Graves 2007; Reed et al. 2008) and in many species, males with more elaborate sexual traits have a longer lifespan (Jennions et al. 2001). Even within a single species, results can be conflicting. For example, in the seed beetle, *Callosobruchus maculatus*, males have a shorter lifespan and age faster than females (Fox et al. 2003; 2006; Maklakov et al. 2007) yet a number of natural evolution experiments have shown only a weak effect of sexual selection on the evolution of male and female lifespan and ageing (Maklakov et al. 2007; 2010; Maklakov and Fricke 2009).

One reason for these inconsistencies across empirical studies may be the lack of information on both male and female age-dependent reproductive effort for most study species. Theory suggests that how the sexes alter their reproductive effort with age, and the effect this has on fitness, is critical in determining the effect of sexual selection on the evolution of lifespan and ageing (Partridge and Barton 1996; Graves 2007; Bonduriansky et al. 2008). Indeed, sexual selection may promote the evolution of slower ageing in males than in females, if reproductive success increases with age in males to a greater extent than it does in females (Partridge and Barton 1996; Graves 2007; Bonduriansky et al. 2008). In agreement with this prediction, I found that male *G. sigillatus* live longer and age more slowly than females and that this pattern is associated with differences in how the sexes invest in reproductive effort across their lifetime. Calling effort is an important determinant of mating success in field crickets (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muňoz et al. 2010) and I found that males increased this form of reproductive effort with age. If this increase in late-life reproductive effort enhances male reproductive success in *G. sigillatus*, the strength of sexual selection on males will increase as they age and this will, in theory, select against genes that have negative pleiotropic effects late in life and the accumulation of deleterious mutations (Graves 2007; Bonduriansky et al. 2008) and promote enhanced somatic maintenance (Kirkwood 1977; Kokko 1997). In contrast, females exhibited clear reproductive senescence. This is expected to reduce the strength of sexual

selection with age and enable genes with negative pleiotropic effects acting late in life and the accumulation of deleterious mutations to persist in the population (Graves 2007; Bonduriansky et al. 2008). This pattern of reproductive senescence is also expected to select against somatic maintenance in females, as there is little benefit to investing in somatic maintenance for a future that is unlikely to be reached (Kirkwood 1977). More work is needed, however, to determine how these evolutionary processes contribute to the evolution of sex-specific ageing in *G. sigillatus*, although it is unlikely that these alternatives (mutation accumulation and antagonistic pleiotropy) will be mutually exclusive (Williams et al. 2006).

Life-history theory, and particularly evolutionary theories of ageing, predicts that current reproductive effort will trade-off against future reproduction and lifespan (Roff 1992). I predicted that these trade-offs would be manifested as negative genetic correlations between early and late-life reproductive effort or between early-life reproductive effort and longevity. Contrary to this prediction, I found no apparent trade-offs between reproductive effort measured at different ages or between age-dependent reproductive effort and lifespan, with the genetic correlations between these traits being largely positive within the sexes. I did, however, find a positive genetic correlation between early-life reproductive effort and the rate of ageing in both sexes providing support for the antagonistic pleiotropy theory of ageing (Williams 1957; Rose 1991) . This trade-off was particularly strong in females, which is consistent with their shorter relative lifespan and decline in reproductive effort with age. In general, trade-offs between age-dependent reproductive effort and lifespan (Rose and Charlesworth 1981; Rose 1984a) or rates of ageing (Tatar et al. 1993; Maklakov et al. 2010) appear well supported in females but fewer studies have successfully explored this relationship in males due to difficulties in quantifying reproductive effort in most species (Hunt et al. 2006; Zajitschek et al. 2007). For this reason, crickets have become a valuable model for ageing research because of the relative ease with which both male and female age-dependent reproductive effort can be measured. For example, in the Australian black field cricket (*Teleogryllus commodus*) a regime of divergent artificial selection on male lifespan resulted in a correlated response in age-dependent reproductive effort (Hunt et al. 2006). Males from lines selected for a shorter lifespan began calling earlier in adulthood and called more in total than males from lines

selected for a longer lifespan (Hunt et al. 2006), suggesting that antagonistic pleiotropy has played an important role in the evolution of male lifespan in this species. A half-sib quantitative genetic study on this same species, however, failed to detect a genetic correlation between early-life reproductive effort and lifespan in either sex but did find a negative genetic correlation between average fecundity and lifespan in females (Zajitschek et al. 2007). Importantly, both of these studies were based on lifespan, which may not always provide an accurate measure of senescence (Pletcher 1999; Monaghan et al. 2008) because high intrinsic frailty may reduce longevity without accelerating the rate of ageing. Indeed, I found little evidence for a genetic association between early-life reproductive effort and lifespan in either sex of *G. sigillatus*. My results therefore illustrate the importance of examining both lifespan and ageing parameters when attempting to understand the evolution of senescence (Pletcher 1999; Bonduriansky et al. 2008; Monaghan et al. 2008).

Intralocus sexual conflict occurs whenever selection on shared phenotypic traits in one sex displaces the other sex from its phenotypic optima, because many traits shared by the sexes have a common genetic basis but are subject to contrasting selection that prevents the sexes from evolving independently to their own phenotypic optima (Bonduriansky and Chenoweth 2009). Consequently, when intralocus sexual conflict is operating in a population, traits shared by the sexes will be characterised by strong and positive intersexual genetic correlations, as well as selection gradients that are different in sign and strength across the sexes (Bonduriansky and Chenoweth 2009). Recently, it has been proposed that sex differences in the optimal timing or relative costliness of reproductive effort may mediate intralocus sexual conflict over lifespan and rates of ageing (Zajitschek et al. 2007; Bonduriansky et al. 2008). Consistent with this view, I found that the trade-off between early-life reproductive effort and the rate of ageing was considerably stronger in females than in males. Moreover, I found a clear difference in the way that the sexes alter their reproductive effort with age that suggests males have more to gain by living longer and ageing more slowly than females. Despite this sexual divergence, I found positive genetic correlations between measures of age-dependent reproductive effort, lifespan and ageing parameters across the sexes suggesting that these traits are not free to evolve independently in the sexes. Together these findings suggest that intralocus sexual

conflict has the potential to play an important role in the evolution of sex differences in lifespan and ageing in *G. sigillatus*. However, confirmation that intralocus sexual conflict is indeed operating, as well as a direct estimate of its strength, requires formal estimation of the sex-specific fitness surfaces for age-dependent reproductive effort, lifespan and ageing (Lewis et al. 2011). While this is likely to prove difficult, Charmantier *et al.* (2006) showed in a natural population of mute swans (*Cygnus olor*) that sexual selection acting on the age at first and last reproduction is opposing in the sexes and that there was a positive genetic correlation between these two important life-history traits. Unfortunately, this study did not estimate the intersexual genetic correlations for these traits so that the importance of intralocus sexual conflict to the evolution of senescence could not be directly estimated (Charmantier et al. 2006). Considerably more work is needed, however, before the operation of intralocus sexual conflict can be confirmed in *G. sigillatus* and the role, if any, that it may play in the evolution of lifespan and ageing in this species.

In conclusion, my results suggest that sexual selection plays an important role in the evolution of sex differences in lifespan and ageing in *G. sigillatus* by promoting divergence in investment in reproductive effort with age by males and females and therefore the fitness consequences associated with lifespan and ageing. This sexual divergence was coupled with strong positive intersexual genetic correlations between lifespan and measures of ageing, raising the potential for intralocus sexual conflict over the evolution of sex differences in lifespan and ageing in this species. However, more research is needed to provide a definitive role for intralocus sexual conflict in this species. My work highlights the need to consider these important, yet largely ignored, mechanisms when studying the evolution of lifespan and ageing (Graves 2007; Bonduriansky et al. 2008).

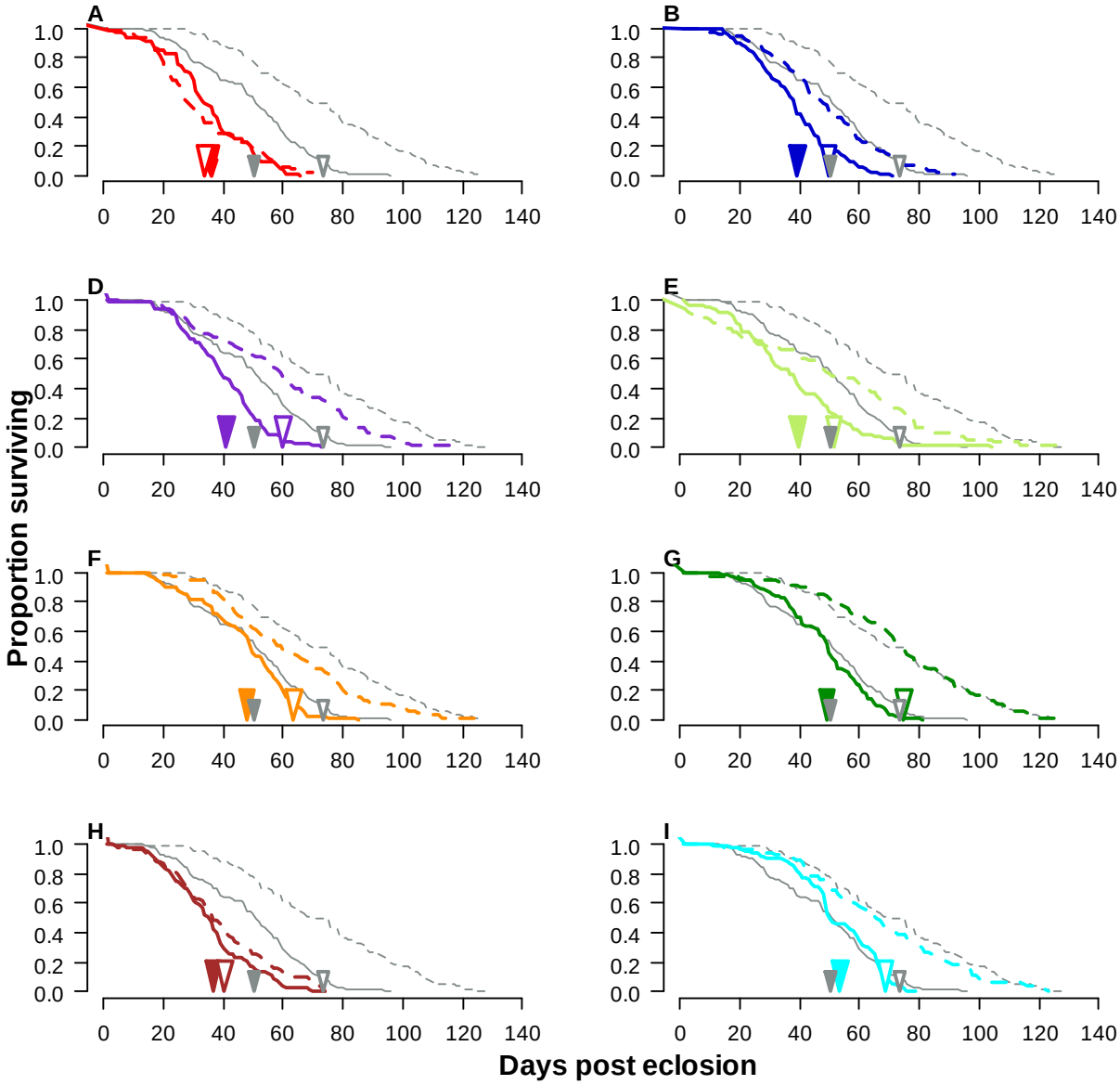

Figure 3-1. Adult survival curves for male (dashed lines) and female (solid lines) *Gryllodes sigillatus* from each of our eight inbred lines (coloured lines) and the outbred population (grey lines). Figures A-I each represents a different inbred line (lines A, B, D-I, respectively). In each panel, the arrow heads represent mean lifespan for males (open symbols) and females (closed symbols) for each inbred line (coloured symbols) and the outbred population (grey symbols).

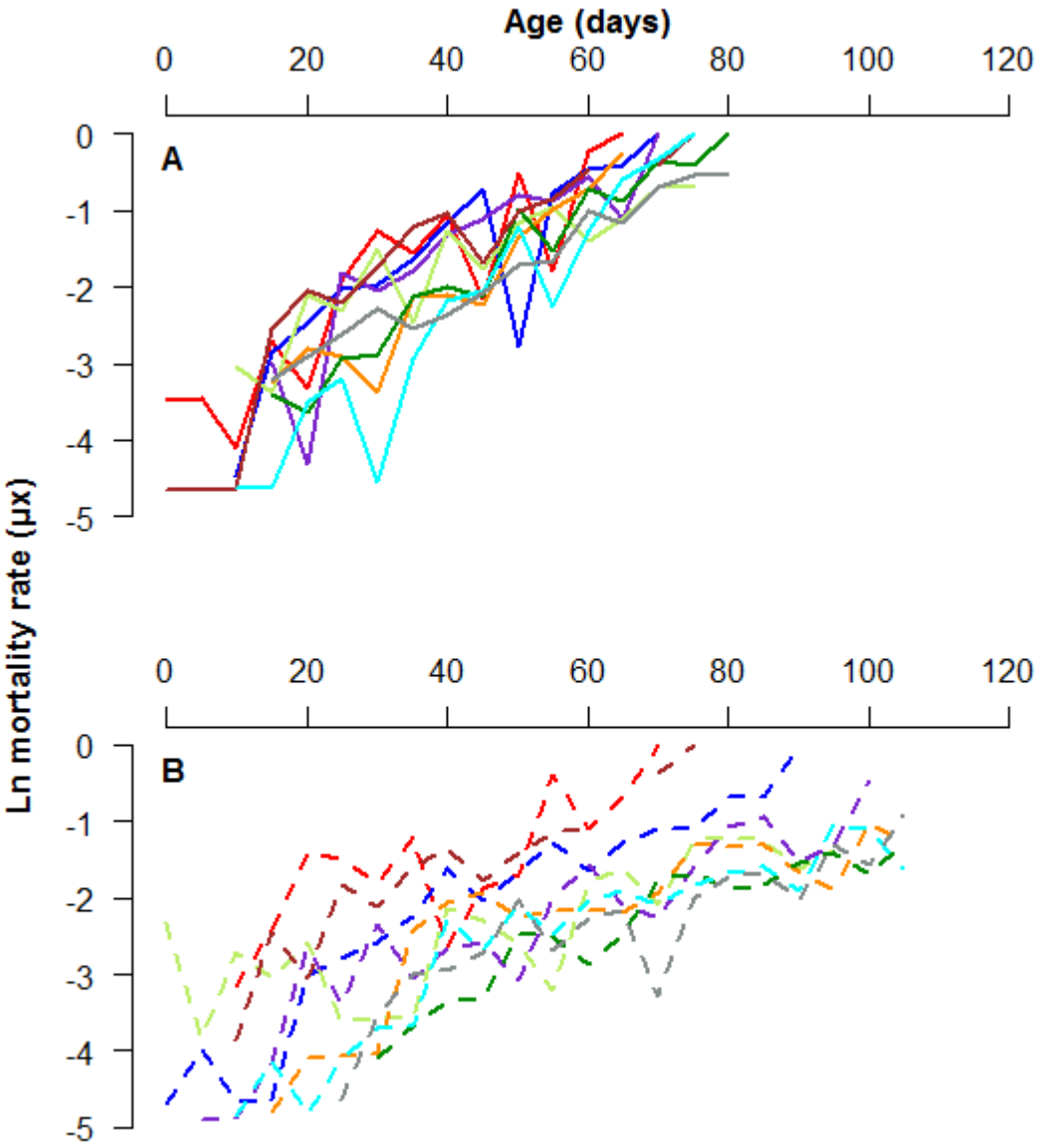

Figure 3-2. Age-dependent mortality rates in (A) female (solid lines) and (B) male (dashed) *Gryllodes sigillatus* from each of my eight inbred lines (coloured lines) and the outbred population (grey lines). Line colours are as presented in Figure 3-1.

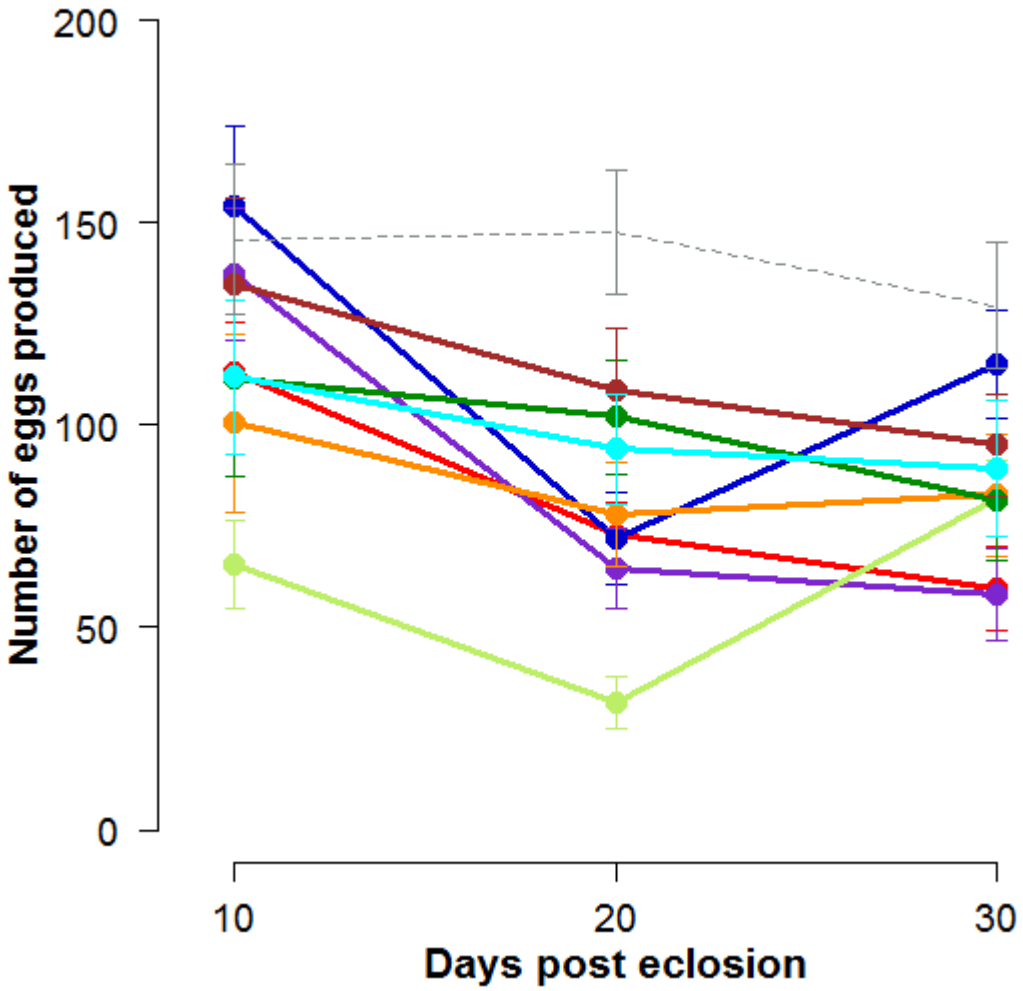

Figure 3-3. Age dependent reproductive effort in female inbred lines (coloured lines) and the outbred population (grey line). I measured female reproductive effort as the number of eggs produced (fecundity) in a 48 hour period commencing on days 10, 20 and 30 post-eclosion to adulthood. Line colours are as presented in Figure 3-1.

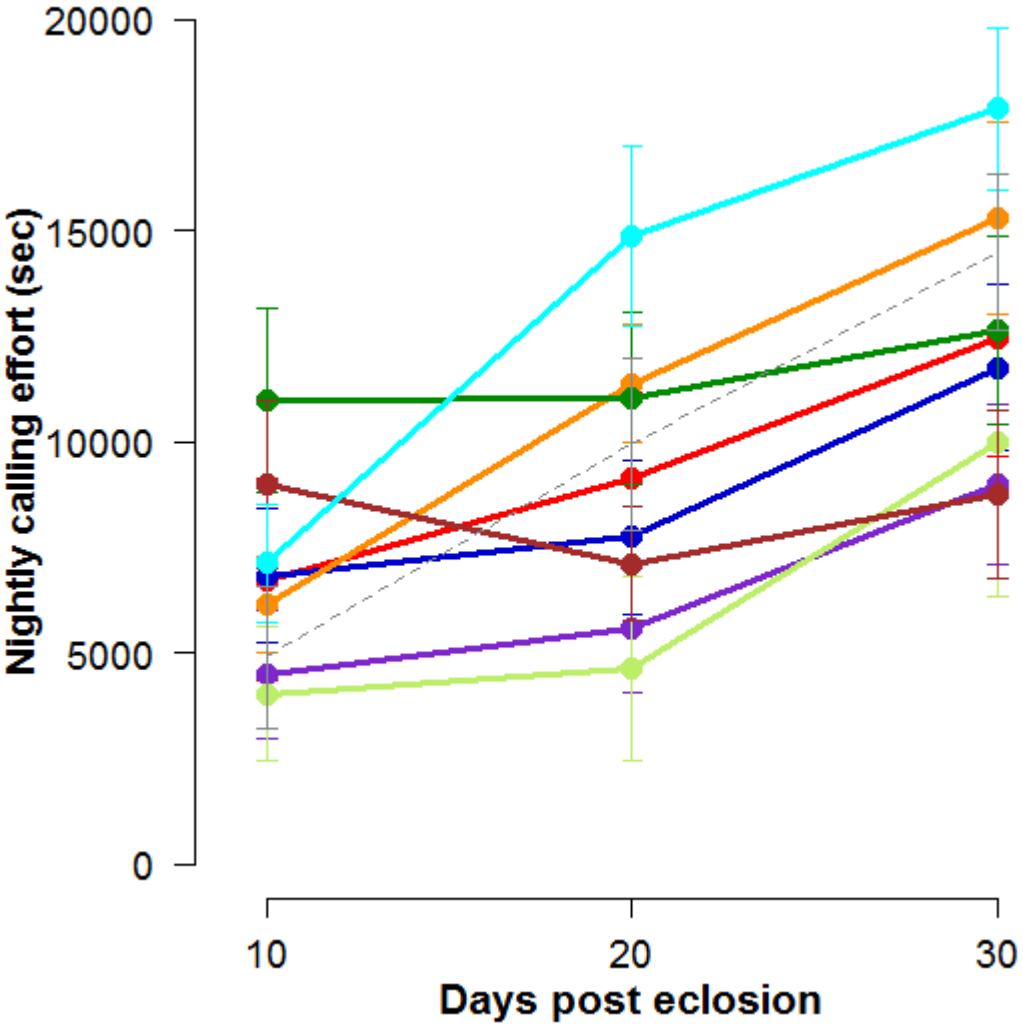

Figure 3-4. Age-dependent reproductive effort (±SE) of male *Gryllodes sigillatus* from each of the eight inbred lines (coloured, solid lines) and the outbred population (grey, dashed lines). I measured male reproductive effort as time spent calling each night on either day 10, 20 and 30 post-eclosion to adulthood. Line colours are as presented in Figure 3-1.

Table 3-1 Mean (±SE) and median lifespan (LS) for male and female *G. sigillatus* for each of the inbred lines and the outbred population from which they were derived.

Notes - The number of individuals alive at days 10, 20, 30 and 40 post-eclosion are also provided to justify the age at which I assayed reproductive effort in the sexes, along with the total number of crickets (n) that were used to estimate ageing parameters.

			Reproductive Effort				
Line	**Mean LS ± SE**	**Median LS**	**Day 10**	**Day 20**	**Day 30**	**Day 40**	***N***
Males							
A	34.06 ± 2.37	29.5	46	39	21	14	48
B	49.75 ± 1.83	49.5	105	102	91	75	108
D	60.04 ± 2.19	61.0	132	128	110	98	133
E	51.55 ± 4.55	52.0	45	40	35	34	51
F	63.60 ± 2.16	60.5	120	118	114	101	120
G	74.87 ± 2.36	75.0	122	121	118	114	125
H	40.44 ± 1.78	38.0	93	84	64	44	96
I	68.73 ± 2.27	67.0	127	124	120	114	127
Outbred	73.62 ± 2.61	71.0	105	104	102	93	105
Females							
A	35.88 ± 1.83	35.0	61	55	45	21	65
B	39.31 ± 1.43	40.0	88	79	62	37	88
D	41.03 ± 1.50	41.0	79	75	59	41	80
E	39.48 ± 2.40	39.0	61	54	42	28	64
F	48.06 ± 1.55	50.0	102	96	84	71	103
G	49.18 ± 1.35	51.0	119	114	104	83	119
H	36.81 ± 1.40	37.0	103	91	70	35	105
I	53.47 ± 1.45	52.0	103	100	93	84	103
Outbred	50.42 ± 1.50	52.0	151	141	120	98	151

Table 3-2. Summary of models used to test for sex and line-specific ageing in *Gryllodes sigillatus,* ordered with the best-fit model at the top. Models include the following Gompertz parameters: baseline mortality (α) and ageing rate (β). Subscripts *m* and *f* refer to sex-specific variables for males and females, respectively, and the subscripts *a*, *b*, *d-i* refer to the 8 inbred lines used in my experiment. The number of parameters fitted in each model is given as *n*, Δ_i is the difference in AIC between model *i* and the model with the lowest AIC value (model A) and Akaike weights are given as w_i (Burnham and Anderson 2002).

Model Name	**Parameters**		***N***	**Δ_i**	**w_i**
(A) Sex-specific baseline mortality and ageing rate	α_m α_f	β_m β_f	4	0.00	0.839
(B) Sex-specific ageing rate	α	β_m β_f	3	3.35	0.161
(C) Line-specific baseline mortality and ageing rate	α_a α_b α_d α_e α_f α_g α_h α_i	β_a β_b β_d β_e β_f β_g β_h β_i	16	97.90	<0.001
(D) Line-specific ageing rate	α	β_a β_b β_d β_e β_f β_g β_h β_i	9	119.31	<0.001
(E) Sex-specific baseline mortality	α_m α_f	β	3	143.28	<0.001
(F) Line-specific baseline mortality	α_a α_b α_d α_e α_f α_g α_h α_i	β	9	151.97	<0.001
(G) Baseline mortality and ageing rate	α	β	2	416.23	<0.001

Table 3-3. Summary of models used to test for line-specific ageing in male and female *Gryllodes sigillatus*, ordered with the best-fit model at the top for each sex. Abbreviations are as in Table 3-2.

Model Name	Parameters		*N*	*Δi*	*wi*
Males					
(A) Line-specific baseline mortality and ageing rate	$\alpha_a\ \alpha_b\ \alpha_d\ \alpha_e\ \alpha_f\ \alpha_g\ \alpha_h\ \alpha_i$	$\beta_a\ \beta_b\ \beta_d\ \beta_e\ \beta_f\ \beta_g\ \beta_h\ \beta_i$	16	0.00	1
(B) Line-specific baseline mortality	$\alpha_a\ \alpha_b\ \alpha_d\ \alpha_e\ \alpha_f\ \alpha_g\ \alpha_h\ \alpha_i$	β	9	14.61	<0.001
(C) Line-specific ageing rate	α	$\beta_a\ \beta_b\ \beta_d\ \beta_e\ \beta_f\ \beta_g\ \beta_h\ \beta_i$	9	22.10	<0.001
(D) Baseline mortality and ageing rate	α	β	2	203.51	<0.001
Females					
(A) Line-specific ageing rate	α	$\beta_a\ \beta_b\ \beta_d\ \beta_e\ \beta_f\ \beta_g\ \beta_h\ \beta_i$	9	0.00	1
(B) Line-specific baseline mortality	$\alpha_a\ \alpha_b\ \alpha_d\ \alpha_e\ \alpha_f\ \alpha_g\ \alpha_h\ \alpha_i$	β	9	22.72	<0.001
(C) Line-specific baseline mortality and ageing rate	$\alpha_a\ \alpha_b\ \alpha_d\ \alpha_e\ \alpha_f\ \alpha_g\ \alpha_h\ \alpha_i$	$\beta_a\ \beta_b\ \beta_d\ \beta_e\ \beta_f\ \beta_g\ \beta_h\ \beta_i$	16	39.03	<0.001
(D) Baseline mortality and ageing rate	α	β	2	74.52	<0.001

Table 3-4. Heritabilities (along diagonal) and genetic correlations (below diagonal) for lifespan, Gompertz ageing parameters and age-dependent reproductive effort in male and female *Gryllodes sigillatus.*

	LS	α	β	ERE	MRE	LRE
Males						
LS	**0.97 ± 0.02**					
α	**-0.79 ± 0.03**	**0.99 ± 0.01**				
β	**-0.42 ± 0.10**	-0.26 ± 0.40	**0.99 ± 0.01**			
ERE	0.35 ± 0.24	**-0.44 ± 0.10**	**0.44 ± 0.17**	**0.36 ± 0.14**		
MRE	**0.51 ± 0.04**	**-0.53 ± 0.07**	0.21 ± 0.27	**0.45 ± 0.06**	**0.70 ± 0.12**	
LRE	**0.49 ± 0.05**	**-0.40 ± 0.05**	-0.06 ± 0.14	**0.15 ± 0.07**	**0.95 ± 0.00**	**0.52 ± 0.14**
Females						
LS	**0.94 ± 0.03**					
α	**-0.40 ± 0.03**	**0.98 ± 0.01**				
β	0.09 ± 0.15	**-1.16 ± 0.23**	**0.99 ± 0.00**			
ERE	-0.11 ± 0.11	**-0.98 ± 0.45**	**0.97 ± 0.06**	**0.48 ± 0.14**		
MRE	**0.32 ± 0.09**	**-0.99 ± 0.46**	1.02 ± 0.88	**0.77 ± 0.35**	**0.76 ± 0.10**	
LRE	0.09 ± 0.11	-0.02 ± 0.04	-0.00 ± 0.16	0.34 ± 0.21	**0.17 ± 0.05**	**0.46 ± 0.14**

Notes - LS refers to adult lifespan, α to the Gompterz α parameter that describes baseline mortality, β to the Gompertz β parameter that describes the rate of ageing, ERE to early-life reproductive effort (measured 10 days post-eclosion), MRE to mid-life reproductive effort (measured 20 days post-eclosion) and LRE to late-life reproductive effort (measured 30 days post-eclosion). Values given in bold are significant at $P < 0.05$.

Table 3-5. Intersexual genetic correlations (±SE) for lifespan, Gompertz ageing parameters and age-dependent reproductive effort in *Gryllodes sigillatus*. Abbreviations are as in Table 3-4 and values in bold are significant at $P < 0.05$.

♂ \ ♀	LS	α	B	ERE	MRE	LRE
LS	**0.88 ± 0.00**	**-0.33 ± 0.09**	0.08 ± 0.04	**-0.10 ± 0.05**	0.19 ± 0.11	0.08 ± 0.17
α	**-0.71 ± 0.01**	**0.76 ± 0.02**	**-0.66 ± 0.24**	**-0.57 ± 0.14**	**-0.62 ± 0.12**	**-0.37 ± 0.10**
β	**-0.28 ± 0.14**	-0.95 ± 0.93	**0.94 ± 0.23**	**0.92 ± 0.38**	**0.81 ± 0.22**	**0.30 ± 0.13**
ERE	**0.31 ± 0.09**	**-0.61 ± 0.21**	0.51 ± 0.38	0.35 ± 0.21	**0.82 ± 0.00**	**0.24 ± 0.07**
MRE	**0.85 ± 0.03**	**-0.63 ± 0.07**	0.43 ± 0.59	0.18 ± 0.34	**0.62 ± 0.08**	0.13 ± 0.07
LRE	**0.85 ± 0.03**	**-0.39 ± 0.03**	0.13 ± 0.27	-0.07 ± 0.16	**0.28 ± 0.13**	**0.15 ± 0.07**

4. OXIDATIVE STRESS AND THE EVOLUTION OF SEX DIFFERENCES IN LIFESPAN AND AGEING IN THE DECORATED CRICKET, *GRYLLODES SIGILLATUS*.

4.1 ABSTRACT

The free radical theory of ageing predicts that oxidative stress, which occurs when cellular levels of Reactive Oxygen Species exceed the capacity of circulating antioxidant defences, directly results in ageing. While this theory offers one of the best supported mechanisms for causing ageing, it has been the subject of criticism because many of its central predictions have received equivocal and/or contradictory support. Instead, recent reviews have suggested that oxidative stress may mediate life-history trade-offs, such as that observed between early reproductive effort, lifespan and rate of ageing, that are key to evolutionary theories of ageing. Here, I use eight inbred lines of the decorated cricket, *Gryllodes sigillatus*, to estimate the genetic (co)variance between age-dependent reproductive effort, lifespan, measures of ageing, as well as oxidative damage to proteins and total antioxidant capacity within and between the sexes. The free radical theory of ageing predicts that oxidative damage should accumulate with age and negatively correlate with lifespan. I found that protein oxidation was greater in the shorter lived sex (females) and was negatively genetically correlated with longevity in both sexes. This negative genetic correlation with lifespan was due to oxidative damage increasing baseline mortality in both sexes and, in female but not males, accelerating the rate of ageing. However, in contrast to predictions of the free radical theory of ageing, oxidative damage did not accumulate with age which may, in males, be because of an increase in total antioxidant capacity with age. In Chapter 3, I found a trade-off between early reproductive effort and ageing rate in both sexes, although this trade-off was more pronounced in females than males. In the current study, I found that elevated female fecundity early in life was associated with greater protein oxidation later in life, which was in turn positively correlated with the rate of ageing. My results therefore provide mixed support for the free radical theory but do suggest that oxidative stress may be mediate sex-specific life-history strategies in *G. sigillatus*.

Keywords; ageing, age-dependent reproductive effort, lifespan, protein oxidation, reactive oxygen species, sexual selection, total antioxidant capacity.

4.2 INTRODUCTION

The concept of constraint driven by resource limitation forms the cornerstone of life-history theory (Roff 1992; Stearns 1992). Recently though it has been recognised that resource-based models may oversimplify the proximate basis of life-history constraints (Barnes and Partridge 2003) and poorly explain the individual variation observed in life-history strategies within and between the sexes (Isaksson et al. 2011). Therefore, a more complete understanding of the evolution of life-history trade-offs requires detailed knowledge of their mechanistic basis (Zera and Harshman 2001; Barnes and Partridge 2003, Nijhout et al. 2003; Lessells 2008). Recently, it has been argued that the direct (i.e. oxidative damage) and indirect (i.e. resources required for protection) costs of oxidative stress may represent a general mechanism that is capable of mediating a range of important life-history trade-offs (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011).

Oxidative stress occurs when the production of Reactive Oxygen Species (ROS), which are produced as by-products of oxidation-reduction (or REDOX) reactions, exceeds cellular antioxidant defences. While, at low levels, ROS are vital to normal physiological functioning (Dickinson and Chang 2011; D'Autréaux and Toledano 2007) under oxidative stress ROS may oxidise biomolecules within the cell (e.g. DNA, RNA, proteins and lipids) and, therefore, have a detrimental effect on organismal performance (Finkel and Holbrook 2000; Lin and Beal 2006). Consequently, managing cellular redox state (i.e. the balance between pro- and anti- oxidants) represents an intricate balance between meeting the functional demands for ROS and preventing and/or repairing any oxidative damage that occurs under oxidative stress (Dowling and Simmons 2009; Monaghan et al. 2009). High levels of ROS will not necessarily result in oxidative stress if this can be balanced by the upregulation of antioxidant defences (Monaghan et al. 2009). Likewise, relatively high levels of antioxidants will not automatically place an individual in a better redox state, as this will depend on the level of ROS that these defences have to neutralize (Monaghan et al. 2009). An

understanding of oxidative stress therefore requires that both sides of this balance are taken into consideration, although this approach is rarely taken in most empirical studies (Monaghan et al. 2009). This is particularly important since this balance is likely to be very dynamic across an organisms lifetime, varying with developmental stage (Blount et al. 2003) and levels of activity (Costantini et al. 2008), as well as with exposure to stressors (Mitler 2002), pathogens (Costantini and Möller 2009) and the quantity of antioxidants in the diet (Cohen et al. 2009). As ROS are produced as an inevitable consequence of aerobic metabolism, managing oxidative stress is likely to be a major determinant of an organism's life-history (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011) that should, in theory, be traded against other important requirements (McNamara and Buchanan 2005; Yearsley et al. 2005). Yet, despite recent calls to do so (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011), few studies have directly tested the role that oxidative stress plays in mediating important life-history trade-offs.

Ageing is an important component of the life-history of most organisms and oxidative stress may play a central role in its evolution (Harman 1956; 1992). Ageing is the progressive, endogenous decline in the physiological and homeostatic function of an organism (Finch 1990) that leads to an increased risk of death with age (Rose 1991; Charlesworth 1994). Evolutionary theories of ageing rely on the fact that the strength of natural selection declines with age (Medawar 1946, 1952; Williams 1957). Weak natural selection late in life allows the accumulation of alleles with negative effects on fitness late in life (*mutation accumulation*, Medawar 1952) and those that enhance early life fitness at the expense of fitness later in life (*antagonistic pleiotropy*, Williams 1957). Whilst there is a well-developed body of evolutionary theory explaining why we age (reviewed by Hughes and Reynolds 2005), our understanding of the mechanistic basis of ageing is far from complete (Partridge and Gems 2006). The free radical theory of ageing (Harman 1956) was the first model to make a direct conceptual link between the production of ROS and the process of ageing. This theory posits that it is the accumulation of oxidative damage, under prolonged or chronic oxidative stress, that causes the functional decline characteristic of ageing (Harman 1956) and that differences in lifespan may be explained by different rates of ROS production,

antioxidant defence or repair of oxidative damage (Harman 1992). While there is substantial correlative evidence to support this theory (reviewed by Beckman and Ames 1998; Sohal et al. 2002), experimental manipulation of antioxidant capacity has repeatedly failed to show a clear effect on lifespan, leading many to question the general role of oxidative stress in mediating ageing (Pérez et al. 2009; Jang and Remmen 2009; Speakman and Selman 2011).

Any link between oxidative stress and lifespan is unlikely to be simple (Speakman and Selman 2011) and how animals manage their oxidative stress, may be affected by their investment in reproductive effort (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011). The trade-off between reproductive effort early in life and lifespan, is central to evolutionary theories of ageing (Williams 1957) and differences in the sign and/or strength of this relationship is likely to change how individuals manage their oxidative stress (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011). If antioxidants or their precursors are used in reproductive effort, for example, as has been shown for elaborate sexual displays (Blount et al. 2003; Pike et al. 2007) and elevated levels of parental care (Wiersma et al. 2004; Christie et al. 2012; Losdat et al. 2011), a trade-off may occur between investment in antioxidant defences and reproductive effort. This may result in a decreased lifespan or more rapid ageing if antioxidants are allocated to reproductive effort at the expense of somatic maintenance (Monaghan et al. 2009). Indeed, theory suggests that this may be favoured if an increase in reproductive effort improves reproductive success more than a longer life does (Kokko 1997). Oxidative stress could also mediate a trade-off between reproductive effort and lifespan if an elevation in reproductive effort requires an increase in metabolic rate (e.g. Kavanagh 1987; Ernsting and Isaaks 1991) resulting in an increase in the rate of ROS leakage from the electron transport chain in the mitochondria. This may push cells into oxidative stress, leading to accelerated ageing and/or a reduction in lifespan. However, while metabolic rate negatively correlates with lifespan in a number of species (e.g. Okada et al. 2011; Seyahooei et al. 2011), evidence for a direct link between an increased metabolic rate and a greater generation of ROS is far from unanimous (Barja 2007; Hulbert et al. 2007; Galtier et al. 2009).

If oxidative stress does mediate the trade-off between reproductive effort and lifespan, then redox states should differ across the sexes because sexual selection affects optimal life-history strategies across the sexes (Bonduriansky et al. 2008). Sexual selection is expected to alter the trade-off between reproductive effort and lifespan differentially in the sexes because females typically invest more heavily in reproduction than males (Trivers 1972). Females are expected to pursue a reproductive strategy with moderate rates of return over a prolonged time period because their reproductive success relies on having time to acquire resources and provision offspring (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). In contrast, male reproductive success depends on the number of matings that can be achieved, and so males are expected to trade current mating success against a long life (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). This pattern means that males should, on average, age faster and live shorter lives than females (Promislow 2003; Graves 2007; Bonduriansky et al. 2008) and this has been shown in a range of taxa (Comfort 1979; Finch 1990; Promislow and Harvey 1990 Promislow 2002). However, if males increase their reproductive effort over time (Mysterud et al. 2004) and this leads to an increase in reproductive success, sexual selection will promote the evolution of slower ageing and longer lifespans in males. Therefore, if the costs of oxidative stress mediate the trade-off between reproductive effort and lifespan, sexual selection and its effects on age-dependent reproductive effort should be expected to generate differences in the balance between ROS production versus antioxidant defence in the sexes. To my knowledge, however, the role of sexual selection in affecting oxidative stress across the sexes has yet to be tested empirically in both males and females, although a number of studies have shown that the sexes do not respond equally to a perturbation in either ROS or antioxidant resources (e.g. Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Magwere et al. 2006).

In this regard, crickets have proven to be an important model for ageing research as both male and female reproductive effort can be easily quantified. Female reproductive effort can be measured as the number of eggs produced (Hunt et al. 2006; Zajitschek et al. 2007) and male reproductive effort can be measured as the time spent calling to attract a mate (Hunt et al. 2004, 2006; Zajitschek et al. 2007; Judge et al. 2008). In a number of cricket species, the number of females attracted to a calling

male on a given night is positively correlated with his calling effort (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muňoz et al. 2010) and is therefore an important determinant of male reproductive success. Furthermore, as calling is metabolically costly (e.g. Kavanagh 1987), nightly calling effort also represents an important form of male reproductive effort. Recently, I used inbred lines of the decorated cricket, *Gryllodes sigillatus*, to show divergent schedules of age-dependent reproductive effort, as well as lifespan and ageing, across the sexes (Chapter 3). In agreement with sexual selection theory, I found that reproductive effort increased with age in males but decreased with age in females, and that males lived longer and aged more slowly than females. These divergent life-history strategies were mediated by a trade-off between early-life reproductive effort and ageing rate in both sexes providing support for the antagonistic pleiotropy theory of ageing, although this relationship was considerably stronger in females.

Here, I examine the role of oxidative damage and antioxidant protection in shaping the evolution of lifespan and ageing in *G. sigillatus*. Using the same inbred lines, I relate measures of oxidative damage (protein carbonylation) and antioxidant protection (total antioxidant capacity) taken early and late in adulthood to lifespan and ageing parameters, as well as patterns of age-dependent reproductive effort, in the sexes. If the management of oxidative stress is important to the evolution of lifespan ageing in this species, I predict that oxidative damage and antioxidant protection will have a genetic basis and differ across the sexes, with oxidative damage being greater in the shorter lived sex (females). I also predict that oxidative damage will accumulate over time (Harman 1956) and that greater reproductive effort early in life will be associated with greater oxidative damage, at least in females. Each of these predictions comes with the obvious *proviso* that the predicted increase in oxidative damage is not associated with the upregulation of antioxidant defences in this species. I discuss my findings in relationship to the role that the management of oxidative stress plays in the evolution of sex-specific differences in lifespan and ageing in *G. sigillatus*.

4.3 MATERIALS AND METHODS

Cricket maintenance and the derivation of inbred lines

G. sigillatus used in this study were descended from ca. 500 adult crickets collected from New Mexico in 2001. These founding animals were allowed to breed panmictically and are referred to as the outbred "USA" population. Nine inbred lines were created from this original stock. To create these lines, designated A-I, crickets selected at random from this outbred population were subjected to over 23 generations of full-sib mating and then 12 generations of panmixis within each line (Ivy et al. 2005). Eight of these inbred lines (A,B,D-I) were used in this study.

Crickets were housed in 15L plastic containers in an environmental chamber (Percival I-66VL) maintained at 32 ± 1ºC on a 14h:10h light/dark cycle. Crickets were provided with cat food pellets (Friskies Go-Cat Senior®) and water in 60ml glass test tubes plugged with cotton wool *ad libitum*, as well as an abundance of egg cartons to provide shelter. As soon as adults were detected, moistened cotton wool was provided in a petrie-dish (10 cm diameter) as an oviposition substrate. Each inbred line was maintained in 2 containers and the outbred population in 8 containers. Each generation, crickets were randomly mixed between containers and were maintained at a density of approximately 300 crickets per container.

Experimental design

In Chapter 3, I measured the genetic variation in and covariation between measures of age-dependent reproductive effort, lifespan and ageing within and between the sexes using my inbred lines of *G. sigillatus*. Full details of the methods are provided in Chapter 3. In brief, on the day of hatching, 270 nymphs from each of the eight lines were established in individual plastic containers (5cm x 5cm x 5cm) and provided with a piece of cardboard egg box for shelter, water in a 2.5ml test tube plugged with cotton wool and fed with catfood pellets (Friskies GoCat Senior). Containers were cleaned and crickets provided with fresh food and water on a weekly basis. Upon reaching final instar, crickets were checked daily for eclosion to adulthood. Adult survival was checked daily from eclosion to death.

I measured male and female reproductive effort on days 10, 20 and 30 post-eclosion and each cricket was measured on only one of these days, being randomly

allocated to these sampling periods prior to adult eclosion. On days 8, 18 and 28 post-eclosion, each experimental cricket was paired with a virgin cricket of the opposite sex taken at random from the outbred population and allowed 48 hours to mate. Pairs were then separated and all experimental females were provided with a small petri-dish (5cm diameter) full of moist sand for oviposition and I measured female reproductive effort by counting the eggs produced in the 48 hours immediately preceding their allotted sampling period. I measured male reproductive effort as the amount of time spent calling on a given night (referred to as nightly calling effort) (Hunt et al. 2004, 2006). I measured nightly calling effort using a custom-built electronic monitoring device (see Hunt et al. 2004, 2006) that samples the calling activity of a series of males by sequentially sampling each male ten times per second using a condenser microphone embedded in the lid of the container. This device records a given male as calling if the sound it receives is ≥ 10 dB louder than the background noise. Nightly calling effort was measured as the number of seconds a male called each night between 18:00 to 09:00. In total, I measured the reproductive effort of approximately 20 males and females at each age from each of the 8 lines and outbred population. My estimates of lifespan and ageing parameters come from a total of 1791 crickets (878 females and 913 males), which represents approximately 90 crickets of each sex per line and the outbred population.

In the current study, I established an additional 100 nymphs from each of the inbred lines, as well as the outbred population, in individual containers on the day they hatched and reared them to adulthood following the protocol outlined above. At eclosion to adulthood, crickets were randomly assigned to one of two treatments, which dictated the age (12 or 32 days post-eclosion) at which oxidative damage and protection from such damage was measured. On day 8 post-eclosion, all crickets were paired at random with an outbred cricket of the opposite sex taken from the stock population for 48 hours to allow mating. For crickets assayed at day 32 post-eclosion, this process was repeated every ten days with another randomly chosen stock animal of the opposite sex being provided. Immediately after mating, females were provided with moist sand in a petri-dish to lay eggs. Each egg pad was left with the females for 48 hours and then replaced with a new one. After their first mating period, females always had access to an egg pad unless they were with a mate. On their allotted day,

crickets were placed in a 1.5ml eppendorf tube and frozen at -80°C prior to the biochemical analysis of oxidative damage and protection.

In total, 6 crickets of each sex per age group were frozen for biochemical analysis from each of the inbred lines and the outbred population (total *N* = 216). However, after accounting for statistical outliers (*n* =3, see below) and one sample batch being contaminated (*n* = 14), I assayed a total of 199 animals.

Biochemical analysis of oxidative damage and protection

All assays were completed within three months of samples being frozen at -80°C. Thawed crickets were homogenised in 1ml of phosphate buffer (pH 7.4) and then centrifuged at 13 000rpm for 20 minutes at 4°C. The supernatant fraction was separated from fat and cuticle and centrifuged for a further 5 minutes. This final supernatant fraction was used for all subsequent biochemical analyses. I measured the degree of oxidative damage in my samples by measuring the concentration of protein carbonyls (PC) and the degree of protection from oxidative damage by measuring total antioxidant capacity (TAC). In each sample, I first measured protein concentration using the Bradford method (Bradford 1976) in samples diluted five-fold in homogenisation buffer. Samples ran in triplicate showed that protein content was highly repeatability across repeated samples from the same cricket (repeatability ± SE: 0.95 ± 0.004). The concentration of PC was assayed in samples using a commercially available kit (Protein Carbonyl Assay Kit, Cayman Chemical, Item no. 10005020) after being diluted 5-fold in homogenisation buffer. Carbonyl groups (CO) are formed on protein side-chains when they are oxidised and are relatively stable, making them amenable to quantification and storage (Dalle-Donne et al. 2003). Moreover since proteins serve a variety of important biological functions (for example, as enzymes), oxidative damage to proteins is likely to be to the general detriment of cellular function (Dalle-Donne et al. 2003). The PC assay relies upon the covalent reaction between protein carbonyl groups and 2,4-dinitrophenylhydrazine (DNPH), which produces the stable product 2,4-dinitrophenyl (DNP) hydrazone. DNP absorbs ultraviolet light and therefore can be quantified using a spectrophotometer (Spectramax Plus384, Molecular Devices) set to 370nm (Levine et al. 1990). The

repeatability of my PC measures between samples run in duplicate was high (0.98 ± 0.002).

TAC was also measured using a commercially available kit (Antioxidant Assay Kit, Cayman Chemical, Item no. 709001), which assays the oxidation of ABTS (2,2'-Azino-di-[3-ethybenzthiazoline sulphonate]) by metmyoglobin, which is inhibited by antioxidants contained in the sample relative to Trolox controls. Oxidised ABTS product can then be measured using a spectrophotometer set to 750nm. All samples were diluted 100-fold and run in duplicate and the repeatability of my TAC measures was high across samples (0.95 ± 0.007).

In all statistical analyses, I used the mean value for PC and TAC measures taken across my repeated measurements of a given sample. To control for differences in the body size of crickets, PC and TAC measures were expressed per milligram of protein analyzed. PC is therefore provided in units of nanomoles per milligram of protein (nmol/mg) and TAC in units of micromoles per milligram of protein (mmol/mg).

Statistical Analyses

Prior to analysis, I $\log_{10}$ transformed my response variables (PC and TAC) to ensure normality. After transformation, I conducted a multivariate outlier analysis using Mahalanobis distances and three crickets were excluded from further analysis as they were greater than two standard deviations from the mean centroid (Tabachnick & Fidell 1989). I examined the effect of line, sex and age on PC and TAC using a mixed-model multivariate analysis of variance (MANOVA), including line as a random effect and sex and age as fixed effects in the model. All interactions between line and the fixed effects were treated as a random effect in the model (Zar 1999). I followed this MANOVA with a series of univariate general linear mixed-models (GLMM) with the same model structure to determine how each of my response variables contributed to the overall multivariate effects. This overall model identified significant differences in PC and TAC across the sexes (see Results) and I therefore examined the effects of line and age on PC and TAC within each sex separately using a mixed-model MANOVA, including age as a fixed effect and line and line x age as random effects. As outlined above, I followed these MANOVAs with a series of univariate GLMMs to determine how each of my response variables contributed to the overall multivariate effects. All

models were implemented in SAS (version 9.1.3, SAS Institute Inc., USA) using the "PROC GLM" procedure with the appropriate *F* values and degrees of freedom being specified according to Zar (1999, Appendix A). In no instance did model simplification alter the outcome of the model, so I present the complete model for each analysis. All data are presented as mean ± 1SE.

The use of inbred organisms in evolutionary research can be problematic, particularly if inbreeding depression results in a large reduction in the fitness of individuals and/or if the inbred lines do not represent a true random sample of genotypes from the outbred population from which they were derived (Hoffman & Parsons 1988). Therefore, in addition to the inbred lines, I also assayed PC and TAC in a sample of crickets from the outbred population they were derived from. In doing this, my aim was not to test how inbreeding influences PC and TAC (which would require replicate outbred populations), rather to provide a baseline for comparison. For this reason, these outbred crickets are not included in any of my statistical analyses but are provided in my figures only for visual comparison.

Quantitative genetic analyses

I estimated the heritabilities of PC, TAC and life-history traits (reproductive effort, lifespan and ageing) from my inbred lines by calculating the intraclass correlation coefficient (*t*) (Hoffmann & Parsons 1988; David et al. 2005) as:

$$t = \frac{nV_b - V_w}{nV_b + (n-1)V_w}$$

where *n* is the number of lines (in this case 8) and V_b and V_w are the between-line and within-line variance components, respectively, estimated directly from a general linear model including line as a random effect. The standard error of the intraclass correlation (SE(*t*)) was calculated according to Becker (1984) as:

$$SE(t) = \sqrt{\frac{2(1-t)^2[1+(k-1)t]^2}{k(k-1)(n-1)}}$$

where *k* is the number of individuals sampled within each line. The heritability (h^2) of each phenotypic trait was then estimated according to Hoffmann & Parsons (1988) as:

$$h^2 = \frac{2}{\left(\frac{1}{t} - 0.5\right)}$$

The standard error of this estimate, (SE(h^2)), was calculated according to Hoffmann & Parsons (1988) as:

$$SE(h^2) = \frac{2}{\left(1 - \frac{t}{2}\right)^2} SE(t)$$

I estimated genetic correlations (and their standard errors) between traits using the jackknife method of Roff & Preziosi (1994). This procedure first estimates the genetic correlation between two traits using inbred line means. Next, a sequence of *N* (in this case 8) pseudo-values is computed by sequentially dropping each of the lines, estimating the resulting correlations using the formula:

$$S_{N,i} = Nr_N - (N-1)r_{N-1,i}$$

where $S_{N,i}$ is the *i*th pseudo-value, r_N is the genetic correlation estimated using the means of all *N* inbred lines, and $r_{N-1,i}$ is the genetic correlation obtained by dropping the *i*th inbred line alone (Roff & Preziosi, 1994). The jacknife estimate of the genetic correlation (r_j) is the mean of the pseudo-values and an estimate of the standard error (*SE*) is given by:

$$SE = \frac{\sum_{i=1}^{i=N} (S_{N,i} - r_j)^2}{N(N-1)}$$

Simulation models have shown that this jacknife approach provides more accurate genetic estimates than those based on conventional inbred line means when fewer than 20 inbred lines are used in the analysis (Roff & Preziosi 1994). These estimates of genetic (co)variance from inbred lines contain variance due to dominance and/or epistasis and therefore should be considered broad-sense estimates (Falconer & Mackay 1996). As crickets were reared individually the variance between lines due to common environment effects was minimised in my experimental design. Estimates of heritability and genetic correlations were considered statistically significant if the estimates divided by their standard errors were greater than 1.96, rejecting the null hypothesis of no correlation based on a two tailed *t*-distribution with infinite degrees of freedom.

4.4 RESULTS

A mixed-model MANOVA indicated that line and sex significantly influenced the linear combination of PC and TAC measures (Table 4-1). There was, however, no overall multivariate effect of age on levels of PC and TAC (Table 4-1). Univariate general linear mixed-models (GLMM) showed that the overall multivariate effect of line was driven by significant line differences in both PC and TAC (Table 4-1). Likewise, the overall multivariate effect of sex was due to sex differences in both PC and TAC (Table 4-1) with females, on average, exhibiting higher levels of both PC and TAC than males (Figure 4-1). The magnitude of sex differences in PC and TAC, however, varied across lines and with age, as indicated by the significant line x sex and age x sex interactions (Table 4-1). Consequently, to facilitate subsequent interpretation I conducted separate analyses on males and females.

In males, there was a significant multivariate effect of line and age on the linear combination of PC and TAC but no interaction between these main effects (Table 4-2). GLMM showed that the overall multivariate effect of line resulted from significant line differences in both PC and TAC (Figure 4-2A and B, Table 4-2A). In contrast, the overall multivariate effect of age was due to the fact that TAC significantly increased with age in males whereas PC did not (Figure 4-2A and B, Table 4-2A). In females, there was a significant multivariate effect of line on the linear combination of PC and TAC but there was no effect of age nor was there an interaction between line and age (Figure 4-2B and C, Table 4-2B). As shown for males, GLMM revealed that the overall multivariate effect of line detected in females was due to significant differences in both PC and TAC across lines (Figure 4-2B and C, Table 4-2B).

Consistent with these line differences, PC and TAC measured early and late in life were significantly heritable in both sexes and these measures were positively genetically correlated within each sex (Table 4-3). In my previous study (Chapter 3), I found that measures of age-dependent reproductive effort, lifespan and ageing all exhibited significant heritabilities in the sexes (Table 4-3). Moreover, I found a significant positive genetic correlation between early-life reproductive effort (ERE) and the rate of ageing (β) in both sexes, albeit this relationship was considerably stronger in females (Table 4-3). Here I show that both PC and TAC are genetically correlated with reproductive effort, although the sign and strength of these genetic correlations

varied with age and across the sexes (Table 4-3). In males, there were consistent negative genetic correlations between early and late-life reproductive effort and both PC and TAC (Table 4-3). In females, there was a negative genetic correlation between early-life reproductive effort and both PC and TAC when expressed early in life but a positive genetic correlation when expressed later in life (Table 4-3). There were, however, consistent negative genetic correlations between late-life reproductive effort and both PC and TAC, irrespective of when they were expressed in life (Table 4-3).

We also found that PC and TAC were genetically correlated with lifespan and measures of ageing in the sexes. Irrespective of whether expressed early or late in life, there were consistent negative genetic correlations between lifespan and levels of PC and TAC in both males and females (Table 4-3). In females, levels of PC and TAC expressed early in life were positively genetically correlated with baseline mortality, while later in life these measures were positively genetically correlated with the rate of ageing (Table 4-3). In males, there were consistent positive genetic correlations between baseline mortality and the levels of both PC and TAC expressed early and late in life (Table 4-3). However, there was only a positive genetic correlation between the level of TAC expressed late in life and the rate of ageing in males (Table 4-3).

Previously, I have shown strong positive intersexual genetic correlations between measures of age-dependent reproductive effort, lifespan and measures of ageing that suggests these traits are unlikely to evolve independently in the sexes (Table 4-4, Chapter 3). Consistent with this, I found that the genetic correlations between development time and measures of age-dependent PC and TAC were generally strong and positive across the sexes, with the exception of PC measured late in life (Table 4-4). The patterns of genetic correlations between these traits across the sexes largely mirrored the patterns observed within each sex (Table 4-4). Notable exceptions include the strong positive genetic correlation between baseline mortality and PC expressed late in life across the sexes, as well as the positive genetic correlation between PC expressed early in life in females and late-life reproductive effort in males (Table 4-4).

4.4 DISCUSSION

Despite tremendous progress in gerontology (Partridge et al. 2010), we still have a relatively poor understanding of the proximate basis of ageing (Partridge and Gems 2006). This is especially true for the role (if any) that oxidative stress plays in mediating the ageing process (D'Autréaux and Toledano 2007). In the decorated cricket, *Gryllodes sigillatus*, the sexes have contrasting patterns of age-dependent reproductive effort with male calling effort increasing with age, whereas female fecundity decreases with age (Chapter 3). As predicted by sexual selection theory (Promislow 2003; Graves 2007; Bonduriansky et al. 2008), these contrasting schedules of reproductive effort are associated with differences in lifespan and ageing between the sexes, with males living longer and ageing more slowly than females (Chapter 3). These divergent life-history strategies are mediated by a positive genetic correlation between early-life reproductive effort and ageing rate in both sexes, although this relationship is considerably stronger in females (Chapter 3). Here, I examined whether oxidative stress is a proximate cost of reproductive effort that is able to mediate these sex-specific rates of ageing and differences in lifespan in male and female *G. sigillatus*. I found that levels of oxidative damage to proteins (protein carbonylation) and antioxidant protection (total antioxidant capacity) were greater in females than in males and that both measures possess significant levels of genetic variation in the sexes. Moreover, these measures were genetically correlated with lifespan, measures of ageing and age-dependent reproductive effort. These relationships were complex, however, with the sign and magnitude differing with age and across the sexes. Thus, while my findings suggest that the management of oxidative stress is likely to play an important role in the evolution of lifespan and ageing in *G. sigillatus*, the exact nature of the role is far from simple.

The free radical theory of ageing (Harman 1956) is one of the best supported mechanistic theories of ageing (Beckman and Ames 1998; Finkel and Holbrook 2000). This theory predicts that the accumulation of oxidative damage to proteins, lipids and DNA, which occurs when the production of ROS exceeds circulating antioxidant defences, causes ageing (Harman 1956). This means that short lived animals should have high levels of oxidative damage due to a chronic or prolonged imbalance between ROS production and antioxidant protection (Harman 1956). Over the last fifty

years, this theory has gained a great deal of empirical support (reviewed by Beckman and Ames 1998). For example comparative studies show that oxidative damage and/or rates of ROS production are inversely related to lifespan across a broad range of species including flies (e.g. Sohal et al. 1993a), mammals (Sohal et al. 1993b; Lambert et al. 2007) and reptiles (Robert et al. 2007). Recently, however, this theory has been heavily criticised because some of its central predictions have not been verified empirically, the most notable being that the knockout of major antioxidant genes (either singly or in combination) appears to have an inconsistent effect on lifespan in a number of laboratory model species (Pérez et al. 2009; Jang and Remmen 2009; Speakman and Selman 2011). For example, of eighteen genetic manipulations of the expression of antioxidant enzymes in mice only one (the deletion of the gene encoding the antioxidant superoxide dismutase) significantly reduced lifespan (Pérez et al. 2009). While this, and similar observations, have been taken as evidence against this theory of ageing (Pérez *et al.* 2009), there is a growing awareness that antioxidants are unlikely to have a simple, direct effect on lifespan. ROS regulate a number of important cellular processes (Dickinson and Chang 2011) including cell growth, migration, apoptosis (Apel and Hirt 2004, Veal et al. 2007) and gene expression (Allen and Tresini 2000; Apel and Hirt 2004). For example, in *Arabidopsis* (Desikan et al. 2001) and *C. elegans* (Schulz et al. 2007) ROS may induce the expression of a number of genes, some of which encode antioxidant enzymes. This regulation of antioxidant enzyme expression by ROS is one mechanism that allows cells to precisely regulate their redox state, i.e. the balance between pro- and anti- oxidants. Such precise regulatory mechanisms explain the observation that antioxidants are often positively correlated with ROS production and/or oxidative damage (Costantini and Verhulst 2009). This means that rather than antioxidants being positively correlated with lifespan, as studies often assume should be the case, the reverse is often true (Buttemer et al. 2010). Additionally, when optimal redox states are perturbed, for example via antioxidant manipulation, cellular function may be impaired (Tapia et al. 2006; Lin & Beal 2006) meaning that antioxidant manipulation may cause pathologies that reduce lifespan, independently of ageing (Magwere et al. 2006). Therefore, the failure of antioxidant manipulations to consistently affect lifespan should not always be taken as absolute evidence against the free radical theory (Lin & Beal 2006).

In fact, the results I present here two lines of support for the free radical theory of ageing. First, I found that oxidative damage was significantly greater in the shorter lived sex (females). Sex differences in lifespan are widespread (Promislow 1992; Clutton-Brock & Isvaran 2007) and several studies have shown that oxidative damage and/or ROS production is greater in the shorter lived sex (e.g. Ide et al. 2002; Borrás et al. 2003; Ali et al. 2005). Oxidative stress may differ across the sexes because of differences in metabolic rate or mitochondrial efficiency in males and females, which may affect rates of ROS production (Magwere et al. 2006). Alternatively, either sex may show different responses to changes in redox status that affect their resistance to oxidative damage (Magwere et al. 2006). Both of these hypotheses have gained some support. For example, female rats, which live longer than males, produce fewer ROS from their mitochondria and show increased expression of antioxidant enzymes, due to the female sex hormone oestrogen up-regulating their expression (Borrás et al. 2003, 2007; Viña et al. 2005). These studies show an important role for sex hormones in mediating intersexual differences in oxidative stress in mammals (Borrás et al. 2003, 2007; Viña et al. 2005; Miller et al. 2007). The mechanistic basis of intersexual differences in oxidative stress has been less thoroughly explored in invertebrates. The greater oxidative damage I show in female crickets may be because sexual selection, which promotes the evolution of longer lives in male than females decorated crickets (Chapter 3), has promoted the evolution of different levels of mitochondrial efficiency across the sexes, differences in the expression of key antioxidant enzymes and/or rates of repair of oxidised molecules. Such differences have been observed across ecotypes of the garter snake, which have evolved differences in lifespan due to different rates of extrinsic mortality (Robert and Bronikowski 2010).

The second line of support that I found for the free radical theory of ageing was that lifespan was negatively genetically correlated with oxidative damage in both sexes. The magnitude of these genetic correlations differed across the sexes and with age. In females, oxidative damage assayed early and late in life was negatively correlated with lifespan but in males, only damage measured early in life was negatively genetically correlated with longevity. While a number of studies have shown negative phenotypic correlations between oxidative damage and/or rate of ROS production (e.g. Sohal et al. 1993a,b; 1995; Lambert et al. 2007; Robert et al. 2007)

fewer have explored the genetic basis of this association. In *Drosophila,* experimental evolution studies have found that divergent selection on lifespan causes correlated changes in the expression and activity of particular antioxidant enzymes and resistance to oxidative stress (Arking et al. 2000; Harshman and Haberer 2000). Furthermore, studies mapping Quantitative Trait Loci (QTL) for oxidative stress, reproduction and lifespan in inbred *Drosophila* lines have found natural and heritable genetic variation for resistance to oxidative stress (Wang et al. 2006) and that QTL for lifespan (Curtsinger and Khazaeli 2002) but not early female fecundity (Wang et al. 2006) overlap with QTL for oxidative stress. Taken together, these results show that resistance to oxidative stress is heritable and genetically correlated with lifespan in *Drosophila.* My study suggests that the same is true in decorated crickets.

In addition to finding negative genetic correlations between lifespan and oxidative damage, I also found that oxidative damage was genetically correlated with ageing parameters. Firstly, I found that there were positive genetic correlations between baseline mortality and oxidative damage early in life in both males and females. This suggests that lines which have higher baseline mortality, which is typically interpreted as a measure of "frailty", tend to have greater oxidative damage early in life. This may arise because lines with higher baseline mortality generate more free radicals during metabolism (Speakman et al. 2004), are less robust to stimuli that can perturb redox homeostasis such as disease (Pal et al. 2010) or are less able to detect, repair or remove oxidised molecules (Caple et al. 2010; Tyson et al. 2010). I also found in females, but not in males, that oxidative damage late in life was positively associated with ageing rate. This means that greater oxidative damage late in life can accelerate rates of ageing in female decorated crickets. Crucially, this finding may provide a proximate mechanism underpinning the more pronounced antagonistic pleiotropy that I have previously detected in female decorated crickets (Chapter 3).

Reproductive effort, however, did not increase oxidative damage in male crickets; in males, I found negative genetic correlations between reproductive effort and oxidative damage. I might expect that female fecundity is more costly than male calling effort for a number of reasons. First, if female mitochondria are less efficient than male mitochondria, they may produce more ROS per unit oxygen. This means

that an increase in metabolic rate associated with reproductive effort could increase ROS production more in females than a comparable (or even greater) increase in metabolic rate in males. Such intersexual differences in mitochondrial efficiency appear to be widespread (e.g. Borrás et al. 2003, 2007; Viña et al. 2005). Second, egg production may increase female metabolic rate more than calling effort does in males. If greater metabolic rate increases rates of ROS production, it could push cells into oxidative stress and accelerate rates of oxidative damage. While increasing metabolic rate does not necessarily increase ROS production (Hulbert et al. 2007), reproductive effort can increase metabolic rate in crickets (e.g. Kavanagh 1987) and in decorated crickets, lifespan and metabolic rate are negatively correlated (Okada et al. 2011). Oxidative stress may mediate this association between metabolic rate and lifespan, although research is needed to test this hypothesis. Finally, female reproductive effort may increase oxidative damage because females divert more antioxidants to producing eggs than in maintaining their soma. Previous studies have shown antioxidants are vital to producing eggs in the Australian field crickets (Chapter 6) and Fire bugs (Jedlička et al. 2009).

While these results offer support for the free radical theory of ageing, a key prediction of this theory is that oxidative damage accumulates over time (Harman 1956). In decorated crickets, oxidative damage did not increase with age in either males or females. Several studies have found age-associated increases in oxidative damage, including protein oxidation (Stadtman 1992; Sohal et al. 1993a,b, 1995; Kasaplogu and Özben 2001). However, because levels of oxidative damage and/or rates of ROS production frequently vary across different tissue types (Sohal et al. 1994a; Kaneko et al. 1997; Radák et al. 2002; Borrás et al. 2003) and age-associated changes in oxidative damage may occur at different rates in different tissues (Sohal et al. 1995), there may have been age-associated changes in oxidation to other molecules (e.g. lipids or DNA) that I did not examine. My results offer some evidence that this may be the case: while I did not find an age-dependent increase in oxidative damage, I did find that older males had greater antioxidant capacity than younger males. In decorated crickets there is a positive correlation between oxidative damage and antioxidant protection. This result, which has been detected in a number of other species (Costantini and Verhulst 2009; Buttermer et al. 2010), occurs because animals

up-regulate their investment in antioxidant protection in response to greater rates of ROS production. However, up-regulated antioxidant defences may not always match elevated production of ROS and, despite greater antioxidant protection, oxidative damage may still occur (Costantini and Verhulst 2009). This appears to be the case in humans whereby old males have greater levels of oxidative damage to both DNA and proteins despite having greater levels antioxidant protection (Gianni et al. 2004). It appears that this is also the case in decorated crickets because lines and the sex (females) with greater levels of antioxidant protection still incur greater oxidative damage. This positive association between oxidative damage and antioxidant protection explains the apparently counter-intuitive result that, in *G. sigillatus,* antioxidant protection was negatively genetically correlated with lifespan. Rather than illustrating that antioxidants *per se* carry a mortality cost, this inverse association between lifespan and antioxidants reflects that shorter lived crickets have to produce more antioxidants to combat higher levels of ROS production. This positive association also means that the age-associated increase in male antioxidants may illustrate an adaptive response to an age-associated increase in pro-oxidant production (as predicted by the mitochondrial theory of ageing – Harman 1972; Fleming 1982), which has been detected via a global measure of antioxidant status but not by an assay of oxidative damage to a single tissue type (e.g. proteins). This result highlights the importance of measuring both antioxidant protection, damage and/or ROS production to make inferences about oxidative stress (Costantini and Verhulst 2009).

My work shows that oxidative stress may explain differences in lifespan and ageing rates across the sexes and the trade-off between early reproductive effort and ageing rate seen in females. Therefore, given that male and female decorated crickets have very different life-history strategies (Chapter 3), each sex should be under selection to manage their redox states very differently. However, I found strong positive genetic correlations between antioxidant protection both early and late in life and between oxidative damage early in life. This suggests that oxidative damage and investment in antioxidants are not free to evolve independently across the sexes. This indicates the potential for intralocus sexual conflict over oxidative stress in decorated crickets. Intralocus sexual conflict occurs whenever selection on shared phenotypic traits in one sex displaces the other sex from its phenotypic optima, because many

traits shared by the sexes have a common genetic basis but are subject to contrasting selection that prevents the sexes from evolving independently to their own phenotypic optima (Bonduriansky and Chenoweth 2009). Consequently, when intralocus sexual conflict is operating in a population, traits shared by the sexes will be characterised by strong and positive intersexual genetic correlations, as well as selection gradients that are different in sign and strength across the sexes (Bonduriansky & Chenoweth 2009). I have previously shown the potential for intralocus sexual conflict over age-dependent reproductive effort, ageing and lifespan in decorated crickets (Chapter 3). The current study shows that intralocus sexual conflict over lifespan and ageing may be mediated by conflict over levels of oxidative damage (early in life) and/or repair. Confirmation that intralocus sexual conflict is indeed operating, as well as a direct estimate of its strength, requires formal estimation of the sex-specific fitness surfaces for age-dependent oxidative damage and protection (Lewis et al. 2011).

In conclusion, my study shows that oxidative damage and antioxidant protection are important determinants of lifespan and ageing in *G. sigillatus* and therefore provides broad support the free radical theory of ageing. In support of this theory I find that oxidative damage is greater in the shorter lived sex (females) and is negatively genetically correlated with lifespan. However, the lack of evidence for an age-dependent increase in oxidative damage undermines a key premise of this theory. I found a positive association between oxidative stress and antioxidant protection, indicative of active upregulation of antioxidants in response to greater production of ROS. This result shows that the relationship between oxidative damage and protection is not a simple, inverse one. I also found that, in females but not males, oxidative damage late in life was a proximate cost of early reproductive effort that accelerates rates of ageing. This illustrates that oxidative stress could mediate this important, sex-specific life-history trade-off as well as explaining intersexual differences in ageing and lifespan. This means that oxidative stress may be a proximate means by which sexual selection shapes sex-specific life-history strategies. However, I also find that inter-sexual genetic correlations between oxidative damage and antioxidant protection are largely strong and positive, showing that either sex is constrained in their ability to regulate oxidative damage and antioxidant capacity. This highlights the importance of

considering sexual selection and sexual conflict in research examining the mechanistic basis of ageing.

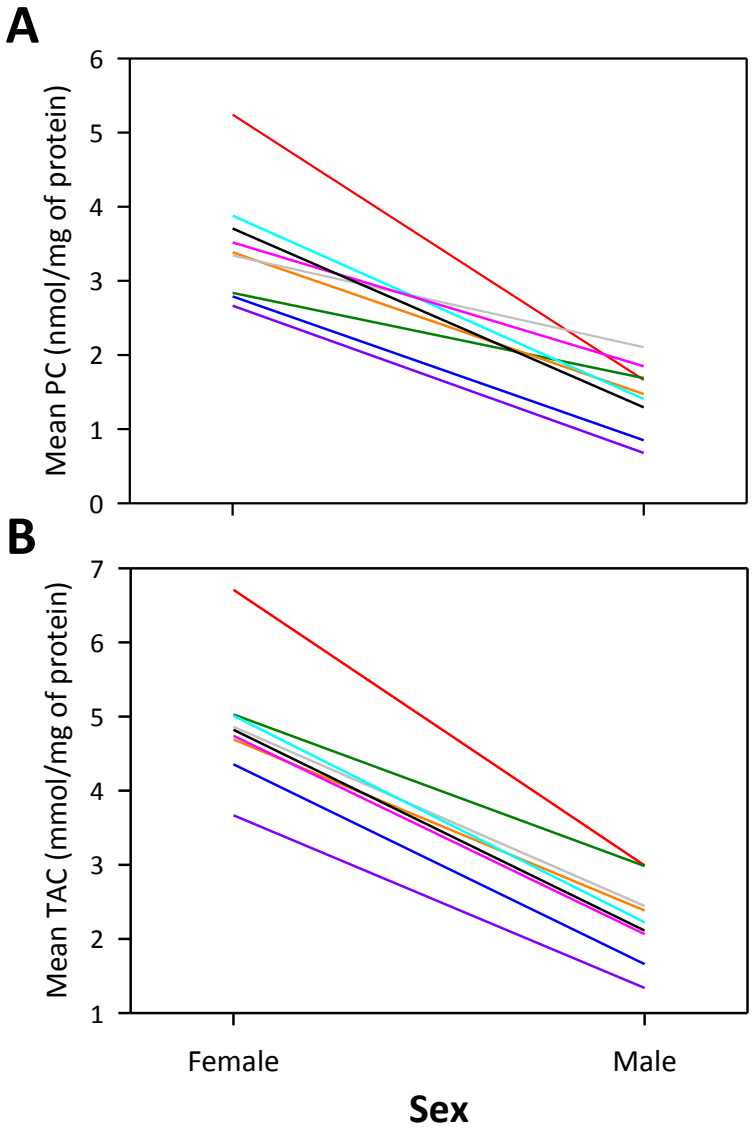

Figure 4-1. Mean sex differences in protein carbonylation (PC) and total antioxidant capacity (TAC) in the different inbred lines and the outbred population of *Gryllodes sigillatus.* The different inbred lines and outbred population are colour coded: line A = red, line B = green, line D = orange, line E = grey, line F = blue, line G = purple, line H = cyan, line I = pink and the outbred base population = black.

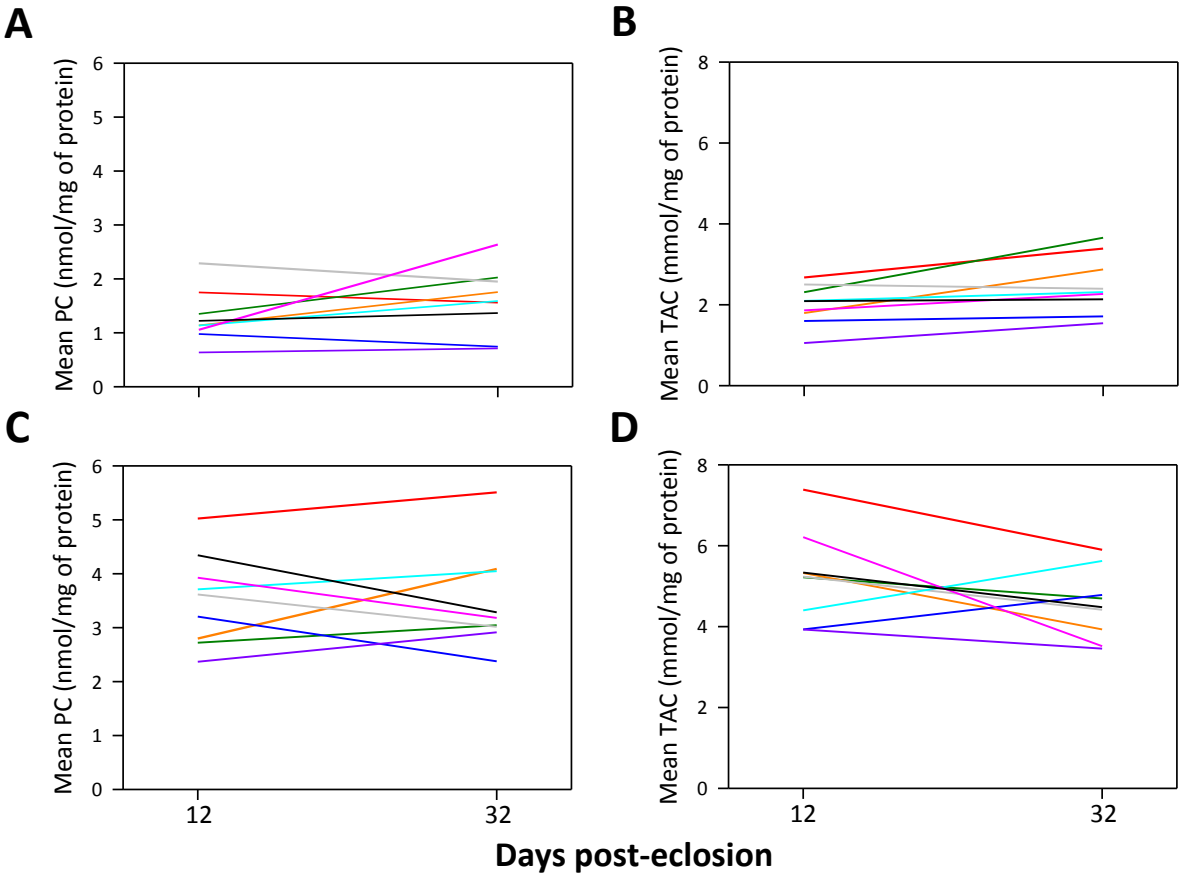

Figure 4-2. Age-dependent protein carbonylation (PC) and total antioxidant capacity (TAC) in the different inbred lines and the outbred population of (A & B) male and (C & D) female *Gryllodes sigillatus*. The different inbred lines and outbred population are colour coded as outlined in Figure 4-1.

Table 4-1. Mixed-model MANOVA examining the effects of line, sex , age and their interactions on levels of protein carbonylation (PC) and total antioxidant capacity (TAC) per mg protein in *Gryllodes sigillatus*. I also present univariate GLMMs to determine how each of these response variables contributed to the overall multivariate effect. As there were significant differences in levels of PC and TAC across the sexes, as illustrated by a significant main effect of sex as well as interactions with line and age, I conducted subsequent analyses for each sex.

	MANOVA			
	Wilks' λ	*F* value	*df*	*P* value
Line (A)	0.675	4.379	14,282	0.0001
Sex (B)	0.026	111.446	2,6	0.0001
Age (C)	0.781	0.845	2,6	0.476
A x B	0.850	1.812	14,282	0.037
A x C	0.907	1.001	14,282	0.451
B x C	0.413	5.229	2,6	0.048
A x B x C	0.911	0.964	14,282	0.490
	Univariate GLMM			
	F value	*df*	*P* value	
PC				
A	4.483	7,7.869	0.0265	
B	42.809	1,7.015	0.0003	
C	1.291	1,7.038	0.294	
A x B	4.428	7,7	0.034	
A x C	1.725	7,7	0.246	
B x C	1.090	1,7.066	0.331	
A x B x C	0.722	7,142	0.657	
TAC				
A	10.010	7,7.034	0.004	
B	202.867	1,7.059	0.0001	
C	1.212	1,7.069	0.307	
A x B	0.709	7,7	0.675	
A x C	0.601	7,7	0.742	
B x C	9.122	1,7.041	0.019	
A x B x C	1.144	7,142	0.340	

Table 4-2. Mixed-model MANOVA examining the effects of line and age on levels of PC and TAC in (A) male and (B) female *Gryllodes sigillatus*. I also present univariate GLMMs to determine how each response variable contributes to the overall multivariate effect.

A. Males	**MANOVA**			
	Wilks' λ	*F* value	*Df*	*P* value
Line (A)	0.467	4.562	14,138	0.0001
Age (B)	0.395	5.178	2,6	0.049
A x B	0.815	1.061	14,138	0.398
	Univariate GLMM			
	F value	*Df*	*P* value	
PC				
A	5.192	7,7	0.023	
B	1.893	1,7.085	0.211	
A x B	1.289	7,70	0.274	
TAC				
A	6.893	7,7	0.010	
B	10.634	1,7.111	0.013	
A x B	0.989	7,70	0.454	
B. Females	**MANOVA**			
	Wilks' λ	*F* value	*Df*	*P* value
Line (A)	0.729	2.199	14,142	0.010
Age (B)	0.720	1.177	2,6	0.373
A x B	0.846	0.892	14,142	0.574
	Univariate GLMM			
	F value	*Df*	*P* value	
PC				
A	4.182	7,7	0.039	
B	0.100	1,7.045	0.766	
A x B	0.708	7,72	0.672	
TAC				
A	3.873	7,7	0.047	
B	2.651	1,7.036	0.147	
A x B	0.875	7,72	0.536	

Table 4-3. Heritabilities (along diagonal) and genetic correlations (below diagonal) for early and late-life reproductive effort (ERE and LRE), lifespan, Gompertz ageing parameters (α and β), early and late-life protein carbonylation (EPC and LPC) and early and late-life total antioxidant capacity (ETAC and LTAC) in male and female *Gryllodes sigillatus*. Standard errors for these estimates are in parenthesis. Values given in bold are significant at $P < 0.05$ and those highlighted in grey are taken from Chapter 3.

	ERE	LRE	LS	α	β	EPC	LPC	ETAC	LTAC
Males									
ERE	**0.36 (0.14)**								
LRE	**0.15 (0.07)**	**0.52 (0.14)**							
LS	0.35 (0.24)	**0.49 (0.05)**	**0.97 (0.02)**						
α	**-0.44 (0.10)**	**-0.40 (0.05)**	**-0.79 (0.03)**	**0.99 (0.01)**					
β	**0.44 (0.17)**	-0.06 (0.14)	**-0.42 (0.10)**	-0.26 (0.40)	**0.99 (0.01)**				
EPC	**-0.74 (0.05)**	**-0.34 (0.03)**	**-0.55 0.07)**	**0.87 (0.00)**	-0.68 (0.61)	**0.77 (0.11)**			
LPC	**-0.48 (0.14)**	0.30 (0.46)	-0.19 (0.22)	0.12 (0.13)	-0.04 (0.06)	**0.39 (0.10)**	**0.43 (0.18)**		
ETAC	**-0.72 (0.19)**	**-0.23 (0.02)**	**-0.87 (0.01)**	**0.80 (0.01)**	0.05 (0.25)	**0.80 (0.01)**	**0.58 (0.11)**	**0.54 (0.17)**	
LTAC	**-0.46 (0.12)**	**-0.28 (0.03)**	**-0.07 (0.04)**	**0.39 (0.13)**	**0.46 (0.06)**	**0.38 (0.13)**	**0.53 (0.10)**	**0.78 (0.02)**	**0.76 (0.11)**
Females									
ERE	**0.48 (0.14)**								
LRE	0.34 (0.21)	**0.46 (0.14)**							
LS	-0.11 (0.11)	0.09 (0.11)	**0.94 (0.03)**						
α	**-0.98 (0.45)**	-0.02 (0.04)	**-0.40 (0.03)**	**0.98 (0.01)**					
β	**0.97 (0.06)**	-0.00 (0.16)	0.09 (0.15)	**-1.16 (0.23)**	**0.99 (0.00)**				
EPC	**-0.20 (0.03)**	**-0.46 (0.21)**	**-0.37 (0.18)**	**0.24 (0.06)**	-0.11 (0.10)	**0.38 (0.18)**			
LPC	**0.16 (0.08)**	**-0.60 (0.06)**	**-0.61 (0.02)**	-0.05 (0.22)	**0.26 (0.02)**	**0.91 (0.19)**	**0.52 (0.17)**		
ETAC	-0.01 (0.01)	**-0.42 (0.13)**	-0.30 (0.25)	**0.13 (0.06)**	0.02 (0.03)	**0.90 (0.07)**	**0.92 (0.13)**	**0.33 (0.15)**	
LTAC	**0.12 (0.02)**	-0.08 (0.17)	**-0.80 (0.02)**	0.15 (0.02)	**0.07 (0.02)**	**0.72 (0.11)**	**0.71 (0.12)**	0.46 (0.34)	**0.34 (0.17)**

Table 4-4 Intersexual genetic correlations (±SE) for lifespan, Gompertz ageing parameters and age-dependent reproductive effort in *Gryllodes sigillatus*. Abbreviations are as outlined in Table 4-3.

♂ \ ♀	ERE	LRE	LS	α	β	EPC	LPC	ETAC	LTAC
ERE	0.35 (0.21)	**0.24 (0.07)**	**0.31 (0.09)**	**-0.61 (0.21)**	0.51 (0.38)	-0.29 (0.18)	-0.09 (0.07)	**-0.36 (0.09)**	-0.18 (0.24)
LRE	-0.07 (0.16)	**0.15 (0.07)**	**0.85 (0.03)**	**-0.39 (0.03)**	0.13 (0.27)	**0.17 (0.04)**	**-0.24 (0.08)**	0.19 (0.11)	**-0.41 (0.12)**
LS	**-0.11 (0.05)**	0.08 (0.17)	**0.88 (0.00)**	**-0.33 (0.09)**	**0.08 (0.04)**	**-0.76 (0.10)**	**-0.79 (0.05)**	**-0.63 (0.20)**	**-0.94 (0.01)**
α	**-0.57 (0.12)**	**-0.37 (0.10)**	**-0.71 (0.01)**	**0.76 (0.02)**	**-0.66 (0.24)**	**0.79 (0.07)**	**0.71 (0.19)**	**0.65 (0.16)**	**0.69 (0.06)**
β	**0.92 (0.04)**	**0.30 (0.13)**	**-0.28 (0.14)**	-0.95 (0.93)	**0.94 (0.23)**	0.18 (0.12)	**0.44 (0.03)**	**0.19 (0.09)**	**0.51 (0.05)**
EPC	**-0.78 (0.38)**	**-0.09 (0.04)**	**-0.53 (0.03)**	**0.99 (0.03)**	**-1.04 (0.44)**	**0.48 (0.06)**	0.25 (0.21)	**0.44 (0.09)**	**0.34 (0.12)**
LPC	0.06 (0.09)	**0.23 (0.03)**	0.10 (0.46)	**0.27 (0.08)**	**-0.27 (0.02)**	**0.31 (0.10)**	0.15 (0.11)	**0.54 (0.10)**	-0.11 (0.21)
ETAC	-0.15 (0.17)	0.01 (0.14)	**-0.69 (0.02)**	**0.58 (0.01)**	**0.41 (0.13)**	**0.76 (0.05)**	**0.62 (0.09)**	**0.74 (0.03)**	**0.71 (0.04)**
LTAC	**0.47 (0.08)**	0.21 (0.42)	**-0.63 (0.03)**	0.04 (0.10)	**0.20 (0.06)**	0.40 (0.25)	**0.58 (0.10)**	**0.68 (0.05)**	**0.48 (0.08)**

5. FLEXIBLE DIET CHOICE IN RESPONSE TO MATING CAUSES SEX-SPECIFIC PATTERNS OF NUTRIENT REGULATION IN THE AUSTRALIAN FIELD CRICKET, *TELEOGRYLLUS COMMODUS.*

5.1 ABSTRACT

In a broad range of species, diet affects both reproductive effort and lifespan. Moreover, sexual selection can affects rates of ageing, lifespan and strategies of age-dependent reproductive effort differently in either sex. Therefore, sexual selection may promote different responses to dietary manipulation and strategies of nutrient regulation across the sexes to maximise male and female fitness. However, sex-specific responses to dietary manipulation are currently poorly understood. I used the geometric framework of nutrition to examine the effect of diet on reproductive effort and lifespan in male and female Australian field crickets, *Teleogryllus commodus.* I also tested how mating affected strategies of nutrient regulation across the sexes. I found that each sex had precise and distinct dietary optima for lifespan and reproduction. In both sexes, diet mediated a trade-off between longevity and reproductive effort, although this trade-off was much more pronounced in females than males. Mating shifted the dietary optima for male reproductive effort and also affected nutrient regulation across the sexes when crickets were provided with a choice of diets. While there was no sexual dimorphism in intake between virgin males and females, intake diverged post mating such that it differed significantly across the sexes and, in males, across mating treatments (i.e. virgin versus mated crickets). My results show that nutrient regulation is a flexible trait that responds to variation in mating status. Moreover, intersexual differences in nutrient regulation may reflect the influence of sexual selection on dietary intake to improve sex-specific fitness.

Keywords; ageing, geometric framework of nutrition, life-history theory, reproductive effort, sexual selection.

5.2 INTRODUCTION

In species ranging from yeast (Jiang et al. 2000) to primates (Colman et al. 2009), when animals eat a restricted diet, but not so restricted as to suffer from

malnutrition, they live longer and age more slowly than fully fed animals. This treatment, termed Dietary Restriction (DR) is the only intervention that is known to extend lifespan in both vertebrate and invertebrate taxa and so is considered an evolutionarily conserved mechanism of lifespan extension (Mair and Dillin 2008). Understanding longer lives under DR is therefore an important aim in gerontology but, despite decades of research, the association between diet, ageing and lifespan is still poorly understood (Mair and Dillin 2008; Flatt 2009; Tatar 2010).

Evolutionary explanations for longer lives under DR predict that by mimicking resource limitation in nature, DR promotes the reallocation of resources away from producing young (whose survival prospects are poor) and towards greater somatic maintenance (Harrison et al. 1989; Holliday 1989; Phelan and Austad 1989; Shanley and Kirkwood 2000; Kirkwood and Shanley 2005). This adaptive explanation is supported by a mathematical model showing that switching resources to somatic maintenance during short periods of famine may increase lifespan (Shanley and Kirkwood 2000). There is also empirical support for this adaptive explanation of lifespan extension under DR. First, DR frequently reduces reproductive effort in a number of species (e.g. Klass 1977; Chippindale et al. 1993; Kenyon et al. 1993; Bruning et al. 2000; Bishop and Guarente 2007). Second, DR may elevate somatic maintenance, for example, by increasing rates of repair of wounds (Reed et al. 1996) or damaged DNA (Haley-Zitlin and Richardson 1993). However, recent studies have challenged a key prediction of these evolutionary theories and shown that DR may increase lifespan without reducing reproductive effort (e.g. Mair et al. 2004, Grandison et al. 2008; Maklakov et al. 2008; O'Brien et al. 2008).

In fact, the traditional view that restriction of a single nutrient (e.g. calories - Masoro 2000) extends lifespan but at a cost to reproductive effort is being replaced by a more developed understanding of how multiple nutrients interact to affect different life-history traits. Recent studies have shown that it is the ratio of particular nutrients (e.g. protein to carbohydrate) that affects lifespan and reproductive effort (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Grandison et al. 2009). Moreover, each of these life-history traits may be maximally expressed at distinct ratios of key nutrients. For example, in female fruit flies (*Drosophila melanogaster*) lifespan is greatest in animals that eat a 1:16 ratio of protein to carbohydrates, however,

fecundity is optimised when females eat a 1:2 ratio of protein to carbohydrate (Lee et al. 2008). Responses to dietary manipulation are also often sex-specific (Carey et al. 2002; Magwere et al. 2004) and dietary optima for a particular life-history trait (e.g. reproductive effort) may differ across the sexes (Maklakov et al. 2008). For example, a study on the field cricket (*Teleogryllus commodus*) showed that female reproductive effort was greatest on a 1:1 ratio of protein to carbohydrate, whilst male sexual advertisement was maximised in males that consumed a higher ratio of carbohydrate (P:C 1:3) (Maklakov et al. 2008). Sex-specific dietary optima for reproductive effort may be the result of the specific nutritional demands of reproduction across the sexes, for example if females require more protein to make eggs (Williams 1996) while males require more carbohydrate to fuel metabolically expensive sexual displays (e.g. Kavanagh 1987).

Strikingly, a growing number of studies have shown that animals can precisely regulate their intake of multiple nutrients simultaneously to consume a diet that is optimal for a particular trait, such as fecundity (fruit flies, Lee et al. 2004), lifespan (field crickets, Maklakov et al. 2008) or attractiveness (cockroaches, South et al. 2010). This raises the possibility that, rather than resource allocation determining investment in lifespan versus reproductive effort (Harrison et al. 1989; Holliday 1989; Phelan and Austad 1989; Shanley and Kirkwood 2000; Kirkwood and Shanley 2005) animals can behaviourally regulate their investment in these traits by adjusting their dietary intake. If this is the case, we should expect each sex to adopt different strategies of nutrient regulation because sexual selection is predicted to drive the evolution of divergent life-history strategies in sexes (Promislow 2003; Graves 2007; Bonduriansky et al. 2008).

Sexual selection occurs because one sex (typically females) invests more resources in producing young than the other sex (typically males). This means that males may allocate extra resources towards competing for females and so reproductive success is more variable amongst males (Bateman 1948; Trivers 1972). Because their reproductive success relies upon having time to rear young and accrue resources, females are predicted to invest moderately in reproductive effort over a prolonged period of time (Bonduriansky et al. 2008). In contrast, sexual selection acting in males may promote intense investment in early reproductive effort in order to increase mating success. However, this increase in early reproductive investment

may carry a cost in terms of survival (Kokko 1997; 1998). This "live fast and die young" strategy of reproductive effort (e.g. Hunt et al. 2004; Punzalan et al. 2010) should promote the evolution of faster ageing and shorter lives in males than females (Bonduriansky et al. 2008). Alternatively, sexual selection may favour the evolution of longer lives in males if they increase their fitness by investing more in reproductive effort with age (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). Sexual selection can therefore affect the optimal life-history strategy in males or females (Promislow et al. 2003; Graves et al. 2007; Bonduriansky et al. 2008; Chapter 4) and so could affect responses to DR across the sexes (LeBourg 2010). Quantifying how males and females regulate their nutritional intake may elucidate how sexual selection promotes different life-history strategies in either sex. For example, males may consume the diet that maximises reproductive investment while females may favour the diet best for lifespan.

Maklakov et al. (2008) used the geometric framework of nutrition, a state-space modelling approach (Simpson and Raubhenheimer 1993; 1995), to test the prediction that that the sexes should be under selection for specific and divergent dietary preferences to maximise their individual fitness. Maklakov et al. (2008) found that both sexes of *T. commodus* optimised their lifespan on a high carbohydrate, low protein diet (P:C 1:3). This diet was also best for male reproductive effort. However, for females to maximise their fecundity they required greater dietary protein intake (P:C 1:1). When provided with a choice of diets, both sexes consumed a diet that increased their lifespan and male rather than female reproductive effort (P:C 1:3). This intake target was detrimental to female (but not male) fitness. Maklakov et al. (2008) concluded that either sex was constrained in their ability to regulate their dietary intake and that this indicated intralocus sexual conflict (IASC) over nutrient regulation. However, Maklakov et al. (2008) used mated females and virgin males to characterise responses to dietary manipulation and so confounded sex and mating status. In crickets, mating and mate availability affect a number of reproductive behaviours (e.g. calling effort or frequency - Hill 1998; Souroukis 1992) and therefore, may affect dietary optima for reproductive effort (Simpson et al. 2010). Mating may also affect nutrient regulation across the sexes because, if sexual selection influences inter-sexual

differences in dietary preferences, in the absence of mates both sexes may optimise their fitness by investing in a longer life until mates become available.

Here, I examine sex-specific responses to dietary manipulation and ask how mating status affects dietary optima and nutrient regulation when individuals have a choice of diets. I construct 24 synthetic diets that vary in their absolute amount of, and ratio of protein to, carbohydrate. These 24 diets correspond to four nutrient concentrations (P+C) of each of six different protein to carbohydrate ratios (P:C) (Figure 1-1, Table 2-1). I then measure the exact intake of each cricket on each of these diets and ask how diet affects lifespan and reproductive effort in each sex. I then assessed the effect of mating on male responses to dietary manipulation by comparing the responses of mated males to dietary manipulation to those of virgin males in Maklakov et al. (2008). By looking at intake of each of these nutritionally imbalanced diets, I can see how crickets manage the trade-off between over- and under- ingesting protein and carbohydrate. In a second experiment, I determined how males and females regulate their nutritional intake when allowed to make choices between alternate diets that differed in their ratio of protein to carbohydrates but with equal nutrient concentration. I then compared the strategies of nutrient regulation in virgin and mated crickets to see if and how mating affects inter-sexual differences in dietary intake.

5.3 MATERIALS AND METHODS

Making diets and measuring intake

I made twenty-four, dry granular diets that varied in both their ratio of protein to carbohydrate (P:C) and absolute amount of protein and carbohydrate (P+C) according to the established protocol detailed in Simpson and Abisgold (1985) and in Chapter 2 of this thesis (Figure 1-1, Table 2-1).

Experimental animals

Approximately 200 males and 200 gravid females were collected from Smith's Lake, New South Wales in Australia, in March 2009. Animals were maintained in 80L plastic containers that contained stacks of egg boxes for shelter, food (Friskies GoCat Senior) and water provided *ad libitum.* Females were able to lay eggs in damp cotton

wool wedged in the bottom of plastic takeaway boxes cut to a depth of ca. 2cm. For six weeks after collection, animals were maintained in these panmictic populations and their eggs air freighted weekly back to the laboratory at the University of Exeter, Tremough Campus. Crickets have since been maintained in large populations, of ca. 500 animals, for three generations.

In this experiment, crickets from this Smith's Lake population were collected on their day of hatching. Individual nymphs were housed in $5cm^3$ plastic containers at 28°C under 12.5L:11.5D and provided with water in a 2.5ml test tube plugged with cotton wool and a piece of egg carton for shelter. Nymphs were cleaned and fed Friskies GoCat Senior weekly: for the first three weeks food was provided ground in the lid of a 1.5ml eppendorf and then each animal was given two whole pellets of cat food each week. When nymphs reached their final developmental instar they were checked daily for eclosion as adults.

On eclosion, adult crickets were weighed and their pronotum measured using a binocular microscope and an eye-piece graticule. Crickets were then randomly assigned to a dietary treatment and provided with one container of food of known dry mass on their first day of adulthood. De-ionized water was provided in the upturned plastic lid of a vial (1.6cm diameter, 1.6cm deep) glued using epoxy resin to the middle of a plastic petri dish (5.5cm in diameter). Animals were given either one (Experiment 1, no choice) or two (Experiment 2, intake under choice) feeding platforms (vial lid: 2.1 cm diameter, 1.2 cm deep petri dish: 5.5cm in diameter, 1.2cm deep) containing a particular food and a one inch length of plastic tubing as shelter. The materials used for providing shelter and for feeding dishes meant that experimental animals could not consume anything other than the diets provided. The design of the feeding vials meant that any food spilt was collected in the petri dish and therefore could be weighed.

Irrespective of whether animals were used for Experiment 1 or 2, the feeding and mating protocols were as follows: food was removed and replaced on day three post eclosion. On day six, food was removed and a stock mate introduced to the container. During this mating window, experimental animals were provided with de-ionised water and new food was only introduced when the mate was removed on day seven post eclosion. This weekly feeding cycle was repeated throughout the

experimental animal's lifetime. Animals were checked and cleaned daily and if their food was running low, new food was provided. This ensured that each cricket had a constant supply of food and could therefore consume as much as required.

Once feeding platforms were removed from the experimental animal, faeces were removed using a pair of fine forceps. The platform was then kept in a drying oven at 30°C for 36 hours to remove moisture prior to weighing. Consumption of diet was calculated as the dry weight of food provided to the experimental animal minus that remaining after feeding. This information was converted to a weight of protein and carbohydrate consumed (e.g., 5 mg of 15P:45C ingested equals 0.75 mg of protein and 2.25 mg of carbohydrates, the remaining 40% of the diet being indigestible cellulose and vitamins). Daily consumption was calculated as the sum of diet consumed by an individual over its lifetime divided by its longevity.

Measuring reproductive effort and survival

Adult mortality was checked daily from eclosion. Reproductive effort of male and female *T. commodus* was measured on days 3 and 7 post eclosion, and weekly thereafter (i.e. day 14, 21, 28 etc.) To ensure female reproductive effort could be assayed and then intersexual comparisons made, both males and females were mated. 24 hours prior to each period that reproductive effort was sampled (i.e. days 6, 13, 20 etc), every experimental animal was paired with a cricket of the opposite sex taken from the Smith's Lake stock population to mate. Pairs were then separated and all experimental females were provided with a small petri-dish (5cm diameter) full of moist sand for oviposition. Females had an egg pad in which to lay on all days apart from that when she was paired with a male. To count eggs, the content of each petri-dish was emptied into a container of water, swirled and the eggs removed with fine forceps and counted.

In male crickets, the amount of time spent calling is a good measure of mating success because females strongly prefer males that call more in both the lab and in the field (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muňoz et al. 2010) and calls are metabolically expensive to produce (e.g. Kavanagh 1987). Consequently, I measured the amount of time each male spent calling on a given night (hereafter referred to as

calling effort) using a custom-built electronic monitoring device and the same protocol as that detailed in Chapter 3.

Experiment 1. No choice on six nutritional rails at four concentrations.

On eclosion, nymphs were randomly allocated to one of the 24 dietary treatments (Figure 1-1, Table 2-1). Animals were excluded from analyses if they escaped or died in the 48 hours following eclosion. The total sample size was 430 animals, of which 208 were males and 222 females. Animals were subject to the feeding and mating regime described previously, with dietary intake and reproductive effort being measured over the animal's entire lifespan.

Experiment 2. Dietary intake under choice.

I provided 40 animals of each sex with two diets of equal nutritional value (P+C) but that differed in their ratio of protein to carbohydrate (P:C). This corresponded to diets 4 and 24 (Figure 1-1, Table 2-1). In each sex, 20 of the experimental animals were either mated once a week, as in Experiment 1, or left as virgins, but otherwise subject to the same feeding protocol as in Experiment 1.

Statistical analyses

Experiment 1. No choice on six nutritional rails at four concentrations.

I used a multivariate response-surface approach (Lande and Arnold 1983) to estimate and visualize the linear and nonlinear effects of protein and carbohydrate intake on my response variables (i.e. lifespan and reproductive effort). Response surfaces were fitted in SPSS (version 15) and visualized using nonparametric thin-plate splines in R (version 2.5.1). I used a sequential model building approach to assess whether the linear and nonlinear effects of protein and carbohydrate ingestion differed for my response variables (Draper and John 1988; Chenoweth and Blows 2005). As my different responses variables were measured in different units (e.g. number of eggs laid versus seconds spent calling), it was necessary to standardize them for statistical comparison. For this reason, I standardized each response variable to a mean of zero and standard deviation of one using a *Z*- transformation prior to analyses. All analyses were conducted on *Z*-transformed data, although figures were

presented using unstandardized data to facilitate interpretation. I then included a dummy variable, response type (*RT*), in a reduced model containing only the standardized linear terms:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \varepsilon \quad \text{(Eq. 1)}$$

where *R* is the standardized response measure, N_i refers to the intake of the *i*th nutrient, *n* represents the number of nutrients contained in the model and ε is the unexplained error. From Eq. 1, the unexplained (i.e. residual) sums of squares for this reduced model (SS_r) was compared to the same quantity (SS_c) from a second (complete) model that included all of the terms in (1) with the addition of the terms $\alpha_i N_i RT$ which represents the linear interaction of *RT* and the *i*th nutrient.

$$W = \beta_0 + \alpha_0 Sex + \sum_{i=1}^{n} \beta_i L_i + \sum_{i=1}^{n} \alpha_i L_i Sex + \varepsilon \quad \text{(Eq. 2)}$$

A partial *F*-test (Bowerman and O'Connell 1990) was used to compare SS_r and SS_c from Eq.1 and Eq.2 respectively:

$$F_{a,b} = \frac{(SS_r - SS_c)/a}{SS_c/b} \quad \text{(Eq. 3)}$$

where *a* is the number of terms that differ between the reduced and complete model and *b* is the error degrees of freedom for SS_c.

To test whether the quadratic effect of nutrient intake differed between response variables, the SS_r from the reduced model:

$$W = \beta_0 + \alpha_0 Sex + \sum_{i=1}^{n} \beta_i L_i + \sum_{i=1}^{n} \alpha_i L_i Sex + \sum_{i=1}^{n} \beta_i L_i^{\,2} + \varepsilon \quad \text{(Eq. 4)}$$

was compared to the SS_c of the complete model:

$$W = \beta_0 + \alpha_0 Sex + \sum_{i=1}^{n} \beta_i L_i + \sum_{i=1}^{n} \alpha_i L_i Sex + \sum_{i=1}^{n} \beta_i L_i^{\,2} + \sum_{i=1}^{n} \alpha_i L_i^{\,2} Sex + \varepsilon \quad \text{(Eq. 5)}$$

using Eq. 3.

To test whether correlational effects of nutrient intake on response variables differed, the SS_r from the reduced model:

$$W = \beta_0 + \alpha_0 Sex + \sum_{i=1}^{n} \beta_i L_i + \sum_{i=1}^{n} \alpha_i L_i Sex + \sum_{i=1}^{n} \beta_i L_i^{\,2} + \sum_{i=1}^{n} \alpha_i L_i^{\,2} Sex + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} \beta_{ij} L_i L_j + \varepsilon$$

(Eq. 6)

was compared to the SS_c of the complete model:

$$W = \beta_0 + \alpha_0 Sex + \sum_{i=1}^{n} \beta_i L_i + \sum_{i=1}^{n} \alpha_i L_i Sex + \sum_{i=1}^{n} \beta_i L_i^{\,2} + \sum_{i=1}^{n} \alpha_i L_i^{\,2} Sex + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} \beta_{ij} L_i L_j + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} \alpha_{ij} L_i L_j Sex + \varepsilon$$

(Eq. 7)

using Eq. 3.

In summary, comparing model Eq. 1 versus Eq. 2, Eq. 4 versus Eq. 5, and Eq. 6 versus Eq. 7 provides a test for the overall significance of the interaction between response type and the linear, quadratic and correlational effects of nutrient intake, respectively. Therefore, significant differences in these model comparisons, as detected with a partial *F*-test, demonstrate that the linear, quadratic and/or correlational effects of nutrient intake on the response variables differ, respectively. I also inspected the interaction of individual nutrients with the response variable terms from the full model (Eq. 7) to determine which of the nutrients were responsible for the significance of the overall partial *F*-test.

Regulation of dietary intake when constrained on a single, nutritionally imbalanced diet

To examine how male and female crickets trade-off over- versus under-ingesting protein and carbohydrate when constrained on a single, nutritionally imbalanced diet, I calculated the average intake array from the male and female data used to create the response surfaces (see Figure 5-5). I first estimated the slope of the average intake array for males and females (and tested this they were significantly different from zero) and then tested whether the actual slope of the intake array (β_a) deviated significantly from the hypothetical slope of -1 (β_h) using a *t*-test where (β_a-β_h)/($SE_{\beta a}$) approximates a *t*-distribution with *n*-2 degrees of freedom (Zar, 1999). A slope of -1 is expected if crickets are maintaining a constant intake of nutrients or energy (as 1 gram of protein and carbohydrates yields the same amount of energy). Analysis of Covariance (ANCOVA), including sex as a fixed effect, protein intake as the

covariate and carbohydrate as the response variable, was used to determine if the slope of the average intake array differed for the sexes.

To further examine how tightly either sex regulate their intake of protein and carbohydrate, I calculated the coefficient of variation (*CV* = SD/mean) of daily protein and carbohydrate intake in each sex and compared these using a variance ratio test, where $F_{a,b} = (CV_a/CV_b)$ or $F_{b,a} = (CV_b/CV_a)$ whichever is the largest *CV* (CV_a or CV_b) (Zar 1999). I also compared the *CV* for daily protein and carbohydrate intake across the sexes to see whether males and females differed in their ability to regulate these nutrients. The reason for using *CV* (instead of variance or standard deviation) was that female daily intake of protein and carbohydrates was significantly greater than for males (see average intake array) and variance increases directly with the mean (Zar 1999).

Experiment 2. Dietary intake under choice

To determine if crickets were eating diets 4 and 24 non-randomly, I calculated the expected daily intake (per mg of cricket) of protein and carbohydrates if crickets fed at random on the two diets. To do this, I halved the total amount of both diets consumed and then converted this to the amount of protein and carbohydrates ingested (following the protocol outlined in South et al. 2011). These expected amounts were subtracted from the observed intake of protein and carbohydrates and the difference tested against a mean of zero using a one-sample *t*-test.

To analyse nutrient intake when animals were provided with a choice of diets I used MANOVA to account for the fact that consumption of protein and carbohydrate were correlated within an individual. I incorporated sex, age and mating treatment (virgin / mated) into my analyses. In all analyses, I used daily intake of protein and carbohydrate as my explanatory variables but scaled this measure for body mass because crickets fed the different diets have different lifespan and longer lived crickets have a high total intake of nutrients in both males (β ± SE, 0.037 ± 0.003, $t_{207} = 11.658$, $P = 0.0001$) and females (β ± SE, 0.040 ± 0.006, $t_{221} = 7.252$, $P = 0.0001$). I also scaled for the body weight of the cricket because the daily intake of diet increased significantly with body mass in both males (β ± SE, 0.051 ± 0.021, $t_{207} = 2.458$, $P = 0.015$) and females (β ± SE, 0.056 ± 0.027, $t_{221} = 2.113$, $P = 0.036$).

Due to the imbalanced number of crickets in each treatment, I analysed the trajectory for the cumulative intake of protein and carbohydrates using a repeated measures MANOVA (O'Brien and Kaiser 1985) implemented in JMP (version 8.0.2, SAS Institute, Inc). Sex and mating status, plus their interaction, were included as fixed effects in the model and the cumulative daily intake (per mg of cricket) of either protein or carbohydrates across the five feeding periods were included as the response variables (i.e. the repeated measures). I first ran this overall model with all possible interactions (Table 5-2) and, due to the significant interaction terms (see below), I followed this with specific paired post-hoc comparisons to determine where differences occurred (e.g. virgin males versus mated males). In instances where there were significant interactions between time and one (or both) of the fixed effects, indicating a difference in the slope of the relationship between daily protein and carbohydrate intake over time, I sequentially added consecutive feeding periods to the model to determine the time at which the two feeding trajectories differed (i.e. when the fixed effect x time interaction became significant). In each of the paired comparisons for intake target ratios, I ran repeated-measures MANOVA that included the single, main effect differing in the comparison (e.g. sex in Figure 5-3A), time and their interaction for both nutrients.

In addition to analysing the nutrient trajectories over time, I also analysed the final intake target of male and female crickets using a MANOVA that included sex and mating status, plus their interaction, as fixed effects and the total daily intake of protein and carbohydrate as the response variables. This MANOVA was followed up with univariate ANOVAs to determine which response variables contributed to the overall multivariate effect.

5.4 RESULTS

Experiment 1. No choice experiment on six nutritional rails at four nutrient dilutions

Linear analyses of lifespan response surfaces showed that greater daily carbohydrate consumption extended female lifespan. Daily carbohydrate also affected female lifespan via a negative quadratic term, illustrating an optima on the nutrient landscape for daily carbohydrate intake. Protein consumption influenced female

survival but only via a negative correlational term with carbohydrate, showing that lifespan was greatest on high carbohydrate, low protein diets (P:C ratio of 1:8) (Figure 5-1B, Table 5-1). However, the nutritional landscape for female fecundity showed that females maximised their egg production on intermediate ratios of protein to carbohydrate (P:C 1:1). Linear analyses showed a significant increase in egg production with greater consumption of both daily protein and daily carbohydrate and that the effects of each of these nutrients on fecundity were of a similar magnitude. There were also significant, negative non-linear effects of both protein and carbohydrate illustrating the presence of fecundity peaks for both protein and carbohydrate (Figure 5-1D, Table 5-1).

In males, the peak for lifespan was intermediate between two nutritional rails (P:C 1:1 and 1:3). Linear analyses showed that both daily protein and carbohydrate intake had a positive effect on male lifespan but that lifespan increased more per unit carbohydrate consumed, than per unit protein. Negative quadratic terms for both protein and carbohydrate consumption on lifespan showed clear peaks for each nutrient (Figure 5-1A, Table 5-1). Male reproductive effort was greatest in the region of 1:1 P:C and improved with greater intake of both nutrients, although the linear effect of carbohydrate was greater than that of protein. Negative quadratic terms for both protein and carbohydrate consumption on reproductive effort showed clear peaks for both protein and carbohydrate (Figure 5-1C , Table 5-1).

Comparison of nutrient landscapes across traits within the sexes

Male lifespan versus daily reproductive effort

There was a significant difference in the linear effects of daily protein and carbohydrate intake on lifespan and daily reproductive effort in males ($F_{2,410}$ = 3.919, P = 0.021), resulting from the fact that lifespan was more responsive to the daily intake of carbohydrates than daily reproductive effort was (daily protein intake: $F_{1,410}$ = 1.028, P = 0.311; daily carbohydrate intake: $F_{1,410}$ = 6.386, P = 0.012). There was also a significant difference in the quadratic effect of daily protein and carbohydrate intake on lifespan and daily reproductive effort in males ($F_{2,406}$ = 6.285, P = 0.0021) due to the fact that curvature of the landscape for daily protein intake was more pronounced for lifespan than daily reproductive effort (daily protein intake: $F_{1,406}$ = 8.459, P = 0.004;

daily carbohydrate intake: $F_{1,406} = 0.136$, $P = 0.713$). However, the correlational effects of daily protein and carbohydrate intake did not differ significantly for lifespan and daily reproductive effort ($F_{1,404} = 0.245$, $P = 0.621$).

Female lifespan versus daily reproductive effort

There was a significant difference in the linear effects of daily protein and carbohydrate intake on lifespan and daily reproductive effort in females ($F_{2,442} = 44.219$, $P = 0.0001$). Examination of the univariate interaction terms from the linear model, showed that this overall difference was the result of daily protein intake having a significant positive effect on female daily reproductive effort but no effect on lifespan ($F_{1,442} = 53.190$, $P = 0.0001$). Additionally, lifespan was more responsive to carbohydrate intake than daily reproductive effort was ($F_{1,442} = 6.631$, $P = 0.010$). There were also significant differences in the quadratic effects of daily protein and carbohydrate intake on lifespan and daily reproductive effort ($F_{2,438} = 4.116$, $P = 0.016$), because the curvature of the landscape for daily carbohydrate intake was more pronounced for lifespan than daily reproductive effort (daily protein intake: $F_{1,438} = 1.287$, $P = 0.257$; daily carbohydrate intake: $F_{1,438} = 6.340$, $P = 0.012$). The correlational effect of daily protein and carbohydrate intake on lifespan and daily reproductive effort differed significantly ($F_{1,436} = 9.404$, $P = 0.002$) as a consequence of significant negative covariation between the effect of these nutrients for lifespan but not for daily reproductive effort (Table 5-1).

Comparison of nutrient landscapes between the sexes

Male versus Female lifespan

There was a significant difference in the linear effects of daily protein and carbohydrate intake on lifespan across the sexes ($F_{2,426} = 23.593$, $P = 0.0001$). This was driven primarily by the fact that male lifespan increased with daily protein intake but female lifespan did not (daily protein intake: $F_{1,426} = 44.840$, $P = 0.0001$; daily carbohydrate intake: $F_{1,426} = 1.974$, $P = 0.161$). There was also a significant difference in the quadratic effects of these nutrients on lifespan across the sexes ($F_{2,422} = 9.642$, $P = 0.0001$) because the curvature of the landscape for daily protein intake and daily carbohydrate intake were more pronounced in females than males (daily protein

intake: $F_{1,422}$ = 15.274, P = 0.0001; daily carbohydrate intake: $F_{1,422}$ = 7.563, P = 0.006). The correlational effects of daily protein and carbohydrate intake on lifespan also differed significantly across the sexes ($F_{1,420}$ = 8.063, P = 0.005) with the negative covariance between these nutrients being stronger in females than in males (Table 5.2).

Male versus Female daily reproductive effort

There was a significant difference in the linear effects of daily protein and carbohydrate intake on daily reproductive effort in the sexes ($F_{2,426}$ = 3.956, P = 0.019) and this was primarily driven by daily reproductive effort being significantly more responsive to the daily intake of protein in females than males (daily protein intake: $F_{1,426}$ = 5.139, P = 0.024; daily carbohydrate intake: $F_{1,426}$ = 1.272, P = 0.260). There was, however, no significant differences in the quadratic ($F_{2,422}$ = 0.061, P = 0.940) or correlational ($F_{1,420}$ = 0.218, P = 0.641) effects of daily protein and carbohydrate intake on daily reproductive effort across the sexes.

Nutrient regulation without diet choice

In both sexes, the slope of the average intake array was negative and differed significantly from a slope of zero (β ± SE, males: -0.852 ± 0.042, t_4 = 20.469, r^2 = 0.991, P = 0.0001; females: -0.760 ± 0.030, t_4 = 25.137, r^2 = 0.994, P = 0.0001). Furthermore, the slope of the average intake array differed significantly from a slope of -1 for both males (t_4 = 3.523, P = 0.024) and females (t_4 = 8.00, P = 0.0012) suggesting that neither sex was regulating their dietary intake to maintain a constant intake of nutrients or energy. The slope of these intake arrays suggest that males and females, when not given the choice of actively choosing between diets, gave greater weight to the regulation of carbohydrates than protein. ANCOVA revealed a significant effect of sex ($F_{1,8}$ = 93.837, P = 0.0001) and daily protein intake ($F_{1,8}$ = 959.181, P = 0.0001) on the daily intake of carbohydrates but the interaction between these variables ($F_{1,8}$ = 3.148, P = 0.114) was not significant demonstrating that the slopes of the average intake array for males and females does not differ.

As predicted from the slope of the intake array, the *CV* for daily protein intake was significantly greater than the *CV* for daily carbohydrate intake in both males (CV_{protein} = 0.832, $CV_{\text{carbohydrates}}$ = 0.599, $F_{208,208}$ = 1.389, P = 0.009) and females (CV_{protein}

= 0.840, $CV_{carbohydrates}$ = 0.594, $F_{222,222}$ = 1.413, P = 0.005). This further suggests that both sexes were prioritising regulating carbohydrates over protein. The CV for daily protein intake ($F_{222,208}$ = 1.009, P = 0.474) and for daily carbohydrate intake ($F_{208,222}$ = 1.008, P = 0.476) did not differ significantly between the sexes, suggesting males and females were equally as efficient at regulating the intake of these nutrients.

Experiment 2. Nutrient regulation when given dietary choice

Non-random diet consumption

A paired *t*-test that compared the absolute amounts of diets 4 and 24 consumed, showed that crickets, irrespective of sex or mating status, consumed significantly more of diet 24 than diet 4 (virgin females: t_{15} = 4.912, P = 0.0001; mated females: t_{15} = 3.902, P = 0.001; virgin males: t_{21} = 3.925, P = 0.001; mate males: t_{18} = 5.081, P = 0.0001; Figure 5-3A). The pattern of dietary choice led to a significantly higher daily intake of carbohydrates than expected if crickets fed at random (virgin females: t_{15} = 4.912, P = 0.0001; mated females: t_{15} = 3.902, P = 0.001; virgin males: t_{21} = 3.925, P = 0.001; mate males: t_{18} = 5.081, P = 0.0001) and a significantly lower daily intake of protein (virgin females: t_{15} = 4.912, P = 0.0001; mated females: t_{15} = 3.902, P = 0.001; virgin males: t_{21} = 3.925, P = 0.001; mate males: t_{18} = 5.081, P = 0.0001) (Figure 5-2B).

Cumulative intake of nutrients under dietary choice

Table 5-2 shows that the cumulative intake of both nutrients increased with time. The intake of nutrients over time, however, did not differ significantly with either sex or mating status *per se*, but did via interactions (Table 5-2). For the daily intake of protein, there were significant interactions between sex and mating status, between mating status and time and between sex, mating status and time (Table 5-2). The latter two interactions demonstrate that the slope of the cumulative intake of protein differs across my main effects. For the daily intake of carbohydrates, there were significant interactions between sex and time and between mating status and time (Table 5-2), which both demonstrate that the slope of the cumulative intake of carbohydrates differs across my main effects. Collectively, these interactions highlight

a complex pattern that requires further *post-hoc* analysis to determine the origin of these differences. These paired comparisons can be visualized in Figure 5-3.

The cumulative intake of protein was higher for mated males than mated females (Figure 5-2A, P:C ratio, mated males = 1:1.91, mated females = 1:2.70), for virgin females than mated females (Figure 5-2C, P:C ratio, virgin females = 1:2.18, mated females = 1:2.70) and for mated males than virgin males (Figure 5-3D, P:C ratio, virgin males = 1:2.00, mated males = 1:1.91)(Table 5-2). The cumulative intake of carbohydrates, however, did not differ significantly across these paired comparisons (Table 5-3). Neither the cumulative intake of protein or carbohydrates differed significantly between virgin male and female crickets (Figure 5-3B, P:C ratio, virgin males = 1:2.00, virgin females = 1:2.18). For those paired comparisons with significant differences in the cumulative daily intake of protein, I ran the same repeated-measures MANOVA model but added data for consecutive feeding periods to the model to determine the time point at which the interaction between the fixed effect (sex or mating status) and time became significant (i.e. the intake trajectory diverged across the fixed effect). In each instance, the interaction between the fixed effect and time became significant with the addition of data from the third feeding period, which corresponds to the first feeding period after mating (mated male vs mated female: feeding periods 1 & 2: $F_{1,33} = 2.630$, $P = 0.114$; feeding periods 1, 2 & 3: $F_{2,32} = 22.243$, $P = 0.0001$; mated female vs virgin female: feeding periods 1 & 2: $F_{1,30} = 0.022$, $P = 0.882$; feeding periods 1, 2 & 3: $F_{2,29} = 11.404$, $P = 0.0002$; male male vs virgin male: feeding periods 1 & 2: $F_{1,39} = 1.781$, $P = 0.190$; feeding periods 1, 2 & 3: $F_{2,38} = 3.351$, $P = 0.046$).

Intake target of nutrients under dietary choice

MANOVA revealed that although sex or mating status did not significantly influenced the total intake of nutrients *per se* (sex: Pillai's Trace = 0.051, $F_{2,68} = 1.823$, $P = 0.169$; mating status: Pillai's Trace = 0.054, $F_{2,68} = 1.940$, $P = 0.152$), there was a significant interaction between these two fixed effects (Pillai's Trace = 0.092, $F_{2,86} = 3.462$, $P = 0.037$). Univariate ANOVAs revealed that this effect was driven by the total cumulative intake of protein ($F_{1,69} = 6.982$, $P = 0.010$) but not carbohydrates ($F_{1,69} = 0.077$, $P = 0.782$). ANOVA and Fisher's LSD post-hoc analyses showed that mated

males had a significantly higher total daily intake of protein than crickets in the other 3 treatment groups (all *P* values < 0.05, Figure 5-4).

5.5 DISCUSSION

In a broad range of species, diet affects lifespan and reproductive effort (reviewed in Mair and Dillin). Recent work has suggested that sexual selection should promote different strategies of age-dependent reproductive investment across the sexes, which in turn, should affect the evolution of ageing and lifespan in males and females (Bonduriansky et al. 2008). If either sex has different life-history strategies, then responses to dietary manipulation and strategies of nutrient regulation should differ across the sexes. However, very little work has asked how either sex manages their dietary intake to affect their sex-specific fitness. I examined how lifespan and reproductive effort respond to dietary manipulation in the Australian field cricket, *Teleogryllus commodus.* I then tested how either sex regulated their dietary intake under choice and how this affected male and female life-history strategies. Finally, I explored how mating affected nutrient regulation across the sexes.

My results show that in both sexes, lifespan was optimised on high concentration diets containing a low ratio of protein to carbohydrate. However, there were inter-sexual differences in the nutritional landscapes for lifespan because male, but not female, longevity was improved by eating moderate amounts of protein. This result supports several recent studies showing that in insect species including flies (Lee et al. 2008; Fanson et al. 2009), ants (Dussutour and Simpson 2009) and bees (Pirk et al. 2010), lifespan is greatest on high carbohydrate, low protein diets. As predicted by evolutionary theory (Williams 1957; Kirkwood 1977; Roff 1992; Stearns 1992), I found some evidence for a trade-off between reproductive effort and lifespan because in both sexes longevity and reproductive effort had distinct dietary optima. However, this trade-off was much more pronounced in females than males. Traditionally, trade-offs have been considered in terms of resource allocation, the assumption being that a finite amount of a particular resource (e.g. calories) is divided up amongst different, competing traits (e.g. fecundity versus lifespan). The greater proportion of this resource allocated to a particular trait, the greater its expression (Roff 1992; Stearns 1992). However, my results add to a growing body of research showing that, rather

than calories mediating the trade-off between reproductive effort and lifespan, each of these traits may have different, multi-nutritional dietary optima (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Grandison et al. 2009). This means that the expression of each of these traits is decided at the point when nutrients are consumed (Cotter et al. 2011). For example, individual *T. commodus* could preferentially consume high dietary protein to improve their reproductive effort or eat a high carbohydrate to improve their survival.

My results are very similar to those of Maklakov et al. (2008) who found that in both male and female *T. commodus,* lifespan was optimised on high carbohydrate, low protein diets (P:C 1:3). As carbohydrate intake increased beyond this dietary optimum, male lifespan began to decline but female lifespan did not. This previous study also found that female fecundity peaked on a 1:1 P:C ratio (Maklakov et al. 2008). The only pronounced difference between the nutritional landscapes produced in my study and that of Maklakov et al. (2008) concerns the diet optimal for male calling effort. Maklakov et al. (2008) found that calling effort peaked on the same diet as that for male lifespan (P:C 1:3). However, I found that investment in calling was greatest in males which consumed a 1:1 P:C ratio. This difference between the current study and Maklakov's et al. (2008) result is most likely because in the latter study males were virgins but in my study, all males were mated to ensure that the full costs of reproduction were experienced by both sexes.

There are good reasons to expect nutritional landscapes to respond to changes in mating status. In male crickets, mate availability affects the expression of a number of costly, reproductive behaviours. Specifically, in the presence of females male crickets are more likely to call (Sadowski et al. 2002), call more intensely (Souroukis et al. 1992; Hill 1998) and increase both the rate (Reinhardt and Siva-Jothy 2005) and quality (Dowling and Simmons 2012) of sperm production. These reproductive traits may have specific nutritional demands, for example, carbohydrate may be needed to fuel energetically expensive advertisement calls (Kavanagh 1987) and protein to manufacture spermatophores (Simmons 1993; Wedell 1994). If mating affects the expression of reproductive behaviours, it could shift the dietary optimum for reproductive effort away from high carbohydrate diets (as in Maklakov et al. 2008) and towards higher protein diets (this study). The greater reliance on dietary protein for

calling effort seen in my study may reflect the nutritional demands of regularly producing protein rich spermatophores (Wedell 1994) as well as metabolically costly advertisement call (Prestwich and Walker 1981; Kavanagh 1987; Hoback and Wagner 1997).

The hypothesis that mating changes the nutritional landscape for male reproductive effort is further endorsed by my finding that mating affects dietary intake under choice. Foremost, I found active regulation of nutritional intake in both sexes because both males and females consistently over-consumed carbohydrate whilst under-consuming protein when allowed a choice of diets. This is in keeping with a number of studies showing that species, including cockroackes (South et al. 2011), predatory beetles (Jensen 2011), spiders (Jensen 2012), caterpillars (Lee et al. 2006), ants (Dussutour and Simpson 2009), cats (Hewson-Hughes et al. 2011) and primates (Felton 2009), actively regulate their nutritional intake when provided with a choice of foods. Importantly, I show that mating caused male and female intake trajectories to diverge. Prior to mating, the intake for male and female crickets was similar across treatment groups (i.e. mated versus virgin animals). Different strategies of nutrient regulation emerged only after animals had mated. These divergent intake trajectories resulted in significant differences in the intake targets (i.e. final ratio of protein to carbohydrate consumed) across the sexes, whereby, mated males consumed greater dietary protein than virgin males and virgin or mated females. Although I only detected very mild divergence in intake trajectories over time, these intersexual differences help highlight the adaptive significance of sex-specific strategies of nutrient regulation and illustrate a mechanism allowing either sex to manage their life-history strategies.

First, my finding that mating changes dietary intake under choice supports recent studies showing that dietary intake is a plastic trait that responds to individual condition (e.g. immune challenges, Lee et al. 2006; Povey et al. 2009) or social environment (e.g. presence of brood, Dussutour and Simpson 2009). However, this study is the first, to my knowledge, to show that mating affects strategies of nutrient regulation. Secondly, the difference in dietary intake under choice between the sexes argues against either sex being constrained in their dietary choices. Maklakov et al. (2008) found that, despite divergent dietary optima for lifetime reproductive effort,

females and male consumed the same diet when allowed choice. This carried a pronounced cost to female, but not male, fitness. The authors hypothesised that females were unable to feed towards the diet that optimised their sex-specific fitness because of intralocus sexual conflict (IASC). IASC occurs whenever traits shared by the sexes are subject to contrasting selection but have a common genetic basis, which prevents the sexes from evolving independently to their own phenotypic optimum (Bonduriansky and Chenoweth 2009). To show the presence (or absence) of IASC over dietary intake, requires formal estimation of both the fitness surfaces for diet and the intersexual genetic correlations between dietary choice (e.g. Lewis et al. 2011). Maklakov et al. (2008) did not examine the genetic basis of dietary choice and so could not test for intralocus conflict. Therefore, while they showed that females chose to eat more carbohydrate than was optimal for their lifetime fecundity, they did not show that this was due to genetic constraints on nutrient regulation. Instead, female feeding decisions could be influenced by some other factor, for example, perhaps eggs provisioned with greater amounts of dietary carbohydrate have a survival advantage in the field, for example, nymphs are larger at eclosion. While I did not examine the genetic basis of nutrient regulation either, the sexual dimorphism I found in dietary intake shows that either sex can make different dietary choices and therefore, argues against IASC over nutrient regulation in this species. Finally, intersexual differences in dietary intake shed some light on the selective pressures shaping intake targets in either sex. The intake target for females falls within the contour lines surrounding the dietary optima for female lifespan, suggesting that females feed towards a peak that is optimal for their lifespan rather than fecundity. In contrast, male intake targets fall somewhere between the peaks for lifespan and reproductive effort. The female strategy is in keeping with predictions of sexual selection, which predict that females are more likely to invest in a long life than intensive reproductive effort because their fitness requires time to acquire resources to produce young (Bonduriansky et al. 2008). In males, the dietary optima for lifespan and reproductive effort do not overlap but are in similar regions of the nutritional landscape. Therefore, by feeding between these two peaks and maximising their lifetime reproductive success by achieving an optimal compromise between longevity and reproduction. However, more work is needed to

examine the adaptive significance of inter-sexual divergence in strategies of nutrient regulation.

In addition to showing regulation of dietary intake when allowed a choice of diets, crickets also regulated their intake when restricted to a single nutritionally imbalanced diet. The intake array showed that crickets regulated their intake of carbohydrate more precisely than their protein intake. This illustrates that crickets fed low carbohydrate diets could over-consume proteins to gain more carbohydrate. In contrast, when crickets were provided with high carbohydrate diets and protein was the limiting nutrient, they were less likely to over-eat carbohydrates to gain protein. This is an unusual strategy of regulation for an omnivore: in many herbivorous and omnivorous species, evolved on protein poor diets, excess protein consumption is detrimental to survival (e.g. Lee et al. 2008; Fanson et al. 2009; Simpson and Raubenheimer 2009; Pirk et al. 2010; Dussutour and Simpson 2012). Species from these feeding guilds are more likely to over-eat carbohydrate than protein (i.e. regulate protein more tightly than carbohydrates) to avoid the costs of excessive protein consumption (e.g. Lee et al. 2002; 2003). This is the opposite of the pattern I see in *T. commodus,* which adopts a pattern of nutrient regulation like that more frequently seen in predatory species. Predators, evolved on protein rich diets (Denno and Fagan 2003), have physiological mechanisms to process high amounts of dietary protein, for example, they may be better able to extra energy from proteins (Hewson-Hughes et al. 2011). Predators are therefore more likely to over-ingest protein to gain limiting nutrients (Mayntz et al. 2005; Hewson-Hughes et al. 2011; Jensen et al. 2011; 2012). While the intake array for predacious species illustrates they have a greater capacity to over-consume proteins than *T. commodus*, these results are still surprising. However, crickets, including *T. commodus*, engage in cannibalism, consume protein rich mammal faeces and eat their shed exoskeleton post-eclosion (Simpson et al. 2006; Maklakov et al. 2008; Archer *pers. obvs.*). This suggests that crickets rectify protein deficits in the field by eating very large volumes of protein rich food (e.g. immobilised conspecifics, Simpson et al. 2006). Crickets must therefore have evolved physiological mechanisms for processing high protein diets. In agreement with this, the costs of elevated protein intake I see in this study are relatively minor. In fact, protein intake has a positive effect on reproductive effort across the sexes and male lifespan.

However, in all traits measured (apart from female fecundity) the benefits of high carbohydrate intake outweigh are greater than any positive effects of protein consumption. The pattern of nutrient regulation shown by *T. commodus* may reflect that the fitness benefits of regulating carbohydrate consumption outweigh the costs of excessive protein intake.

In conclusion, my experiment demonstrates for the first time that mating status affects responses to dietary manipulation and shapes differences in how the sexes behaviourally regulate their dietary intake and, in turn, expression of life history traits. These findings challenge the hypothesis that IASC constrains either sex from reaching their specific dietary optimum in *T. commodus*. However, future work should track male and female nutrient regulation over their lifetime to better infer the life-history consequences of these divergent strategies. Studies should also explicitly examine the role (if there is one) of IASC in nutrient regulation. However, my results highlight the importance of considering mating and sexual selection when examining the association between nutrition and life-history strategies. I show that behavioural regulation of diet may be a proximate mechanism by which sexual selection shapes life-history strategies across the sexes.

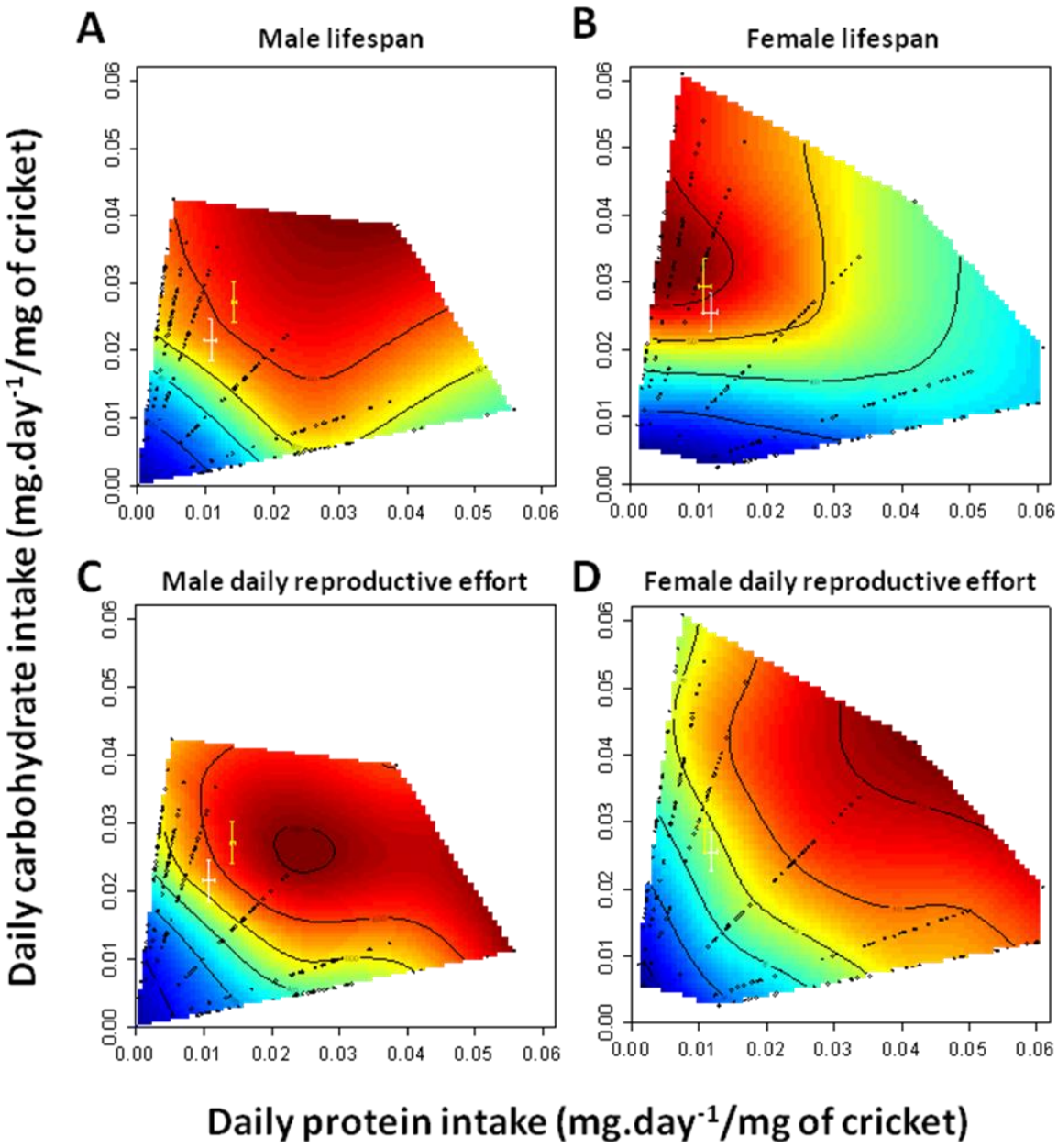

Figure 5-1. Nutritional landscapes illustrating the effects of daily protein and carbohydrate intake (per mg of cricket) on lifespan and daily reproductive effort in male and female crickets *(Teleogryllus commodus).* (A) male lifespan, (B) male daily reproductive effort; time spent calling, (C) female lifespan and (D) female daily reproductive effort; egg production. On each landscape, high values for these response variables are given in red and low values in blue, whilst black dots represent the intake values for individual crickets. The white symbols on each landscape represents the mean regulated intake point (±SE) when virgin crickets are given a choice between diets (diets 4 and 24) in my choice experiment, while the yellow symbols represent this same point for mated crickets.

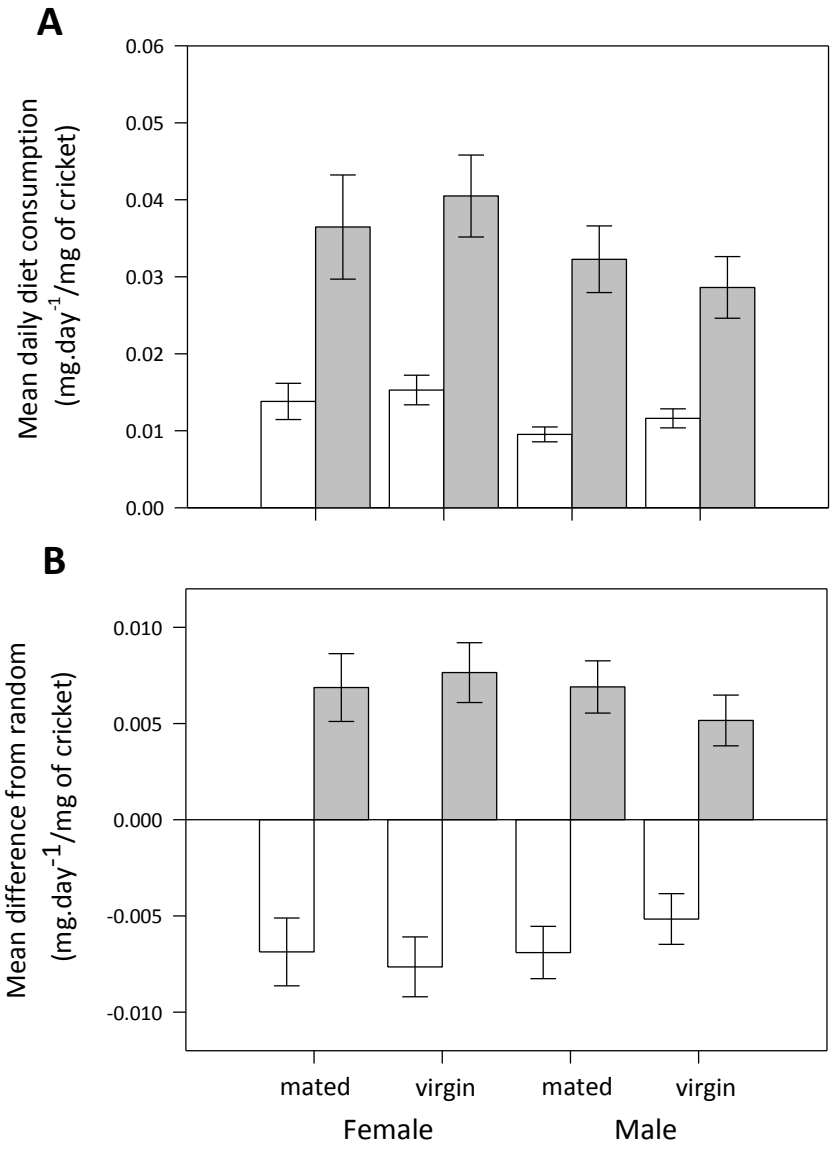

Figure 5-2. (A) The mean (±SE) daily consumption (per mg of cricket) of each diet by mated and virgin crickets (*Teleogryllus commodus*) of each sex. Grey bars represent the consumption of the high carbohydrate diet (diet 24) and the white bars the consumption of the high protein diet (diet 4). (B) The difference in daily protein (white bars) and carbohydrate (grey bars) intake from that expected if crickets fed at random on the two diets.

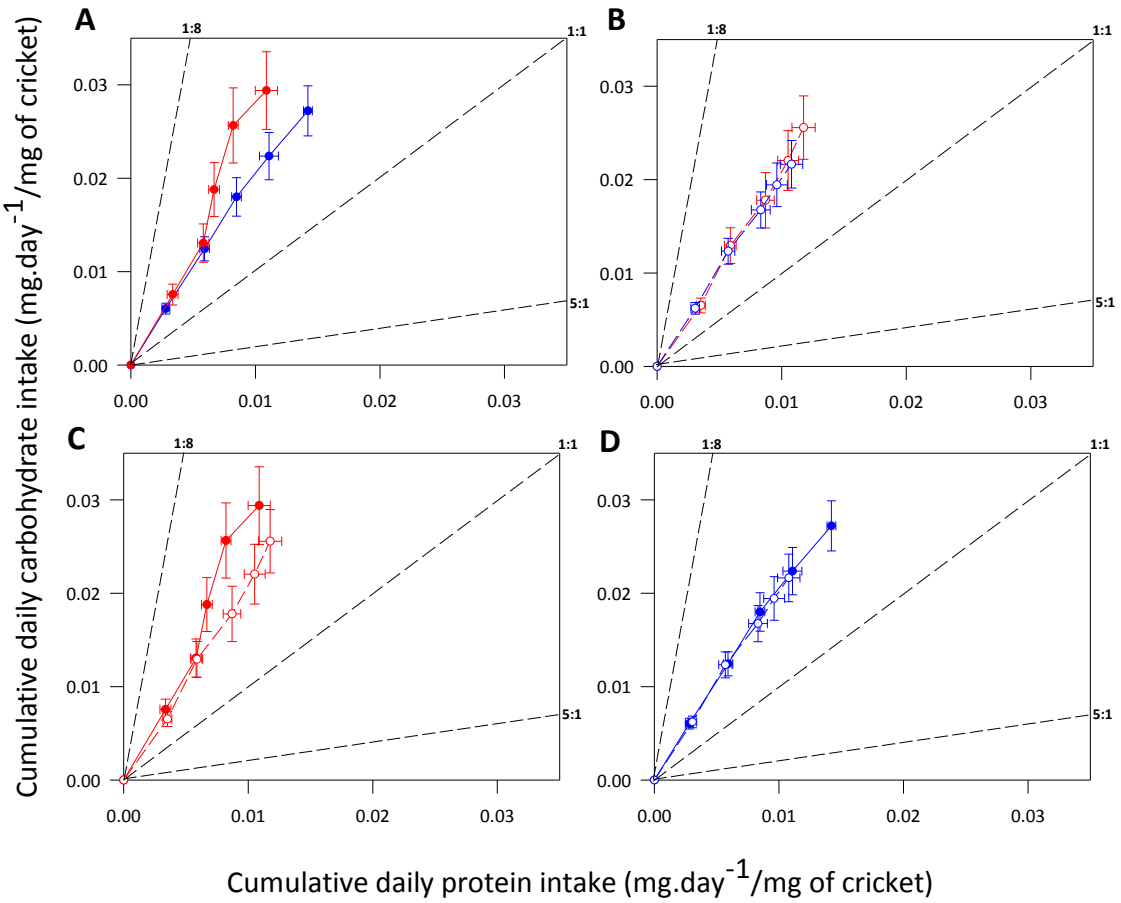

Figure 5-3. Cumulative daily intake (per mg of cricket) of protein and carbohydrates by crickets (*Teleogryllus commodus*) when provided the choice between two alternate diets (diet 4 and diet 24). The comparison of (A) mated females (red closed symbols, solid red lines) and mated males (blue closed symbols, solid blue lines), (B) virgin females (red open symbols, dashed red lines) and virgin males (blue open symbols, dashed blue lines), (C) mated and virgin females and (D) mated and virgin males.

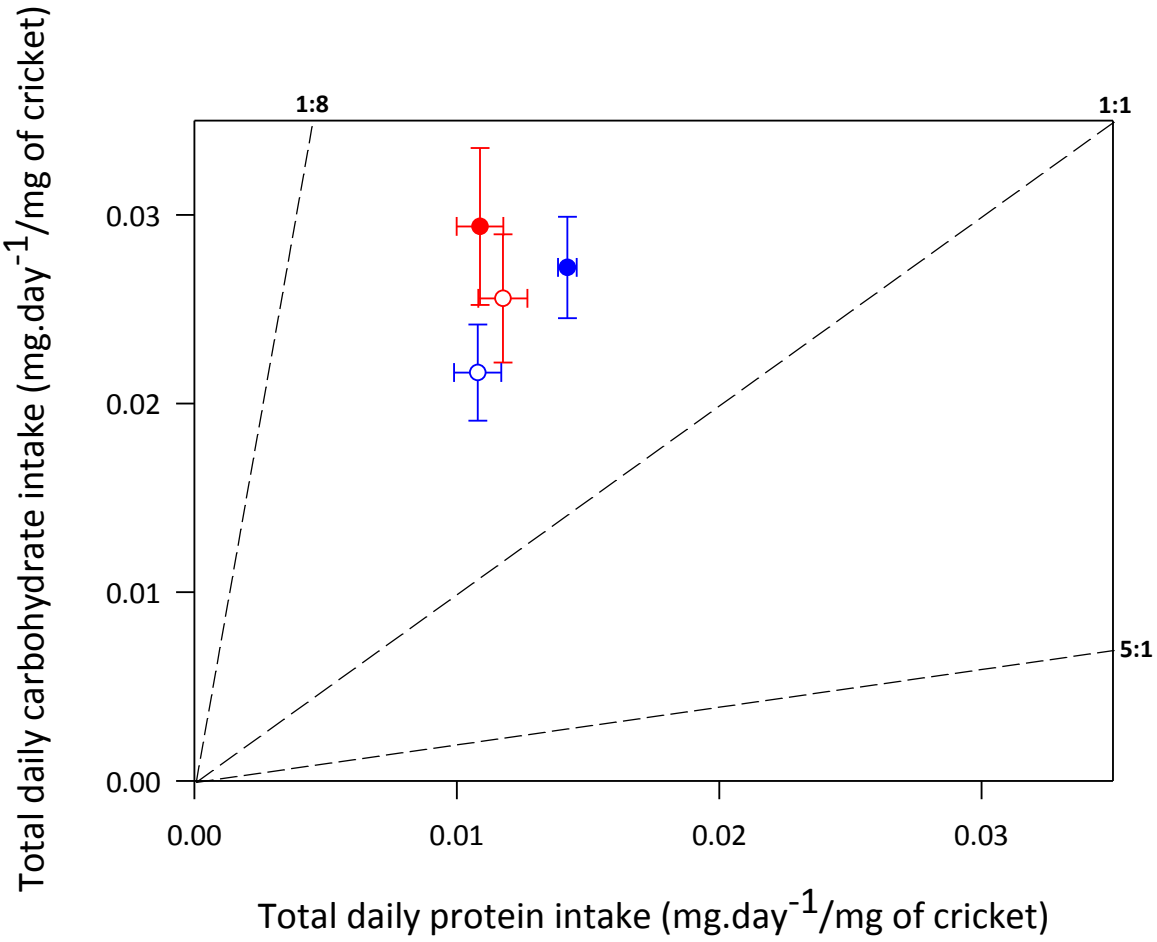

Figure 5-4. Intake targets i.e. total daily intake (per mg of cricket) of protein and carbohydrates by crickets (*Teleogryllus commodus*) when provided the choice between two alternate diets (diet 4 and diet 24). Mated females are represented by red closed symbols

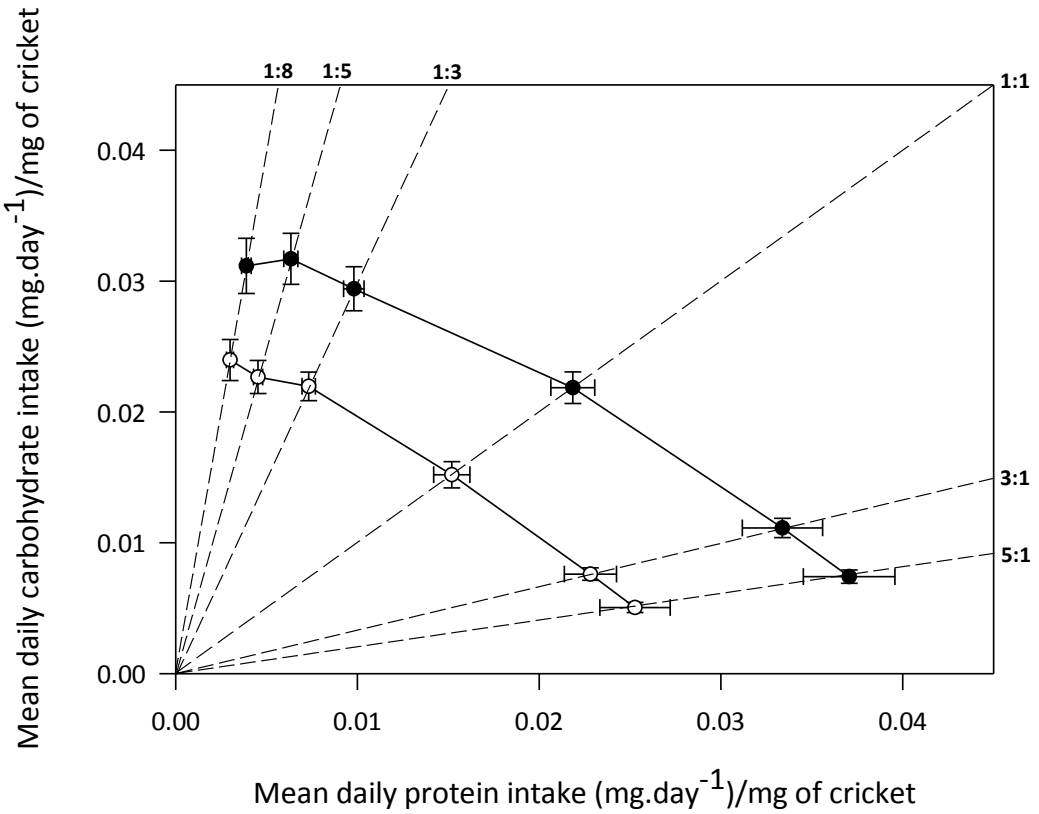

Figure 5-5. Average intake array of male (open symbols) and female (closed symbols) crickets *(Teleogryllus commodus)* fed each of the 24 artificial diets in my "no choice" experiment.

Table 5-1. Linear and nonlinear effects of daily protein (P) and carbohydrate (C) intake per milligram cricket on the lifespan and daily reproductive effort of male and female *T. commodus*. The linear regression coefficients (i.e. P and C) describe the linear slope (given by $\boldsymbol{\beta_i}$) of the relationship between nutrient intake and the response variable, whereas the quadratic regression coefficients (i.e. P x P and C x C) describes the curvature (given by $\boldsymbol{\gamma_{ii}}$) of this relationship, with a negative $\boldsymbol{\gamma_{ii}}$ indicating a convex relationship (i.e. a peak on the response surface) and a positive $\boldsymbol{\gamma_{ii}}$ indicating a concave relationship (i.e. a trough on the response surface). The correlational regression coefficients (i.e. P x C) describe how the covariance between the two nutrients ($\boldsymbol{\gamma_{ij}}$) influences the response variable, with a negative $\boldsymbol{\gamma_{ij}}$ indicating that a negative covariance between nutrients increases the response variable and a positive $\boldsymbol{\gamma_{ij}}$ indicating that a positive covariance between nutrients increases the response variable. Full details of this approach are provided in Lande & Arnold (1983).

	Linear effects		**Nonlinear effects**		
Response variables	**P**	**C**	**P x P**	**C x C**	**P x C**
(A) Males					
Lifespan					
Coefficient* ± SE	0.48 ± 0.06	0.71 ± 0.06	-0.27 ± 0.04	-0.15 ± 0.05	-0.11 ± 0.06
T	8.12	12.13	7.35	3.01	1.75
Df	205	205	202	202	202
P	0.0001	0.0001	0.0001	0.002	0.082
Daily Reproductive Effort					
Coefficient ± SE	0.38 ± 0.07	0.48 ± 0.07	-0.10 ± 0.04	-0.12 ± 0.05	-0.06 ± 0.08
T	5.58	7.00	2.53	2.41	0.70
Df	205	205	202	202	202
P	0.0001	0.0001	0.012	0.017	0.49
(B) Females					
Lifespan					
Coefficient ± SE	-0.06 ± 0.06	0.60 ± 0.06	-0.09 ± 0.06	-0.33 ± 0.05	-0.44 ± 0.10
T	1.02	10.43	1.61	6.90	4.55
Df	219	219	216	216	216
P	0.31	0.0001	0.110	0.0001	0.0001
Daily Reproductive Effort					
Coefficient ± SE	0.53 ± 0.07	0.38 ± 0.06	-0.17 ± 0.07	-0.13 ± 0.05	-0.00 ± 0.11
T	8.13	6.07	2.45	2.38	0.03
Df	219	219	216	216	216
P	0.0001	0.0001	0.015	0.018	0.979

Table 5-2. Repeated measures MANOVA examining the effects of sex, mating status, time and their interactions on the cumulative daily intake of protein and carbohydrates (mg.day-1) scaled to the body mass of the cricket (per mg of cricket).

Daily protein intake			
Model term	*F* value	*Df*	*P* value
Sex (A)	0.803	1,69	0.373
Mating status (B)	0.006	1,69	0.939
Time (C)	144.252	4,66	0.0001
A x B	4.966	1,69	0.029
A x C	1.539	4,66	0.201
B x C	6.698	4,66	0.6360001
A x B x C	3.459	4,66	0.013
Daily carbohydrate intake			
Model term	*F* value	*Df*	*P* value
Sex (A)	0.634	1,69	0.429
Mating status (B)	0.828	1,69	0.366
Time (C)	58.981	4,66	0.0001
A x B	0.001	1,69	0.996
A x C	2.934	4,66	0.027
B x C	3.286	4,66	0.016
A x B x C	1.172	4,66	0.331

Table 5-3. Post-hoc repeated measures MANOVAs demonstrating differences between the sexes and with mating status in the cumulative daily consumption (per mg of cricket) of protein and carbohydrates.

Comparison	***F* value**	***df***	***P* value**
A. Mated male vs mated female			
Daily protein intake			
Sex (A)	9.357	1,33	0.0044
Time (B)	123.518	4,30	0.0001
A x B	11.716	4,30	0.0001
Daily carbohydrate intake			
Sex (A)	0.284	1,33	0.598
Time (B)	30.111	4,30	0.0001
A x B	1.638	4,30	0.191
B. Virgin male vs virgin female			
Daily protein intake			
Sex (A)	0.408	1,36	0.527
Time (B)	53.012	4,33	0.0001
A x B	0.577	4,33	0.682
Daily carbohydrate intake			
Sex (A)	0.354	1,36	0.555
Time (B)	26.362	4,33	0.0001
A x B	1.185	4,33	0.336
C. Mated female vs virgin female			
Daily protein intake			
Mating status (A)	0.138	1,30	0.138
Time (B)	44.995	4,27	0.0001
A x B	8.581	4,27	0.0001
Daily carbohydrate intake			
Mating status (A)	0.280	1,30	0.601
Time (B)	24.026	4,27	0.0001
A x B	1.123	4,27	0.367
D. Mated male vs virgin male			
Daily protein intake			
Mating status (A)	1.833	1,39	0.184
Time (B)	136.124	4,36	0.0001
A x B	8.452	4,36	0.0001
Daily carbohydrate intake			
Mating status (A)	0.622	1,39	0.435
Time (B)	34.494	4,36	0.0001
A x B	2.443	4,36	0.060

6. OXIDATIVE STRESS DOES NOT EXPLAIN SEX-SPECIFIC LIFE-HISTORY RESPOSES TO DIETARY MANIPULATION IN THE AUSTRALIAN FIELD CRICKET, *TELEOGRYLLUS COMMODUS*

6.1 ABSTRACT

The free radical theory of ageing predicts that oxidative stress, which occurs when cellular levels of Reactive Oxygen Species (ROS) exceed circulating antioxidant defences, causes ageing. Recent reviews have highlighted that oxidative stress may also mediate the trade-off between reproductive effort and lifespan predicted by evolutionary theory. However, very few studies have tested this hypothesis. Here, I use diet to manipulate lifespan and reproductive effort in the Australian field cricket *Teleogryllus commodus* separately across the sexes. I then supplemented half of these animals with the antioxidant, vitamin E, and assayed lifespan, reproductive effort, oxidative damage and antioxidant protection. I predicted that if oxidative stress causes ageing, oxidative damage should be greatest in the shortest lived animals. If oxidative stress is a proximate cost of reproduction, damage should be greatest in animals that invest intensely in reproduction. Finally, antioxidant supplementation should either reduce oxidative damage or increase lifespan or reproductive effort. In keeping with the free radical theory, I found that oxidative damage was greater in the shorter lived sex (females). However, in contrast, crickets fed diets that extended their lifespan had high levels of oxidative damage. In females, vitamin E improved fecundity only when coupled with a high carbohydrate intake, while in males reproductive effort was associated with reduced antioxidant protection. These results offer equivocal support for the free radical theory and suggest that oxidative stress does not mediate life-history trade-offs in *T. commodus*.

Keywords; free radical theory of ageing, geometric framework of nutrition, oxidative stress, sexual selection

6.2 INTRODUCTION

Ageing (or senescence) is the irreversible decline in organismal performance with age that progressively increases an individual's risk of dying (Finch 1990).

Evolutionary theory predicts that senescence occurs because so few animals in nature survive environmental hazards to reach old age (e.g. Austad 1997) that natural selection acting on the few surviving animals is weak (Haldane 1942). This weak natural selection allows the accumulation of late acting, deleterious mutations (*mutation accumulation* - Medawar 1952) and alleles that improve fitness early in life but have negative pleiotropic effects expressed later on (*antagonistic pleiotropy* - Williams 1957). Specifically, the antagonistic pleiotropy theory of ageing predicts that there is a trade-off between early and late life fitness. This means that increasing reproductive investment early in life should accelerate ageing, reduce lifespan or reproductive effort later in life (Williams 1957). Whilst this prediction has been met in a variety of species both in the laboratory (e.g. Rose 1984a; Luckinbill et al. 1984; Partridge and Fowler 1992) and in the field (e.g. Bonduriansky and Brassil 2005; Charmantier et al. 2006; Nussey et al. 2008b) we still do not understand the mechanistic basis of this trade-off for many species (Isaksson et al. 2011).

Moreover, while there is a well developed body of theory explaining the evolution of ageing, we do not fully understand its proximate causes (Kenyon 2010; Partridge 2010). Several lines of evidence suggest that Reactive Oxygen Species (ROS) may contribute to causing ageing (reviewed by Beckman and Ames 1998), however, whether ROS play a major, or minor, causal role is unclear (reviewed by Beckman and Ames 1998; Finkel and Holbrook 2000; Sohal et al. 2002; Balaban et al. 2005; Jang and Remmen 2009; Speakman and Selman 2011). ROS are formed on the incomplete reduction of oxygen, primarily in the mitochondria during aerobic metabolism (Barja 2007). The high reactivity of ROS make them excellent signalling molecules that are used to regulate a number of cellular processes including growth, development and apoptosis (D'Autreaux and Toledano 2007; Veal et. al. 2007). However, this high reactivity also carries costs and ROS may readily react with biological molecules and, by doing so, impair their function. For example, lipid oxidation reduces the integrity of lipid membranes (Brigelius-Flohe and Traber 1999) and oxidation of proteins impairs enzyme function (Dalle-Donne et al. 2003). ROS homeostasis is therefore a compromise between meeting the functional demands for ROS and preventing the damage that they cause when present in excess (Finkel and Holbrook 2000). Antioxidants help maintain this balance by detoxifying ROS, however, when ROS

production exceeds antioxidant defences, cells enter a pro-oxidant state called oxidative stress and cellular damage accumulates (Finkel and Holbrook 2000).

However, if oxidative stress affects the expression of other life-history traits (e.g. reproductive effort) we should not always expect that antioxidant supplementation will improve lifespan (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2008; Isaksson et al. 2011). Recent reviews have highlighted that oxidative stress may be a proximate cost of reproductive effort that accelerates ageing and reduces lifespan (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2008; Isaksson et al. 2011). This means that oxidative stress may, in theory, mediate the trade-off between reproductive effort and lifespan, predicted by evolutionary theories of ageing (Williams 1957; Kirkwood 1977). This may happen if reproductive effort increases metabolic rate (e.g. Kavanagh 1987; Bergeron et al. 2011) and, in turn, ROS generation, then greater investment in reproduction would push cells into oxidative stress and accelerate senescence (but see Balaban et al. 2005; Hulbert et al. 2007). Alternatively, antioxidants, which are frequently used during reproduction, could drive a resource-based trade-off between investment in somatic protection from ROS and reproductive effort (e.g. Salmon et al. 2001; Wang et al. 2001; Alonso-Alvarez et al. 2004; Bertrand et al. 2006; Torres and Velando 2007; Garratt et al. 2011a; 2011b). In this case, if greater reproductive effort improves fitness more than a long life (e.g. Hunt et al. 2004), supplementary antioxidants may be allocated to increasing reproduction rather than lifespan.

If oxidative stress does mediate the trade-off between reproduction and ageing, ROS homeostasis should differ across the sexes because sexual selection may promote very different life-history strategies in the sexes (Bonduriansky et al. 2008). Female reproductive success relies on having time to acquire resources and rear young and so natural selection promotes a strategy of reproductive investment that yields modest returns over a longer time period. In males, sexual selection may promote intense investment in early reproductive effort to gain additional mates (Vinogradov 1998; Hunt et al. 2004; Bonduriansky et al. 2008), which may be achieved by sacrificing lifespan (Kokko 1997; 2001). Where males adopt this "live fast and die young" life-history strategy, selection is expected to promote the evolution of longer lives and slower ageing in females than males (Bonduriansky et al. 2008). However, this

prediction may be reversed if a male shows an age-dependent increase in sexual advertisement (e.g. Mysterud et al. 2004). This may occur because with advanced age the chance of surviving to mate again decreases and so it may pay to invest increasingly in reproduction (Kokko 1997). Where an age-dependent increase in male reproductive effort improves fitness, selection should favour the evolution of longer lives and slower ageing in males relative to females (Bonduriansky et al. 2008). Hence, sexual selection can affect the evolution of ageing, lifespan and reproductive effort across the sexes (Bonduriansky et al. 2008). Oxidative stress could underpin these sex-specific life-history strategies: for example, where males "live-fast and die-young", we might expect to see high oxidative damage early in life as a consequence of intense early reproductive effort but little investment in antioxidant defences and/or repair. However, very few studies have explored the mechanistic basis of sex-specific rates of reproductive effort, ageing and lifespan.

Here, I use Dietary Restriction (DR) to examine the relationship between oxidative stress, ageing and reproductive effort across the sexes. Strikingly, in a broad range of species, animals fed a restricted diet (but not so restricted as to cause malnutrition) live longer and age more slowly than fully fed animals (e.g. McCay et al. 1935; Ball et al. 1947; Weindruch et al. 1986; Jiang et al. 2000; Magwere et al. 2004; Mair et al. 2005; Colman et al. 2009). However, this increased longevity is often associated with reduced reproductive effort (e.g. Klass 1977; Chippindale et al. 1993; Bishop and Guarente 2007). Initially it was thought that the restriction of calories was responsible for the increased longevity seen under DR (e.g. Ball et al.1947; Ross 1972; Masoro 1990) by prompting the adaptive reallocation of resources to somatic maintenance away from reproductive effort (Harrison and Archer 1989; Holliday 1989; Phelan and Austad 1989; Shanley and Kirkwood 2000; Kirkwood and Shanley 2005). However, more recent studies have shown that it is the ratio of particular nutrients in the diet, rather than the absolute calorie content, that affects reproductive effort and lifespan (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Grandison et al. 2009). For example, female *Drosophila melanogaster* live longest when they eat a 1:16 ratio of protein to carbohydrate (P:C), but lay the most eggs when fed a 1:2 ratio of P:C (Lee et al. 2008). Dietary optima for particular life-history traits may also differ across the sexes (e.g. Maklakov et al. 2008). For example, a previous study on crickets found

that female fecundity was greatest in animals that consumed a 1:1 P:C ratio, but in males, reproductive effort was maximised on a higher carbohydrate diet (1:3 P:C) (Maklakov et al. 2008). These sex-specific dietary optima may reflect the different nutritional demands of reproduction across the sexes. For example, dietary protein may limit the ability of females to produce eggs (Williams 1994) but not male sperm production. In summary, because diet affects reproductive effort and lifespan, if oxidative stress causes ageing or is a proximate cost of reproduction, it should respond to dietary manipulation. To date, studies that have examined the relationship between DR and oxidative stress have employed very few dietary treatments, manipulated one nutrient in one sex (males) and in a very few species (Table 6-1). Moreover, these studies have invariably overlooked reproductive effort (but see Burger et al. 2007).

In the black field cricket, *Teleogryllus commodus,* I manipulated the ratio of protein to carbohydrate (P:C) and absolute nutritional value (P+C) in four diets, that each differentially affect lifespan and reproductive effort in males and females (Maklakov et al. 2008; Chapter 5). Field crickets are particularly amenable to this type of research because reproductive effort can be readily assayed in both females (e.g. counting eggs: Head et al. 2005; Hunt et al. 2006; Zajitschek et al. 2007) and males (recording time spent calling: Hunt et al. 2004; 2006; Zajitschek et al. 2007; Judge et al. 2008). Following the manipulation of the ratio of nutrients in diets I then supplemented half of these animals with the antioxidant vitamin E. I subsequently measured lifespan and reproductive effort in animals fed each diet, and in a subset, assay total antioxidant capacity (TAC) and oxidative damage (protein carbonyl group formation (PC), which form on the oxidation of proteins; Dalle-Donne et al. 2003). If the free radical theory (Harman 1956) is supported, oxidative damage should be greater in the shorter lived sex and animals that are fed a diet suboptimal for lifespan. If this is due to a trade-off between lifespan and reproductive effort, I predict that increased investment in reproduction should be associated with greater oxidative damage. I predict that sexual selection will promote different resolution of the trade-off between oxidative damage and antioxidant protection in males and females.

6.3 MATERIALS AND METHODS

Experimental animals

On the day of hatching, each of 500 nymphs from a population of black field crickets, *Teleogryllus commodus,* originating from Smith's Lake, eastern Australia, were put into individual plastic containers (5cm x 5cm x 5cm). These crickets had been maintained in the laboratory at the University of Exeter for five generations in 20 litre plastic tubs, at 27°C under a 13:11 L:D cycle. Each nymph was provided with a piece of cardboard egg box for shelter and water in a 2.5ml test tube plugged with cotton wool. For the first three feedings, food was provided in ground form in the lid of a 2.5ml eppendorf. For subsequent feedings, two cat food pellets (Friskies Go-Cat Senior) were provided weekly. Once nymphs reached their final developmental instar, they were checked daily for eclosion as adults. Upon eclosing animals were weighed and their pronotum measured using a binocular microscope and eyepiece graticule. They were then randomly allocated to one of the four dietary treatments (designated in keeping with Maklakov et al. 2008, Table 6-2), with half of the animals supplemented with vitamin E as per the protocol detailed below and the other half unsupplemented (i.e. 8 different treatment groups). Each of these eight groups were then allocated to either Experiment 1 or 2, whereby animals in Experiment 1 had their lifespan and reproductive effort measured, whilst animals in Experiment 2 were sacrificed 17 days post eclosion and biochemical analyses performed. I set up 10 survival and 10 assay animals for each level of diet and supplementation for each experiment, such that I set up 320 animals. However, a very few crickets escaped, died due to a handling death or did not survive to day 17 to be assayed such that I used 312 animals in total.

Making artificial diets and measuring dietary preference under choice

I made four, dry granular diets according to the geometric framework of nutrition (Simpson and Raubenheimer 1993) and the methods detailed in Chapter 2 of this thesis. The diets used in each experiment are shown in Table 6-2 whilst Figure 6-1 shows the nutritional values of these diets. I chose these diets because I have found that they have a pronounced effect on reproductive effort and lifespan in either sex (Chapter 5; Figure. 6-2).

Vitamin supplementation

I supplemented diets with the antioxidant, vitamin E. The term vitamin E encompasses lipid soluble molecules called tocopherols and tocotrienols (Brigelius-Flohe and Traber 1999). These compounds have specific functional roles in cellular signalling and in regulation of gene expression (Azzi et al. 2004) but function primarily as chain breaking antioxidants (Brigelius-Flohe and Traber 1999). Of all forms of vitamin E, the most widely available in nature is alpha tocopherol (Sheppard et al. 1993). Bound DL-alpha tocopherol (a synthetic, stable form of vitamin E) was added to each of the four diets at 12mg per 100g of diet. This supplementation level was chosen because this matches the concentration of vitamin E in Friskies Go Cat Senior, which the crickets have evolved under for five generations. To add vitamin E to the diets, bound DL-alpha tocopherol (hereafter vitamin E) was dissolved in 2ml of ethanol and then vortexed for five minutes at 15hz in a glass vial. This solution was dissolved in chloroform and introduced to the diets alongside the other water insoluble ingredients. Where diets were not supplemented with vitamin E, 2ml of ethanol was added to the chloroform as a control. Each diet was stored under inert gas at -80˚C and new batches of diet were made each month.

Feeding protocol

Experimental animals were kept in a plastic container (17 x 12 x 6 cm) and provided with one container of food of known dry mass on their first day of adulthood. Water was provided in the upturned plastic lid of a vial (1.6cm diameter, 1.6cm deep) glued using epoxy resin to the middle of a plastic petri-dish (5.5cm in diameter) and food was provided in a slightly larger feeding platform (vial lid:2.1 cm diameter, 1.2 cm deep petri dish: 5.5cm in diameter, 1.2cm deep). A one inch length of plastic tubing was provided for shelter. The materials used in housing the crickets meant that experimental animals could not consume anything other the artificial diets and the design of feeding platforms meant that any food split was collected in the petri-dish and so could be weighed.

Food of known dry mass was provided on the day of eclosion. Three days later, this food was removed and replaced with new food of known mass. On day six, the food was removed and a stock animal of the opposite sex introduced to the container.

This mate was removed 24 hours later (day 7) when new food was provided. This weekly feeding cycle was repeated throughout the experimental animal's lifetime in Experiment 1, or until day 17 in Experiment 2 when animals were killed. Animals were checked for mortality and water daily and if their food was running low, was replaced and a new batch of food of the same diet and known mass provided.

When feeding platforms were removed from the experimental animal faeces were carefully removed using a pair of fine forceps. The platform was then kept in a drying oven at 30°C for 36h to remove moisture prior to weighing. Consumption of diet was calculated as the dry weight provided to the experimental animal minus that reweighed after feeding. This information was converted to a weight of protein and carbohydrate consumed (e.g. 5 mg of 15P:45C ingested equals 0.75 mg of protein and 2.25 mg of carbohydrates, whilst the remaining diet (40%) is indigestible carbohydrate). Daily consumption was calculated as the sum of diet consumed over a lifetime divided by its longevity.

Measuring reproductive effort

Reproductive effort of male and female *T. commodus* was measured on days 3 and 7 post eclosion, and once a week thereafter (i.e. day 14, 21, 28 post eclosion etc.). Prior to each sampling period, each experimental animal was randomly paired with a cricket of the opposite sex taken from the Smith's Lake stock population and allowed 24 hours to mate. For quantification of female reproductive effort pairs were then separated and all experimental females were provided with a small petri-dish (5cm diameter) full of moist sand for oviposition. This oviposition substrate was only removed when a male was introduced and then replaced when the male was removed. To count eggs, the content of each petri-dish was emptied into a container of water, swirled and the eggs removed with fine forceps and counted.

In male crickets, the amount of time spent calling is a good measure of mating success because females strongly prefer males that call more in both the laboratory (Hunt et al. 2006) and the field (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muñoz et al. 2010). It is also a good measure of reproductive effort because calls are metabolically expensive to produce (Kavanagh 1987). Consequently, I measured the amount of time each male spent calling on a given night (hereafter referred to as

calling effort) using a custom-built electronic monitoring device and the protocol outlined in Chapter 3.

For assay animals, the protocol was as for survival animals, but on day 17 post eclosion the animals were re-weighed and killed by freezing at -80˚C in a 1.5ml eppendorf. Animals were killed at day 17 because on the basis of prior work (Chapter 5), this is just below the mean longevity seen for females fed high protein diets (e.g. diet 4) so should allow sufficient time for physiological differences between animals on different diets to accumulate but not select for those that are particularly long lived (i.e. exceed mean age of animals fed high protein diet from Chapter 5). Where animals did not survive to day 17, they were replaced.

Biochemical assays

All assays were completed within three months of freezing at -80°C. Animals were thawed and their gut and crop dissected out using fine scissors and forceps to ensure that only antioxidants and proteins that had been assimilated into tissues were assayed and not those contained within undigested food. Post dissection, the crickets were re-weighed and then immediately homogenised for thirty seconds in 2ml of phosphate buffered saline (pH 7.4) in falcon tubes using an Ultra-Turrax T-28 homogeniser. Homogenate was centrifuged at 13 000rpm for 20 minutes at 4°C in 2ml eppendorfs. The supernatant fraction was separated from fat and cuticle and centrifuged for a further 5 minutes at 13 000rpm. This final supernatant fraction was used for all analyses. Supernatant was divided into aliquots for each particular assay such that samples need not be thawed more than once more prior to analyses and stored in 1.5ml eppendorfs at -80˚C.

Protein concentration was determined using the Bradford method (Bradford 1976) using a spectrophotometer (Spectramax Plus384, Molecular Devices). Samples were ran in duplicate on a 96 well, flat bottomed plate and were diluted five-fold in homogenisation buffer. The Bradford method relies on the binding of the dye Coomassie Blue G250 to proteins: by measuring the amount of bound dye at 595nm using a spectrophotometer, the amount of protein may be calculated. Whilst any measure of oxidative damage to a single cellular component cannot give a complete picture of oxidative damage, I measured protein carbonyls, which are stable moieties

produced by most types of ROS on oxidation of proteins (Dalle-Donne et al. 2006). Because proteins often have important biological roles, e.g. as enzymes, protein oxidation is likely to have a pronounced influence on cellular function (Dalle-Donne et al. 2006). Concentration of protein carbonyls was assayed using a commercially available kit (Protein Carbonyl Assay Kit, Cayman Chemical, Item no. 10005020). In summary, the PC assay relies upon the covalent reaction between protein carbonyl groups and 2,4-dinitrophenylhydrazine (DNPH). This reaction produces the stable product DNP, that absorbs UV and so it may be quantified using a plate reader at a wavelength between 360-385nm. Samples were diluted five-fold with homogenisation buffer such that protein content was within 1-10mg/ml and ran in duplicate.

Total antioxidant capacity (TAC) was also measured using a commercially available kit (Antioxidant Assay Kit, Cayman Chemical, Item no. 709001). These kits measure the oxidation of ABTS (2,2'-Azino-di-[3-ethybenzthiazoline sulphonate]) by metmyoglobin. This oxidative reaction is inhibited by antioxidants, and so by comparing the degree of inhibition to a standard curve constructed using known amounts of the inhibitor (Trolox), the total antioxidant capacity of sample can be ascertained (as used in Buduneli et al. 2006; Kowluru et al. 2006; Kowluru and Kanwar 2007). Samples were run in duplicate and diluted 100-fold. PC and TAC results were presented and analysed per mg protein, to control for the confounding effect of body size: TAC and PC henceforth refer to these measures i.e. per mg protein.

To measure vitamin E, 5ml of pyrogallol was added to 300µl of whole cricket homogenate in a 40ml glass vial. Pyrogallol prevented any oxidation of vitamin E. Samples were then saponified to remove lipids, which entailed adding 1ml of KOH to each sample. Samples were then recapped under nitrogen and placed in a water bath at 70˚C for 30 minutes. 5ml of hexane was then added to extract vitamin E from the sample. 10ml of Milli-Q was added to vitamin E, all samples were recapped under nitrogen and put on a bench top mixing plate for five minutes. They were then centrifuged at 10000RPM at 4˚C for 4 minutes. 3ml of the hexane layer was extracted and dried down in an evaporating rotary chamber for 20 minutes. Once completely evaporated, 100ul of 0.05% butylated hydroxytouene (BHT) in ethanol was added to dissolve the vitamin E. Samples were then vortexed for 30 seconds at 15Hz. 100ul of sample was injected into a Dionex HPLC system fitted with a Waters Spherisorb 3um

ODS2 column (4.6x150mm). The mobile phase was methanol to water (97:3), run isoratically for 15 minutes. Vitamin E was detected using a fluorescence detector, with an excitation wavelength of 330nm and an emission wavelength of 480nm. Peak area was quantified using a standard curve prepared from alpha-tocopherol standard. The peaks allowed for quantitative amounts of µg of vitamin per gram of cricket to be calculated.

Statistical analyses

I manipulated diet and vitamin supplementation in a factorial design. However, because each diet varied in its ratio of protein to carbohydrate (P:C) and absolute amount of protein and carbohydrate (P+C), crickets could regulate their intake of one or other of these nutrients by eating more or less diet. This meant that animals fed different diets could consume the same volume of one nutrient (e.g. protein) but as a consequence, ingest very different amounts of the other (e.g. carbohydrate). Therefore, to determine the effects of specific nutrients I incorporated daily intake of protein and carbohydrate into my statistical models. I discuss my results with respect to the intake of protein and carbohydrate but highlight which diet (i.e. 4, 8, 10 or 14) was optimal for a particular trait, such that results may be readily compared to earlier work (e.g. Maklakov et al. 2008).

All analyses were conducted in R version 2.14.1. Where necessary, response variables were first transformed using box-cox ("powerTransform" and "bcPower" functions in the package "car" - Fox and Weisberg 2011) or square root transformations. First, to determine whether supplementation increased levels of vitamin E in cricket homogenate, I used the "glm" (General Linear Model) function in R where sex, vitamin supplementation, carbohydrate and protein intake were included as fixed effects. This model structure (i.e. fixed effects) was also used to examine the effects of nutrition on different response variables (i.e. lifespan and reproductive effort).

PC and TAC were positively correlated and so I analysed the effects of daily protein and carbohydrate intake, sex and vitamin supplementation on PC and TAC using Multivariate Analysis of Co-Variance (MANCOVA) by using the "manova" function in the R package "car" (Bates et al. 2012). This overall MANCOVA model identified

significant differences across the sexes (see Results) so I also conducted-sex specific analyses. For each sex, I followed the MANCOVA with a series of univariate general linear models (GLM) with the same model structure to determine how each of my response variables contributed to the overall multivariate effects.

6.4 RESULTS

Nutrition and lifespan

Analyses of lifespan data using General Linear Models (GLMs), revealed significant variation in lifespan across the sexes ($F_{153,154}$ = 19.22, P = 0.0001) with males living on average 11 days longer than females (male lifespan: 47.70 ± 2.85 days, female lifespan 36.70 ± 1.95 days). There were also marginally non-significant interaction terms between sex and daily intake of carbohydrate ($F_{149,150}$ = 3.53, P = 0.06) and sex and daily consumption of protein ($F_{150,151}$ = 3.01, P = 0.08). To better understand how diet affected lifespan across the sexes, I conducted sex-specific analyses.

In males and females, average lifespan was greatest in animals that consumed a 1:3 ratio of protein to carbohydrate (females: 48.95 ± 3.13 days, males: 55.89 ± 6.12 days) and poorest in crickets fed a high protein, low carbohydrate diet (P:C 3:1 - females: 25.16 ± 2.40 days; males, 40.40 ± 5.61 days). In females, only greater daily intake of carbohydrate significantly improved female longevity (Figure 6-3, Table 6-3), however, male lifespan was significantly affected by both carbohydrate and protein (Figure 6-3, Table 6-3). While greater intake of both of these nutrients had a positive effect on male lifespan, male lifespan increased more per unit carbohydrate consumed than per unit protein ingested (Figure 6-3).

Nutrition and daily reproductive effort

Reproductive effort was analysed separately across the sexes because my responses variables were measured in different units in either sex (e.g. eggs laid in females versus seconds spent calling in males). Female fecundity, measured as the number of eggs laid per female per day, was greatest when crickets consumed a 1:1 ratio of protein to carbohydrate in both supplemented and unsupplemented animals. In females that were not supplemented with vitamin E, both protein and carbohydrate

intake significantly improved daily reproductive effort (Figure 6-4, Table 6-3).

However, the effects of vitamin E supplementation on female fecundity were complicated. Vitamin E supplementation only affected fecundity via an interaction with carbohydrate (Table 6-3), whereby the slope of the positive regression line between carbohydrate intake and daily fecundity was much steeper in supplemented animals (β ± SE = 69.67 ± 23.20) than unsupplemented animals (β ± SE = 51.09 ± 19.64) (Figure 6-4). Without vitamin E supplementation, females on diets optimal for daily fecundity (diet 10, 14.88 ± 1.72 eggs) laid on average 6 eggs per day more than those on suboptimal diets (diet 4, 9.37 ± 1.17 eggs). When females were fed supplemented diets, the magnitude of this difference in daily fecundity doubled, with females on optimal diets (diet 10, 20.46 ± 2.66 eggs) laying on average 12 more eggs per day compared to females fed suboptimal diets (diet 4, 8.41 ± 1.84 eggs) (Figure 6-4, Table 6-3).

In males, nightly calling effort was greatest in males that consumed a high carbohydrate, low protein diet (P:C 1:3). Both greater daily intake of carbohydrates and protein significantly elevated calling effort (Figure 6-5, Table 6-3). However, the slope of the relationship between calling effort and carbohydrate intake (β± SE = 48.30 ± 14.94) was much steeper than the slope between protein intake and reproductive effort (β± SE = 28.65 ± 11.81), showing that male calling effort increased more per unit carbohydrate consumed than per unit protein. Whilst vitamin E supplementation qualitatively improved how long males spend calling across all dietary treatments, the effect was not statistically significant (Figure 6-5,Table 6-3).

Nutrition, oxidative damage and antioxidant protection

Firstly, I tested the assumptions of my experimental procedures. GLM analyses where vitamin E in whole tissue homogenate was my response variable showed that circulating levels of vitamin E were higher in supplemented than unsupplemented animals ($F_{159,160}$ = 41.07, P = 0.0001) but that levels of vitamin E in the homogenate did vary significantly as a function of any other explanatory variable. This shows that vitamin E supplemented in the diet was significantly incorporated into cricket homogenate and that my supplementation protocol was successful.

GLM analyses showed that protein levels of crickets was significantly greater in animals that consumed greater amounts of daily protein ($F_{153,154}$ = 69.81, P= 0.0001) and carbohydrate ($F_{153,154}$ = 4.47, P =0.036) even once the crop and gut had been removed. This is not particularly surprising: animals that ate more food, particularly on high protein diets, incorporated more protein into the homogenate. To account for this variation in protein content oxidative damage to proteins (PC) and total antioxidant capacity (TAC) are scaled per unit of protein. All subsequent results and analyses of PC and TAC refer to these scaled values.

I analysed the effect of nutrition and sex on PC and TAC using a MANCOVA model, to account for the correlation in these measures of oxidative damage and protection. I found that sex significantly influenced the linear combination of PC and TAC measures in my full MANCOVA model (Table 6-4). Univariate general linear models showed that this was because PC was significantly greater in females than males (males: 1.52 ± 0.25 PC groups / mg protein, females: 1.70 ± 0.25 days) but that TAC did not differ significantly across the sexes. Univariate GLM analyses, also revealed a marginally non-significant three-way interaction between daily protein intake, vitamin supplementation status and sex (P = 0.08) on PC group formation. Given this marginally significant interaction term, strong intersexual differences in PC across the sexes and sex-specific responses of lifespan and reproductive effort to diet, I conducted sex-specific analyses of PC and TAC to facilitate interpretation of analyses.

Sex specific MANCOVA models showed that in both males (Figure 6-6, Table 6-6) and females (Figure 6-6, Table 6-5), greater daily consumption of protein was significantly associated with reduced levels of oxidative damage. In males, there was also a significant interaction between vitamin E consumption and protein intake, whereby the slope of the negative relationship between protein intake and oxidative damage was steeper in unsupplemented than supplemented animals (Figure 6-6, Table 6-6). Female TAC was not affected by intake of protein, carbohydrate or by vitamin E supplementation (Figure 6-7, Table 6-5). In males, there was a significant negative association between daily carbohydrate intake and TAC, showing that individuals that consumed greater dietary carbohydrate also had reduced TAC (Figure 6-7, Table 6-6).

6. 5 DISCUSSION

The free radical theory of ageing, which proposes that oxidative damage to cellular molecules such as lipids and DNA causes senescence (Harman 1956), is the most widely supported mechanism for ageing (reviewed in Beckman and Ames 1998; Finkel and Holbrook 2000; Sohal et al. 2002). However, this theory has received equivocal and often mixed empirical support (reviewed in Beckman and Ames 1998). Recently, the free radical theory has been heavily criticised because its central predictions have often not been met (Howes 2006; Jang and Remmen 2009; Pérez et al. 2009; Speakman and Selman 2011). Instead, studies have suggested that we should examine the role of oxidative stress in mediating life-history trade-offs (Costantini 2008; Monaghan et al. 2008; Dowling and Simmons 2009; Isaksson et al. 2011) such as that between lifespan and early reproduction effort predicted by evolutionary theories of ageing (Williams 1957). However, very little research has tested this hypothesis. Here, I used diet to manipulate lifespan and reproductive effort across the sexes in Australian field cricket, *T. commodus*, to examine how this affected oxidative damage and antioxidant protection. This allowed us to examine the association between oxidative stress, lifespan and reproductive effort in male and female crickets to test the free radical theory of ageing and the role of oxidative stress in mediating life-history strategies.

I found that diet affected lifespan and reproductive effort in both male and female crickets. In both sexes, lifespan was greatest in crickets that consumed high carbohydrate, low protein diet (P:C 1:3). This result supports past studies showing that in field crickets (Maklakov et al. 2008; Chapter 5 of this thesis), flies (Lee et al. 2008; Fanson et al. 2009), ants (Dussutour and Simpson 2009) and bees (Pirk et al. 2010), lifespan is greatest on high carbohydrate, low protein diets. In females, I found that fecundity was greatest in animals that consumed an equal ratio of protein to carbohydrates (P:C 1:1) but in contrast, males calling effort was maximised in males that consumed the same diet as that for lifespan (P:C 1:3). These differences in dietary optima for reproductive effort across the sexes probably reflect the specific nutritional demands of reproductive effort in males and females, for example, males require

greater dietary carbohydrate to fuel energetically expensive sexual displays (e.g. Kavanagh 1987). Crucially, because female lifespan and reproductive effort had distinct dietary optima, diet imposed a trade-off between these life-history traits. However, in contrast to predictions from evolutionary theories of ageing (Williams 1957; Kirkwood 1977), I did not find evidence for a trade-off between male lifespan and reproductive effort.

While the dietary optima for lifespan in both sexes and female fecundity is the same in this study as in Chapter 5, the results for male reproductive effort do not agree across chapters. In Chapter 5, male calling effort was optimised on a 1:1 ratio of protein to carbohydrate but in the current study, males called more on the very highest carbohydrate diet (P:C 1:3). This difference is likely because in the current study, a nutrient ratio was represented by a single diet (i.e. 1:3 = diet 14, 1:1 diet 10), while in Chapter 5, each ratio of nutrients corresponded to four different diets diluted more or less with indigestible carbohydrates (Figure 1-1). In both chapters, the calling effort for males on diets 10 and 14 (Figure 6-2) was statistically indistinguishable. However, in Chapter 5, when performance of all males feeding along a particular rail was accounted for then a clear peak for calling effort on a 1:1 ratio of protein to carbohydrate was identified. This disagreement between chapters illustrates that either study measured something slightly different, i.e. calling effort on one diet on a nutritional rail, versus four different diets on each different rail.

I then examined whether oxidative damage and/or antioxidant protection explained differences in lifespan across and within the sexes. I found that males lived longer than females and that this inter-sexual difference in lifespan was associated with significant differences in oxidative damage across the sexes. Specifically, oxidative damage was significantly greater in the shorter lived sex (females). This result supports the free radical theory of ageing, which predicts that animals that live longest should have the lowest levels of oxidative damage (Harman 1956). This result adds to a large body of evidence that oxidative damage and/or ROS production is often greater in the shorter lived sex (e.g. Ide et al. 2002; Borrás et al. 2003; Ali et al. 2005; Chapter 3 of this thesis). These sex differences in levels of protein oxidation may be due to different metabolic rates or mitochondrial efficiency across the sexes, differences in the expression of antioxidant enzymes or rates of repair of oxidised

molecules (Viña et al. 2003, Magwere et al. 2006, Borrás et al. 2007). My result shows that lower levels of oxidative damage in males may be a proximate mechanism by which sexual selection promotes different rates of ageing across the sexes.

However, in direct contrast to the predictions of the free radical theory, I found that crickets which consumed diets that improved their lifespan, did not have lower levels of oxidative damage. In fact, the diet that maximised lifespan was associated with the greatest levels of oxidative damage in both females and males (P:C 1:3). This result may reflect a challenge associated with testing the free radical theory of ageing. Namely, the rate at which oxidative damage accumulates is often tissue specific (Sohal et al. 1994a; Dubey et al. 1996; Radák et al. 2002). This means that diets which increased lifespan in *T. commodus* may have reduced oxidative damage to other tissue types (e.g. DNA, rather than proteins). In this case, reduced oxidative stress could still provide a proximate mechanism by which diet extends lifespan in *T. commodus*. Alternatively, diet may affect lifespan via a different mechanism altogether.

While a large number of studies carried out in mice and rats, show that DR may extend lifespan whilst reducing ROS production and/or oxidative damage (Gredilla et al. 2001; Jeon et al. 2001; Guo et al. 2002; López-Torres et al. 2002; Radák et al. 2002; Diniz et al. 2003; Sanz et al. 2004), other studies have found that DR has no effect (e.g. Sohal et al. 1994a; Kaneko et al. 1997; Gredilla et al. 2001; López-Torres et al. 2002; Radák et al. 2002) or even increases oxidative damage in particular tissues (Dubey et al. 1996). The relationship between diet, oxidative stress and lifespan also appears to be species specific. In *Drosophila melanogaster,* DR delayed the onset of oxidative damage (Zheng et al. 2005) but also reduced the ability to withstand chemically induced oxidative stress (Burger et al. 2007) and in nematodes, DR significantly increased ROS generation (Schulz et al. 2007). These results show that while in mammals DR frequently reduces oxidative damage and/or ROS production, this may not be the case in invertebrates (e.g. Burger et al. 2007; Schulz et al. 2007).

Instead, I found that greater protein consumption was associated with lower levels of oxidative damage in both males and females. High protein consumption is detrimental to survival in a number of species (e.g. Lee et al. 2008; Fanson et al. 2009; Simpson and Raubenheimer 2009; Pirk et al. 2010; Dussutour and Simpson 2012) including *T. commodus* (Maklakov et al. 2008). The mortality costs of high protein

intake has often been attributed to elevated oxidative damage (Sanz et al.2004; Ayala et al. 2007). However, my study supports research showing that oxidative damage is not always elevated when animals consume high protein diets (Petzke et al. 2000; Lacroix et al. 2004). Instead, protein intake may increase rates of degradation and repair of oxidised proteins (Garlick et al. 1991; Petzke et al. 2000; Kapahi et al. 2004) and therefore, reduce oxidative damage to proteins (Petzke et al. 2000). The low rates of oxidative damage seen in *T. commodus* that consume high protein diets, may therefore reflect greater rates of repair of damaged proteins. However, protein turnover itself can reduce lifespan (Hands et al. 2009) and the currently unidentified costs of protein synthesis may be a mechanism by which protein intake reduces lifespan independently of oxidative stress. More research is clearly needed to identify the physiological costs of high protein intake and protein turnover (Hands et al. 2009).

My experiments found little support for the hypothesis that oxidative stress is a proximate cost of reproduction (Dowling and Simmons 2009), although my results show clear evidence of a relationship between antioxidant availability in the diet and reproductive effort in both males and females. In females, antioxidant supplementation significantly increased fecundity but only when coupled with high carbohydrate intake. This adds to a large body of research showing that vitamin E supplementation improves female fertility in species including rats (Evans and Bishop 1922), fish (e.g. Serezli et al. 2010; Palace and Werner 2006; El_Gamal et al. 2007), hens (Scheideler and Froning 1996), rotifers (Enesco and Verdane Smith 1980), moths and beetles (Canavoso et al. 2001), dipteran parasites (House 1966), bees (Shekiladze 1971) and fire bugs (Jedlička et al. 2009). However, in my study, vitamin E only improved female fecundity when coupled with high carbohydrate consumption. Such synergistic interactions between different antioxidants are widespread (Niki 2010). For example, Orledge et al. (2012) showed that there were positive synergistic effects of vitamin E and carotenoid supplementation on body size, but not sexual ornamentation, in pheasants. Studies have also found interactions between antioxidants and carbohydrates because sugars can help preserve particular antioxidants (Peinado et al. 2010). Synergistic interactions between vitamin E and diet may help explain why studies testing the role of antioxidant supplementation on lifespan, disease and reproductive effort have found mixed and sometimes

contradictory results. For example, some studies have found that vitamin E supplementation improves semen motility in human males, while others have found that vitamin E does not affect semen at all (reviewed in Kefer et al. 2009).

In males, vitamin E supplementation did not significantly affect how long a male called to attract a female. That vitamin E supplementation significantly improved female fertility but not male calling effort may reflect the specific mode of action of this antioxidant. Vitamin E breaks cascades of lipid oxidation along lipid membranes, helping preserve their structural integrity (Packer 1994). This may be particularly important in females producing lipid rich, membrane bound eggs (e.g. Jedlička et al. 2009) but less so in influencing how long a male spends calling. However, I found that as a male's daily carbohydrate intake increased, males invested more in calling to attract females, but had reduced antioxidant defences. This result indicates that in *T. commodus* there was a trade-off between male reproductive effort and antioxidant protection. A negative association between reproductive effort and antioxidants has previously been identified in birds (Alonso-Alvarez et al. 2004; Wiersma et al. 2004). However, in *T. commodus,* this trade-off does not appear to be functionally important because greater calling effort and reduced antioxidant protection did not reduce lifespan.

That males which consumed high carbohydrate diets called more reflects the high energetic demands of reproductive effort in *T. commodus* (Kavanagh 1987) but why high carbohydrate intake was associated with reduced antioxidant defences is less clear. One possibility is that because high carbohydrate intake improved survival and male reproduction, males that called more were more able to tolerate high levels of oxidative damage and so did not need to invest as heavily in antioxidant defences. Instead, extra antioxidants or energy may instead be allocated to other fitness determining traits, such as ensuring sperm viability (e.g. Kefer et al. 2009; Helfenstein et al. 2010). It is worth noting that I did not find a direct, significant association between calling effort and protein damage but qualitatively, diets that progressively improved male calling effort were associated with increasing levels of oxidative damage. This result offers some preliminary support for oxidative damage being a cost of male reproductive effort in *T. commodus*.

Evidently, vitamin E supplementation had a weak and inconsistent effect on life-history traits across the sexes. However, the homogenate of vitamin E supplemented crickets had significantly higher levels of vitamin E than unsupplemented animals, showing that the supplementation protocol was successful. Although vitamin E supplementation may extend longevity in nematodes (Harrington and Harley 1988), rotifers (Enesco and Verdone-Smith 1980; Sawada and Eriesco 1984), fruit flies (Driver and Georgeau 2003) and mice (Navarro et al. 2005), the effects of supplementation depend heavily on the dose and timing of supplementation (Driver and Georgeou 2003). For example, in *Drosophila melanogaster,* vitamin E can both increase lifespan and also have toxic effects: when vitamin E was supplemented at 20 μg/ml, average life span was increased by 16%. However, vitamin E did not affect lifespan at higher or lower concentrations (Driver and Georgeou 2003). My results may reflect simply that I did not supplement crickets at the very precise dosage that might improve the expression of any life-history traits. This illustrates one reason why antioxidant supplementation may not be a suitable means to improve human health; over a narrow range of dosages antioxidants may have a negligible, positive or negative effect on organismal performance (Driver and Georgeou 2003).

Understanding the relationship between male reproductive effort, oxidative damage and antioxidant protection allows us to test the hypothesis that oxidative stress ensures the honesty of male sexual signals (von Schantz 1999). If expression of a sexually selected trait is sensitive to oxidative stress, which is elevated in response to immune challenges, investment in that sexually selected trait reliably indicates a male's health status. If this resistance to oxidative stress is heritable, a female may improve the survival of her offspring by mating with males that invest more in that sexually selected trait (von Schantz et al. 1999). This immuno-dependence model of pre-copulatory sexual selection has received empirical support (e.g. Blount et al. 2003) but has seldom been tested in species other than birds (Dowling and Simmons 2009). Past work on *T. commodus,* has shown that male calling song is affected by his immune status (Simmons et al. 2005) and both female preferences for male song, and components of male calling are heritable (Simmons 2004). Hence, this species meets some prerequisites of this model of pre-copulatory sexual selection. Whilst I did not explicitly aim to test this hypothesis, I found that males that invested more in sexual

display had lower levels of antioxidant defences and also lived longer. Whilst calling more did not signal lower levels of damage or higher antioxidant defences, it may show how resistant a male is to oxidative damage. However, my results showed that calling effort, antioxidant protection and oxidative damage were significantly affected by diet, and so female choice based on these traits may be difficult, particularly in heterogeneous environments (Tolle and Wagner 2011).

Understanding the mechanistic basis of senescence is a central aim in biological research. In particular, our current understanding of the role that oxidative stress plays in causing senescence is incomplete. My study provides equivocal support for the free radical theory of ageing. While I found sexual differences in oxidative damage that corresponded to the free radical theory (i.e. damage was greater in the shorter lived sex), oxidative damage did not respond to dietary manipulation in the direction predicted by this theory. The failure of oxidative damage to respond to empirical manipulations that affect lifespan is a frequently cited criticism of the free radical theory (Howes 2006; Jang and Remmen 2009; Pérez et al. 2009). I also found a relationship between antioxidants and reproductive effort, albeit one which differed across the sexes. This supports accumulating research showing that the association between reproductive effort and oxidative stress is sex-specific (Garratt et al. 2011a,b) and depends on the specific reproductive behaviours being measured. For example, in zebra finches (*Taeniopygia guttata*) although there is a positive genetic correlation between antioxidant protection and how many times an individual breeds across their lifetime (Kim et al. 2010), greater brood size within each of these breeding events reduces antioxidant capacity (Alonso-Alvarez et al. 2004). Although diet imposed a trade-off between reproductive effort and lifespan in females, I found no support for the hypothesis that oxidative stress mediated this life-history trade-off. My results illustrate that unravelling the mechanisms underlying dietary effects on life-history trade-offs may prove to be a very difficult task.

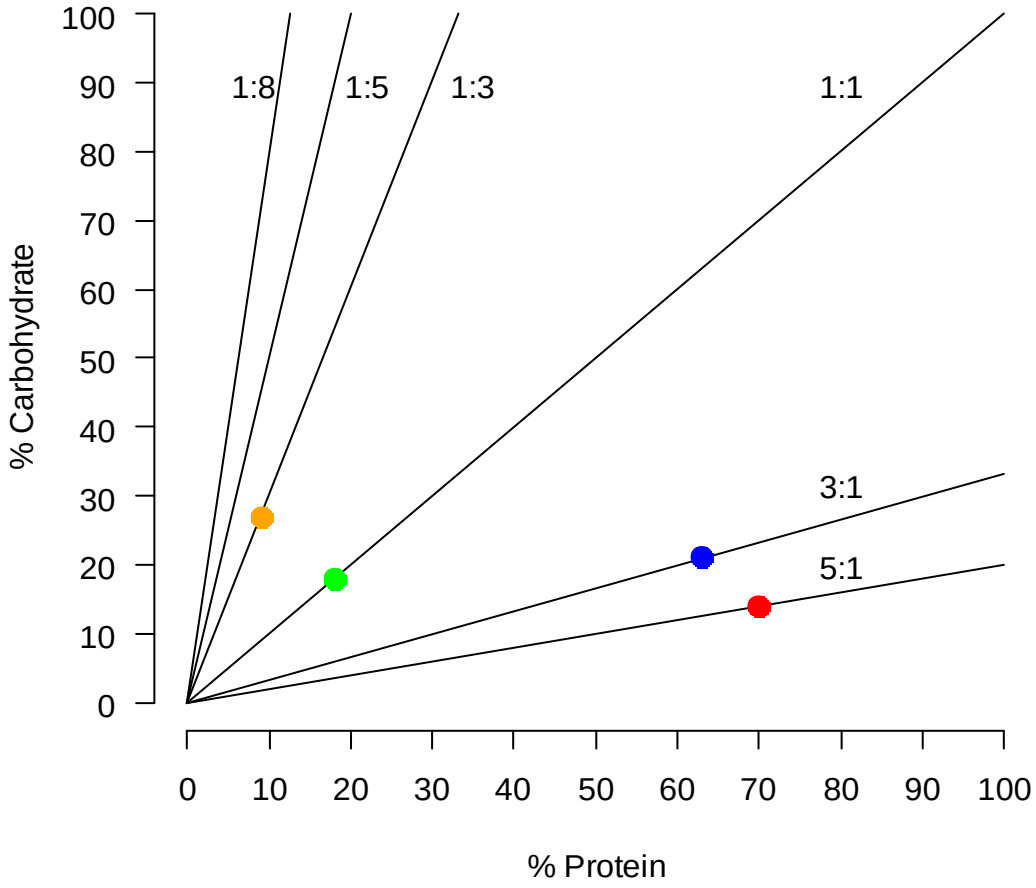

Figure 6-1. The location of the four diets used on the nutritional landscape. These ratios of protein to carbohydrate are represented by black lines. The diets used in this study are marked in coloured circles which correspond to Figure 6-2, showing how these diets affected lifespan and reproductive effort in male and female crickets in Chapter 5 of this thesis.

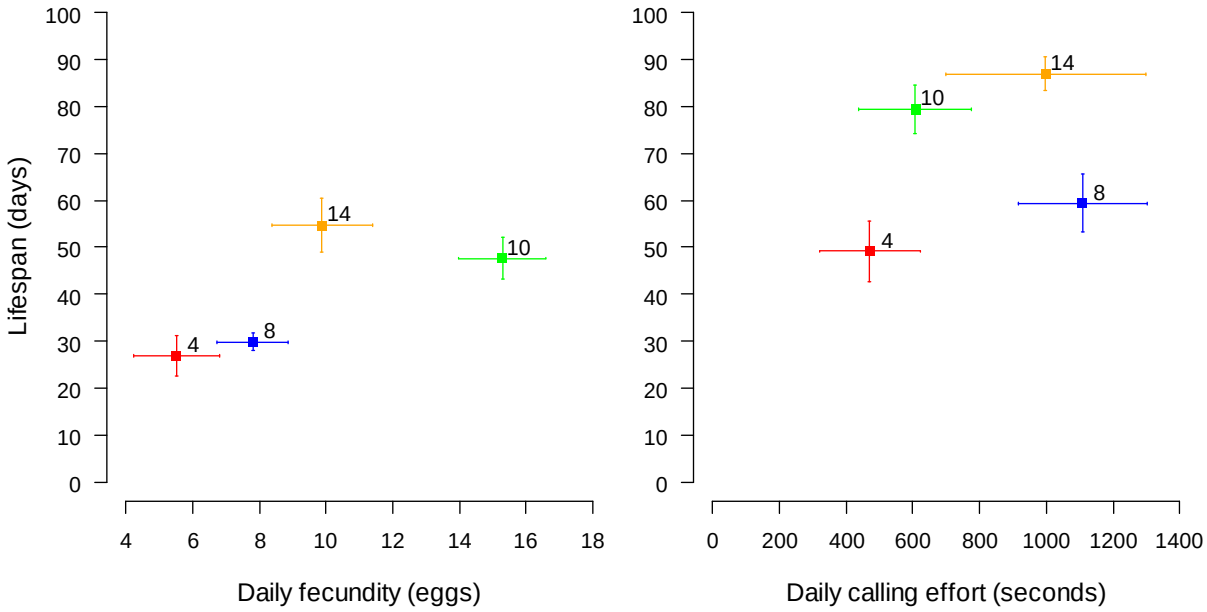

Figure 6-2. The effects of each diet on lifespan in either sex (females: left, males: right) and daily reproductive effort in Chapter 5 of this thesis. This figure illustrates how these diets allow us to compare oxidative damage in long and short lived animals (e.g. females: compare diets 10/14 with 4/8, males compare diets 10/14 with 4/8) and across animals that invest heavily or very little in reproductive effort (e.g. females 10 > 8/4, males 8/14 > 4).

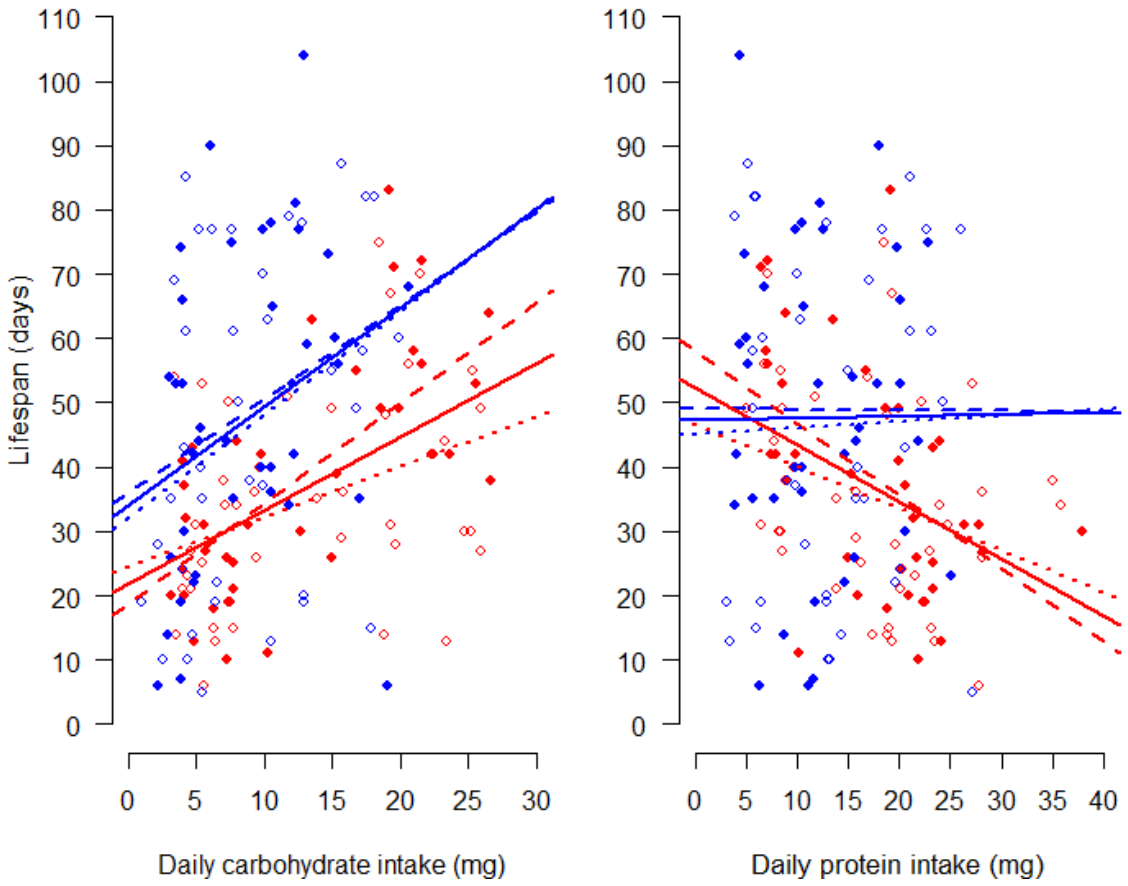

Figure 6-3. The effect of daily intake of carbohydrate (mg) and protein (mg) on lifespan in males (blue) and females (red). Solid points represent unsupplemented animals and animals supplemented with vitamin E are represented by open points. Solid regression line represents average data for supplemented and unsupplemented animals.

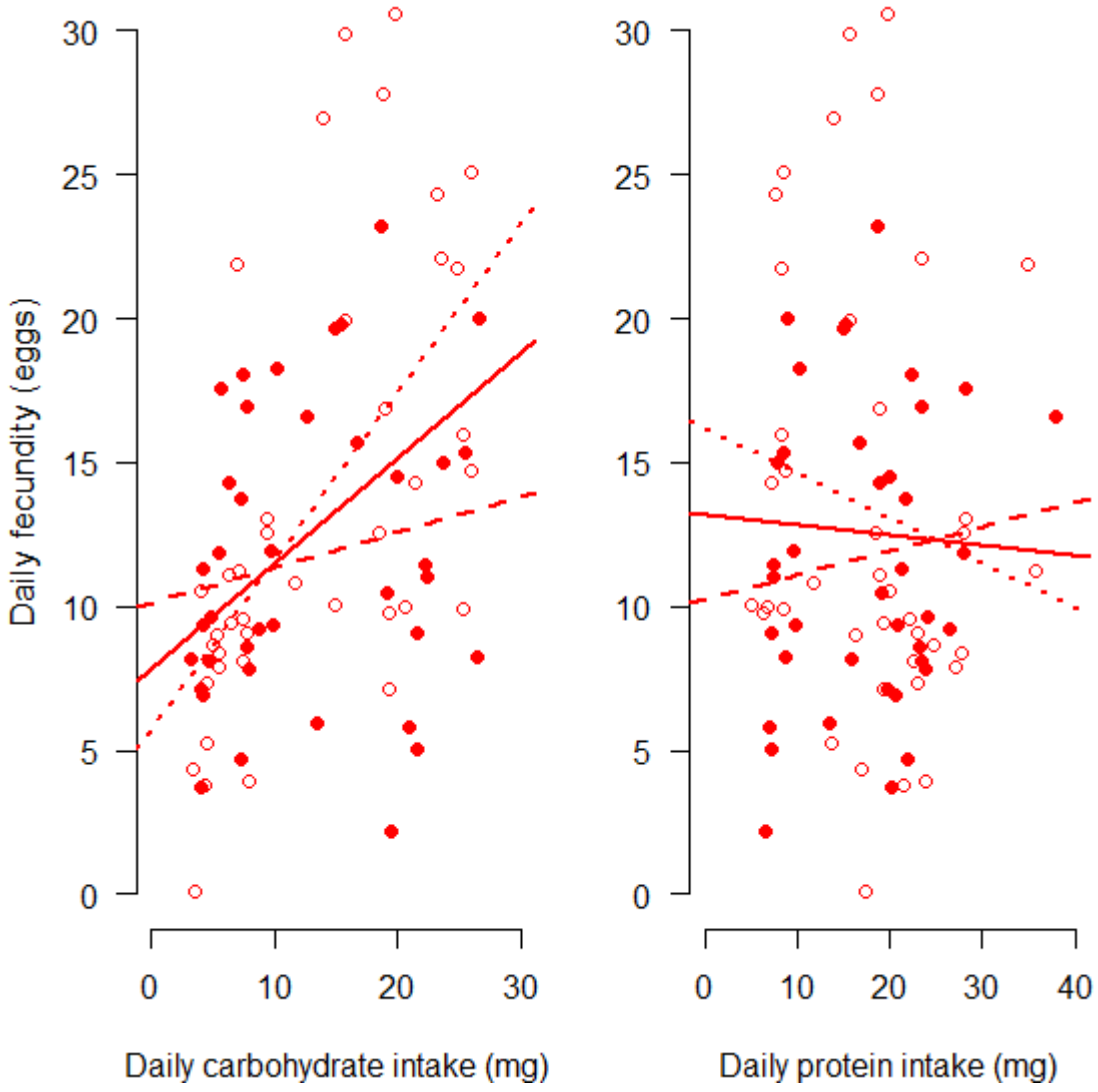

Figure 6-4. The effect of daily intake of carbohydrate (mg) and protein (mg) on the female fecundity. Symbols are as in Figure 6-3.

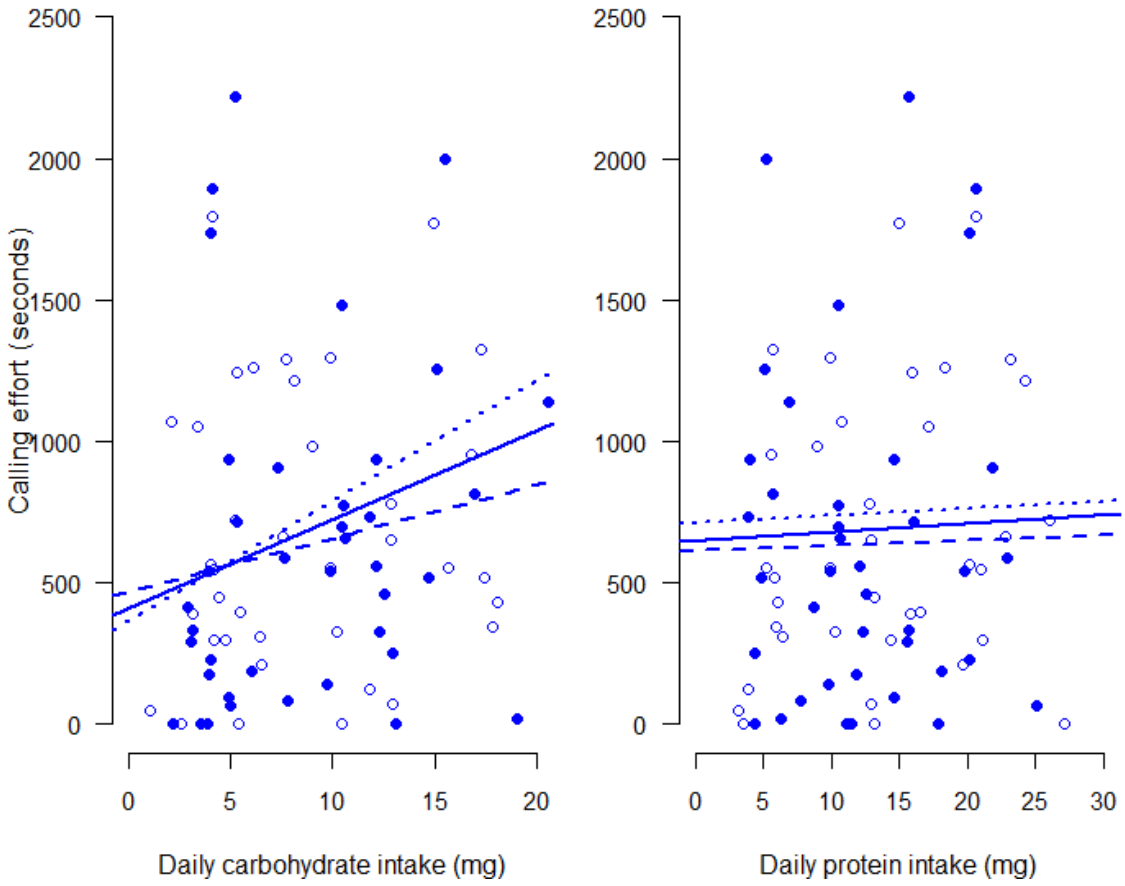

Figure 6-5. The effect of daily intake of carbohydrate (mg) and protein (mg) on the daily reproductive effort in males. Symbols are as in Figure 6-3.

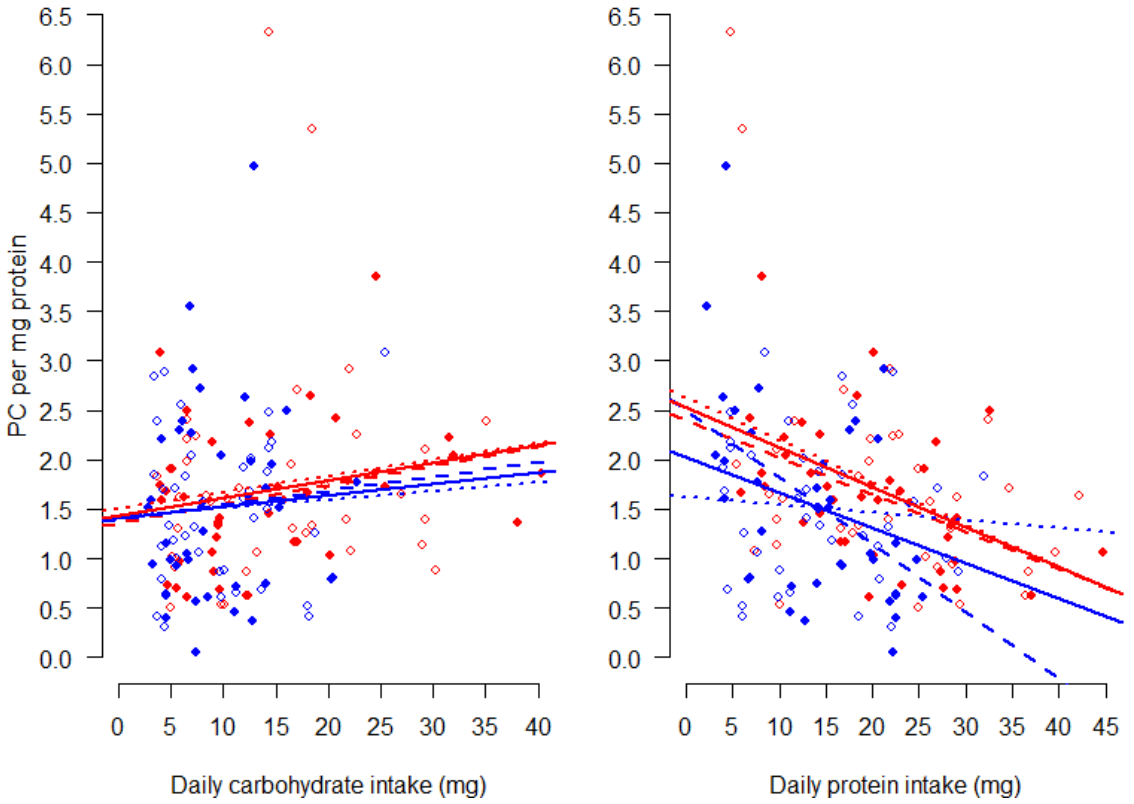

Figure 6-6. The effect of daily intake of carbohydrate (mg) and protein (mg) on protein carbonyl group formation (nMol) per mg of protein. Symbols are as in Figure 6-3

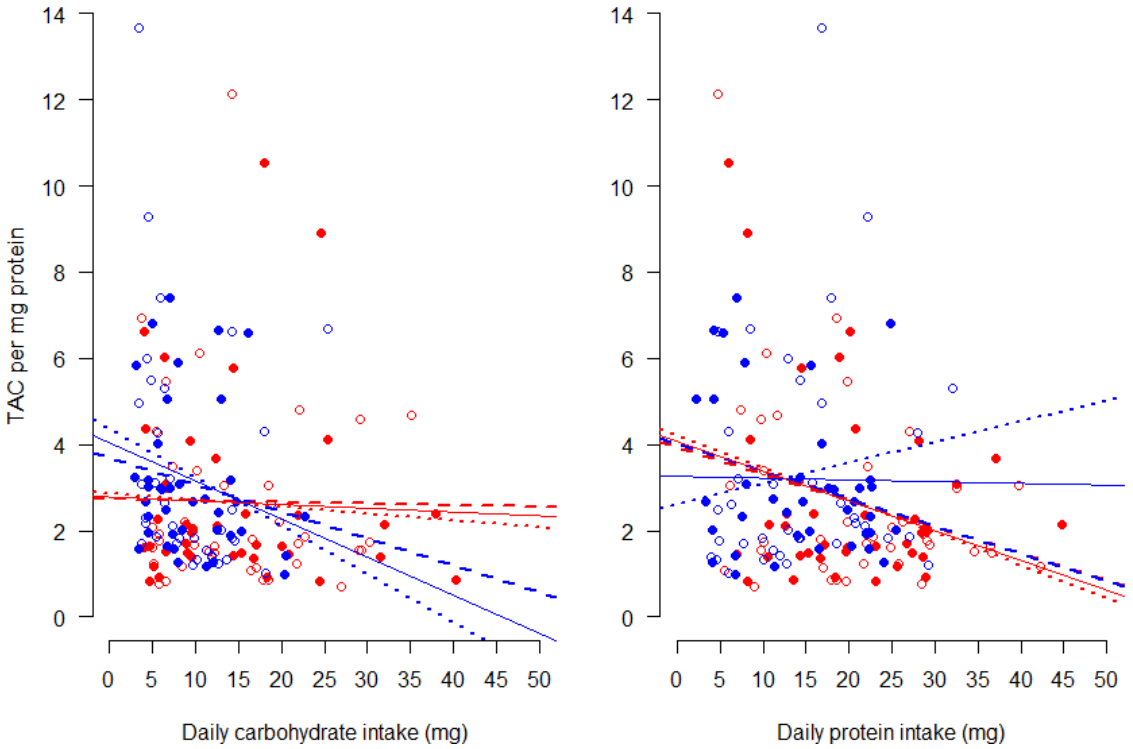

Figure 6-7. The effect of daily intake of carbohydrate (mg) and protein (mg) on total antioxidant capacity (mM) per mg of protein. Symbols are as in Figure 6-3.

Table 6-1. Results of some key studies examining how DR affects oxidative stress. **Abbreviations**: AOX-antioxidants, CAT-catalase, damage-oxidative damage, GSH-glutathione, GPX-glutathione peroxidase, mtDNA-mitochondrial DNA, nDNA-nuclear DNA, OS-oxidative stress, ROS- ROS production, SOD-superoxide dismutase. **Symbols:** '↑' means DR increased damage, ROS or AOX, '↓' DR reduced damage, ROS or AOX, and '–' no significant difference between DR and control animals. If two symbols are provided, results differed across tissue types or over time.

Species	Sex	Dietary restriction regime	Results	Ref
Primates	M	30% previous	Damage: protein ↓	1
Mice	M	60% *ad libitum*	Damage: DNA ↓ / –	2
			AOX: SOD, CAT, GPX ↓ / ↑ / – Damage: protein ↓ ROS: ↓	3
			Damage: protein ↓ / ↑	4
			Damage: lipid ↓ ROS: ↓	5
Rats	M	AL +/- exercise DR +/- exercise	AOX: CAT ↑,GPX↑, GSH –, SOD– ROS: ↓ / –	6
		Protein restriction Caloric restriction Total food restriction	Damage: protein ↓	7
		Intermittent feeding	Damage: DNA ↓ / –	8
			AOX: SOD ↓, GPX ↓, CAT↓ Damage: protein ↓ / –	9
		60% *ad libitum* animals	Damage: DNA ↓	10
			AOX: CAT –, SOD – Damage: lipid ↓	11
			Damage: nDNA –, mtDNA ↓ ROS: ↓	12
			Damage: protein ↓ ROS: –	13
			Damage: DNA ↓ ROS: ↓	14
		40% methionine restriction 80% methionine restriction	ROS :↓ Damage: DNA↓, lipid↓, protein↓	15
		40% amino acid restriction (except methionine)	Damage: DNA –, lipid ↓, protein↓ ROS: –	16
		8.5% caloric restriction 25% caloric restriction	Damage: mtDNA – , protein ↓ ROS: ↓ / –	17
		40% Protein restriction	Damage: DNA ↓ protein ↓ ROS: ↓	18
		DR - 50% *AL* animals AL+F - Fibre supplemented DR+Fibre	AOX: SOD - ↑, CAT - ↑, GSH - ↑ Damage: lipid ↓ (DR/DR-F/F)	19
		Short term: 40% *ad libitum*	Damage: Short term –	20

		Long term: 40% *ad libitum*	Long term: mtDNA ↓, nDNA − ROS: Short term −, Long term ↓	
	M F	60% *ad libitum* animals	Damage: protein ↓	21
D. melanogaster	F	Caloric restriction	Damage: lipid delayed	22
	M F		Resistance to OS − / ↓	23
C. elegans	-	Axenic media	AOX: SOD ↑, CAT ↑	24
		Glucose restriction	AOX: CAT ↑, SOD−, GPX − ROS: ↑	25
S. cerevisiae	-	Caloric restriction	AOX: CAT ↑, SOD ↑, GPX ↑	26

Refs: 1. Zainal et al. 2000, 2. Sohal et al. 1994a, 3. *S*ohal et al. 1994b, 4. Dubey et al. 1996; 5. Guo et al. 2002, 6. Kim et al. 1996; 7. Youngman et al. 1992, 8. Kaneko et al. 1997, 9. Radák et al. 2002, 10. Chung et al. 1992, 11. Jeon et al. 2001, 12. Lopez-Tores et al. 2002, 13. Pamploma et al. 2002, 14. Sanz et al. 2005, 15. Caro et al. 2008; 16. Caro et al. 2009, 17. Gómez et al. 2007, 18. Ayala et al. 2007, 19. Diniz et al. 2003, 20. Gredilla et al. 2001, 21. Aksenova et al. 1998, 22. Zheng et al. 2005, 23. Burger et al. 2007, 24. Houthoofd et al. 2002, 25. Schulz et al. 2007, 26. Argawal et al. 2005.

Table 6-2. Protein and carbohydrate composition of the artificial diets used in my feeding experiments. The total nutrients present in each diet is given as the sum of the percentage protein and percentage carbohydrate (i.e. P+C = 36), the remaining percentage consists of indigestible carbohydrate.

Protein (P)	Carbohydrate (C)	P + C	P : C	Diet Number
70	14	84	5:1	4
63	21	84	3:1	8
18	18	36	1:1	10
9	27	36	1:3	14

Table 6-3 Summarized output for sex-specific GLM analyses of the affect of daily intake of protein (P), carbohydrate (C) and vitamin E (factor) (V) on lifespan and daily reproductive effort in male and female *T. commodus*. The significance of each explanatory variable was determined via step-wise model simplification and so if an explanatory variable had a significant affect on a particular response via an interaction term, its individual affect could not be ascertained (e.g. female reproductive effort, vitamin E and carbohydrate intake). These values are represented by NA.

	Test statistics General Linear Models		
Explanatory variables	***F***	***DF***	***P***
Males			
Lifespan			
Vitamin E (V)	0.89	73, 74	0.349
Carbohydrate (C)	14.12	74, 75	0.001
Protein (P)	5.96	74, 75	0.017
V x C	0.01	70, 71	0.922
V x P	0.12	71, 72	0.728
C x P	0.14	72, 73	0.713
C x P x V	1.18	69, 70	0.282
Reproductive Effort			
Vitamin E (V)	0.22	73, 74	0.641
Carbohydrate (C)	10.64	74, 75	0.002
Protein (P)	5.87	74, 75	0.018
V x C	0.01	72, 76	0.930
V x P	0.00	70, 71	0.987
C x P	0.01	71, 72	0.928
C x P x V	0.05	69, 70	0.832
Females			
Lifespan			
Vitamin E (V)	1.62	76, 77	0.207
Carbohydrate (C)	25.97	77, 78	0.001
Protein (P)	0.48	75, 76	0.490
V x C	1.94	74, 75	0.168
V x P	0.09	72, 73	0.769
C x P	2.02	73, 74	0.159
C x P x V	1.14	71, 72	0.289
Reproductive Effort			
Vitamin E (V)	NA	NA	NA
Carbohydrate (C)	NA	NA	NA
Protein (P)	11.45	74, 75	0.001
V x C	8351	74, 75	0.005
V x P	0.06	72, 73	0.813
C x P	2.78	73, 74	0.100
C x P x V	0.47	71, 72	0.494

Table 6-4. MANCOVA examining the effects of daily intake of protein and carbohydrate and their interactions on levels of protein carbonylation (PC) and total antioxidant capacity (TAC) per mg protein in *Teleogryllus commodus*.

	MANCOVA			
Model terms	Pillai	*F* value	*Df*	*P* value
Supplementation (V)	0.002	0.174	2, 139	0.840
Carbohydrate intake (C)	0.115	9.074	2, 139	0.000
Protein intake (P)	0.092	7.016	2, 139	0.002
Sex (S)	0.081	6.164	2, 139	0.003
V x C	0.000	0.125	2, 139	0.988
V x P	0.021	1.487	2, 139	0.230
V x S	0.007	0.501	2, 139	0.604
C x S	0.014	1.019	2, 139	0.364
P x C	0.007	0.523	2, 139	0.594
P x S	0.011	0.775	2, 139	0.163
V x C x P	0.009	0.630	2, 139	0.534
V x C x P	0.016	1.147	2, 139	0.321
V x P x S	0.023	1.619	2, 139	0.202
C x p x S	0.018	1.285	2, 139	0.280
V x P x C x S	0.004	0.962	2, 139	0.385

Table 6-5. MANCOVA examining the effects of line and age on levels of PC and TAC in female *Teleogryllus commodus.* I also present univariate GLMs to determine how each response variable contributes to the overall multivariate effect.

	MANCOVA			
Model terms	Pillai	*F* value	*Df*	*P* value
Supplementation (V)	0.000	0.013	2, 69	0.987
Carbohydrate (C)	0.039	1.406	2, 69	0.252
Protein (P)	0.170	7.077	2, 69	0.001
V x C	0.001	0.042	2, 69	0.959
V x P	0.010	0.334	2, 69	0.718
C x P	0.000	0.014	2, 69	0.987
V x C x P	0.016	0.555	2, 69	0.576
	Univariate GLM			
	F value	*Df*	*P* value	
PC				
V	0.005	1, 70	0.943	
C	0.002	1, 70	0.970	
P	14.191	1, 70	0.0001	
V x C	0.003	1, 70	0.955	
V x P	0.401	1, 70	0.589	
C x P	0.024	1, 70	0.876	
V x C x P	1.092	1, 70	0.300	
TAC				
V	0.016	1, 70	0.900	
C	2.763	1, 70	0.101	
P	1.330	1, 70	0.253	
V x C	0.086	1, 70	0.770	
V x P	0.152	1, 70	0.698	
C x P	0.001	1, 70	0.979	
V x C x P	0.001	1, 70	0.978	

Table 6-6. MANCOVA examining the effects of line and age on levels of PC and TAC in male *Teleogryllus commodus*. I also present univariate GLMs to determine how each response variable contributes to the overall multivariate effect.

	MANCOVA			
Model terms	Pillai	*F* value	*Df*	*P* value
Protein (P)	0.075	2.788	2, 69	0.068
Carbohydrate (C)	0.114	4.444	2, 69	0.015
Supplementation (V)	0.019	0.650	2, 69	0.525
P x C	0.048	1.751	2, 69	0.181
P x V	0.063	2.300	2, 69	0.108
V x C	0.016	0.573	2, 69	0.567
V x C x P	0.079	2.537	2, 69	0.086
	Univariate GLM			
	F value	*Df*	*P* value	
PC				
V	0.789	1, 70	0.378	
C	0.818	1, 70	0.371	
P	4.501	1, 70	0.037	
V x C	0.709	1, 70	0.403	
V x P	3.990	1, 70	0.0497	
C x P	0.139	1, 70	0.711	
V x C x P	0.443	1, 70	0.508	
TAC				
V	0.124	1, 70	0.721	
C	8.979	1, 70	0.004	
P	0.054	1, 70	0.817	
V x C	0.868	1, 70	0.354	
V x P	2.220	1, 70	0.141	
C x P	2.521	1, 70	0.116	
V x C x P	3.175	1, 70	0.079	

7. GENERAL DISCUSSION

While ageing is nearly ubiquitous across multicellular organisms it is not inevitable; some species age and die over a couple of weeks (e.g. fruit flies, Finch 1990) while others do not appear to senesce at all (e.g. hydra, Martinez et al. 1998). Understanding how evolution has shaped such variation in lifespan and rates of ageing both across and between species, poses a considerable challenge to biological research (Nussey et al. 2008a; Kenyon 2010; Partridge 2010). Recent reviews have highlighted that to develop our understanding of ageing requires that we adopt an integrated approach to gerontology, combining classic predictions of evolutionary theories of ageing with ecology (Abrams 1993; Bonduriansky and Brassil 2002; 2005; Wilkinson and South 2002; Williams and Day 2003; Reznick et al. 2004; Norry et al. 2006; Sparkman et al. 2006; Möller et al. 2007), sexual selection (Promislow 2003; Graves 2007; Bonduriansky et al. 2008; Maklakov and Fricke 2009) and kin selection and social environment (Lee et al. 2003; Dytham and Travis 2006; Bourke 2007; Cant and Johnstone 2008; Sharp and Clutton-Brock 2010). Such an integrated approach has already begun to fill in missing pieces of the ageing puzzle, however, there remain many gaps in our understanding of how and why we age. I have attempted to address some of these in my doctoral research. In particular, I have examined how the evolution and mechanistic basis of ageing is affected by sexual selection and diet. In this discussion I summarise my key findings and illustrate how they fit into the field of gerontology as a whole.

7.1 SEXUAL SELECTION AND THE EVOLUTION OF SENESCENCE

Recent work has highlighted that sexual selection and sexual conflict can shape divergent strategies of age-dependent reproductive effort and in turn, affect the evolution of ageing and lifespan across the sexes (Promislow 2003; Graves et al. 2007; Bonduriansky et al. 2008). However, sexual selection has been poorly integrated into ageing research (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). In Chapter 3, I conducted the first study to quantify the genetic (co)variance between ageing, lifespan and reproductive effort across the sexes and found clear evidence for an

important role for sexual selection in the evolution of lifespan and ageing in *G. sigillatus*. This study illustrates the importance of considering sexual selection and sexual conflict when examining the evolution of ageing and lifespan.

However, our understanding of how sexual selection affects ageing is still largely incomplete. To develop our understanding further studies should characterise how sexual selection affects age-dependent reproductive effort, ageing and lifespan in a broad range of species. In particular, research should be carried out in species that differ in their mating systems (e.g. monogamous vs polyandrous) or strategies of parental care (e.g. maternal vs paternal vs biparental). These will vary in the opportunity for, and intensity of, sexual selection and sexual conflict across the sexes. Such research may therefore elucidate the interaction between life-history strategies, sexual selection and sexual conflict across the sexes (Bonduriansky et al. 2008). More research should also be carried out in natural populations because ageing, lifespan and mortality rates often differ across wild and captive populations (e.g. Kawasaki et al. 2008). Moreover, predictions of evolutionary theories of ageing may not be upheld in wild populations (Nussey et al. 2008a). For example, in the wild extrinsic mortality is frequently not condition independent (Bronikowski and Promislow 2005) and this affects predictions about how mortality rates should affect the evolution of ageing and lifespan (Williams and Day 2003; Williams et al. 2006). Research in the wild could therefore help further develop our understanding of the interaction between ageing, environment and sexual selection and so allow us to assess the effects of measuring ageing in an artificial laboratory environment (Bonduriansky et al. 2008).

To understand how sexual selection affects ageing and lifespan also requires that we better understand the interaction between natural and sexual selection. Predictions about how sexual selection affects senescence depend critically on whether sexual and natural selection act antagonistically or synergistically in males (Bonduriansky et al. 2008). For example, if male-male competition selects for greater male condition (reviewed by Lorch et al. 2003; Lailvaux and Irschick 2006) then sexual and natural selection may act synergistically to reduce mortality rates in males (Bonduriansky et al. 2008). This would reverse the typical prediction that intense sexual selection will cause more rapid senescence in males than females. Studies that manipulate both natural and sexual selection and ask how these affect lifespan,

actuarial (mortality risk) and functional (physiological performance) senescence, may develop our understanding of how sexual and natural selection interact to affect patterns of ageing across the sexes.

Finally, to understand how sexual selection and sexual conflict affect the evolution of ageing and lifespan, it is important to examine the mechanistic basis of sex differences in senescence. The remainder of my thesis aimed to do just this. I first asked if sexual selection could promote sex-specific life-history responses to dietary manipulation and if either sex could regulate their nutritional intake to optimise their sex-specific fitness.

7.2 FOOD, SEX AND DEATH: THE ROLE OF NUTRITION IN LIFE-HISTORY STRATEGIES

In a broad range of species, reproductive effort and lifespan are optimised at very precise ratios of specific nutrients (e.g. protein to carbohydrate) (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009). These dietary optima are often different for each trait, for example, reproductive effort may require protein to allocate to gametes (Williams 1994) but excessive protein intake is often detrimental to longevity perhaps because of the toxic effects of nitrogenous wastes (Simpson and Raubenheimer 2009). This means that there may be a trade-off between investment in reproduction and lifespan that is decided at the point at which nutrients are consumed (Cotter et al 2011). In Chapter 5, I asked if sexual selection promoted sex-specific resolutions of this trade-off in *T. commodus* and then asked how this trade-off was affected by mating.

My results showed that mating can shift the dietary optima for life-history traits, most notably male reproductive effort. I also found that crickets can regulate their dietary intake in response to a changing environment (e.g. mating status). Finally, I found sexual dimorphism in dietary intake under choice with females feeding towards the diet optimal for lifespan, while the male intake fell between the diets optimal for lifespan and reproductive effort. These results suggest that intersexual difference in nutrient regulation may be a proximate mechanism by which sexual selection promotes sex-specific life-history strategies.

However, much more work is needed to develop our understanding of nutrient regulation across the sexes and its consequences for male and female fitness, of which lifespan and ageing are vital components. Foremost, nutritional landscapes need to be better parameterised, for example, confidence intervals surrounding nutritional peaks for particular traits would enable us to better determine the biological consequences of intake targets. For example, confidence intervals would illustrate whether two peaks that are in similar, but non-overlapping regions of the nutritional landscape (e.g. male lifespan and reproductive effort in Chapter 5) are indeed, statistically distinct. While such techniques exist in the statistics literature, they are computationally demanding and require specialist software (del Castillo and Cahya 2001). They are therefore not readily available to most biologists. Second, to determine the adaptive significance of nutrient regulation also requires that we track an individual's nutrient intake for its entire lifetime. Had I done this in Chapter 5, I may have seen greater intersexual divergence in intake targets and therefore, been better able to infer their biological significance in either sex.

Moreover, although animals in a laboratory environment can show fine-scale regulation of nutritional intake, animals in the field may not do so because the costs of choosiness outweigh any benefits of exerting choice in an environment where food is patchy and ephemeral. Whilst collecting nutritional intake data in the field poses a considerable challenge, recent studies have shown that the geometric framework may be applied in the wild. For example, Simpson et al. (2006) provided migratory bands of Mormon crickets a choice of synthetic diets and, by examining their intake via video recordings, showed a clear preference for dietary protein. Felton et al. (2009) monitored the foraging behaviour and dietary intake of wild spider monkeys and then analysed the nutritional content of every food item the monkeys were seen to consume. By doing so, the authors found that monkeys regulated their dietary consumption of protein rather than energy. Studies in the field that asses how dietary choices affect life-history strategies would be particularly interesting, for example, we could determine if rates of extrinsic mortality affect intake targets. We might predict that, in populations evolved under higher levels of environmental mortality, animals eat to optimise their reproductive effort rather than their lifespan. In species evolved where there are fewer sources of environmental mortality, intake targets may shift to

promote a longer lifespan. To date, very few studies have explore the mechanisms by which ecology shape life-history strategies (but see Robert and Bronikowski 2010).

A major challenge of conducting field based studies of nutritional ecology is that accurately gathering data on the consumption of a particular nutrient is incredibly difficult. A solution to this problem is to model the proportions of nutrients that are consumed rather by an individual than the absolute amounts of each nutrient. Mixture triangles, whereby 3-D compositions of a diet are represented as a single point within an equilateral or right angled triangle, provide a tool to do this and so may make studies testing how animals manage their nutrition in the field much more feasible (reviewed in Raubenheimer 2011).

A major advantage of mixture triangles is that, because they consider the mixtures or proportions of particular nutrients in the diet rather than the absolute amount, they enable a '*n*' dimensional nutritional problem to be represented on a '*n-1*' nutritional space (Raubenheimer 2011). For example, any mixture of two nutrients can be plotted on a single axis, a three nutrient mixture can be represented in a 2-D space (i.e. within a triangle) (Figure 7-1) and any mixture of four-nutrients can be plotted in a 3-D space. Effectively, by not measuring "amount" we gain an additional vector that enables us to address higher order nutritional problems. For example, a recent study used mixture triangles to examine how cats regulate their intake of lipid, protein and carbohydrate simultaneously (Hewson-Hughes 2011). We could also incorporate micronutrients (e.g. amino acids or vitamins) into this framework, for example, in Chapter 6 I could have manipulated proportions of vitamin E, protein and carbohydrate in the diet simultaneously, which would have allowed us to determine the effect of vitamin E dose on life-history traits. Mixture triangles also allow us to readily quantify utilisation efficiency, by comparing the nutrient composition of faeces and comparing this to the composition of food consumed (Raubenheimer 2011). Utilisation efficiency is one important means of nutritional regulation (e.g. Dussutour and Simpson 2009), albeit one which is usually very difficult to measure and so often ignore. Finally, performance can be plotted onto a mixture triangle and therefore, the effect of three nutrients on a given trait could be analysed using response surface methodologies (as in Chapter 5). While mixture triangles are not appropriate to all nutritional problems, for example where the absolute intake of particular nutrients is

important, they will facilitate research in the field and allow us to adopt a truly multi-nutritional approach to nutritional ecology, moving beyond the manipulation of just protein and carbohydrate (Raubenheimer 2011).

Finally, although the geometric framework has been used to great effort to explore how diet affects reproductive effort, ageing and lifespan, this technique had not been used to explore the mechanistic basis of ageing and lifespan. In Chapter 6, I used the geometric framework to examine the role of oxidative stress in senescence and in mediating life-history strategies in *T. commodus* across the sexes.

7.2 OXIDATIVE STRESS, SENESCENCE AND REPRODUCTION: TESTING THE FREE RADICAL THEORY

In Chapters 4 and 6 I examine the role of oxidative stress in causing senescence to test the free radical theory of ageing, and, more generally, to determine if oxidative stress is a proximate constraint on life-history evolution. In Chapter 4, I quantified the genetic (co)variance of oxidative damage, antioxidant protection, ageing parameters, lifespan and age-dependent reproductive effort to examine how oxidative damage affects sex-specific life-history strategies. I found that oxidative stress mediated trade-offs between traits expressed at different ages (e.g. early reproductive effort and ageing rate in females). This offers support for the hypothesis that oxidative stress is a proximate cost of reproductive effort that accelerates ageing and reduces lifespan. Moreover, oxidative damage was greater in the shorter lived sex (females) and was negatively genetically correlated with lifespan, which provides strong support for the free radical theory of ageing. However, in this study, I could not determine whether oxidative damage was a cause of senescence or a consequence of senescence in *G. sigillatus*. To really test the free radical theory of ageing it is important to manipulate lifespan and determine how this affects oxidative stress (or *vice versa*).

I did this in Chapter 6 where I used diet to manipulate lifespan and reproductive effort in both sexes of *T. commodus* to ask how this affected oxidative damage and antioxidant protection. I found that damage was greater in the shorter lived sex but that the diet which was also optimal for lifespan was associated with the greatest levels of oxidative damage. This result directly contradicts the free radical theory of ageing. In females, vitamin E improved fecundity when coupled with high

carbohydrate intake and in males, high calling effort was associated with reduced antioxidant protection. This study shows that the association between oxidative stress, lifespan and reproductive effort is complicated and likely to vary across the sexes. Whilst this chapter offers some equivocal support for the free radical theory, unlike in *G. sigillatus* it does not appear that oxidative stress mediates life-history reposes to dietary manipulation in *T. commodus*. In summary, my doctoral research offers equivocal support for the free radical theory of ageing (Harman 1956).

The free radical theory offers one of the best supported mechanisms for causing ageing (Beckman and Ames 1998; Finkel and Holbrook 2000) but this theory has recently been heavily criticised and recent review articles have advocated that we abandon it altogether (e.g. Howes et al. 2006; Pérez et al. 2009). While several criticisms have been levied at the free radical theory, the two most frequently cited are that (1) some very long-lived species have high levels of oxidative damage, rates of ROS production and/or poor antioxidant defences (Speakman and Selman 2011) and (2) genetic manipulations of antioxidants do not consistently affect longevity in the direction predicted by the free radical theory of ageing (Jang and Remmen 2009; Pérez et al. 2009; Howes 2006). For example, of eighteen genetic manipulations that affected the expression of antioxidant enzymes, only one influenced lifespan in the direction predicted by the free radical theory (Pérez et al. 2009). However, do these criticisms really warrant our abandoning the central premise of the free radical theory of ageing that the accumulation of oxidative damage causes senescence?

First, I address the general criticism that long lived species with high levels of oxidative damage and/or ROS production undermine the free radical theory of ageing. A frequently cited example of such a species is the naked mole rat (Speakman and Selman 2011). This eusocial mammal generates high levels of ROS from its mitochondria (Lambert et al. 2007) and has severe oxidative damage in particular tissues (Andziak et al. 2006) but appears to be very good at repairing and tolerating this damage (Pérez et al. 2009). However, despite apparently high levels of oxidative stress, the naked mole rat is the longest lived rodent (Buffenstein et al. 2005). This has been declared evidence against the free radical theory (e.g. Speakman and Selman 2011).

However, naked mole rats also exhibit very unusual patterns of ageing. This species shows negligible senescence because its risk of dying does not increase with age (Mele et al. 2010). Moreover, it remains in good health for around 85% of its lifespan (around 30 years) and female naked mole rats can continue to breed into their third decade (Mele et al. 2010). Moreover, there is no evidence for either mutation accumulation or the disposable soma theory in this species, while the antagonistic pleiotropy theory has only received equivocal support (reviewed by Mele et al. 2010). There is much about the evolution of ageing (or the apparent lack of) in the naked mole rat that we do not understand. The failure of a species which, according to established definitions (Finch 1990) does not age (Mele et al. 2010), to conform to any mechanistic theory of ageing does not constitute compelling evidence against that theory. Species that buck otherwise widespread trends (i.e. an inverse relationship between ROS production and lifespan) (Lambert et al. 2007) are important and interesting and should be the target of intensive research. However, they do not provide grounds to entirely dismiss any one theory of the mechanistic basis of ageing.

The second frequently cited criticism of the free radical theory is the failure of antioxidant manipulation to consistently affect lifespan (Jang and Remmen 2009; Pérez et al. 2009). This prediction is based on the assumption that there will be a simple, direct relationship between antioxidants and lifespan. This assumption is most likely a gross oversimplification. ROS perform a number of vital physiological functions (Dickinson and Chang 2011), including the regulation of cell growth, development and apoptosis (Apel and Hirt 2004; Veal et al. 2007; Palmer and Paulson 2009). A cell's redox state (i.e. its balance between anti- and pro-oxidants) affects the expression of several genes (Allen and Tresin 2000; Tapia et al. 2006). However, the balance of ROS and antioxidants needed to perform a particular function varies across cell types (Allen and Tresin 2000; Veal et al. 2007). The optimal redox state may also shift in response to a changing environment, for example, immune challenges, season or age (Lopez-Martinez et al. 2008; Cohen et al 2009). This means that manipulations of antioxidants could disrupt cellular redox state to the detriment of organismal function and performance (Lin and Beal 2006; Tapia 2006). For this reason, antioxidant manipulation could cause pathologies that themselves affect lifespan, independently of ageing (Magwere et al. 2006).

The possibility that antioxidant manipulation may disrupt cellular function is particularly true of studies that knock out or over-express antioxidant genes. Disrupting the expression of any gene may have downstream, unforeseen effects at other loci (Gingrich and Hen 2000). Similarly, knockout studies may disrupt genes that have pleiotropic effects i.e. regulate more than one function (Gingrich and Hen 2000). Given that a widely accepted evolutionary theory of ageing relies on the presence of alleles with pleiotropic effects on lifespan (*antagonistic pleiotropy*, Williams 1957), alleles with pleiotropic effects on lifespan may be widespread across the genome. Both pleiotropy and epistatic interactions between genes complicate the interpretation of the phenotypes of knockout organisms (Gingrich and Hen 2000).

Studies citing the failure of antioxidants to consistently affect oxidative stress and lifespan also overlook that antioxidants are not the only line of defences against oxidative stress. Different species have evolved a complex suite of mechanisms to protect against oxidative stress (Buttemer et al. 2010). For example, a long lived garter snake ecotype has DNA that is easily damaged by ROS, however, these snakes are able to rapidly and efficiently repair this damage (Robert and Bronikowski 2010). Currently, the repair of oxidised molecules is poorly understood and comparatively seldom investigated. To fully understand the association between oxidative stress and lifespan it is important to measure, wherever possible, rates of ROS production, levels of damage, antioxidant defences and repair.

Finally, my research also shows that reproductive effort can affect oxidative damage and antioxidant capacity and therefore, endorses the importance of incorporating an understanding of reproductive effort into predictions about the relationship between oxidative damage, antioxidant protection and lifespan (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011). If antioxidants are allocated to reproductive investment (e.g. sexual signals, Blount 2003) rather than somatic maintenance because greater reproductive effort improves fitness more than a long life does (e.g. Hunt et al. 2004) then antioxidant supplementation may not affect lifespan. However, this does not mean that oxidative stress does not affect senescence. Moreover, this association may be sex-specific because sexual selection promotes different life-history strategies in males and

females (Bonduriansky et al. 2008). This illustrates the importance of considering sexual selection in research examining the mechanistic basis ageing.

In summary, predictions about how manipulations of any antioxidant should affect lifespan need to be grounded on a better understanding of sex-specific life-history strategies and the roles of particular ROS, antioxidants and redox state in maintaining normal physiological function (Lin and Beal 2006). Until we have such a well developed understanding, the failure of antioxidant manipulation to consistently affect oxidative damage and lifespan should be interpreted with care. The wealth of correlative evidence in support of the free radical theory suggest that the perturbation of redox states affect ageing and lifespan (e.g. Sohal et al. 1993a,b; 1994; 1995; Beckman and Ames 1998; Lambert et al. 2007; Robert et al. 2007; Buttemer et al. 2010; Robert and Bronikowski 2010). However, while oxidative stress appears to have a part to play in causing ageing, other processes are likely at work too. For example, whilst long-lived species such as the naked mole rat that have high rates of ROS production (Lambert et al. 2007) and levels of oxidative damage (Andziak et al. 2006) are not sufficient grounds to dismiss the free radical theory entirely, they do show that other mechanistic processes likely contribute to senescence. Additionally, the association between ROS, oxidative stress and lifespan is unlikely to be quite as straightforward as the free radical theory predicts (Speakman and Selman 2011), primarily because of the complex interactions between particular pro- and anti-oxidants (Veal et al. 2007) and the important role that redox state plays in maintaining physiological function (Apel and Hirt 2000; Lin and Beal 2006; Tapia 2006; Dickinson and Chang 2011). Unravelling this complex association and how it is affected by the environment (Robert and Bronikowski 2010) and sexual selection (Chapter 4), poses a considerable challenge to ageing research. Fortunately, a number of single gene mutations have been discovered that extend the lifespan of laboratory animals and these are likely to provide a framework against which we may piece together parts of the ageing jigsaw. Already investigation into the insulin/insulin-like growth-factor-like signalling (IIS) pathway (Kimura et al. 1997; Lin et al. 1997; Ogg et al. 1997) shows how diet (Apfeld and Kenyon 1999), reproduction (reviewed in Partridge and Gems 2002), oxidative stress (Honda and Honda 1999; Kops et al. 2002) may interact to cause ageing. This research heralds an exciting era for gerontology and one which may, over

fifty years since Harman conceived the free radical theory, clarify the role of oxidative stress in ageing, lifespan and life-history trade-offs.

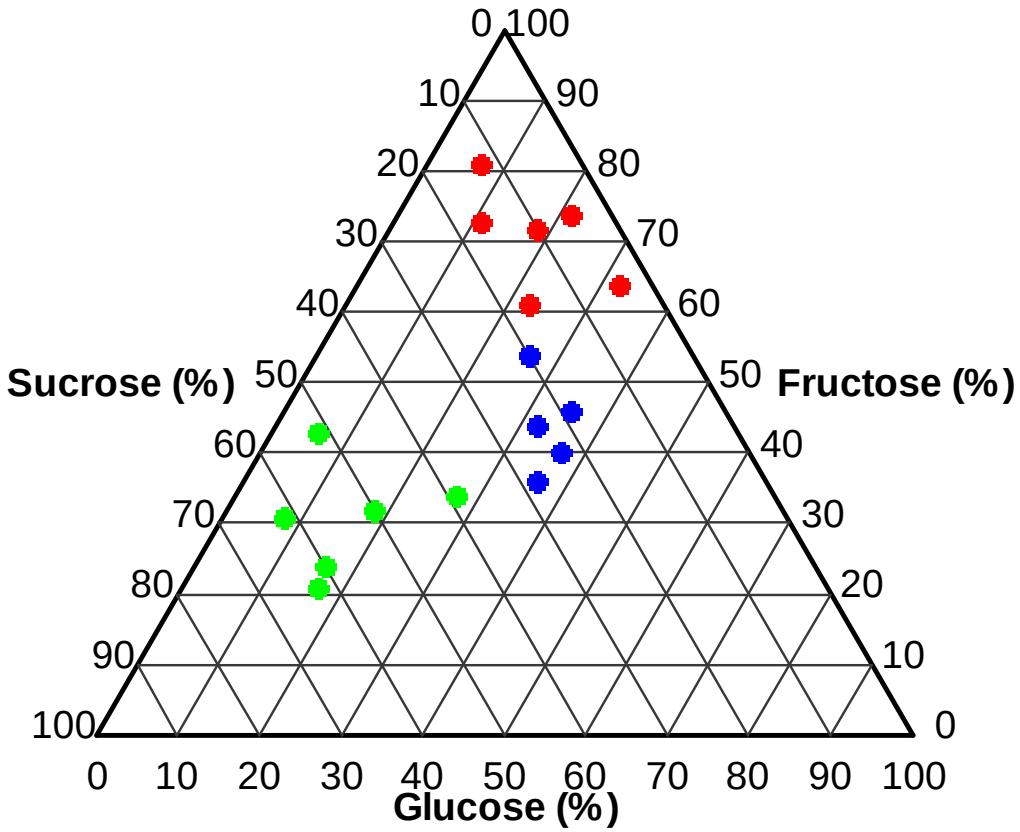

Figure 7-1. A hypothetical mixture triangle . This figure is loosely based on one taken from Raubenheimer (2011), which shows the fructose, sucrose and glucose composition of nectar of flowers pollinated by different pollinator groups (represented by different colours). This example shows how a three nutrient mixture (i.e. fructose, glucose and sucrose), may be represented in a two-dimensional space to answer a particular nutritional problem (i.e. to examine plant-pollinator relationships.)

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