Sex, diet, health and lifespan in the fruit fly,

Drosophila melanogaster

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Abstract

Nutrition is a vital determinant of lifespan, reproduction, health and ageing. Much has been done to investigate the lifespan consequences of short-term (proximate) nutritional manipulation, but much less is known about long-term (evolutionary) nutritional manipulation and nutritional mismatches. In this thesis I addressed this important omission, using the fruit fly, *Drosophila melanogaster*, model system. I empirically tested two evolutionary theories: the Thrifty Phenotype and Thrifty Genotype hypotheses, which predict the general life history consequences of nutritional mismatches over the lifetime or over evolutionary time, respectively. I also tested how the latter interacted with long-term nutritional selection regimes. Contrary to predictions, I showed that the costs of nutritional mismatches between developmental and adult diets were not universal, but instead dependent on the nature of the mismatch, sex and the components of life history measured (Chapter 2). Similarly, the costs of mismatches between evolved and proximate nutrition were dependent on evolved feeding regime, sex, life history component measured and proximate diet (Chapter 3). I discovered that there was enhanced sexual dimorphism for lifespan in nutritionally selected lines, which was associated with sex-specific life history patterns and a partial resolution of sexual conflict (Chapter 4). Transcriptome-wide analysis of these nutritionally selected lines revealed differential expression in genes with functions related to lifespan, post-mating responses, regulation and epigenetic modification (Chapter 5). Finally, I found that manipulation of another important component of altered lifestyles, activity level, had no effect on lifespan or reproduction (Chapter 6). Overall, my results make a novel contribution to the study of nutritional mismatches and long-term nutritional selection. The results also highlight the importance of simultaneously studying both sexes and several age-specific components of life history, in different proximate environments, to fully elucidate the fitness consequences of nutritional manipulation.

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Chapter 1: General Introduction

1.1 Nutrition, lifespan, ageing, health and disease

Nutrition is vitally important for lifespan, ageing, health and disease susceptibility. As such, a significant body of work has examined the life history consequences of short-term nutritional manipulation, across a huge variety of taxa (e.g. Chapman & Partridge, 1996; Partridge & Gems, 2002; Skorupa et al., 2008; Barnes et al., 2008; Gese et al., 2016). Concurrent with this growing empirical knowledge of the effects of short-term nutritional change, unprecedented, rapid and long-term changes in modern human nutrition and lifestyles in industrialised countries, have brought the drastically understudied areas of long-term nutritional manipulation and nutritional mismatches (switches) into stark focus (e.g. Cordain et al., 2005; Myers & Williamson, 2014). Shifts towards the consumption of high calorie diets and more sedentary, less active lifestyles have been associated with a rapid rise in obesity and associated negative health consequences (e.g. Mokdad et al., 2000; Speakman & Selman, 2003). Furthermore, mismatches in reproductive scheduling between ancestral and modern humans, such as shifts towards a later age at first reproduction in modern, industrialised nations (e.g. Kuzawa & Bragg, 2012) have further raised questions over the impacts of altered lifestyles, for life history and fitness. Despite this attention, little has been done to thoroughly investigate the life history consequences, in a tractable model system.

In this thesis, I used the powerful fruit fly model system, *Drosophila melanogaster*, to investigate the effects on lifespan, reproduction and ultimately, fitness, arising from dietary mismatches over lifetime, evolutionary manipulations of nutritional feeding regimes and finally, altered activity levels. I also examined the mechanisms (differential gene expression patterns) associated with the life history patterns that were observed following the evolutionary manipulation of feeding regime.

In this thesis introduction, I review and evaluate the evidence for the role of nutrition on lifespan, ageing, health and disease, and the life history consequences of nutritional manipulation.

1.2 Life history consequences of short-term (proximate) nutritional manipulation

There is widespread consensus that the manipulation of dietary quality, quantity or composition can lead to changes in life histories. For example, in *D. melanogaster*, a large number of studies highlight the close association between nutrition, lifespan and reproduction (e.g. Chapman &

Partridge, 1996; Partridge & Gems, 2002; reviewed by Barnes et al., 2008). Many studies of dietary manipulation in the literature have focused on the short-term (proximate) manipulation of diet within a single generation.

Dietary restriction and caloric restriction

Dietary and caloric restriction (DR, CR, respectively) are prime examples of the manipulation of dietary quality and quantity, which result in profound life history consequences. DR and CR involve the reduction in nutrient availability, but without malnutrition, such as via food dilution (e.g. Tatar, 2007; Fontana et al., 2010). Both DR and CR have been linked to lifespan extension in a wide range of studies across yeast, nematodes, invertebrates (including insects), mammals and even humans (e.g. Maroso, 2005; Fontana et al., 2010). The underlying mechanisms involve nutrient sensing pathways, such as the insulin (or insulin-like, IIS) and TOR (Target Of Rapamycin) signalling pathways (see recent reviews by Mair & Dillin, 2008; Teleman, 2010; Partridge et al., 2011). Interestingly, recent research has shown that DR is also associated with mitochondrial ageing, perhaps via mutations in mitochondrial DNA (Guarente, 2008).

Manipulation of nutrient composition

Manipulation of the composition, ratios or content of one or more nutrients in the diet (such as proteins or sugars) has also been widely conducted empirically (e.g. Chapman et al., 1994; Chapman & Partridge, 1996; Droney, 1996; Magwere et al., 2004; Fricke et al., 2008; Fricke et al., 2010). Such manipulations can lead to pronounced effects on the lifespan and reproductive success of one or both sexes, across a range of taxa and also affect larval and adult growth, morphology, behaviour, physiology and indeed overall fitness, as outlined below.

The protein content of the adult diet is often reported to be a strong determinant of lifespan, reproduction and fitness. For example, a high protein adult diet leads to increased adult body size, survival, reproductive output and fitness, in comparison to a low protein adult diet, in *D. melanogaster* (e.g. Chapman et al., 1994; Chapman & Partridge, 1996; Magwere et al., 2004; Fricke et al., 2010). These patterns would be particularly expected in females, as protein is important for maintaining egg production (Bownes et al., 1991), metabolism and growth. Altered fecundity patterns in response to altered adult dietary content are driven by underlying changes to the expression and activity of nutrient sensing genes (e.g. Teleman, 2010; Partridge et al., 2011). Shifts in egg laying behaviour in response to diet could also be driven by processes akin to host-sensing (e.g. Lindstrom, 1999; Bateson et al., 2004).

The effects of protein are similarly important for male reproductive success. The protein content of the diet affects the amount of male courtship, mating and the quantity and quality of sperm production (Droney, 1996; Fricke et al., 2008). The effects of diet on reproductive traits are not always straightforward, but in general, low protein adult diets lead to fewer courtships and matings (e.g. Fricke et al. (2008), but see Fricke et al. (2010)).

The effects of nutritional composition can also be experienced indirectly. For example, the diet fed to adult males can influence the extent of post-mating responses (e.g. egg and offspring production) stimulated in their female mates (Fricke et al., 2008). Furthermore, such effects can be non-linear, with lowered reproduction in females observed at both high and low levels of protein in the male diet.

The body of research into the manipulation of different dietary components is not reviewed in detail here (for recent detailed reviews see: e.g. Skorupa et al., 2008;). However, broadly speaking, proximate manipulations of major diet components (e.g. protein and carbohydrate levels) result in the following general patterns: (i) loss of lifespan and reproductive success at very low nutrient levels (Chapman & Partridge, 1996; Magwere et al., 2004; Partridge et al., 2005; Piper & Partridge, 2007; Barnes et al., 2008), (ii) extended lifespan and moderate reproductive success at intermediate nutrient levels (e.g. Magwere et al., 2004), (iii) shortened lifespan and increased reproductive output at high nutrient levels, (iv) shortened lifespan and decreased reproduction under very high nutrient conditions (e.g. Magwere et al., 2004).

Genetic basis of life history responses to nutrition

A significant body of research shows that life history responses to proximate (short-term) dietary manipulation are underpinned by candidate genes and pathways linked with nutrient signalling [refs]. The insulin-signalling (IIS) and TOR-signalling pathways are particularly important in mediating the response of lifespan to nutrition (e.g. Teleman, 2010; Partridge et al., 2011). A large body of experimental work, in *Drosophila* and other model species has quantified the effects of nutrient-sensing gene manipulation (in knockout or knockdown studies) of candidates in these pathways, on lifespan, life history and the interaction with diet (e.g. nutritional manipulation studies and gene manipulation studies in the study of ageing; as reviewed by Piper & Bartke, 2008).

Interestingly, many candidate genes involved in mediating the role of proximate diet on lifespan also show antagonistic pleiotropy (Williams, 1957). Antagonistic Pleiotropy (AP) is an evolutionary genetic theory of ageing, proposing that single genes can have opposing early benefits and later

costs over the lifetime. Where the benefits outweigh the costs, in evolutionary terms, these genes persist, allowing the occurrence of genes whose effects can reduce later life fitness and cause late life pathologies. AP was proposed as a theory (Williams, 1966) to provide a general explanation of ageing, the intrinsic deterioration of individuals over time, as reflected within populations by an increased likelihood of mortality and decreased reproductive output, with age. Experimental work has confirmed the theory and recent studies have identified many individual genes showing AP as predicted. Such genes are often linked with increased early life fecundity or body size and decreased longevity (e.g. Clancy et al., 2001; Tatar et al., 2001). Furthermore, these trade off relationships can be condition-dependent, i.e. affected by the nutritional environment (see Piper & Bartke, 2008). For example, the increased longevity of *Drosophila* mutants carrying a loss of function in the insulin receptor substrate homolog, *chico,* traded-off with reduced early life fecundity and body size only when flies were held on concentrated food (Clancy et al., 2001; Tatar et al., 2001; Clancy et al., 2002).

Insights from genetic trade-offs over lifetime (such as antagonistic pleiotropy) highlight the importance of studying several components of life history in an age-specific manner, so as not to overlook temporal trade-offs. Life history effects that are only manifested at specific life history stages, may be overlooked by focusing on total reproductive output over the lifetime, or just lifespan itself, rather than age-specific survival and reproduction. This thesis employs age-specific assays in order to gain a comprehensive picture of the life history.

Life history trade-offs and proximate nutrition

The life history consequences of altered nutrition can also be governed by physiological or life history trade-offs between different life history traits, which may be overlooked in the absence of measurements of several life history components (e.g. survival and reproductive output). Traits may be correlated, co-evolve or show pleiotropy over lifetime. Theory proposes that different life history components may trade-off, due to the allocation of limited resources, acquired from nutrition, between them. This raises the question of the role of nutrition in mediating the extent or presence of trade-offs and the fitness implications.

Life history theory predicts that differences in survival may arise from the re-allocation of limited resources across trade-offs between soma and gametes (Trivers, 1972; Bonduriansky et al., 2008; reviewed by Magwere et al., 2004) and from the physiological allocation of limited resources and energy across life history components (e.g. Reznick, 2010). Trade-offs can also arise from the genetic coupling of traits, across lifetime, such as via antagonistic pleiotropy (Williams, 1957), as

described above. Theoretical and empirical work supports the existence of a trade-off between extended lifespan and reduced early life reproduction (e.g. Zwaan et al., 1995; Flatt, 2011), and between lifespan and reproductive rate (Stearns, 1992). Furthermore, reduced survival can be compensated for by increased fitness (Maklakov & Lummaa, 2013).

However, there is growing empirical support that trade-offs between life history components, such as lifespan and reproductive output, are not inevitable (e.g. Grandison et al., 2009). Instead, the presence or absence of trade-offs can be dependent upon condition, diet, sex or life stage. Many studies have found no lifespan-fecundity trade-off (e.g. Mair et al., 2004; Barnes et al., 2006; see Barnes et al., 2008). Adler et al. (2013) also found survival differences in the absence of trade-offs with reproduction. As yet, it is uncertain whether the presence of plentiful resources is required for the trade-off to be absent, or whether trade-offs can also be absent if resources are limited. However, lifespan extension observed under dietary restriction, is not always dependent on trade-offs with reproduction and so does not necessarily represent a cost to other life history traits (Grandison et al., 2009).

Measurement of fitness consequences

Different measures of fitness are used in the dietary manipulation literature, for *D. melanogaster* and other species. Fitness is often defined as a measure of the contribution of an individual to the next generation. Fitness measures that are either dependent or independent from population growth rate can be calculated (Edward et al., 2010). Many studies use lifetime reproductive success (LRS) as a measure of fitness that is independent from population dynamics. However, a powerful, but less frequently used fitness measure, is Euler's r (Malthusian parameter, intrinsic rate of population increase). This is a measure of fitness that accounts for population dynamics (Gotelli, 2001; Wigby & Chapman, 2005; Edward et al., 2010). Euler's r is calculated using the Euler equation, from the sum of the product of age-specific measures of survival and reproductive output (Gotelli, 2001; Wigby & Chapman, 2005). As such, Euler's r is weighted towards early life reproduction, a period during which selection is most strong. In this thesis, Euler's r was used as the measure of fitness and compared with LRS.

Sex-specific responses to nutritional manipulation

Interestingly, the responses of male and female life histories to proximate diet manipulations can vary significantly. For example, Magwere et al. (2004) produced lifespan and fecundity curves, across a range of dietary protein concentrations in *D. melanogaster* and showed that the response of female lifespan to DR was much more striking than was the case for males.

There is a growing acknowledgement of the importance of studying both sexes in life history and fitness assays. Surprisingly, measurement of sex-specific effects is still often lacking from empirical work, or is only conducted across separate, time-staggered experiments, between which there can be inevitable variability in life history responses. The sexes clearly differ in their biology and often differ in their evolutionary interests (Parker, 1979), so would be expected to show divergent, sex-specific life history patterns. Hence empirical study of both sexes simultaneously, is important.

Many factors could drive sex-specific life history responses to diet, including the greater specific requirement or sensitivity of one sex to nutritional composition or mismatches, based on their biology. It follows that females may show a greater sensitivity to dietary protein content than males, as protein is vital to maintain egg production (Bownes et al., 1991) and related female-specific metabolism and growth. This increased protein sensitivity may be reflected in sex-specific patterns of lifespan, or of reproduction and in part, may explain the observation of condition-dependent patterns of sexual dimorphism for lifespan (SDL). For example, Maklakov et al. (2008), reports sex-specific effects of nutrition on reproduction. Similarly, Wit et al. (2015) found male but not female lifespan, in *Drosophila,* to be correlated with environmental variation.

Despite numerous theoretical predictions surrounding life history trade-offs, relatively little is currently known about the sex-specific impact of reproductive costs on survival trajectories in both sexes simultaneously (reviewed by Lemaitre *et al.*, 2015), or in response to nutritional manipulation.

The different evolutionary interests of the sexes, arise from their divergent reproductive roles. These sex-specific interests can impact on many different reproductive traits, such as mating frequency, fertilisation success and lifespan differences (Parker, 1979; Dean et al., 2007). Sex-specific patterns of reproduction may be associated with sex differences in lifespan, due to adaptive, sex-specific optimisation of trade-offs between lifespan and reproductive, mating or developmental traits, leading to sex-specific life history strategies (e.g. Trivers, 1972; Bonduriansky et al., 2008, reviewed by Maklakov & Lummaa, 2013).

Differences in female and male lifespan are widely documented across the majority of animal taxa (e.g. Promislow, 1992; Moore & Wilson, 2002; Liker & Szekely, 2005; Clutton-Brock & Isvaran, 2007). Much less is known about the possible role of nutrition on patterns of sexual dimorphism for lifespan (SDL), or factors that could influence the extent of SDL (e.g. Regan & Partridge, 2013). One leading hypothesis is that enhanced SDL could be a mechanism to resolve sexual conflict and

allow females and males to achieve increased sex-specific fitness (as reviewed by Cox & Calsbeek, 2009).

It is known that the extent of SDL can also be altered by diet and may show a complex relationship with increasing dietary restriction (DR), within a single species. For example, in *D. melanogaster*, SDL is maximised by a 60% reduction in the standard dietary yeast and sugar content (the DR level which optimised female lifespan) and SDL is minimised or absent at extreme food concentrations (below 30%, or above 130% of the standard dietary yeast and sugar content) (Magwere et al., 2004).

The direction of SDL also has the potential to be altered by diet, although there has been little test of this hypothesis. Female lifespan frequently exceeds male lifespan, across a wide range of species, although this direction is reversed in some species (such as the red flour beetle, *Tribolium castaneum*). Even within species, it is predicted that SDL direction can be diet- or context-dependent. For instance, widely documented patterns for *Drosophila*, raised on standard (SYA) food, show females live longer than males (e.g. Magwere et al., 2004); however, this direction of lifespan dimorphism reversed (males lived longer than females) when the social environment of *Drosophila* on standard food was altered (Wit et al., 2015). Similarly, Magwere et al. (2004) demonstrated that graded manipulation of both the protein and sucrose content of the diet, simultaneously, could altered the direction of SDL, and also peak survival and baseline mortality rate (an ageing parameter).

The extent of expression of sex-specific life history effects may be constrained by the shared genetic basis of the sexes and governed by the genetic correlation for a particular trait between the sexes. Hence, the shared genome places a constraint on sex-specific expression of divergent phenotypes and can lead to sexual conflict.

Sexual conflict can be expressed within genes (intra locus sexual conflict), or between genes (inter locus sexual conflict) (e.g. Rice & Holland, 1997; Bonduriansky & Chenoweth, 2009). When a different allele of a focal locus is favoured in each sex, selection arising from sexual conflict may act in opposite directions in males and females (sexually antagonistic selection). The potential for sex-specific phenotypic divergence may therefore be constrained by the shared genome of the sexes, leading to intralocus sexual conflict (as reviewed by Chapman et al., 2003; Chapman, 2006). This may place an evolutionary constraint on sex-specific adaptation (Delph et al., 2004; Poissant et al., 2010) for traits whose expression is an emergent property of the interactions between the sexes (e.g. mating frequency) or for traits for which there is an underlying genetic correlation

between the sexes (e.g. body size, locomotory activity; e.g. Long & Rice, 2007, but see also Fuchikawa & Okawa, 2013).

It has been proposed that sexual conflict may influence the evolution of sexual dimorphism (SD), such that the expression of SD may act to relax evolutionary constraints on the sexes imposed by the effects of their shared genome (reviewed by Cox & Calsbeek, 2009). SD is widely documented in life history, behavioural and morphological traits, as well as in gonad and gamete development (Fairbairn, 2013). The nature of SD can be highly species-specific or diet-dependent. For example, the direction of SD for adult body size reverses between two species of dung fly and between different larval environments (Ding & Blanckenhorn, 2002).

Life history traits, such as SDL, life history trade-offs, or sex specific life histories, under a particular nutritional environment, may be associated with underlying transcriptomic (gene expression) changes. In a sense, this is a method in which the constraints on the sexes, from their shared genome, could be circumvented, allowing sex-specific phenotypic expression. Gene expression patterns have been implicated in the existence of sexual dimorphism more generally. It is likely that SDL results from transcriptomic changes, although little is currently known and further investigation is needed.

Nutritional geometry approaches to proximate nutritional manipulation

In the significant body of research into the effects of proximate nutritional variation that has been summarised so far, usually only one or a few major diet components are varied. However, a more biologically realistic scenario is to vary nutritional composition along across several different nutrients and a larger number of concentrations for each nutrient, and assess the resulting outcomes (e.g. Partridge et al., 2005).

More recently, theoretical modelling (nutritional geometry) approaches have complemented the growing body of experimental studies on the consequences of proximate manipulation of nutrition (Simpson & Raubenheimer, 2007). Nutritional geometry models can be used to predict the responses of lifespan or reproduction to fine-scale manipulation of the content of several nutrients concurrently and the required conditions to achieve theoretical fitness optima (Simpson & Raubenheimer, 2007; Archer et al., 2009). Such predictions would then need testing in tightly-controlled experimental contexts. It would also be important to use biologically-realistic diets, which the study species may be likely to encounter in the field or which lie within the normal physiological realm of the model species being tested. For example, protein levels in the diet should be varied only between the level that causes starvation but below that which represents

toxicity (‘overfeeding’) (e.g. Chapman et al., 1994; Chapman & Partridge, 1996; Magwere et al., 2004; Fricke et al., 2010). Nutritional geometry approaches are outside the scope of this thesis, but an interesting avenue for future work, as explored in the General Discussion.

1.3 Life history consequences of long-term (ultimate) nutritional manipulation

In contrast to the significant body of research into the proximate effects of nutritional variation, much less is known on the effects of long-term nutritional variation on life history. Nutritional manipulation can be applied over the long-term, via experimental evolution studies and the application of nutritional selection regimes (see review: Burke & Rose, 2009). However, only very few of these studies have studied the consequences of nutritional manipulation on life history in any rigorous depth. In fact, there has been more investigation of the effect of nutritional selection on reproductive isolation (e.g. Dodd, 1989), than on longevity and other life history traits.

One study investigated the consequences on developmental traits, from evolution under chronic larval malnutrition, for 112 generations (Vijendravarma et al., 2012). Another study has shown that evolution under an unpredictably fluctuating feeding regime, led to a significant reduction in the body size of both sexes; when compared with evolution under a predictable, constant feeding regime (Perry et al., unpub.).

Further thorough investigation is therefore required to investigate the life history consequences of long-term nutritional manipulation, in both sexes simultaneously, also given the relevance to modern human societies.

1.4 Nutritional mismatches

Another area of nutritional research that is significantly under studied is that of the effects of dietary mismatches (switches) on life history and fitness. Nutritional mismatches can be defined as temporal switches in diet or feeding regime, which occur over the short-term (within a single generation, between different life history stages), or the long-term (across generations or evolutionary time).

Given the large body of study into the proximate effects of different diets supplied for the whole lifetime on adult life history, there are remarkably fewer studies that investigate either the generality of diet-induced effects on life history across all life stages, or the implications of

developmental (larval) diet, specifically, on adult life history. Both are major topics addressed in my thesis research.

The effects of introduction of a particular dietary regime at particular life stages can influence the life history consequences. For example, whilst DR in adults is widely observed to lead to adult lifespan extension, several studies have found that DR just applied to larvae, followed by a non-restricted adult diet, did not result in any lifespan effects (e.g. in *D. melanogaster* (Zwaan et al., 1991; Tu & Tatar, 2003)). However, this absence of life history consequences from a restricted developmental diet is not consistently found across species. For example, in the house cricket (*Acheta domesticus*), development on a low protein diet, followed by a standard adult diet led to an extended lifespan (Lyn et al., 2012).

Larval dietary protein content is also independently important for the expression of larval and adult life history. For example, a high protein larval diet can lead to an increase in adult *D. melanogaster* body size (e.g. May et al., 2015), developmental rate and viability, when compared to a low protein larval diet. In addition, rearing *D. melanogaster* males on a low protein larval diet led to a reduction in the quantity of sperm transferred to females during mating (McGraw et al., 2007).

Manipulation of the larval diet can be used to assess the overall life history effects of developmental diet. This may be especially important in insect species in which feeding during development, occurs only during the larval stage. Adult structures that develop as imaginal discs within the larvae, e.g. the wing imaginal discs, are significantly affected by larval nutrition prior to the major developmental remodelling that occurs in the subsequent, non-feeding, pupariation (pupal) stage. The entire period of development from early first instar larvae to final adult eclosion occurs on the ‘larval diet’, so this is akin to the ‘developmental diet’ in other species. The larval versus adult diet division is therefore relevant for comparing the consequences of developmental versus adult nutrition.

The stage is therefore set for investigating the interaction between developmental and adult diets and, importantly, the life history implications of nutritional mismatches between these life history stages. The effects of within-lifetime (single-generational) switches in exposure to different diets certainly has the potential to lead to rapid switches in life history responses. For example, in as little as 6hours after a dietary switch, *D. melanogaster* has previously shown the capacity for altered egg laying behaviour (e.g. Chapman et al., 1994; Mair et al., 2004). However, despite this potential, few studies have fully studied the life history consequences of single-generational mismatches in nutrition.

The nature of temporal nutritional mismatches can be deviations away from evolved, standard or optimal dietary nutrient content or feeding regime. Such mismatches are widely regarded to be costly to fitness, manifested as survival or reproductive costs to individuals.

Two prominent evolutionary hypotheses, originally proposed in the context of human evolution, predict the life history consequences of nutritional mismatches. These hypotheses concern switches in nutrition, either within a single generation between the developmental and adult stages (Thrifty Phenotype (TP) Hypothesis, tested in Chapter 2), or between generations- between evolved ('ancestral') and proximate ('modern') nutrition (Thrifty Genotype (TG) Hypothesis; Neel, 1962; tested in Chapter 3). I explore these two theories, their limitations and describe the limited empirical evidence underlying both hypotheses below.

Short-term nutritional mismatches and the Thrifty Phenotype hypothesis

Nutritional mismatches in the quality or quantity of diets between developmental and adult stages (or within either life stage) are predicted to result in costs for survival, reproductive output, or overall fitness. A large body of experimental work in *Drosophila* and other species has quantified the effects of short-term variation in nutrition on lifespan and life history within the adult life stage, as described above (e.g. nutritional manipulation studies, often within in the field of ageing). However, much less research has been done to test the effects of temporal switches in dietary composition, in the context of the TP (and TG) mismatches theory.

Many factors can influence the expression of dietary mismatches in nutrition between life history stages. Depending on the species, developmental nutrition is often determined by parental nutritional status. Changes in nutritional status could arise from environmental change between the maternal (developmental) diet and the nutritional environment into which the offspring emerge. Short-term nutritional change is more likely in a variable environment. Single-generational nutritional mismatches could also arise for animals which migrate to an environment which is nutritionally different from the maternal (and developmental) environment, after birth.

The Thrifty Phenotype (TP) hypothesis is an evolutionary theory, first conceived in a human context, which proposes that mismatches between developmental and adult environments, within a single generation, can be costly to fitness and increase susceptibility to later life pathologies (Hales & Barker, 1992; Hales et al., 1997). The central idea is that phenotypes expressed in response to the developmental environment, e.g. traits such as insulin sensitivity or body size, become 'fixed' or 'set' in anticipation of a matching adult environment (Hales & Barker, 1992; Hales et al., 1997; Bateson et al., 2004). These phenotypes may be beneficial under the

developmental conditions encountered but maladaptive in the mismatched adult environment, leading to life history costs (Hales & Barker, 1992; Hales et al., 1997; Ravelli et al., 1998; Bateson et al., 2004).

A handful of empirical studies have demonstrated the life history consequences of such nutritional mismatches, which are in line with predictions arising from the TP hypothesis. For example, a poor quality developmental diet, followed by a good quality adult diet, led to reduced offspring production by females (e.g. Huck et al., 1987).

However, little is known about the consequences of single-generation mismatched diets on age-specific survival and reproduction patterns in both sexes simultaneously. Furthermore, the TP hypothesis does not address whether particular combinations of mismatches between developmental or adult diets, could ameliorate the costs of mismatched nutrition and very little research has been conducted on this topic (May et al., 2015). Knowledge is also lacking on whether costs are expressed equally across different life history traits. It would be expected that if such single-generational nutritional mismatches are common, then there should be selection to counter their effects, so it is uncertain over the persistence or extent of costs under certain conditions. These ideas are explored in the experimental work conducted in Chapter 2.

Long-term nutritional mismatches and the Thrifty Genotype hypothesis

Long-term nutritional mismatches can result if the nutritional environment to which organisms are adapted changes rapidly, across generations. Life history consequences arising from mismatches of this nature are understudied and require further study, motivated by the relevance to large-scale recent changes in human nutrition in industrialised countries.

The Thrifty Genotype (TG) hypothesis proposes the potential fitness consequences of mismatches between evolved ('ancestral') and proximate ('modern') nutritional environments. The TG hypothesis specifically predicts that mismatches between an evolutionary history of unpredictable cycles of feast and famine, and a modern diet of *ad libitum* feeding on diets of consistently increased nutritional content, will carry fitness costs (Neel, 1962; Prentice et al., 2005).

The TG hypothesis was first conceived as an evolutionary explanation for the prevalence of modern human obesity, arising from mismatches between ancestral and modern diets (Neel, 1962). The TG hypothesis proposes that the ancestors of modern humans living from around 10,000 years ago relied on agriculture for their nutrition and experienced an unpredictable, fluctuating history of food availability (including 'feast' and 'famine' periods) linked to a fluctuating climate (Neel, 1962; Prentice et al., 2005). Critics argue that the extent or existence of

these ancestral feast-famine fluctuations was not uniform, but varied between geographical regions and demographic groups (Sellayah *et al*., 2014).

Populations that evolved under a history of unpredictable food availability, may have been subject to positive selection for 'thrifty' genes associated with increased fat deposition and energy storage during 'feasts', to increase resilience to subsequent 'famines' (Neel, 1962; Prentice et al., 2005). However, it has also been suggested that these 'thrifty genes' may have accumulated via genetic drift rather than selection (Speakman, 2008). The transition to agriculture may have led to the relaxation of selection pressures on predator evasion and hunter-gather traits. Hence genes linked with fat and energy storage were no longer maladaptive and 'drifted' into the population (Drifty Phenotype Hypothesis/Predation Release Theory; Speakman, 2008; reviewed by Sellayah *et al.*, 2014).

Regardless of the mechanism, a genetic propensity to rapidly accumulate fat when food was plentiful, may be detrimental under conditions where food is always plentiful. This idea is now proposed to help explain the modern human predisposition to obesity. These mismatches between the nutritional environment under which ancestral life histories evolved and the current nutritional environment, left individuals maladapted to modern conditions. It is possible that these mismatches may also have shifted genes from their evolutionary optima, proving detrimental to fitness.

Specific examples of candidate genes associated with TG (Neel, 1962) have been highlighted (Prentice *et al.*, 2005). For example, the insulin microsatellite locus (INS-VNTR), which is involved in the nutrient sensing insulin-signalling pathway, fetal growth and survival, has been identified as a possible thrifty gene that is potentially linked with diabetes (Prentice et al., 2005). Positively selected thrifty genes have been proposed to have a range of metabolic, physiological and behavioural effects, including energy-efficient metabolism, inactivity, rapid fat gain, switching off 'non-essential' physiological processes, over-eating and food hoarding (Prentice *et al.,* 2005).

Several authors have criticised the TG hypothesis (Speakman, 2008, Sellayah *et al.*, 2014). Some question whether 'famines' may have been sufficiently severe to exert strong selection pressures on 'thrifty genes' and if these genes had reached fixation, why modern obesity is not even more prevalent (Speakman, 2008).

Another criticism is that many regions show less of a contrast (mismatch) between 'ancestral' and 'modern' nutritional environments (Sellayah et al., 2014). Perhaps the extent of feast-famine periods during evolutionary history (in terms of size and frequency of fluctuations) may be linked

with the strength of selection acting and hence the propensity to modern obesity observed. A greater unpredictability and severity of 'famine' occurrence, would perhaps then have exerted a stronger selection pressure on 'thrifty genes', leading to a tendency for higher obesity levels in those regions.

Whilst there is disagreement over the geographical uniformity of the environments under which life histories evolved and the mechanisms of 'thrifty gene' accumulation, there is general consensus on two important points. Firstly, evolution in an unpredictably fluctuating environment will lead to selection for genes that enhance the fitness of individuals under those evolved conditions. These adaptations could allow a greater resilience and fitness under novel or fluctuating environments and thus, a gain in plasticity to environmental change. Secondly, mismatches between evolved and modern environments can result in fitness costs.

Little is known to date about whether particular evolved feeding regimes can reduce the costs of nutritional mismatches by enhancing resilience and plasticity to poor quality or to novel proximate nutritional environments. Furthermore, little empirical work has focused on testing these TG predictions or determining the life history response (in terms of survival, reproduction and fitness) to an evolutionary history of fluctuating, unpredictable food supply. There been little study of the life history consequences of the interaction between an evolutionary feeding regime manipulation and mismatched proximate diets. This topic is investigated in Chapter 3.

1.5 Reducing the costs of nutritional mismatches: plasticity and life history strategies

Mismatches may not incur the costs predicted by the TP and TG hypotheses if their resulting effects can be reduced by an individual's capacity for developmental, phenotypic or life history plasticity. I outline the theory behind the potential role of plasticity in ameliorating the costs of nutritional mismatches below and the current empirical work to address these ideas.

The extent to which the potential fitness costs arising from mismatched diets can be reduced will depend upon the extent of phenotypic or life history plasticity expressed (Sultan, 2003; Bateson et al., 2004, reviewed by Flatt & Schmidt, 2009). Life history costs from mismatched nutrition can, in part, be reduced by an individual's capacity for phenotypic and life history plasticity, or adaptability, in the face of nutritional change (e.g. Stearns, 1992; Pigliucci, 2001).

Indeed, *D. melanogaster* has the potential for rapid lifespan plasticity to short-term dietary manipulation within adult life (e.g. Mair et al., 2003; Flatt & Schmidt, 2009). It is therefore possible that when environmental fluctuations are frequent, selection could counter the costs of

mismatches. Traits would therefore not be permanently 'set' at development, but could change. Furthermore, it is possible that a high quality developmental environment might ameliorate the costs of a low quality (mismatched) adult diet.

Fitness costs arising from single-generational dietary mismatches can potentially be reduced via three main strategies. First, phenotypic changes associated with a poor quality developmental diet could be, in part, ameliorated by compensatory feeding and catch-up growth after a dietary switch to improved conditions (e.g. Metcalfe & Monaghan, 2001; Ozanne & Hales, 2004; Innes & Metcalfe, 2008). Second, a good quality developmental diet could lead to carry-through ('silver spoon') benefits into adulthood (e.g. Bateson et al., 2004; Hopwood et al., 2014). For example, a larger body size accrued through the carry over effects of increased developmental nutrients might also result in greater fat reserves (e.g. Bateson et al., 2004). These traits could ameliorate any costs from the nutritional mismatch and poor diet encountered in adulthood. Finally, a harsh developmental environment could promote the survival of only the most resilient individuals that possess adaptations to allow them to persist through a harsh developmental viability selection filter (as reviewed by May et al., 2015). These individuals would then emerge as a cohort of fitter adults in comparison to those that developed on good quality food that were not subject to viability selection at the developmental stage. Together, these effects could ameliorate potential fitness costs arising from mismatched nutritional environments, within a single generation.

There is as yet little experimental evidence to show the extent to which phenotypic and life history plasticity can ameliorate the costs of single-generational nutritional mismatches (but see: Mair et al., 2003; Flatt & Schmidt, 2009). There are only a few direct empirical tests of the three potential strategies outlined above for the reduction of fitness costs and of how signals from the developmental environment may influence adult life history (e.g. Ozanne & Hales, 2004; Bateson et al., 2004; May et al., 2015).

Furthermore, selection on nutritional environments can lead to the evolution of fixed life history strategies (Stearns, 1992), which can better predispose individuals to altered nutritional environments. For example, an increased resilience to starvation and an enhanced plasticity to novel diets might be predicted from evolution under an unpredictable feeding regime (Rion & Kawecki, 2007), so ameliorating some of the costs predicted from mismatched nutrition (Neel, 1962). Additionally, evolution under a regime of unpredictable feeding could select for individuals able to capitalise on resources when they became available and exhibit increased fecundity when food was *ad libitum*, to avoid energetically expensive egg production when food was scarce (Rion & Kawecki, 2007). As yet, there have been no direct empirical tests of this hypothesis and little is

still known about the factors influencing the extent and nature of the life history consequences of nutritional mismatches, or the possibility for sex-specific patterns.

1.6 Study system

The fruit fly, *Drosophila melanogaster,* is a valuable model system for studying the life history consequences of nutritional mismatches, the gene expression changes associated with nutrition-induced life history patterns and the role of lifestyle (activity levels) on life history. Powerful tools are available such as defined diets, genetic tools and a fully sequenced genome. In addition, the ease of rearing, short generation time, and relatively short lifespan, together provide a strong foundation for nutrition and ageing studies.

There is also a considerable body of literature for *D. melanogaster* on the role of diet and dietary restriction on lifespan, providing a strong foundation for this research. Furthermore, many nutrient sensing genes, ageing and developmental pathways in *D. melanogaster,* share high homology across taxa (e.g. Katewa & Kapahi, 2010; Wangler et al., 2015). For example, 60% of genes are conserved between flies and humans and around 75% of genes implicated in human disease are known to have genetic homologues in the fruit fly genome (e.g. Jennings, 2011). Fruit flies also share key metabolic pathways with mammals and so can be used to model many human diseases (Jennings, 2011). Fruit flies are therefore a useful and relevant model species, for experimentally testing hypotheses derived in a human context.

1.7 Thesis aims and outline

This research in this thesis addressed four important, interconnected areas. First, to determine the life history consequences of nutritional mismatches, by testing predictions arising from the TP and TG hypotheses (Chapters 2 and 3). Secondly, the sex-specific fitness consequences associated with the manipulation of evolved feeding regimes, which altered the extent of sexual dimorphism for lifespan (Chapter 4). Following on from this, I aimed to determine gene expression patterns associated with the evolved manipulation of feeding regime (Chapter 5). Finally, I tested the role of lifestyle (activity levels) on life history (Chapter 6), as dietary and activity patterns were often both associated with life history and implicated in healthy ageing. The work has wider relevance for the evolution of sexual dimorphism for lifespan, life history trade-offs, plasticity and, more broadly, for human medicine and healthy ageing.

I simultaneously studied both sexes of *D. melanogaster* and obtained age-specific measures of several life history traits, for different fine-scale mating regimes; to more comprehensively determine the life history consequences of nutritional or activity level manipulation (as recommended by e.g. May et al., 2015). This thesis measured reproductive output in terms of 'egg production' and 'offspring production', for clarity, to avoid the different definitions of the term, 'fecundity', used in the literature.

Each data chapter of the thesis is a discrete piece of work, written with the intention of submitting as separate manuscripts for publication.

In Chapter 2, I tested predictions arising from the TP hypothesis, by manipulating the protein content (high or low) of developmental (larval) versus adult diets of populations of *D. melanogaster*, within a single generation. I compared the age-specific survival, reproductive and fitness responses of both sexes to a mismatched or a constant dietary protein content, under once-mated versus weekly-mating regimes.

In the Chapter 3, I tested predictions arising from the TG hypothesis, by using replicated selection lines of *D*. *melanogaster*, maintained for over 360 generations on either unpredictable ('Random') or predictable ('Regular') feeding regimes. I assayed the life history responses (in terms of survival, reproduction and fitness) of these lines to a common garden diet and across several proximate diets (starvation, low and high protein), which were mismatched to the 'evolved' diet.

In Chapter 4, I built on and extended the work from Chapter 3, to test the hypothesis that increased SDL allows both females and males to achieve greater sex-specific fitness and hence that the expression of SDL minimises sexual conflict. The same feeding regime selection lines were used as in Chapter 3. The life history consequences for both sexes were tested on common garden diet and the mating regime for each sex was equivalent.

In Chapter 5, I investigated the gene expression (transcriptomic) patterns associated with the evolutionary manipulation of feeding regime, for both sexes reared in a common garden environment and derived from the life history assay outlined in Chapter 4. This allowed inference of possible transcriptomic patterns underlying the differences in SDL and sex-specific fitness, between the evolved feeding regime lines.

In Chapter 6, I extended investigations of the life history consequences of environmental manipulation. I designed a method to directly, robustly and consistently elevate activity levels and then tested the life history consequences of elevated activity levels, in comparison to controls. This determined the effect of another aspect of an altered lifestyle.

The General Discussion summarises and links together the results from the 5 data chapters, places them in the wider context and outlines recommendations for future investigation.

1.8 Statement of contribution

All work detailed in the thesis was conducted and written by myself, with the exception of the bioinformatics analysis in Chapter 5 which was conducted by Dr. Irina Mohorianu and Dr. Rachel Rusholme Pilcher.

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Chapter 2: Life history consequences of mismatches between developmental and adult diets: testing the "Thrifty Phenotype" hypothesis

2.1 Abstract

Nutritional mismatches between different life history stages can result in fitness costs. For example, the Thrifty Phenotype (TP) hypothesis proposes that mismatches between diets that are ingested at developmental versus adult stages within a single generation can be detrimental to health and lead to late-life pathologies. However, it is not currently known whether there is any combination of mismatch in the developmental versus adult environment that can reduce the costs of a dietary mismatch, or indeed whether costs are expressed equally across different life history traits. For example, it is possible that a high quality developmental environment might reduce the costs of a low quality (mismatched) adult diet. I tested these ideas by manipulating the protein content (high or low) of developmental (larval) versus adult diets of populations of the fruit fly, *Drosophila melanogaster*. I compared the survival, reproductive and fitness responses of both sexes to a mismatched or a constant dietary yeast (protein) content. The main finding was that not all mismatched diets were costly, contrary to the predictions of the Thrifty Phenotype hypothesis. Individuals raised on a high protein larval and low protein adult diet, exhibited higher survival, reproductive output and fitness than did individuals reared on a constant low protein diet. A high protein adult diet resulted in consistent benefits over a low protein adult diet in both sexes. In contrast, for mismatched diets I found that outcomes varied across different traits, sexes and mating environments. For example, a low protein larval and high protein adult diet (LH) reduced the reproductive output and fitness of weekly-mated focal females in comparison to a constant high protein diet. Survival of both sexes and the reproductive output and fitness of males and once-mated females did not differ between the mismatched LH diet and the constant high protein diet. Overall, the results showed that exposure to mismatched diets was not always more costly than constant diets. Hence, some fitness costs can potentially be reduced by switching to alternative diets at the adult stage.

2.2 Introduction

Mismatches in the quality or quantity of diets between developmental and adult stages, within a single generation, are expected to be costly, leading to detrimental effects on survival and reproductive output. The extent to which the potential fitness costs arising from mismatched diets can be reduced will depend upon the extent of phenotypic or life history plasticity expressed (Sultan, 2003; Bateson et al., 2004, reviewed by Flatt & Schmidt, 2009).

The Thrifty Phenotype (TP) hypothesis proposes that mismatches between developmental and adult environments can influence disease susceptibility and lead to later life pathologies and reduced fitness (Hales & Barker, 1992). Originally derived in a human health context, the key premise is that beneficial phenotypes expressed in response to developmental conditions (e.g. body size and insulin sensitivity) become 'fixed' in anticipation of a matching adult environment. However if the adult environment is altered ('mismatched') from developmental conditions, the phenotypes are now maladaptive, resulting in life history costs (Hales & Barker, 1992; Hales et al., 1998; Ravelli et al., 1998; Bateson et al., 2004).

If such mismatches are common, then there should be selection to counter their effects. There are three main ways in which fitness costs arising from single-generational dietary mismatches could be reduced. First, a switch from a poor quality developmental diet to improved nutrition, could lead to compensatory feeding and catch-up growth after the dietary switch, reducing the costs of a 'poor start' (e.g. Metcalfe & Monaghan, 2001; Ozanne & Hales, 2004; Innes & Metcalfe, 2008). Second, carry-through ('silver spoon') benefits from a good quality developmental diet could ameliorate the costs of a poor quality adult diet, despite the nutritional mismatch (e.g. Bateson et al., 2004). For example, increases in body size and fat reserves from a nutritionally-rich developmental diet could be carried through to adulthood (e.g. Bateson et al., 2004). These traits could reduce life history costs arising from a mismatched, poor quality adult diet. Finally, a harsh developmental environment could act as a strong filter on developmental viability, selecting for only the most resilient individuals, with higher average fitness than individuals reared under good quality conditions during development, and not subjected to a developmental viability selection filter (as reviewed by May et al., 2015). Together, these examples of plasticity could theoretically reduce fitness costs expected from mismatched nutrition between developmental and adult stages.

There is as yet little experimental evidence about the extent to which phenotypic and life history plasticity can alter the costs of nutritional mismatches (but see Mair et al., 2003; Flatt & Schmidt, 2009). To the best of my knowledge, there are only a few direct empirical tests of the three

potential strategies outlined above, to determine how interaction between the developmental and adult environment may influence adult life history and reduce fitness costs from mismatched nutrition (e.g. Ozanne & Hales, 2004; Bateson et al., 2004; May et al., 2015).

The age-specific consequences of a mismatched diet on concurrent survival and reproduction are also poorly studied. It is not known whether the effects of single-generational mismatched nutritional environments are uniform across different life history traits (May et al., 2015). With the exception of one study on females (May et al., 2015), there has been little work to directly manipulate developmental and adult diets simultaneously and to test the life history and fitness consequences of both sexes, nor to vary mating regimes within such a framework.

I tested these ideas by experimentally varying the protein content of the developmental (larval) and adult diets supplied to cohorts of the fruit fly, *Drosophila melanogaster*, within a single generation. These manipulations created a fully factorial design of mismatched or constant diets between larval and adult stages. I assayed the life history consequences of mismatched versus constant diets on the age-specific survival, reproductive output and fitness of both sexes, across two experiments. In the first, the sexes differed in their mating regimes. Females were once-mated and males were mated each week (i.e. 'weekly-mated'). In the second, both sexes were mated each week so that the fitness of both sexes could be directly compared. These mating regime manipulations also enabled me to gain insight into the life history consequences of fine-scale mating regime manipulation on mismatched and constant diets. I compared developmental parameters and body size between low and high protein larval diets, to determine whether phenotypes expressed at the developmental stage influenced adult life history patterns and fitness.

I tested the prediction from the Thrifty Phenotype hypothesis (Hales & Barker, 1992) that individuals on mismatched diets would have lower fitness than those on constant diets. I predicted that a high protein larval diet would minimise costs of a mismatched low protein adult diet, due to carry-through benefits from early good nutrition. Overall, I expected the high protein adult diet to confer higher fitness than a low adult diet in line with published dietary manipulation studies in *Drosophila* (e.g. Chapman et al., 1994; Chapman & Partridge, 1996; Magwere et al., 2004; Fricke et al., 2010). In addition, I expected high protein larval diet individuals to reach a larger adult body size than for those raised on the low protein larval diet.

2.3 Materials and Methods

2.3.1 Survival and reproductive success to mismatched or constant larval to adult diets in once-mated females and weekly-mated males

I first determined the survival and reproductive responses to mismatched or constant larval to adult diets for females and males. The fitness of each sex was tested concurrently but in separate experiments. Females were mass mated in one episode at the start of their lives. Males were similarly treated, but in addition were given access to females every 7 days, in order for an index of reproductive success to be determined.

The larval and adult nutritional environments were varied by manipulating the protein (yeast) content of the diet. I used low (20% SYA) and high (120% SYA) protein diets (see Suppl. Mat. for recipes). Diets were selected based on published lifespan and fecundity curves from Magwere et al. (2004). The low protein diet was chosen as a stressful, but above starvation, diet. The high protein diet was selected on the basis that it provided greater nutrition than the standard diet, but it did not appear to represent an 'overfeeding' diet. Four fully factorial diet treatments were set up for the male and female experiments: LL, LH, HL and HH (low (L) or high (H) protein larval then adult diets respectively).

Experimental individuals were generated from eggs collected on yeasted red grape agar oviposition plates from the same WT Dah population cage as used in the pilot experiment to determine development times (Suppl. Mat.). A narrow 4hour egg collection period was used to maximise the resolution of developmental timings that were recorded. First instar larvae were transferred at a density of 100 larvae/vial to either low or a high protein diets, 26h after oviposition. Using the developmental timings determined in the pilot work as a guide (Suppl. Mat.) the low protein larvae (n=3700) were set-up 193h (8 days and 1 hour) before high protein larvae (n=600) so that adults from both larval diets would eclose at the same time. This staggered set-up corresponded to the difference in mean egg to adult development time between the two diets, derived from the pilot work. The number of larvae required was calculated after taking into account egg to adult survival and the percentage eclosion during 24h (Suppl. Mat.). A separate cohort of standard WT larvae (n=1300) were set up on standard food (100% SYA) at the same density of 100 larvae/vial, to generate standard WTs for mass-mating with focal adults.

Upon eclosion, virgin focal adults from the low and high protein diets were collected during two 4h periods (10am-2pm and 3pm-7pm), sexed on ice, and designated randomly to either the same diet as their larval environment (i.e. 'constant' environment) or the opposite ('mismatched') diet.

This established the 4 adult treatment populations (LL, LH, HL, HH) for each sex. Each treatment had an initial sample size of 35.

In preparation for mating, adult focal females were held in 4 treatment population bottles (two of high protein food and two of low protein food) at a density of 35 females/bottle. Adult focal males were held in individual vials (1 male/vial). Similarly, newly eclosed standard WT males (n=212) were stored in 4 flasks of 100% SYA (53/flask) and standard WT females were stored at 10 adults/100% SYA vial for 3 days in advance of matings.

Focal females were mass mated on their adult treatment diets at 2 days post-eclosion for 24h by placing them together with 3-4 day old standard WT males. A 60:40 standard WT male to focal female ratio in the mass mating bottles introduced moderate levels of biologically-relevant male-male competition. Focal males were individually mated with individual, 3-4 day old, standard WT virgin females, at a 1:1 ratio, on the focal male adult diet, also at 2 days post-eclosion.

Following mating, focal females were transferred to individual vials (1 focal female per vial), on the allocated adult diet and had no further exposure to males over their lifetime. Focal males were retained in their mating vials and WT females transferred into individual vials of standard 100% SYA media. These vials were labelled with the unique identifier of the focal male mate, for later egg counting. Every week focal males were mated for 24h with a new standard WT females, to give an estimate of male reproductive output.

Weekly 24h egg counts were taken per focal female and from the WT females that were placed for 24h each week with each male. The egg counts from the WT females were taken from the 24h that these females were placed onto standard food (100% SYA) following matings with the focal males on high or low protein food. Egg count vials were retained and first generation (F1) offspring counts were taken 13 or 20 days later for the high and low protein vials, respectively. Egg counts provided a measure of reproductive investment and offspring counts a measure of realised reproductive investment.

Each day focal female and focal male mortality was recorded. This allowed determination of lifespan, age-specific survival (the number of individuals surviving in a population at a given age) and Kaplan-Meier survivorship curves, for each treatment population.

2.3.2 Survival and reproductive success to mismatched or constant larval to adult diets in weekly-mated females and males

A second life history assay was conducted to determine the survival and reproductive responses to a mismatched or constant diet between larval and adult stages, when the mating regime of both focal sexes was identical.

The same four diet treatment populations were used (LL, LH, HL and HH). Sample sizes were increased to 45 per treatment to increase statistical power. Experimental individuals were generated as in the experiment described above. I used a time-staggered set up of larvae (n=5800) on low protein food 193h before the set-up of larvae (n=1000) on high protein food. All larvae were placed in standard densities of 100 larvae/vial. The number of new puparia formed each day, and the sex and time of emergence of all newly eclosed adults, was recorded. This enabled me to calculate larval to puparium and larval to adult viability (Suppl. Mat.). A separate cohort of standard larvae (n=1600) was set up at 100 larvae/SYA vial, to generate WT adults for mating with focal adults.

Matings for focal female and focal male experiments ran concurrently. Each individual from each focal sex was mated for 3 hours to a standard, 3-4 day old WT mate in a 1:1 ratio at weekly intervals over their lifetime. Hence, WT flies were generated on a weekly basis for these matings. The initial mating was performed at 2 days post-eclosion. All matings were conducted on the diet of the focal adult, as before. In this experiment I reduced the mating period from 24h to 3h to minimise the proximate responses in WT female reproductive output to the focal diets. Mating frequency was observed and recorded every 20 minutes for each focal individual during each weekly 3h mating. From this, I calculated the weekly proportion of each sex and diet treatment that mated across the lifetime.

Following mating, individual focal females and focal males were transferred into fresh vials of their assigned low or high protein adult diets. WT females were transferred to individual vials of standard SYA medium, labelled with the unique identifier of their focal male mate. Egg counts were taken from focal females and the standard WT females, to which the focal males had been mated, from the 24h period immediately following the 3h mating.

Numbers of focal female and focal male mortalities were recorded daily for each treatment population and each sex. Focal flies were transferred onto fresh food, without CO_2, every 2-3days.

2.3.3 Statistical analyses

All statistical analyses were performed in R version 3.2.1 (R Core Development Team, 2015).

Survival Analysis

Survival analyses were performed using Cox Proportional Hazards regression analysis, on age-specific mortality data, separately for focal females and focal males. All age-specific mortality data satisfied the proportional hazards assumption of Cox analysis, using both graphical and analytical tests. A Cox model was fitted using the 'coxph' function from the 'survival' package. Individuals that were lost or died during experimental manipulation, were treated as censors in the Cox model. The four diet treatment populations (LL, LH, HL, HH) were partitioned into binary larval and adult diet categorical factors (0=low, 1=high protein) for the analysis. Model simplification was conducted via factor level reduction from a maximal model including both main effects (larval diet and adult diet) and their interaction, to a minimal model containing only significant terms.

Age-Specific Reproduction Analysis

Age-specific egg counts and offspring counts were analysed using generalised linear mixed effects models ('glmer' function from the 'lme4' package), to account for the temporal pseudoreplication arising from taking repeated counts from the same individuals over time. The sexes were analysed separately. Poisson error structure was used for count data. Egg count or offspring count was the integer response variable. Larval diet and adult diet and their interaction (larval:adult) were fitted as categorical fixed effects. The number of days post-eclosion each count was taken was fitted as a continuous random effect and a unique identifier assigned to each individual, was also fitted as a random effect.

The data were overdispersed in all cases. To account for this, an observation level random effect was added to each 'glmer' model (the log-normal Poisson distribution) (Bolker et al., 2009; Harrison, 2014). Maximum likelihood model comparison showed that this provided best model fit and accounted for zero-inflation in the dataset.

Egg to adult viability was calculated as the proportion of eggs laid that hatched as viable offspring, at each timepoint. Proportion data was arcsine transformed and then analysed with a glmer, with Gaussian errors from the 'lme4' package (same output as lmer).

Lifetime Reproduction Analysis

Indices of total lifetime egg production and total lifetime offspring production were calculated separately for each sex and each treatment population by summing weekly 24h egg or offspring counts, respectively, across the lifetime, for each individual. Lifetime egg and offspring production data violated the normality and homogeneity of variances assumptions, so the non-

parametric Kruskal-Wallis test was used to compare median egg and offspring production values between diet treatment populations for each sex. The Mann-Whitney U test was used to determine the possible significance of pairwise comparisons of treatment levels.

Lifetime offspring production, also termed lifetime reproductive success (LRS) was used as a measure of individual-level fitness.

Mating data analysis

The proportion of individuals that mated from each diet treatment population, for each sex and each weekly mating was calculated. An index of mean lifetime proportion mated was calculated from the total number of matings divided by the sum of total number of pairs surviving at each weekly mating over lifetime; for each sex and each treatment population. Mating proportion data were analysed separately for each sex using a generalised linear model with binomial errors. Overdispersion was accounted for by using quasi-binomial errors. A maximal GLM model including larval diet, adult diet, sex and their interaction was fitted and stepwise model reduction conducted, to determine the minimal adequate model.

2.4 Results

2.4.1 Survival and reproductive success to mismatched or constant larval to adult diets in once-mated females and weekly-mated males

Survival

Adult diet had a significant effect on focal female survival, with females on the high protein adult diet (HH and LH treatments) living significantly longer than those on a low protein adult diet (LL and HL treatments) (coxph: $z=6.73$, $p<0.001$; median lifespan=45days, 24days, respectively; Figure 1A; Table S2). There was no significant effect of either larval diet (coxph: $z=0.70$, $p=0.484$; Figure 1A) or the adult:larval diet interaction (coxph: $z=1.60$, $p=0.110$) on focal female survival.

In contrast, the adult:larval diet interaction and the main effects of adult diet and larval diet, all had a significant effect on focal male survival. Like focal females, focal males lived significantly longer on the high in comparison to low protein adult diet (coxph: $z=7.67$, $p<0.001$; median lifespan=67days, 35days, respectively; Figure 1B; Table S3). There was a significant effect of the adult:larval diet interaction (coxph: $z=2.81$, $p=0.005$) and larval diet (coxph: $z=2.66$, $p=0.008$) on focal male survival, manifested on the low protein adult diet, with males living significantly longer on the mismatched high protein larval diet (HL), than on the constant low protein diet (LL) (Figure 1B).

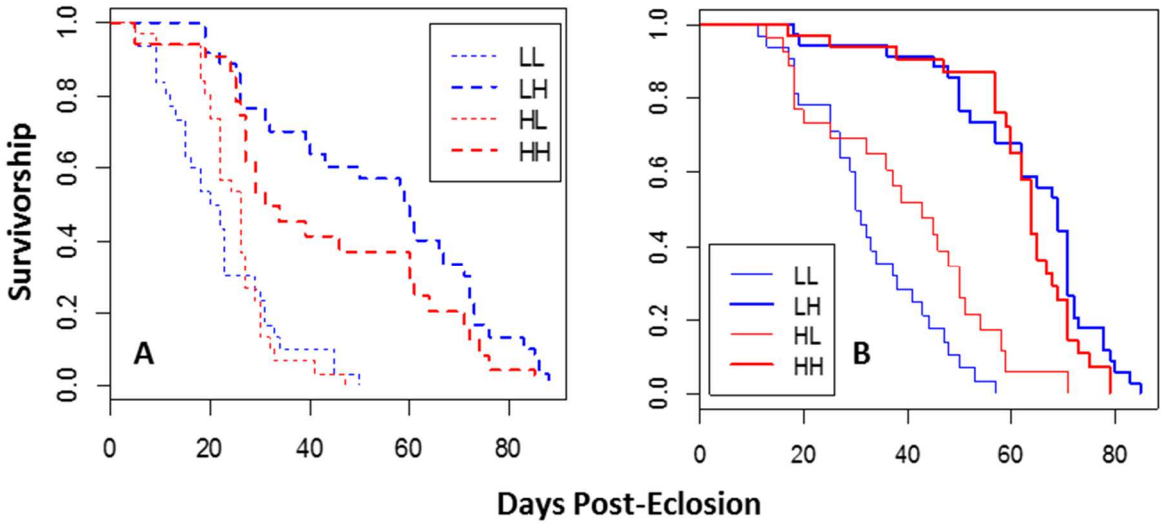

Figure 1. Age-specific survivorship against days post-eclosion, across dietary treatments (LL, LH, HL and HH), for: (A) focal females and (B) focal males. Dietary treatments were: constant low protein (20% SYA), LL; constant high protein (120% SYA), HH; low protein larval diet and high protein adult diet, LH; or high protein larval diet and low protein adult diet, HL. Focal females were once-mated and focal males were weekly-mated.

Focal Female Reproduction

The adult:larval diet interaction and the adult diet main effect both had a significant effect on focal female egg production (glmer: $z=2.103$, $p=0.0354$; $z=10.057$, $p<0.001$, respectively; Figure 2A) and offspring production (glmer: $z=1.140$, $p<0.001$; $z=6.787$, $p<0.001$; Figure 2B) over the lifetime. Focal female reproductive output was significantly higher on the high (LH, HH) than on the low protein adult diet (LL, HL) and was higher on the mismatched HL diet than the constant LL diet (Figure 2A, 2B, insets). Larval diet alone had no significant effect on focal female egg (glmer: $z=0.903$, $p=0.367$) or offspring production (glmer: $z=1.187$, $p=0.235$). Focal female egg to adult viability did not differ significantly between adult or larval diets across the entire lifetime (glmer: $t=0.419$, d.f.=1, $p=0.674$; $t=0.179$, d.f.=1, $p=0.865$).

Egg production, offspring production and egg to offspring viability all significantly declined with age across all diet treatments (glmer: $z=14.750$, $p<0.001$; $z=12.603$, $p<0.001$; $t=9.111$, d.f.=1, $p<0.001$; respectively). Mean focal female offspring production declined rapidly, after peaking at 14 days post-eclosion, presumably due to the depletion of sperm stores in the once-mated focal females.

Total lifetime egg production and total lifetime offspring production differed significantly across focal females (Kruskal-Wallis test: 'egg', K-W chi-sq. = 90.3, df = 3, $p<0.001$; 'offspring', K-W chi-sq.= 107.3, df = 3, $p<0.001$). Median lifetime production was significantly greater for focal females on the high protein adult diet (LH, HH) in comparison to the low (LL, HL) (Mann-Whitney U test: 'egg', $W=347$, $p<0.001$; 'offspring', $W=89$, $p<0.001$) and was significantly greater on the mismatched HL than the constant LL diet (M-W U test: 'eggs', $W=522$, $p= 0.042$; median=0, 0, respectively; 'offspring', $W = 262$, $p < 0.001$; median=1, 0, respectively). There was no significant difference in lifetime progeny production between LH and HH females (M-W U test: 'eggs', $W=744$, $p= 0.125$; median=38, 26, respectively; 'offspring', $W = 712$, $p=0.245$; median= 31, 25, respectively).

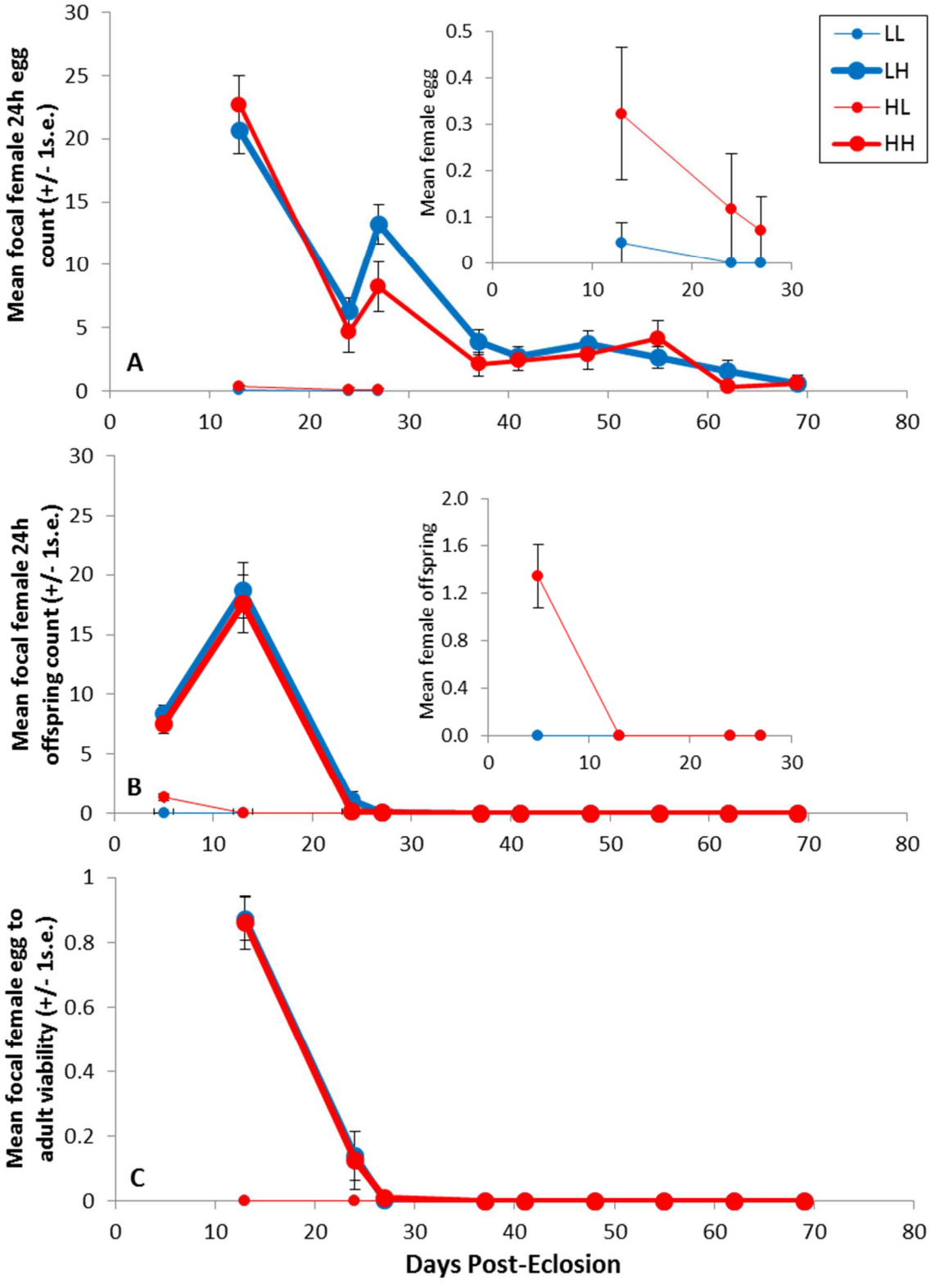

Figure 2. Mean focal female egg production (A), offspring production (B) and egg to adult viability (C), per female, per 24h, against days post-eclosion, for the four dietary treatment populations (LL, LH, HL and HH). Initial n=35 females/treatment. Egg to adult viability is defined as the proportion of eggs which eclosed as adults (C). For illustration, insets on panels (A) and (B) show mean female egg counts and

offspring counts for the two low protein adult diet treatments: HL (red) and LL (blue). All error bars display +/- 1 standard error.

Focal Male Reproduction

Adult:larval diet interaction and adult diet both had a significant effect on focal male egg production (glmer: 'adult:larval', z=1.96, p=0.050; 'adult', z=14.419, p<0.001; respectively; Figure 3A). However, there was no significant effect of larval diet on focal male egg production (glmer: z=1.238, p=0.216). In contrast, adult diet alone had a significant effect on focal male offspring production (glmer: z=13.663, p<0.001; Figure 3B). Focal male egg and offspring production were significantly greater on the high than on the low protein adult diet. There was no significant effect of the larval:adult diet interaction (glmer: z=812, p=0.153) or larval diet (glmer: z=1.784, p=0.0744) on focal male offspring production. Only larval diet had a significant effect on focal male viability (glmer: t=2.481, d.f.=1, p=0.0146; Figure 3C) and there was no significant effect of either adult diet (glmer: t=1.822, d.f.=1, p=0.0676) or the adult:larval diet interaction (glmer: t=0.175, d.f.=1, p=0.853).

Focal male egg and offspring production both declined significantly with age across all treatments (glmer: z=6.688, p<0.001; z=10.269, p<0.001; respectively). Focal male viability also altered significantly over the lifetime (glmer: t=5.151, p<0.001). Reproduction dropped to zero 20-30 days earlier for focal males on the low protein adult diet than on the high protein adult diet.

Total lifetime egg and offspring production also differed significantly across the four diet treatments (K-W test: 'eggs', K-W chi-sq. = 91.2, df = 3, p < 0.001; 'offspring', K-W chi-sq. = 92.2, df = 3, p < 0.001). Focal males on the high protein adult diet had significantly greater lifetime reproductive output than those held on the low protein adult diet (M-W U test: 'eggs', W=209, p<0.001; 'offspring', W=197, p<0.001). There was no significant difference in male lifetime egg production or in male lifetime offspring production between the mismatched HL and the constant LL diets (M-W U test: 'eggs', W=760, p=0.208; median=8, 10, respectively; 'offspring', W=699, p=0.572; median=8, 9, respectively) or between the mismatched LH versus the constant HH diets ('eggs', W=632, p= 0.861; median=95, 96, respectively; 'offspring', W=560, p =0.324; median=70, 78, respectively).

In summary, adult diet had a significant effect on both focal female and focal male egg and offspring age-specific and lifetime production, but there was no significant effect of larval diet on the reproductive output of either sex. There was a significant effect of the adult:larval diet interaction on focal female age-specific and lifetime production, leading to significant differences in female output between mismatched and constant diets. For focal males, there was a significant adult:larval diet interaction for age-specific egg production, but not for age-specific offspring production nor lifetime productivity.

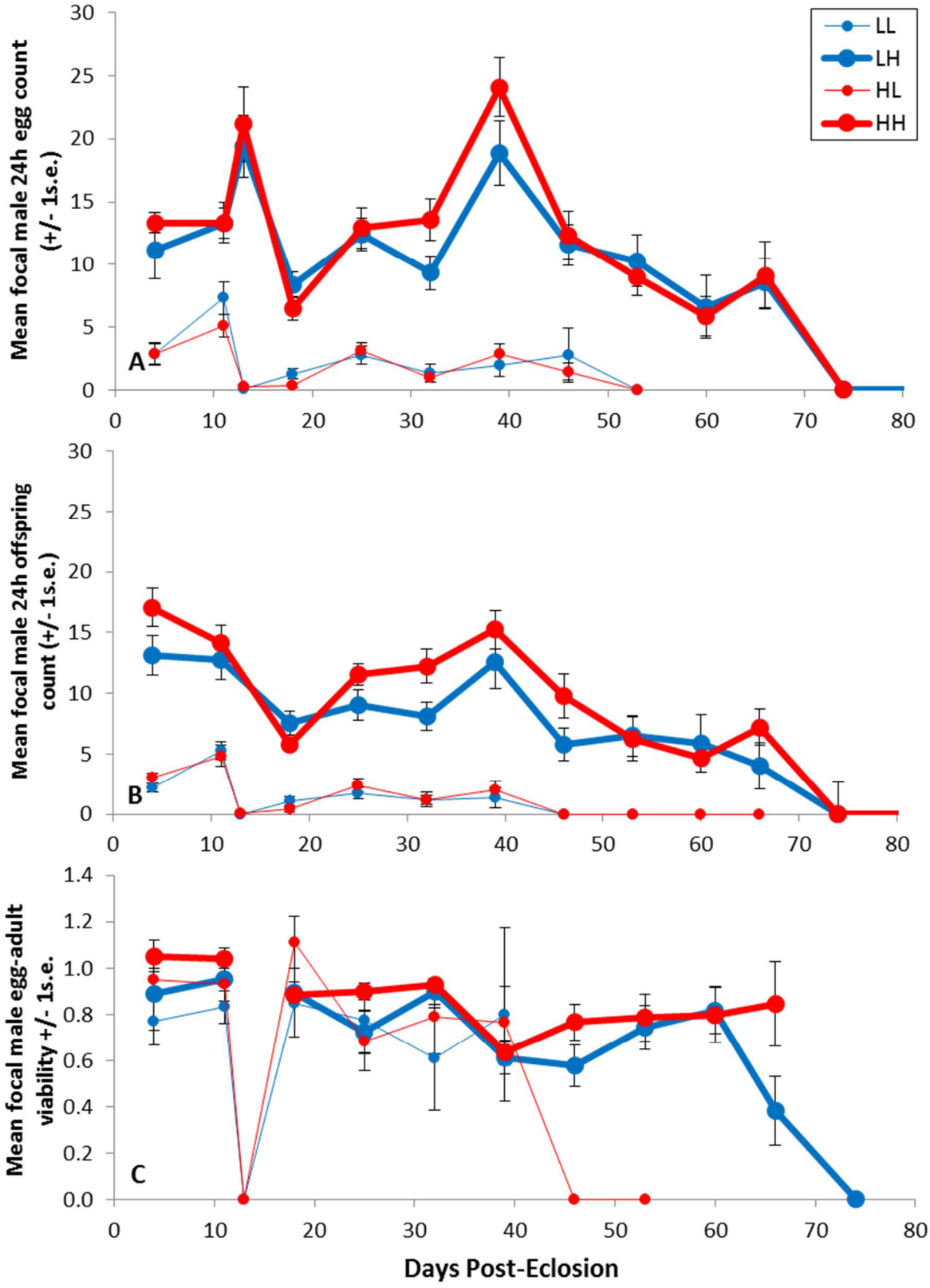

Figure 3. Mean focal male egg production (A), offspring production (B) and egg to adult viability (C), per male, per 24h, against days post-eclosion, for the four dietary treatment populations (LL, LH, HL and HH). Initial n=35 males/treatment. Egg to adult viability is defined as the proportion of eggs laid by the standard

WT female that had been mated to the focal male, during 24h, which eclosed as adults (C). All error bars display +/- 1 standard error.

2.4.2 Survival and reproductive success to mismatched or constant larval to adult diets in weekly-mated females and males

Survival

Both adult diet and larval diet had a significant effect on focal female survival (coxph: z=2.382, p<0.001; z=9.468, p=0.0172; respectively; Figure 4A; Table S4). Focal females lived significantly longer on the high than on the low protein adult diet. Focal females on a low protein adult diet lived significantly longer if they developed on a mismatched high protein larval diet (HL > LL). However there was no significant effect of the adult:larval diet interaction on focal female lifespan (z=1.343, p= 0.179).

The adult:larval diet interaction, adult and larval diet all had significant effects on focal male lifespan (coxph: z=3.317, p<0.001; z=6.894, p<0.001; z=3.796, p<0.001; respectively; Figure 4B; Table S5). Focal males lived longer on the high protein over the low protein adult diet. The survival advantage of a mismatched diet for male survival was more pronounced in adults on a low protein adult diet (HL > LL) than on the high protein adult diet.

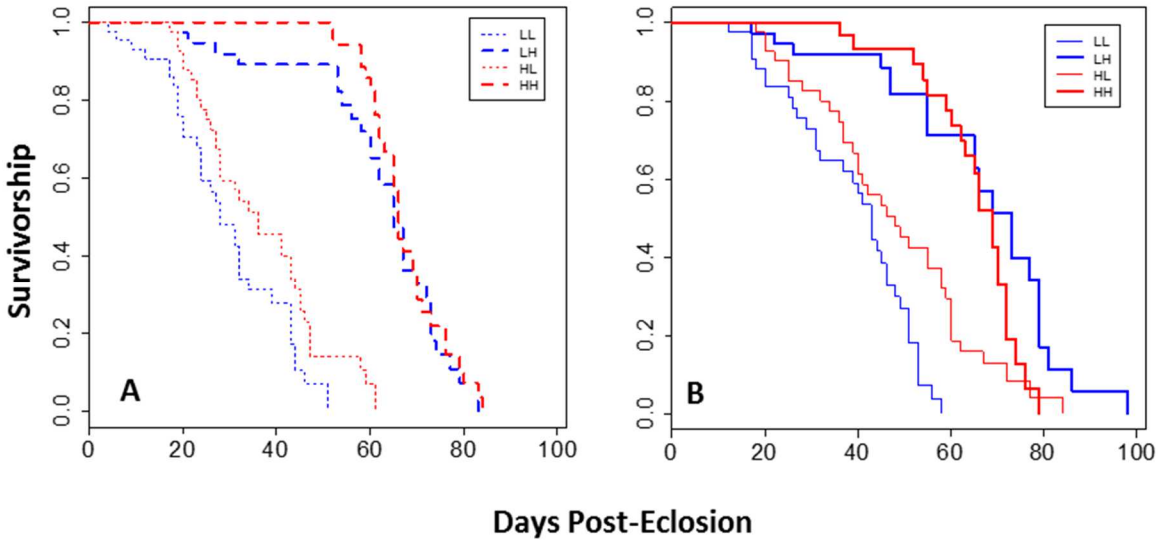

Figure 4. Age-specific survivorship against days post-eclosion, across dietary treatments (LL, LH, HL and HH), for: (A) focal females and (B) focal males. Dietary treatments were: constant low protein (20% SYA), LL; constant high protein (120% SYA), HH; low protein larval diet then high protein adult diet, LH; or high protein larval diet then low protein adult diet, HL. Focal females and focal males were both weekly-mated to standard WT individuals.

Focal Female Reproduction

There were significant effects of adult:larval diet interaction, adult diet and larval diet on focal female egg production (glmer: $z=4.290$, $p<0.001$; $z=23.973$, $p<0.001$; $z=2.424$, $p=0.0153$; respectively; Figure 5A) and offspring production (glmer: $z=3.600$, $p<0.001$; $z=19.492$, $p<0.001$; $z=2.135$, $p=0.0328$; respectively; Figure 5B). Focal female reproductive output was greater on the high than on the low protein adult diet. On both adult diets, it was the high protein larval diet that conferred greater reproductive output for focal females. This led to greater reproductive output on the constant HH diet than on the mismatched LH diet, but greater reproductive output on the mismatched HL diet than the constant LL diet. There was no significant effect of adult diet, larval diet or their interaction on focal female egg to adult viability (glmer: $t=0.607$, d.f.=1, $p=0.559$; $t=1.672$, d.f.=1, $p=0.0954$; $t=1.819$, d.f.=1, $p=0.0674$; respectively; Figure 5C).

Focal female egg production, offspring production and egg to offspring viability all significantly declined with age across all diet treatments (glmer: $z=28.830$, $p<0.001$; $z=28.270$, $p<0.001$; $t=10.80$, d.f.=1, $p<0.001$; respectively).

Total lifetime egg and offspring production differed significantly across the four diet treatments (K-W test: 'eggs', K-W chi-sq. = 141, df = 3, $p<0.001$; 'offspring', K-W chi-sq.= 136, df = 3, $p<0.001$). Median lifetime egg and offspring production were both significantly greater for focal females on a high protein adult diet (LH, HH) than a low protein adult diet (LL, HL) (M-W U test: 'eggs', $W=117$, $p<0.001$; 'offspring', $W=196$, $p<0.001$). Focal female lifetime reproductive output was also significantly higher on the mismatched HL diet than the constant LL diet (M-W U test: 'eggs', $W=135$, $p<0.001$; median=25, 5, respectively; 'offspring', $W = 164$, $p < 0.001$; median=24, 4, respectively). Female lifetime reproduction was also significantly lower on the mismatched LH diet than the constant HH diet (M-W U test: 'eggs', $W=696$, $p= 0.0106$; median=168, 194, respectively; 'offspring', $W = 680$, $p=0.00729$; median= 151, 182, respectively).

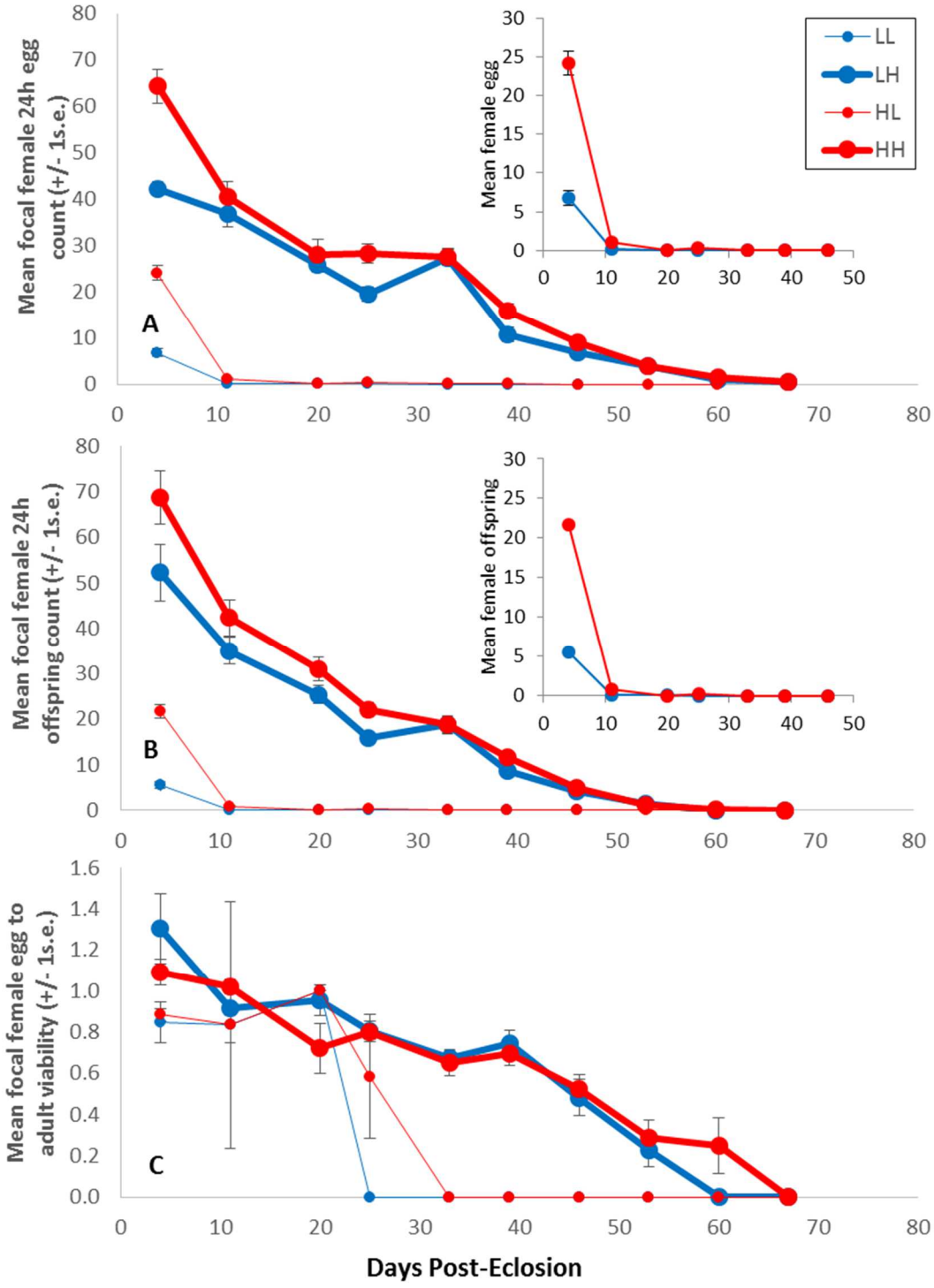

Figure 5. Mean focal female egg production (A), offspring production (B) and egg to adult viability (C), per female, per 24h, against days post-eclosion, for the four dietary treatment populations (LL, LH, HL and HH). Initial n=45 females/treatment. Egg to adult viability is defined as the proportion of eggs which eclosed as adults (C). For illustration, insets on panels (A) and (B) show mean female egg counts and offspring counts, respectively, for the two low protein adult diet treatments: HL (red) and LL (blue). All error bars display +/- 1 standard error.

Focal Male Reproduction

Adult diet alone had a significant effect on focal male egg production (glmer: $z=3.267$, $p=0.00109$; Figure 6A) and offspring production (glmer: $z=4.162$, $p<0.001$; Figure 6B). Focal males on the high protein adult diet had overall greater egg and offspring production than those on the low protein adult diet. Larval diet and the adult:larval diet interaction had no significant effect on focal male egg production (glmer: $z=0.465$, $p=0.642$; $z=0.140$, $p=0.888$; respectively) or on focal male offspring production (glmer: $z=1.402$, $p=0.161$; $z=0.803$, $p=0.422$; respectively). Adult and larval diets both had a significant effect on focal male egg to adult viability (glmer: $t=2.987$, d.f.=1, $p=0.00154$; $t=1.847$, d.f.=1, $p=0.0311$; Figure 6C) but there was no significant effect of the adult:larval diet interaction (glmer: $t=0.692$, d.f.=1, $p=0.489$).

Focal male egg production, offspring production and egg to adult viability all significantly declined over the lifetime, across all diet treatments (glmer: $z=22.740$, $p<0.001$; $z=20.042$, $p<0.001$; $t=18.68$, d.f.=1, $p<0.001$; respectively).

Total lifetime egg and offspring production differed significantly across the four diet treatments (K-W test: 'eggs' K-W chi-sq. = 9.319, df = 3, $p=0.0253$; 'offspring', K-W chi-sq. = 9.058, df = 3, $p=0.0285$). Focal males on the high protein adult diet had significantly higher egg and offspring production than those on the low protein adult diet (M-W U test: 'eggs', $W=3046$, $p=0.00407$; 'offspring', $W=3150$, $p=0.010$). There was no significant difference in male lifetime egg or offspring production between the mismatched HL and the constant LL diets (M-W U test: 'eggs', $W=874$, $p=0.265$; median=168, 156, respectively; 'offspring', $W=784$, $p=0.0651$; median=102, 83, respectively). There was also no significant difference in lifetime reproduction between the mismatched LH diet and the constant HH diet ('eggs', $W=1052$, $p= 0.753$; median=228, 217, respectively; 'offspring' $W=1016$, $p =0.981$; median=125, 140, respectively).

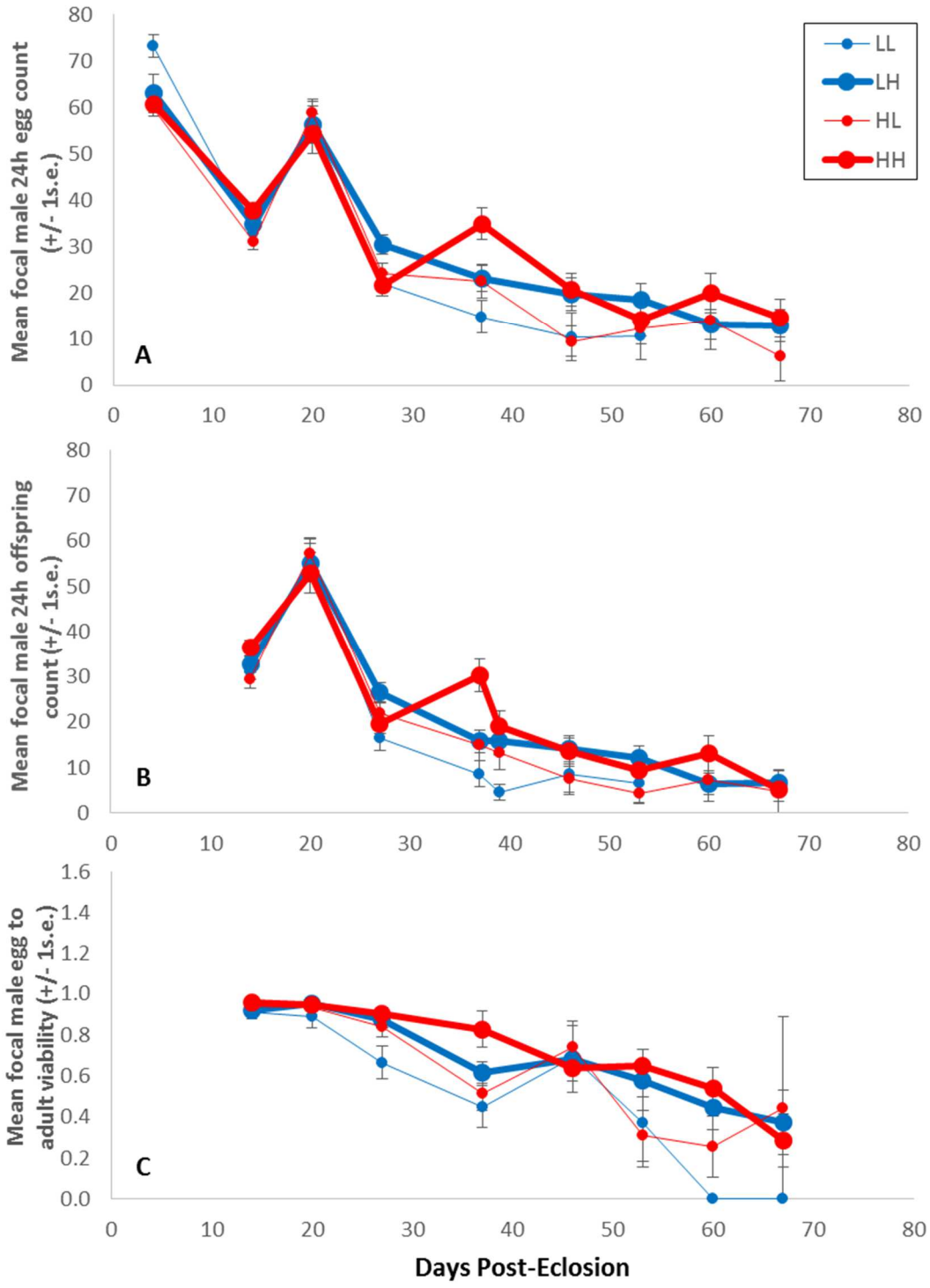

Figure 6. Mean focal male egg production (A), offspring production (B) and egg to adult viability (C), per male, per 24h, against days post-eclosion, for the four dietary treatment populations (LL, LH, HL and HH). Initial n=45 focal males. Egg to adult viability (C) was the proportion of eggs laid per individual standard WT female, which eclosed as adults. All error bars display +/- 1 standard error.

Mating Frequency

Focal females mated significantly more frequently on the low protein adult diet (GLM: $z=2.385$, $p=0.0171$; mean= 0.652, 0.582, respectively), whereas focal males mated more on the high protein adult diet (GLM: $z=3.075$, $p=0.00211$; mean= 0.899, 0.837, respectively). For both sexes there was no significant effect of larval diet (GLM: 'females', $z=0.555$, $p=0.579$; 'males', $z=0.510$, $p=0.610$) or the adult:larval diet interaction (GLM: 'females', $z=0.236$, $p=0.813$, 'males', $z=0.674$, $p=0.500$) on the proportion of individuals that mated.

The use of a combined statistical model revealed a highly significant interaction effect between sex and adult diet (GLM: $z=3.889$, $p<0.001$), showing that the effect of adult diet on lifetime mating proportion differed between the sexes. Males mated in significantly higher proportions than did females (GLM: $z=11.631$, $p<0.001$; mean=0.868, 0.617, respectively).

2.5 Discussion

Nutritional mismatches between developmental and adult nutrition can be detrimental to fitness. The Thrifty Phenotype (TP) hypothesis predicts the theoretical costs of mismatches between the quality of developmental and adult diets (Hales & Barker, 1992; Hales et al., 1997). However much less is known about the age-specific consequences of single-generational mismatched diets on survival and reproductive success of both sexes, or whether particular developmental or adult diets can ameliorate the costs of mismatched nutrition (May et al., 2015). Here I addressed these questions by directly manipulating the protein content of developmental (larval) and adult diets and assaying the life history consequences of mismatched versus constant diets, under once-mated and weekly-mating regimes, in both sexes.

The results showed that a mismatched diet was not universally costly when compared with a constant diet, contrary to predictions from the Thrifty Phenotype hypothesis (Hales & Barker, 1992). In some cases a mismatched diet was either neutral or beneficial to survival, reproductive output and fitness. In other cases there were condition-dependent or sex-dependent costs to reproductive output and fitness.

A 'good start' to life (high protein larval diet) was observed to be beneficial in a later harsh adult environment (low protein adult diet) in terms of survival, age-specific reproduction and fitness, in agreement with theory (Bateson et al., 2004). Hence a mismatched diet of high protein larval then low protein adult food (HL) was beneficial to all life history components measured in comparison to a constant low protein diet (LL). These patterns held for the survival of both sexes under the weekly-mated regime, for female reproductive output and fitness of females (from both mating regimes). However, there was no such difference in male reproduction or fitness (from either mating regime) in the HL versus LL comparison. Other studies also report sex-specific effects of nutrition on reproduction (e.g. Maklakov et al., 2008) or mating regime-specific effects of nutrition on lifespan (e.g. May et al., 2015). A high protein larval diet may have been beneficial as it led to faster development and higher egg to adult developmental viability. This may have led the resulting adult body to be physiologically more resilient to dealing with stresses experienced during adulthood. These findings are contrary to the costs of a mismatched diet as predicted from TP theory (Hales & Barker, 1992) and suggest that a high protein larval diet can ameliorate the costs of a mismatched low protein adult diet.

Also contrary to the TP hypothesis (Hales & Barker, 1992), I found that a 'poor start' (low protein larval diet) did not lead to significant survival costs in a mismatched high protein adult environment (LH), in comparison to the 'good start' (high protein larval diet) (HH) treatment. For

both sexes and both mating regimes there was no significant survival difference between individuals from LH and HH populations. In fact the LH diet treatment conferred a subtle (but non-significant) survival benefit in once-mated females, as May et al. (2015) found was the case for virgin females. This is also in agreement with previous studies in *D. melanogaster* that found no lifespan effects from a restricted larval diet in the context of pre-adult dietary restriction (Zwaan et al., 1991; Tu & Tatar, 2003). The subtle effects I observed were in line with the finding that delayed maturation, and longer development time on low protein diets, is associated with increased longevity (e.g. in the house cricket, *Acheta domesticus*, Lyn *et.al.*, 2012).

The LH mismatched diet reduced weekly-mated (but not once-mated) female reproduction and fitness, relative to the constant HH diet, in line with the TP hypothesis (Hales & Barker, 1992). A poor quality maternal diet during development has also been shown to reduce in offspring production in other species, despite a good quality maternal diet in adulthood, perhaps suggesting conserved mechanisms (e.g. Huck et al., 1987). However, male age-specific reproduction and fitness were unaffected by the mismatched diet. The female-specific fitness costs of a mismatched (LH) diet may have arisen as a consequence of low protein larval diet acting as a stronger developmental viability selection filter on male fitness than female fitness, as the adult eclosion ratio from the low protein larval diet was female biased in my pilot work (although there was no sex-biased eclosion ratio in the second developmental assay).

The most universal determinant of survival, reproduction and fitness was the protein content of the adult diet. For both sexes and for both mating regimes, the high protein adult diet always increased survival, reproductive output and fitness over the low protein adult diet. This would be expected on the basis that the high protein diet was selected to be below toxic protein levels and the low protein diet to below the levels of dietary restriction that lead to lifespan extension and hence stressful (e.g. Chapman et al., 1994; Chapman & Partridge, 1996; Magwere et al., 2004; Fricke et al., 2010).

Similarly, a high protein larval diet always increased developmental rate and viability over a low protein diet, in line with published data. The puparium to adult stage of development was most sensitive to the detrimental effects of a poor quality (low protein) larval diet, as would be expected on the basis of the energetically expensive developmental re-modelling that occurs during this life stage (in agreement with theory, Bateson et al., 2004). Surprisingly, female body size did not differ between larval diets, contrary to predictions that body size should have been smaller on the low protein larval diet (e.g. May et al., 2015). It is possible that the extended time period of development enabled the same final adult body size to be reached by the emerging

cohort. Alternatively, perhaps body size differences between high and low protein larval diets were only manifested in males (not measured here), which may be affected to a greater extent by developmental diet quality. I conclude that life history consequences of mismatched nutrition in females were not driven by body size differences.

The effect of larval diet on egg to adult viability was inconsistent across the different assays (the pilot developmental assay and the main life history assays). It was expected that there would be developmental benefits from a high protein larval diet. The absence of a consistent effect in this regard may have resulted from differences in maternal diets, the food type which eggs were laid onto, or differing maternal investment into number versus quality of eggs laid, as discussed below.

In the pilot developmental assay, all females were reared on standard food and eggs were laid onto yeasted red grape agar plates of good nutritional content, so adjustments in egg laying behaviour due to maternal diet or egg-laying substrate were unlikely. Subsequent allocation of equal numbers of first instar larvae from the plates to vials of low protein or high protein food, meant that larvae on each diet developed at equal densities and in equal numbers. Differential maternal investment in larvae developing on the low and high protein diets seems unlikely, as the maternal diet and egg laying substrates were constant and hence excluded maternal effects and ensured that only larval diet influenced egg to adult developmental viability.

Contrastingly, in the main life history assays, maternal effects differed between larval diet treatments. Larvae developing on a low protein diet were from eggs laid by focal females fed on a low protein adult diet and laying onto this poor quality substrate. Low protein females laid considerably fewer eggs than those fed and laying onto high protein food. This meant that larvae developed in considerably lower numbers and at potentially beneficial lower densities on low than on high protein food. Furthermore, each female may have invested more into the fewer number of eggs laid onto the low protein diet in a process akin to host sensing (e.g. Lindstrom, 1999; Bateson et al., 2004). This could explain why the viability of eggs laid by focal females from all diet treatments onto low or high protein food did not differ.

Egg to adult viability of eggs laid by the standard WT females mates of the focal males did not differ between focal male diet treatments in the first life history assay. In contrast, egg to adult viability was higher in the eggs fertilised by males on the high protein adult diet in the second life history assay. In both assays standard WT females all had the same dietary background (of SYA) and were all laying onto the same diet type (SYA), hence maternal diet and the egg laying substrate was consistent between larval diets. Standard females laid more eggs onto standard

food when mated to focal males from the high protein adult diets than on the low protein adult diets for both assays (as Fricke et al., 2008). This meant that larvae developed in higher numbers and at higher densities from high protein than low protein focal male treatments.

The increased numbers of offspring sired by males from the high over the low protein diet may have been due to the effects of a low protein adult diet on focal male courtship, on mating and the quantity and quality of sperm production (Droney, 1996). However, data on the link between adult dietary protein content and male mating behaviour are equivocal. Fricke et al. (2008) found that males on a low protein adult diet had reduced successful courtship (for re-matings with non-virgins), but in Fricke et al. (2010) there was no difference between male *c*ourtship on low versus high protein adult diets. Furthermore, a low protein larval diet is known to reduce the quantity of sperm males transfer to females, which may impact on its viability (McGraw et al., 2007).

The key difference between the life history assays was the period of exposure of the standard WT female to the low or high protein diet of the focal male. It is likely that the 24h exposure to the focal male diet was sufficient for female proximate effects and a subsequent shift in egg laying behaviour (e.g. Chapman et al., 1994). Proximate effects have previously been observed after 6hour exposure to a different diet. In contrast, the 3h exposure of standard females to the focal male diet (in the second life history assay) minimised such proximate effects. Therefore, the only difference between treatments was the diet of the focal male to which the standard female had been mated. Hence, differences were solely due to focal male effects. In this case the egg to adult viability of offspring was higher when the focal male was reared on a high protein diet, suggesting viability benefits from the good quality paternal diet.

The mating frequency of individuals differed between the sexes and between the adult diets. Focal males mated in higher proportions than focal females, as expected. Focal males were mated to young, virgin standard females that were likely to be more receptive to mating than the ageing focal females. Focal females mated in higher proportions on the low than the high protein adult diet. In contrast, focal males mated in higher proportions on the high than the low protein adult diet, in agreement with Fricke et al., 2008. Further behavioural assays might be useful to determine the underlying reasons.

More broadly, my results provide empirical evidence to support the conclusion that fitness (or life history) costs of nutritional mismatches between life stages are not inevitable and can be minimised by the type of mismatch between developmental and adult diets. Not all phenotypes expressed in response to the developmental environment are 'set' and life history plasticity to a mismatched adult environment can be expressed. This is also observed in the potential of *D.*

melanogaster for rapid lifespan plasticity to short-term dietary manipulation within adult life (e.g. Mair et al., 2003; Flatt & Schmidt, 2009). Hence, under conditions where mismatches are common (e.g. fluctuating environments), there may be selection to counter the potentially deleterious effects of mismatches. These findings have wider relevance for how alteration of developmental or adult environments can minimise costs of mismatches and their associated pathologies. They also add to the body of evidence to show that a reduction in nutrient intake can lead to reduced survival (Chapman & Partridge, 1996; Magwere et al., 2004; Partridge et al., 2005; Piper & Partridge, 2007; Barnes et al., 2008).

In summary, I conducted an empirical test of the life history consequences of mismatches between developmental and adult diets, within a single generation, to test predictions arising from the Thrifty Phenotype hypothesis. Contrary to predictions, I showed that a mismatched diet is not universally costly. A high protein larval diet reduced the costs of a mismatched low protein adult diet and led to enhanced survival, reproduction and fitness over a constant diet of low protein across larval and adult stages. Furthermore, costs of mismatched nutrition were dependent on the life history trait measured, were sex-dependent and differed between mating regimes. A mismatched low protein larval then high protein adult diet was not costly to the survival of either sex, but was costly only to weekly-mated focal female reproduction and fitness. Life history consequences observed for females did not appear to be the result of differences in adult body size between larval diets. As expected, a high protein adult diet universally enhanced survival, reproductive output and fitness over a low protein adult diet, for both sexes. Together the results highlight the importance of measuring multiple life history traits in both sexes and accounting for the effects of different mating regimes when assessing the life history consequences of mismatched nutrition.

The results of this study offer the opportunity to investigate the life history consequences of nutritional mismatches and the factors which can ameliorate the costs of mismatches over a broader range of diets (as recommended by Partridge et al., 2005) or over a broader cross-generational time-scale. This latter topic is pursued in Chapter 3.

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2.7 Supplementary Material

2.7.1 Pilot Developmental Assay

Materials and Methods

I conducted assays of development time and developmental survival on 20% and 120% SYA food medium (20 or 120g yeast, respectively, 15g agar, 50g sugar, 30ml Nipagin solution, 3ml propionic acid per litre). These tests were conducted to inform the experimental design of the two subsequent life history assays by determining the difference in development time, developmental viability and adult body size between low protein and high protein larval diets.

Experimental individuals were generated from eggs collected on yeasted red grape agar oviposition plates, from a large population cage of wild type (WT) *Drosophila melanogaster* flies. Laboratory-caged flies had been reared on standard (100% SYA) food for multiple overlapping generations, at 25°C, 50% relative humidity and a 12:12h light:dark cycle, since the 1970s. Eggs were collected over a narrow period of 4h, to facilitate precise monitoring of subsequent developmental timings. Larvae were picked from the plates 26h after oviposition and transferred to vials of either low (20% SYA) or high protein food (120% SYA) at a density of 100 larvae/vial. 10 vials of each food type were set up.

Numbers of puparia (i.e. immobile puparia with everted spiracles) were recorded twice daily (at 9am and 5pm) from Day 5 onwards. The number and sex of eclosed adults from the same larval vials were also recorded twice daily, until Day 28. From these measurements I determined egg to pupariation and egg to adult time.

Wing vein length was measured as a standard, well-recognised proxy for adult body size (Gidaszewski et al., 2009). Twenty eclosed females per larval diet treatment were frozen, both wings removed, mounted on a microscope slide and then photographed under light microscopy. I measured the length of the L3 wing vein (from its intersection with the anterior cross vein to the wing edge) and the anterior cross vein, using the image analysis software 'Image J'. Each measurement was repeated twice and the mean of both measures was calculated.

Statistical Analyses

Developmental viability was expressed as proportion data and analysed using a generalised linear model (GLM) with quasi-binomial errors to account for overdispersion. Differences in the number of eclosed adults per sex per replicate vial were analysed using a paired t-test, separately for each diet treatment (normality and equality of variances assumptions were met). Development time

data were tested for normality with the Shapiro Wilk test and for equality of variances with the Levene's test. Differences in development time between diet treatments were analysed using the two sample t-test, where the assumptions were met, and the Welch two sample t-test, when variances were unequal. The range of developmental times within each replicate vial, for each diet treatment, was calculated from the difference between the earliest and latest recorded pupariation or eclosion event, respectively. Differences in the range of developmental times between high and low diets were analysed with a two sample t-test.

GLMs with Gaussian errors (same output as the linear model, 'lm') were used to test for effects of larval diet (low or high protein), wing side (left or right wing) and their interaction on wing vein length, in a combined model. Model simplification of the maximal model via stepwise removal of the most non-significant terms was conducted, to determine the minimal model. Model comparison was performed with likelihood ratio tests (using the 'anova' function).

Quantifying Measurement Error

Measurement error in wing vein length was quantified, by calculation of the 'technical error of measurement' (Dahlberg, 1926; equation 1), and from this, the 'relative technical error of measurement' was determined (as reviewed by Harris & Smith, 2009; equation 2). The relative technical error of measurement expresses the size of the measurement error, relative to the mean length of the wing vein being measured.

Equation S1: Technical error of measurement (d) = $\sqrt{\frac{\sum D^2}{2N}}$

D = difference between value of replicates for a measurement

N = number of measurements (adapted from Harris & Smith, 2009).

The technical error of measurement calculates the standard deviation from the two repeated sets of 'N' measurements.

Equation S2: Relative technical error of measurement = $\frac{\text{Technical error of measurement (d)}}{\text{Sample mean}}$

Results

Developmental Viability

Developmental viability was significantly higher on the high in comparison to low protein larval diet, for overall egg to adult viability (GLM: t = 3.907, p = 0.00103; Figure S1.A) and for puparium to adult (GLM: t = 3.796, p = 0.00132; Figure S1.C). There was no significant difference in egg to puparium viability (GLM: t = 1.378, p = 0.185; Figure S1.B) between larval diets. These viability results were independently replicated in the second of the main experiments for egg to adult (e-a), puparium to adult (p-a) and egg to puparium (e-p) stages (GLM: t=12.95, p<0.001, for e-a; t=13.01, p<0.001, for p-a; t=1.966, p=0.0586, for e-p; data not shown).

Significantly more females than males emerged from the low protein larval diet (paired t-test: t=4.554, d.f.=9, p=0.00138; mean=38, 27, respectively) in the pilot assay, but this effect was not apparent on either low (paired t-test: t=0.219, d.f.=21, p=0.829) or high protein larval diets (paired t-test: t=1.288, d.f.=9, p=0.230), in the main life history experiment.

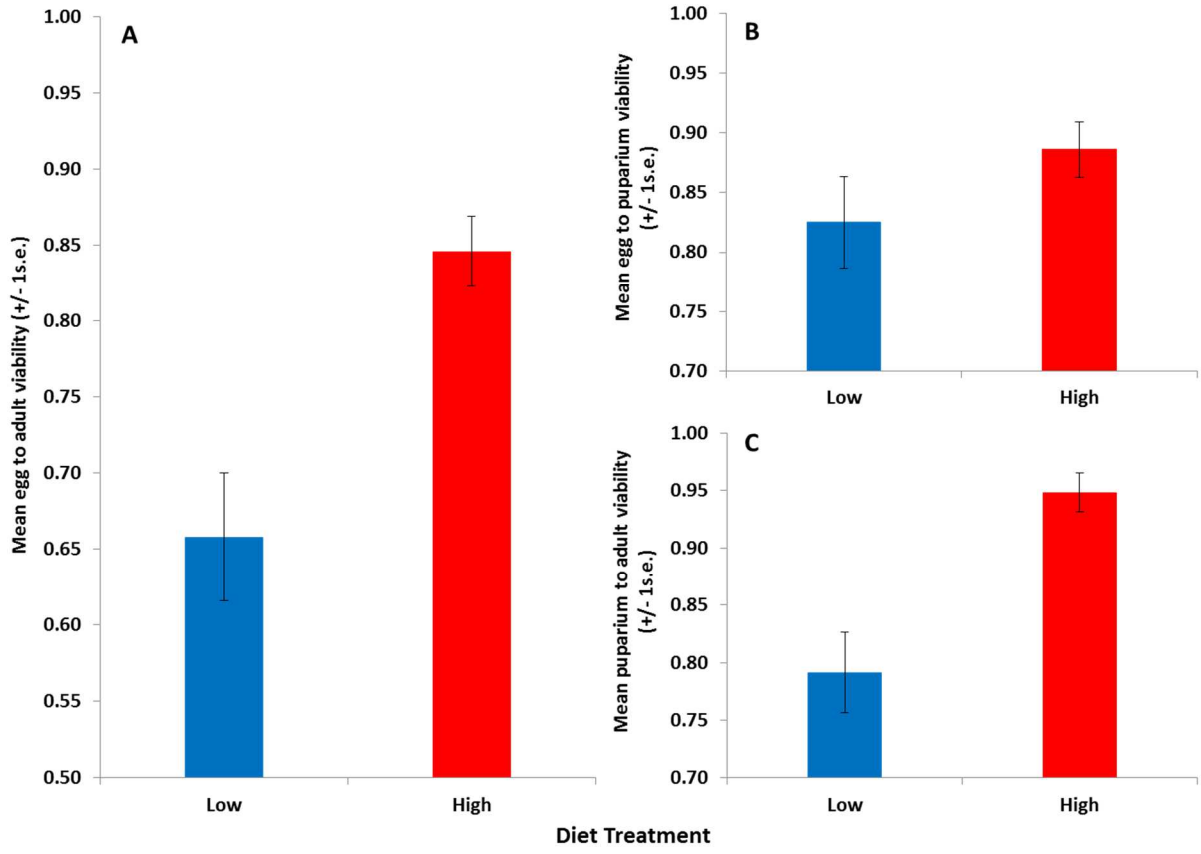

Figure S1. Mean developmental viability (+/- 1s.e.) on low (20%) or high protein (120%) larval diets, at egg to adult (A), egg to puparium (B) and puparium to adult (C) developmental stages. Sample size of 10 vials each with 100 first instar larvae per diet treatment.

Development Time

As expected, development time was significantly longer on the low in comparison to high protein larval diet, for overall egg to adult development time (Two Sample t-test: t=30.825, d.f.=9.807, p<0.001; Figure S2.A), egg to puparium (Two Sample t-test: t=32.803, d.f.=9.084, p<0.001; Figure S2.B) and puparium to adult (Two Sample t-test: t=5.0815, d.f.=11.079, p<0.001; Figure S2.C) stages. Mean egg to adult development time was 193h longer on the low in comparison to high protein larval diet.

Similarly, the time window (range) of development times (from first to last eclosion) was significantly greater on the low in comparison to high protein larval diet, for both egg to adult (Two Sample t-test: t=12.071, d.f.=18, p<0.001; 'low' range=290h, 'high' range=137h) and egg puparium stages (Two Sample t-test: t=11.363, d.f.=11, p<0.001; 'low' range=330, 'high' range=70h). To account for this wider range of eclosion times on the low protein larval diet, it was determined that >8.5 times the number of low as opposed to high protein larvae needed to be collected in the main experiment, to produce equal numbers of adults eclosing in a 24h period around mean eclosion time.

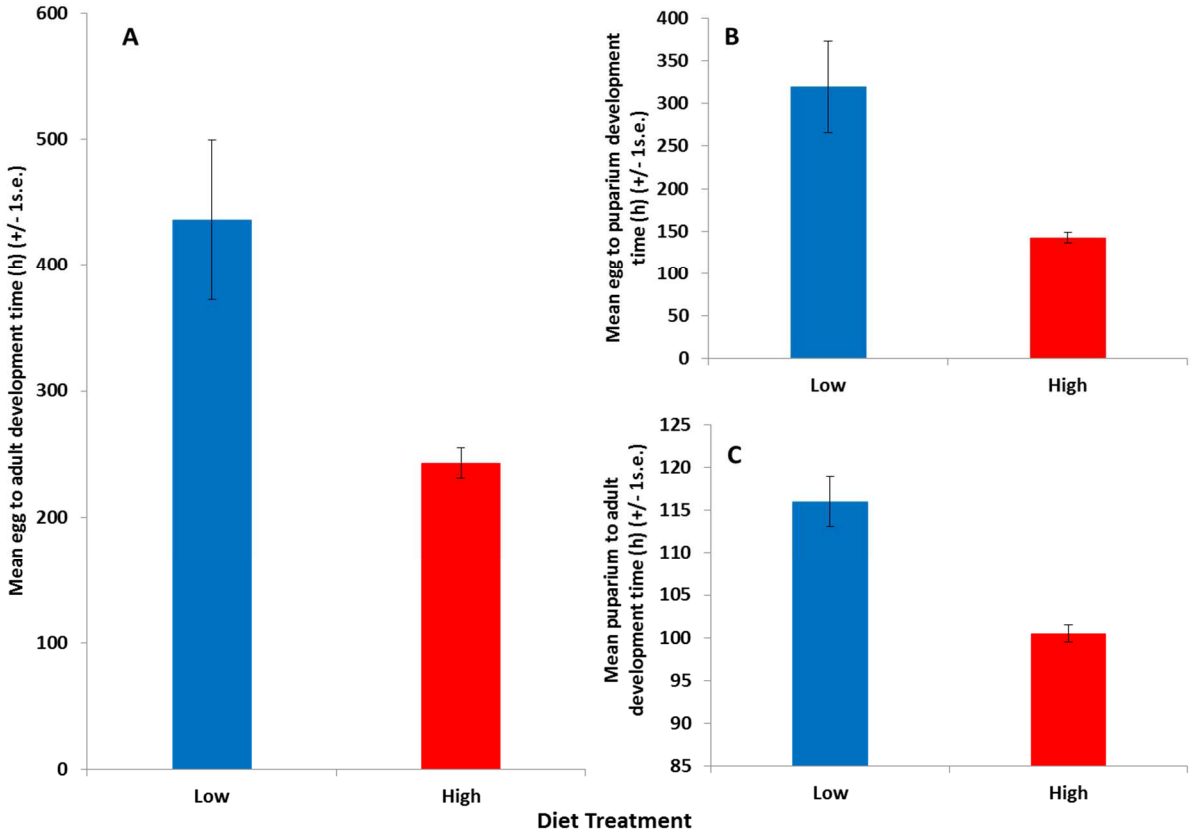

Figure S2. Mean development times (+/- 1s.e.) on low (20% SYA) and high protein (120%) larval diets, at egg to adult (A), egg to puparium (B) and puparium to adult (C) developmental stages. N=10 vials of 100 larvae per diet treatment.

Body size (wing vein length)

There was no significant effect of the larval diet, wing side (left versus right wing) or their interaction on the length of the L3 wing vein (glm: t=0.266, p=0.791; t=0.207, p=0.836; t=0.180, p=0.858; respectively) (Figure S3.A) or on the length of the anterior cross-vein (glm: t=0.682, p=0.498; t=1.232, p=0.222; t=1.200, p=0.234; respectively) (Figure S3.B).

Measurement error for both wing measures was minimal. The relative technical error associated with measurement of the anterior cross vein, was over 15 times greater than the relative technical error associated with measuring the longer L3 wing vein (Table S1). This suggested that the shorter anterior cross vein was more difficult to measure accurately, and so the L3 wing vein is a more suitable and less error-prone measure to use.

Table S1. Relative technical error of measurement (%). Percentage of measurement error relative to the size of the length of the L3 wing vein and the anterior cross vein, on both left (n=20) and right (n=19) wings, across high (120% SYA) and low (20% SYA) protein diets. Calculation as Harris & Smith (2009).

Larval Diet	Wing Vein	Left Wing	Right Wing
High	L3	0.307	0.234
	Anterior	5.358	5.083
Low	L3	0.170	0.181
	Anterior	3.699	4.441

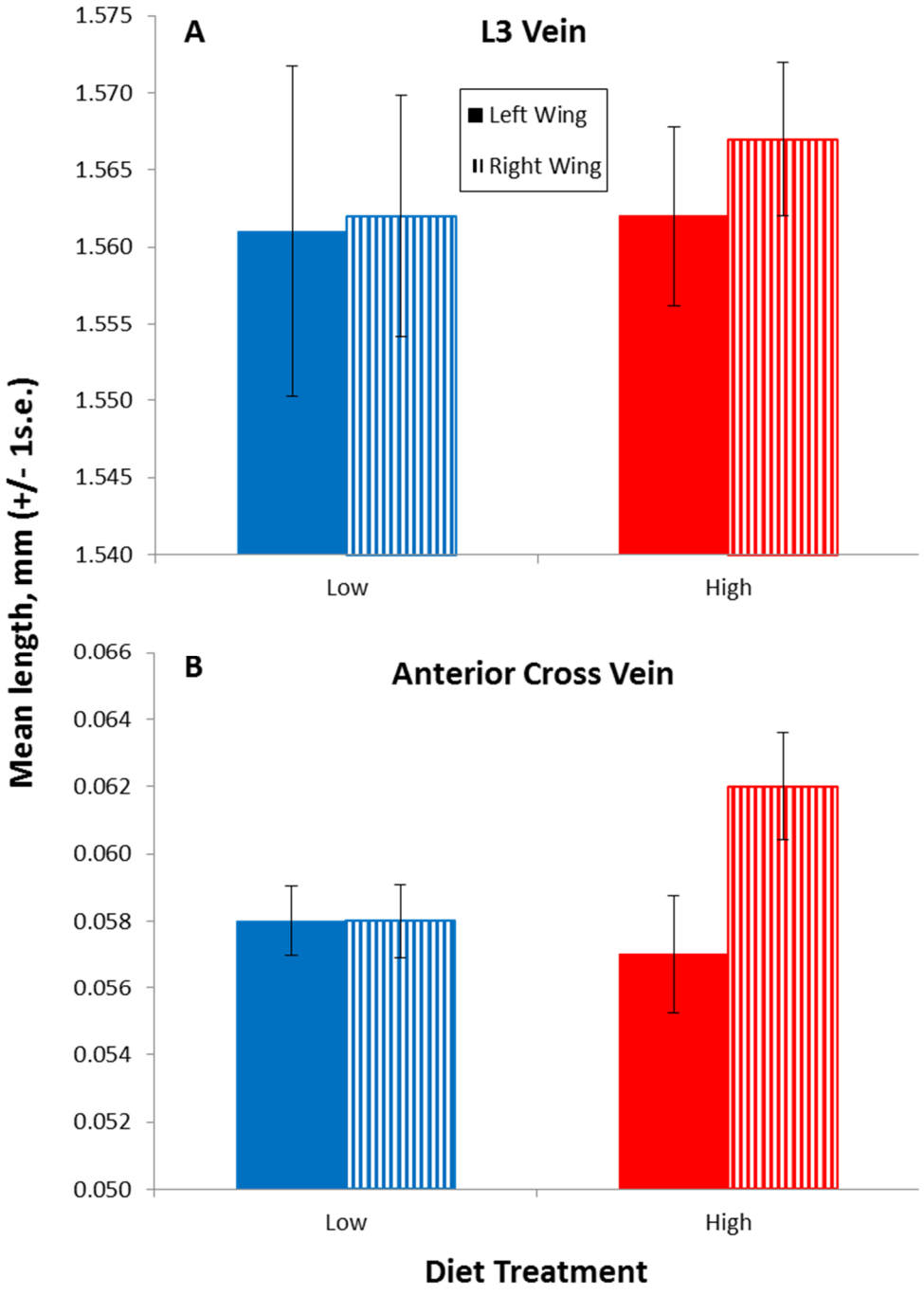

Figure S3. Mean female wing vein length (+/- 1s.e.) of the L3 vein (A) and the anterior cross vein (B), for both wings, after developing on a low (20% SYA) protein or a high protein (120% SYA) larval diet. Sample size of 20 females per diet treatment were measured, using Image J. Left and right wing measurements were paired, derived from the same fly. Each measurement was taken twice and an average calculated, to account for measurement error. Wing vein length was a standard proxy for body size.

2.7.2 Focal Female and Focal Male Median Lifespan

Table S2. Average survival for focal females, when once-mated, across treatment populations (LL, LH, HL, HH)

	LL	LH	HL	HH
Median lifespan (days)	21	59	26	31
Interquartile range (days)	16	44	8	35

Table S3. Average survival for focal males, when mated for 24h weekly, across treatment populations (LL, LH, HL, HH)

	LL	LH	HL	HH
Median lifespan (days)	30	69	39	64
Interquartile range (days)	16	19	31	11

Table S4. Average survival for focal females, when mated for 3h weekly, across treatment populations (LL, LH, HL, HH)

	LL	LH	HL	HH
Median lifespan (days)	27	65	32	66
Interquartile range (days)	18	17	20	9

Table S5. Average survival for focal males, when mated for 3h weekly, across treatment populations (LL, LH, HL, HH)

	LL	LH	HL	HH
Median lifespan (days)	41	66	46	66
Interquartile range (days)	22	30	24	12

Chapter 3: Life history consequences of evolutionary manipulation of feeding regime: testing the "Thrifty Genotype" hypothesis

3.1 Abstract

Nutritional mismatches across evolutionary time or between different life history stages are widely regarded to be costly to fitness. For example, the Thrifty Genotype (TG) hypothesis centres on the potential costs of mismatches between evolved ('ancestral') and proximate ('modern') nutrition. Little is known about whether particular evolved feeding regimes can ameliorate the costs of nutritional mismatches or the life history consequences of manipulating such regimes. I hypothesised that an evolutionary history of unpredictable feeding would ameliorate the costs of a mismatched proximate diet, by enhancing resilience and plasticity to poor and novel nutrition. I tested this idea by using replicated selection lines of the fruit fly, *Drosophila melanogaster*, maintained for over 360 generations on either unpredictable ('Random') or predictable ('Regular') feeding regimes. I assayed the life history consequences of these lines on a common garden diet and over several proximate diets (starvation, low and high protein), that were mismatched to the 'evolved' diet. Contrary to predictions, an evolutionary history of unpredictable feeding did not lead to an enhanced resilience to starvation or increased plasticity to novel diets, at least in terms of survival. In fact, Random male survival was reduced relative to Regular male survival on all but the low protein diet. Only Random female fecundity was increased relative to Regular females, in line with TG predictions. Interestingly, evolutionary manipulation of feeding regime altered the extent of sexual dimorphism for lifespan (SDL). Proximate diet manipulations also altered the direction of SDL. Overall the results show that nutritional mismatches between evolved feeding regimes and proximate diets may lead to survival costs, which are not reduced by a history of unpredictable feeding. A co-authored manuscript (Elizabeth Duxbury, Tracey Chapman & Wayne Rostant), based on the baseline life history assay from this thesis chapter and combined with the contents of thesis chapter 4, has been accepted for publication by *Proceedings of the Royal Society B: Biological Sciences* (Appendix).

3.2 Introduction

3.2.1 Nutritional mismatches

Nutritional mismatches (deviations) away from evolved, standard or optimal nutrition are generally proposed to be costly. That is, switches (mismatches) in dietary nutrient content or in feeding regime are predicted to result in survival or fitness costs to individuals. Life history costs from mismatched nutrition can, in part, be ameliorated by an individual's capacity for phenotypic and life history plasticity, or adaptability, in the face of nutritional change (e.g. Stearns, 1992; Pigliucci, 2001). Furthermore, selection on nutritional environments can lead to the evolution of fixed life history strategies (Stearns, 1992), which can better predispose individuals to altered nutritional environments. However, little is still known about the factors influencing the extent and nature of the life history consequences of nutritional mismatches.

Two prominent evolutionary hypotheses, originally proposed in the context of human evolution, predict the life history consequences of nutritional mismatches. These hypotheses concern manipulations within a single generation between the developmental and adult stages (Thrifty Phenotype Hypothesis (Chapter 1), or between generations between an evolved ('ancestral') diet and a proximate ('modern') diet (Thrifty Genotype (TG) Hypothesis; Neel, 1962). The TG hypothesis is outlined in more detail, below.

3.2.2 The Thrifty Genotype hypothesis

The TG hypothesis was first conceived as an evolutionary explanation for the prevalence of modern human obesity, arising from mismatches between ancestral (evolved) and modern (proximate) nutrition (Neel, 1962). The TG hypothesis proposes that the ancestors of modern humans, who relied on agriculture (since around 10,000 years ago), experienced an unpredictably fluctuating history of food availability (including 'feast' and 'famine' periods), linked with a fluctuating climate (Neel, 1962; Prentice et al., 2005). Critics argue that the extent or existence of these ancestral feast-famine fluctuations was not uniform, but varied between geographical regions and demographic or socio-economic groups (Sellayah *et al*., 2014).

Populations that did evolve under a history of unpredictable food availability, because of fluctuating agricultural production, may have been subject to positive selection for 'thrifty' genes responsible for increased fat deposition and energy storage during 'feast' periods to increase resilience to subsequent 'famine' (Neel, 1962; Prentice et al., 2005). However, it has also been suggested that these 'thrifty genes' may have accumulated via genetic drift rather than selection

(Speakman, 2008). The transition to agriculture may have led to the relaxation of selection pressures on predator evasion and hunter-gather traits. Hence genes linked with fat and energy storage were no longer maladaptive and 'drifted' into the population (Drifty Phenotype Hypothesis/Predation Release Theory; Speakman, 2008; reviewed by Sellayah *et al.*, 2014).

Regardless of the mechanism, a genetic propensity to rapidly accumulate fat when food is plentiful, may be detrimental if food is always plentiful (*ad libitum*) and this idea is now proposed to explain some of the modern human predisposition to obesity. These mismatches between the nutritional environment under which ('ancestral') life histories evolved and a proximate (or 'modern'/more recently altered) nutritional environment, left individuals maladapted to a new nutritional environment. It was possible that these mismatches may have shifted genes from their evolutionary optima, proving detrimental to fitness.

Identification of specific examples of candidate genes associated with TG patterns (Neel, 1962), is now underway and possible targets have been highlighted (Prentice *et al.*, 2005). For example, the insulin microsatellite (INS-VNTR) which is involved in the nutrient sensing, insulin-signalling pathways, fetal growth and survival, has been identified as a possible thrifty gene that is potentially linked with diabetes (Prentice et al., 2005). Positively selected thrifty genes have been proposed to have a range of metabolic, physiological and behavioural effects, including energy-efficient metabolism, inactivity, rapid fat gain, switching off 'non-essential' physiological processes, over-eating and food hoarding (Prentice *et al.,* 2005).

More recently, there have been several criticisms of the TG hypothesis (Speakman, 2008, Sellayah *et al.*, 2014). Some question whether 'famines' may have been sufficiently severe to exert strong enough selection pressures on 'thrifty genes' (Speakman, 2008). Assuming that selection did drive these genes to fixation, this idea proposes that we would expect modern obesity to be even more widespread than currently observed (Speakman, 2008).

Another criticism is that ancestral patterns of feast and famine may not have been uniform across all geographical regions and also that many regions show less of a contrast (mismatch) between 'ancestral' and 'modern' nutrition (Sellayah et al., 2014). Perhaps the extent of feast-famine periods during evolutionary history (in terms of size and frequency of fluctuations) may be linked with the strength of selection acting and hence the propensity to modern obesity observed. A greater unpredictability and severity of 'famine' occurrence, would perhaps then have exerted a stronger selection pressure on 'thrifty genes', leading to a tendency for higher obesity levels in those regions.

Whilst there is disagreement over mechanisms of 'thrifty gene' accumulation and the geographical uniformity of the environments under which life histories evolved, there is general consensus on two important points. Firstly, evolution in an unpredictably fluctuating environment will lead to selection for genes which enhance the fitness of individuals under those evolved conditions. These adaptations could allow a greater resilience or adaptability and increased plasticity and fitness to novel or changing environments and thus, a gain in plasticity to environmental change. Secondly, mismatches between evolved and modern environments can be detrimental for fitness.

However, little empirical work has focused on testing these TG predictions or determining the life history response (in terms of survival, reproduction and fitness) to an evolutionary history of fluctuating, unpredictable food supply. Neither has there been much study of the life history consequences of the interaction between an evolutionary feeding regime manipulation and mismatched proximate diets.

Here I used the fruit fly, *Drosophila melanogaster*, to address these omissions and test key Thrifty Genotype predictions. I investigated the evolved responses of life histories to diet and feeding regime manipulations, using a set of replicated populations experimentally evolved on different feeding regimes for >360 generations (over 15 years). The evolutionary treatments were populations (lines) with an evolutionary history of standard food supplied at either regular ('Regular') or unpredictable ('Random') intervals (the latter simulating periods of feast and famine).

Pilot work (Perry *et al.*, unpub.) revealed an evolved body size difference between Random and Regular flies. In both sexes, Random flies were consistently smaller than Regular flies, on both low protein and high protein proximate diets. However, there was no interaction between evolved regime and proximate diet, both Random and Regular lines had similar increases in body mass on the high protein over the low protein diet.

This evolved decrease in body size in Random flies over Regular flies gave the first evidence for an evolved phenotypic difference between the lines in a condition dependent trait. This set the stage for investigating whether evolved differences in life history traits existed between the lines and the possible condition dependence of these traits.

I first tested the life history responses of Random and Regular populations on a common garden (standard) diet, to test for the baseline level of evolved differences between the regimes, in the absence of nutritional biases. I then tested for life history responses of both regimes across a

range of mismatched proximate diets (starvation, low protein, high protein). This enabled me to determine the life history consequences of nutritional mismatches between evolved feeding regimes and proximate nutrition (both proximate diet and proximate feeding regime). Life history assays were composed of survival assays for both sexes and also assays of weekly reproductive output for females and initial reproductive for males, to ascertain possible associations or trade-offs with survival and to estimate fitness. This enabled me to assess the degree of life history plasticity and resilience to new nutritional environments, which the evolutionary histories may have conferred.

I predicted that evolution under an unpredictable feeding regime (in the Random lines), would select for increased resilience to starvation and an enhanced plasticity to novel diets (Rion & Kawecki, 2007), hence ameliorating costs from a mismatched diet predicted by the TG hypothesis (Neel, 1962). I predicted that the increased resilience and plasticity would be manifested in enhanced survival, reproduction or fitness, across the proximate diets. It was expected that Random lines would capitalise on resources when they became available and exhibit increased fecundity when food was *ad libitum*, to avoid energetically expensive egg production when food was scarce, in comparison to Regular lines (Rion & Kawecki, 2007).

3.3 Materials and Methods

3.3.1 Experimentally Evolved Lines

Experimentally evolved lines of flies used for all life history assays had been reared in the laboratory on standard food (100% standard yeast agar, SYA) supplied at 'regular' or 'random' intervals, for 15 years (360 generations). Three replicated populations of each feeding regime line (Regular and Random) were established. The 3 Regular lines, housed in separate cages (Regular 1, Regular 2, Regular 3, respectively) each received 3 fresh bottles of SYA weekly, on the same day each week, creating a predictable or 'regular' food supply. The 3 Random lines (Random 1, Random 2, Random 3), each received 3 fresh bottles of SYA, supplied at randomly generated intervals (interval=0-14days) creating an unpredictable food supply approximating feast and famine periods. Over the course of 28 days, all lines received the same total quantity of food.

3.3.2 Baseline Life History Assay

Experimental individuals were the second generation of offspring (F2) originating from eggs laid by grandparents (P1) derived from the 3 replicated populations of Regular and Random feeding regime cages (Figure 1). Two generations of rearing under standard conditions were conducted

to minimise maternal effects. Eggs were collected from females (P1) by introducing a single yeasted red grape juice agar plate into each of the 3 Regular and 3 Random cages, for 24h. First instar larvae were transferred to SYA vials at controlled density of 150 larvae/vial. Adult flies (F1 generation) were allowed to emerge and freely mate in their larval vials for 24h and then tipped (without CO_2 anaesthesia) onto fresh SYA bottles for another 12-24h of free-mating. This ensured all F1 individuals were sexually mature (aged between 12h and 48h). 50 F1 females from each of the 6 experimental lines were then transferred into a mini-cage with yeasted red grape juice agar plate, using light CO_2 anaesthesia, and allowed to egg-lay for 24h.

First instar F2 larvae (n=300, per mini-cage agar plate) were transferred to SYA vials, at a density of 150 larvae/vial. Adults emerging from the F2 larval vials were collected as the F2 generation 'focal' flies for the adult fitness experiment. Sample sizes of 45 focal adults/sex/line per line, were used for the life history assay.

Virgin wild-type (WT) Dahomey flies of both sexes (n=60/sex/line), derived from standard density cultures (150 larvae/vial) were generated, for a single mating with the focal flies, 12h post-eclosion, in a 45 focal:60 WT mating ratio. Emerging WT flies were collected as virgins and held in single sex bottles of SYA (60 WT flies per bottle, per sex, per experimental line of focal flies) until mating.

Matings between virgin focal flies and virgin WT flies were set-up 12h post-eclosion (to ensure sexual maturity). Under light CO_2 anaesthesia, each SYA bottle of 60 WT adults was tipped into a SYA bottle of 45 focal adults of the opposite sex, for each of the 6 experimental lines, and allowed to mate for 24h. This mass-mating set-up introduced biologically-relevant male-male competition and aimed to ensure all focal adults were mated. The mating schedule of focal females and focal males was therefore identical.

After mating, focal females and focal males were transferred to single sex vials of standard food (SYA) at a density of 3 flies/vial, under light CO_2 anaesthesia. WT females used in the mating were also stored at a density of 3 flies/vial, in SYA vials, to determine the initial reproductive output of focal males. WT males were discarded after mating. Focal adults received no further matings and no further exposure to the opposite sex after the initial mating.

Initial egg counts for both focal sexes were determined 3 days post-eclosion, by allowing groups of 3 focal females and the retained groups of 3 WT females to lay onto fresh SYA vials, for 11h. An 11h egg laying period was used to avoid egg-overcrowding. Egg vials were retained to determine

egg-adult viability and frozen 13 days after egg laying, for later counting of number of offspring. WT females were discarded after egg laying.

Weekly egg and offspring counts were taken from the once-mated focal females, for the remainder of the experiment following the same protocol as the initial egg count. This allowed me to compare age-related decline in female reproductive output between regimes. An extra food transfer was inserted at the end of the 11h egg lay to maintain consistency of handling across all treatments. Egg counts for the final two weeks of the experiment were recorded for 48h egg-laying periods due to very low egg counts and converted to per 11h egg lays for analysis.

Every 2-3 days (Monday, Wednesday, Friday) food vials were exchanged and the groupings of 3 focal flies per vial were shuffled, to randomise the positioning of focals in vials with fewer than 3 flies (due to mortalities or censors). Focal female and focal male mortalities were checked daily and Kaplan Meier survivorship curves were plotted.

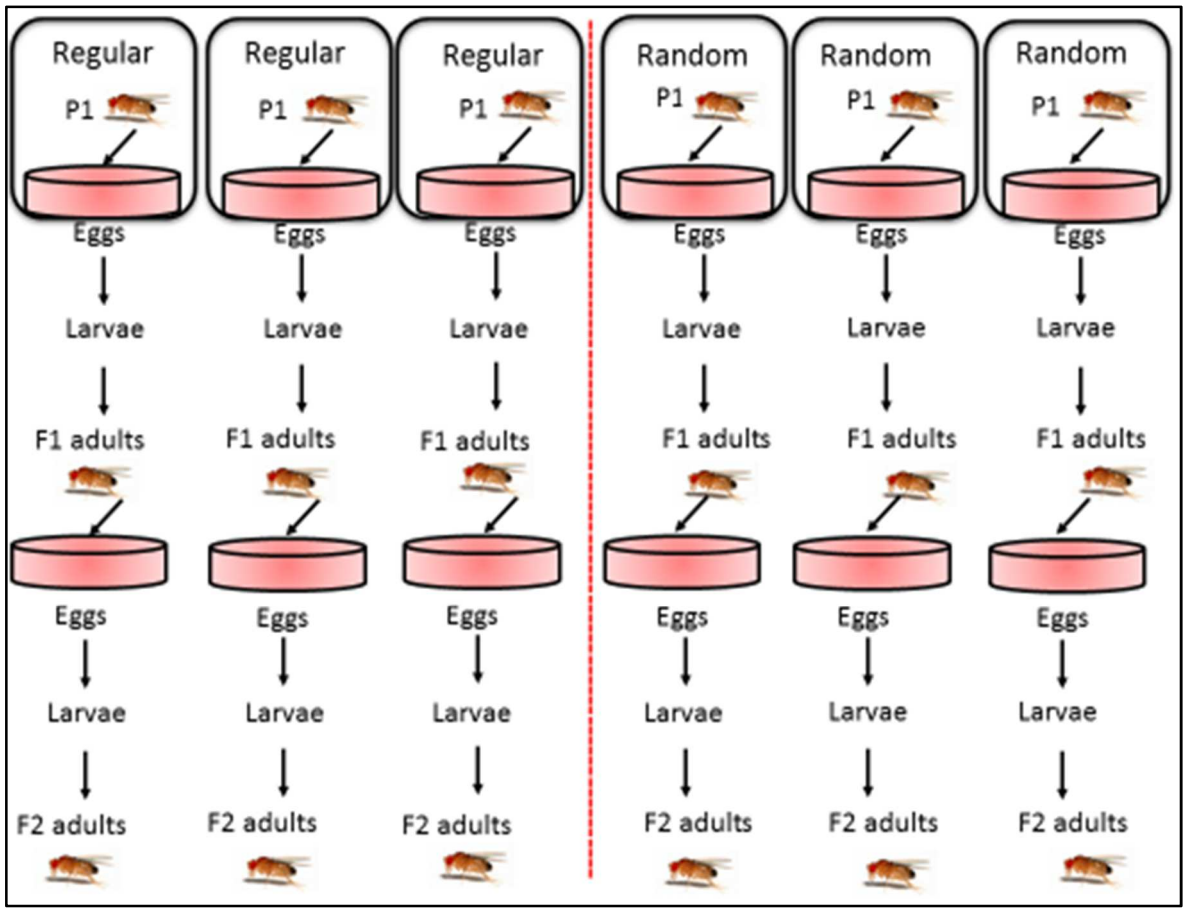

Figure 1. Experimental design for generation of focal individuals. Flies in the 'Regular' and 'Random' cages, sustained on standard yeast agar (SYA), were the grandparents of F2 flies used for experimentation. Eggs for the F1 generation were collected on red grape juice agar plates for 24h and larvae developed at a

standard density of 150 larvae/vial on SYA. F1 adults were mass-mated for 36-48h, with mates from their own feeding regime line.

3.3.3 Starvation Survival Assay

Focal females and focal males used in this experiment were generated as in the baseline life history assay (Figure 1). Focal F2 adults (n=45, per sex, per experimental line) were collected on ice, as virgins, within 6 hours of eclosion and stored in separate single sex bottles of agar-only (starvation) media, for each line. The tight window of eclosion was used to ensure virginity and minimise exposure to the standard SYA food in the F2 larval vials, in advance of the starvation regime. The starvation regime therefore started at a maximum of 6h post-eclosion.

Virgin WT flies of both sexes (n=60/sex/line) were generated and stored in separate, single-sex SYA bottles until mating as in the baseline life history assay. Matings between virgin focal flies and virgin WT flies were set-up 12h post-eclosion (to ensure sexual maturity). Under light CO_2 anaesthesia each SYA bottle of 60 WT adults was tipped into an agar-only bottle of 45 focal adults of the opposite sex, for each of the 6 experimental lines, and free mating allowed for a period of 3h. The short 3h mating period was used to minimise any proximate effects of the starvation diet on the WT mates. WT flies were used, rather than individuals from the F2 generation of cage populations, to allow standardisation of mating partners across all focal flies.

After mating, focal flies were transferred into single-sex, agar-only vials (3 flies/vial). Mortalities were recorded 4 times per day (9am, 1pm, 5pm, 9pm). Dead flies were removed on agar exchange days only (3 times per week: Mon, Wed, Fri). Transfers and shuffling of flies were done using light CO_2 as for the baseline life history assay.

3.3.4 Dietary Life History Assay on Low Protein and High Protein Food

Focal females and focal males used in this experiment were also generated as in the baseline life history assay (Figure 1). Focal F2 adults (n=90, per sex, per experimental line) were collected on ice, within 6 hours of eclosion, to ensure virginity and minimise exposure to standard SYA food. For each sex and for each experimental line, half of these focal virgins (n=45) were randomly allocated to bottles of low protein food (20% SYA: 20 grams of yeast per litre of SYA) and half to bottles of high protein food (120% SYA: 120 grams of yeast per litre of SYA) (Figure 2).

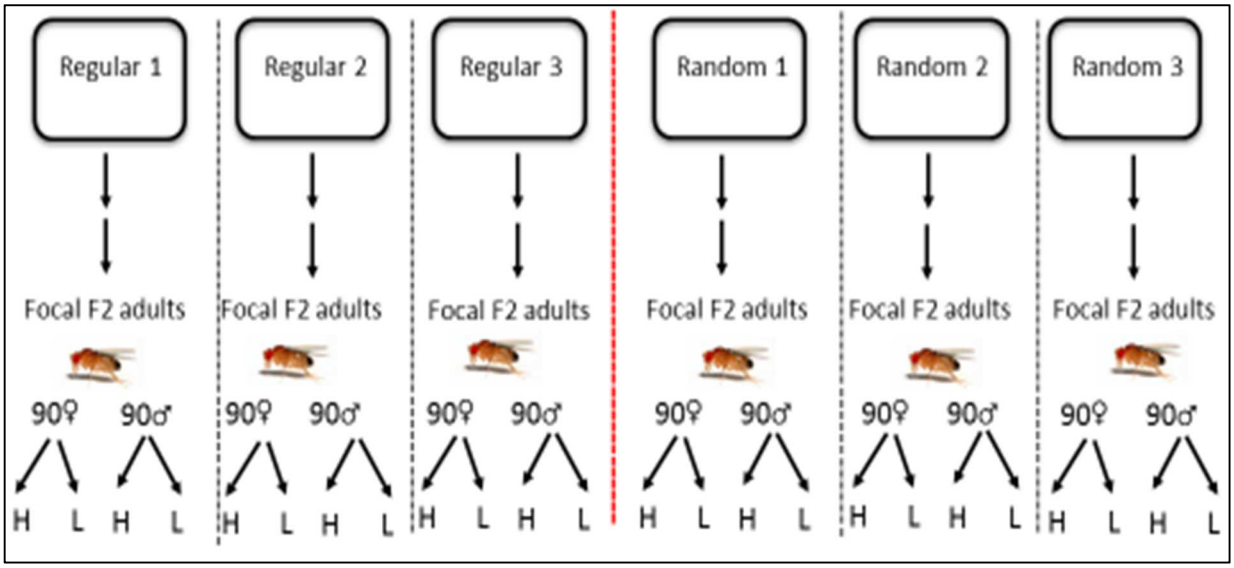

Figure 2. Experimental design for high and low quality diet assay. Focal adults for experimentation were the F2 offspring from the 6 lines (Regular 1, Regular 2, Regular 3, Random 1, Random 2 or Random 3), reared through two generations (double arrows) on standard SYA food. For each sex, half (n=45) were reared, post-eclosion, on high protein (H) and half (n=45) on low protein (L) food.

Virgin WT flies of both sexes (n=120/sex/line) were generated and stored in separate, single-sex SYA bottles (60 WT adults/bottle) until mating, as in the baseline life history assay. Matings between virgin focal flies and virgin WT flies were set-up 12h post-eclosion (to ensure sexual maturity). Under light CO_2 anaesthesia each SYA bottle of 60 WT adults was tipped into a bottle of 45 focal adults of the opposite sex, for each of the 6 experimental lines, and individuals were allowed to mate freely for 3h. Matings were therefore conducted on the low or high protein diets of the focal adults. The 3h mating period was used to minimise WT female proximate responses in egg-laying from a switch in diet (between SYA and the focal diet).

After mating, focal flies were transferred (under light CO_2 anaesthesia) to single sex vials of either low (20% SYA) or high (120% SYA) protein (matching the diet on which they mated), at a density of 3 flies/vial. WT females were also stored at a density of 3 flies/SYA vial, for determining the initial reproductive output of focal males, to which they had mated. WT males were discarded. Focal flies had no further matings or exposure to the opposite sex, following the initial mating.

Initial focal female and focal male egg counts were determined 3 days post-eclosion using a 24h laying period, but otherwise the protocol matched that used for the baseline life history assay. The longer 24h egg-laying period did not result in egg overcrowding. Egg vials were retained to determine egg-adult viability and frozen at 13 days after egg-lay for high protein vials and 18 days after egg-lay for low protein vials (to account for different development times between the diets

(Chapter 1) for later counting of number of offspring. WT females were discarded after egg-laying.

Weekly egg and offspring counts were taken from the once-mated focal females for the remainder of the experiment, following the same protocol as the initial egg count. This allowed me to compare age-related decline in female reproductive output between regimes. An extra food transfer was inserted at the end of the 11h egg lay, for both sexes, to maintain consistency of handling across all treatments.

Every 2-3 days (Monday, Wednesday, Friday) food vials were exchanged and groupings of 3 focal flies per vial were shuffled, to remove any possible vial effects and to randomise the positioning of focals in vials with fewer than 3 flies (due to mortalities or censors). Focal adults were kept on their respective low protein or high protein diet treatments at each transfer. Order of transfers was randomised between treatments at each timepoint. Mortalities were checked daily.

3.3.5 Statistical analyses

All statistical analyses were performed in R version 3.2.1 (R Core Development Team, 2015). Statistical analyses were performed separately for each of the experiments (baseline life history assay, starvation survival assay and for the life history assays on low and high protein diets).

Survival Analysis

Survival analyses were performed using nested, mixed effects Cox Proportional Hazards regression analysis on age-specific mortality data, separately for focal females and focal males. A mixed effects Cox model was fitted using the 'coxme' function from the 'coxme' package. Feeding regime (Random, Ra or Regular, Re) was fitted as a fixed effect and line (replicate cage: Ra1, Ra2, Ra3, Re1, Re2, Re3) nested within feeding regime, as a random effect. Likelihood ratio tests (anova) showed that for all data, the nested coxme model had greater explanatory power and better model fit than either the simple Cox PH model, or a non-nested coxme model.

Sex-specific survival differences were also tested for by combining the female and male datasets and fitting new 'coxme' models. Sex was fitted as a fixed effect and a new term, 'NewLine' (the unique cage identifier: Re1F, Re2F, Re3F, Re1M, Re2M, Re3M) nested within Sex, as a random effect. Again, the nested coxme model had greater explanatory power than either the simple Cox PH model or a non-nested coxme model.

All age-specific mortality data were first tested for the proportional hazards (PH) assumption of Cox analysis, using both graphical and analytical tests. The majority of data satisfied the PH

assumption. Parametric survival analysis was performed for the two datasets with the largest potential violation of the PH assumption and the results compared with the mixed effects Cox 'coxme' analysis to find best model fit. A maximum likelihood approach was used to compare 11 different parametric models and find the best model fit (adapted from Archer et al., 2015). Linear mixed effects models were used to analyse lifespan data. Parametric survival analyses returned the same results as the mixed effects Cox models and hence supported the use of 'coxme' analysis on all survival data.

Age-Specific Reproduction Analysis

Female age-specific egg count and offspring count data were analysed using a generalised linear mixed effects model ('glmer' function from the 'lme4' package) with Poisson error structure for count data. Replicate line (Random 1, Random 2, Random 3, Regular 1, Regular 2, Regular 3) and the number of days post-eclosion were fitted as categorical random effects and feeding regime (Regular or Random) was fitted as a fixed effect. No individual-level random effect was included in the model, as individuals were not uniquely identifiable (measures were taken from randomised groupings of 3 individuals, at each time point). The data were overdispersed in all cases. To account for this, an observation-level random effect was added to each 'glmer' model and maximum likelihood model comparison used to determine best model fit.

Male initial (day 3) egg count and initial offspring count data was tested for normality using the Shapiro Wilk test and for equality of variances, using the Levene's test, separately for each treatment level. Differences in male reproductive output between lines and regimes were analysed using a parametric ANOVA, if the normality and equality of variances assumptions were met, or using a Kruskal-Wallis rank sum test, where assumptions were violated. Initial (day 3) egg and offspring data for focal females was also analysed separately to give comparability with the focal male reproduction data.

Egg to adult viability was calculated as the proportion of eggs laid by groups of 3 focal adults which hatched as viable offspring, at each timepoint. For focal females the age-specific viability data across lifetime was analysed using the 'glmer' function using Binomial errors to account for the proportional data. Similarly, focal male initial egg to adult viability was also analysed with a general linear model (GLM) with binomial errors.

Lifetime reproduction analysis

Indices of female total lifetime egg production and female total lifetime offspring production, were calculated separately for each treatment population by summing egg or offspring counts,

respectively, across lifetime. Mean and standard errors for total lifetime reproduction values for each feeding regime were determined. Differences in total lifetime egg or offspring production between regimes were analysed as for the initial count data.

Female and male fitness analysis

An index of female fitness was calculated as the Malthusian parameter (Euler's r, the intrinsic rate of population growth) using the Euler equation (Gotelli, 2001; Wigby & Chapman, 2005). The Euler equation calculates an index of fitness from age-specific survivorship and age-specific reproduction values and is weighted towards early life reproduction. It uses age-specific survivorship and age-specific reproduction values and is weighted towards early life reproduction.

Female 'potential fitness' was calculated from age-specific egg counts and female 'realised fitness', from age-specific offspring counts. Offspring counts and egg counts were halved, to account for the genetic contribution of one parent (the mother) to the offspring generation. Point estimates of male initial fitness were also calculated using the initial day 3 egg and offspring counts and day 3 survivorship using the Euler equation.

3.4 Results

3.4.1 Baseline Survival

There was no significant difference in focal female survival between the Regular and Random regimes on standard food (nested coxme: z=0.45 , p=0.65; median lifespan=62days, 64days, respectively; Figure 3A; Table S1). In contrast, Regular focal males lived significantly longer than Random males (nested coxme: z= 2.50, p=0.012; median lifespan=57days, 42days, respectively; Figure 3B; Table S2).

There were highly significant sex differences in survival within the Random feeding regime. Random focal females lived significantly longer than Random focal males (nested coxme: z=6.74 , p<0.001; median lifespan females=64days, males=42days; Figure 3C). This pronounced sex difference in survival was absent in the Regular feeding regime in which there was no significant difference between Regular female and male survival (nested coxme: z=0.78, p=0.440, median lifespan females=62days, males=57days, respectively; Figure 3D).

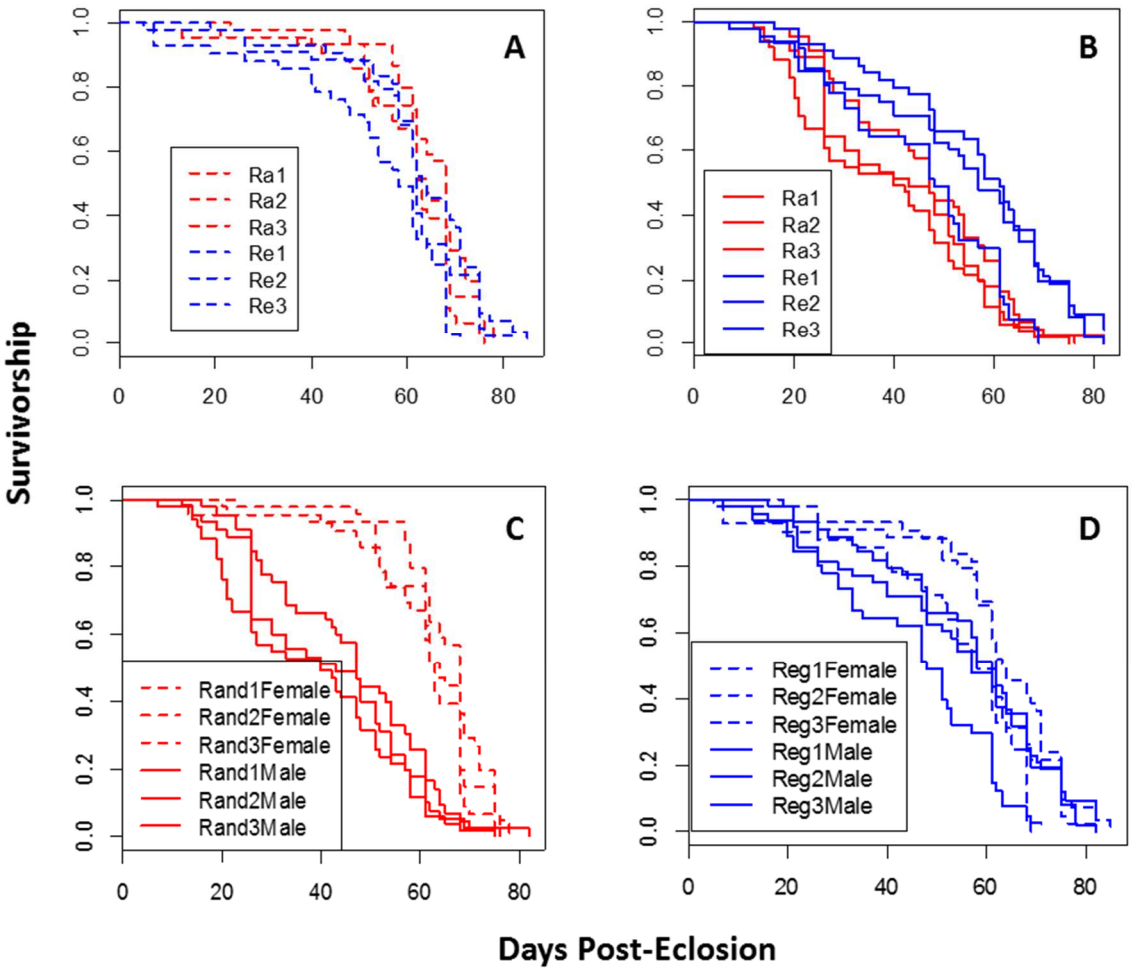

Figure 3. Age-specific survivorship against days post-eclosion, across replicates 1-3 of Random (Ra) and Regular (Re) feeding regimes, held on standard (SYA) food, for: (A) Random vs Regular focal females; (B) Random vs Regular focal males, (C) Random females vs males, (D) Regular females vs males.

3.4.2 Baseline Focal Female Reproduction

There was no significant difference in focal female age-specific egg or offspring production between the Random and Regular regimes over the lifetime on standard food (glmer: z=1.16, p=0.244, Figure 4A; z=0.774, p=0.244, Figure 4B). Egg production and offspring production both significantly decreased with age across both regimes (glmer: z= 78.45, p<0.001, Figure 4A; z=60.55, p<0.001, Figure 4B).

There was also no significant difference in egg to adult viability between Random and Regular females over time (glmer: t=0.490, d.f.=5, p=0.586; Figure 4C) and egg to adult viability also significantly decreased over the lifetime (glmer: t=23.416, d.f.=5, p<0.001).

For comparability with the focal male data, and due to the importance of early egg counts in weighting the estimate of fitness (Euler's r), I tested for differences in the early (day 3) counts for both egg and offspring data. There was no significant difference in early egg counts between Random and Regular females (Two Sample t-test: t = 2.048, df = 4, p-value = 0.110; Figure 4A inset), or in early offspring counts (Two Sample t-test: t = 2.060, df = 4, p-value = 0.109; Figure 4B inset). Consequently, egg to adult viability did not differ significantly, between regimes (GLM: z = 0.496, df = 5, p = 0.627; Figure 4C inset).

Total lifetime egg production and total lifetime offspring production did not differ significantly between Random and Regular females (Welch Two Sample t-test: t=1.322, df=2.167, p=0.308; mean=2087, 1553 eggs, respectively; t=1.309, df=2.205, p=0.310; mean=883, 654 offspring, respectively).

3.4.3 Baseline Focal Male Initial Reproduction

An index of early male reproductive output was calculated from recording initial 24h egg counts and offspring counts, at 3 days post-eclosion, from standard WT females that had been mated to the focal males. There was no significant difference in initial egg counts or offspring counts, between Random and Regular males, held on standard food (Two Sample t-test: t = 1.135, df = 4, p = 0.320; mean egg count= 38, 45, respectively; t = 1.972, df = 4, p = 0.120; mean offspring count

= 25, 34, respectively). Initial focal male egg to adult viability also did not differ between the Random and Regular regimes (GLM: z=1.851 , df=5, p=0.0642 ; mean=0.644, 0.766, respectively).

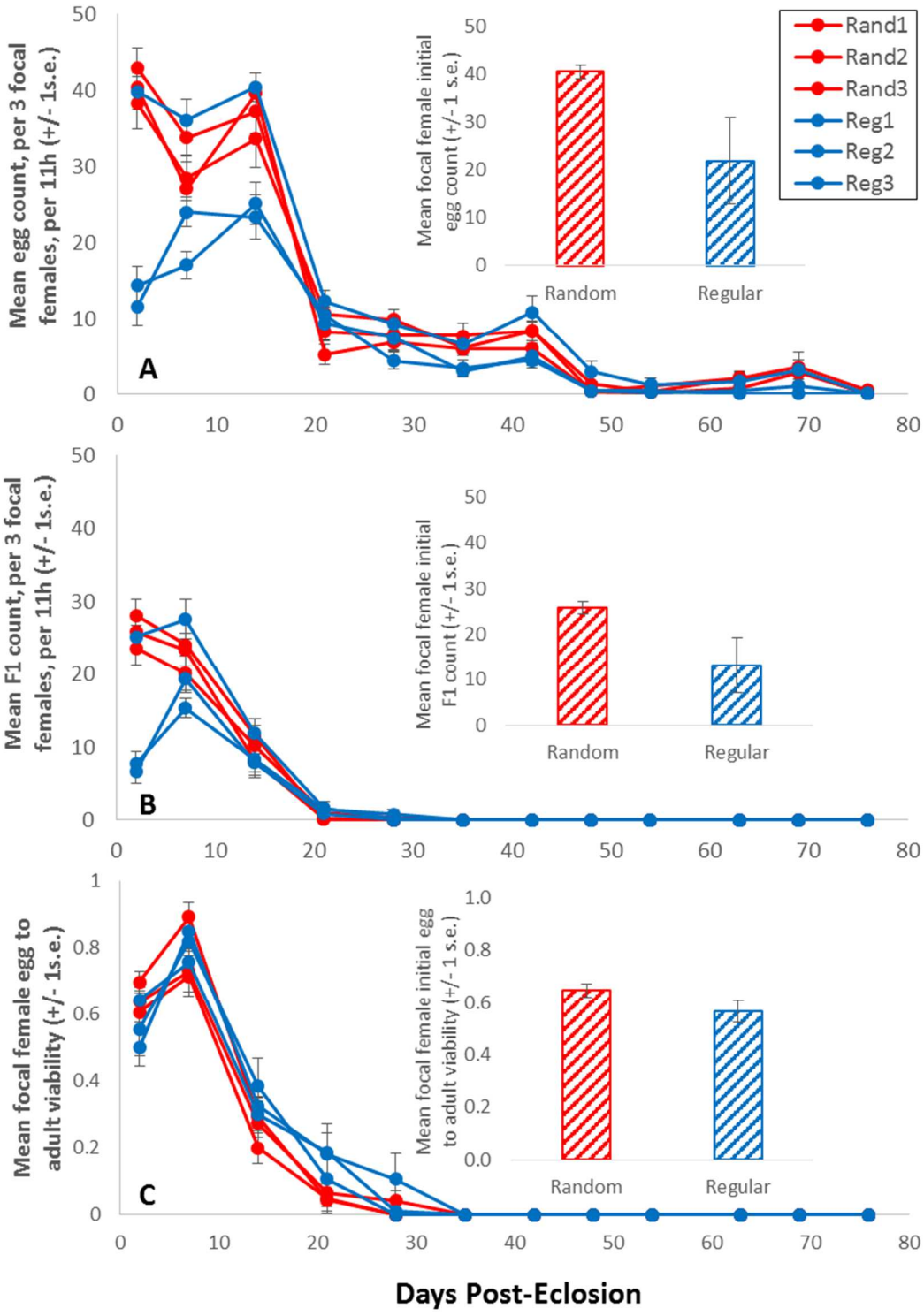

Figure 4. Mean focal female egg production (A), F1 offspring production, (B) and egg to adult viability (C), per 3 females, per 11h, against days post-eclosion, for replicates 1-3 from each of the Rand and Reg

feeding regimes; held on standard (SYA) food. Mean number of offspring that emerged from the 11h egg lay vials (A), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (B). Egg to adult viability is defined as the mean proportion of eggs laid by groups of 3 females during 11h which eclosed as adults (C). Insets for (A), (B) and (C) show mean initial (day 3) egg and offspring counts, respectively. All error bars display +/- 1 standard error.

3.4.4 Baseline Focal Female Fitness and Focal Male Initial Fitness

There was no significant difference in female potential fitness (calculated from egg counts) or female realised fitness (calculated from offspring counts), between Random and Regular regimes, held on standard food (Two sample t-test: t = 2.011, df =4 , p-value = 0.115, Figure 5A; t = 2.030, df = 4, p-value = 0.112, Figure 5B; respectively).

Point estimates of male initial fitness were calculated using the initial day 3 egg and offspring counts and day 3 survivorship. There was no significant difference in male initial fitness, between Random and Regular regimes, for estimates of either potential fitness (Two Sample t-test: t = 1.09, df = 4, p = 0.337; mean = 1.473, 1.547, respectively) or realised fitness (Two Sample t-test: t = 2.043, df = 4, p = 0.111, mean = 1.261, 1.409, respectively).

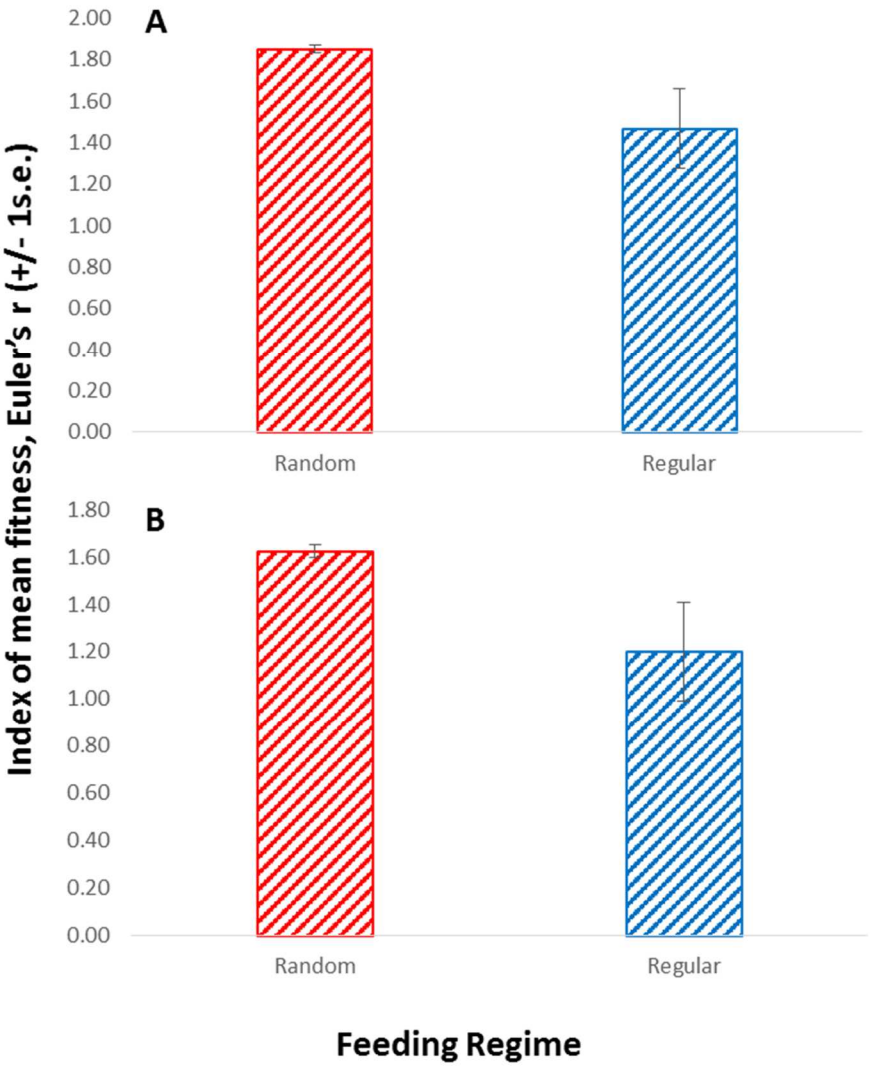

Figure 5. Index of mean fitness (+/- 1s.e.) for focal females from Random and Regular regimes, calculated as Euler's r using age-specific egg counts (A) or age-specific offspring counts (B). Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3); n=45 individuals per line.

3.4.5 Starvation Survival

There was no significant difference in focal female survival between the Random and Regular regimes, under agar-only starvation (nested coxme: z=2.14, p=0.070; median lifespan=272h, for both regimes; Figure 6A; Table S3). In contrast, Regular males lived significantly longer than Random males under starvation (nested coxme: z= 3.74, p=0.010; median lifespan=285h, 272h, respectively; Figure 6B; Table S4).

There was no significant survival difference between females and males, when starved, for either the Random regime (nested coxme: z=0.06, p=0.950; median lifespan=272h, for both sexes; Figure 6C), or the Regular regime (nested coxme: z=0.67, p=0.500; median lifespan=272h, 285h, respectively; Figure 6D).

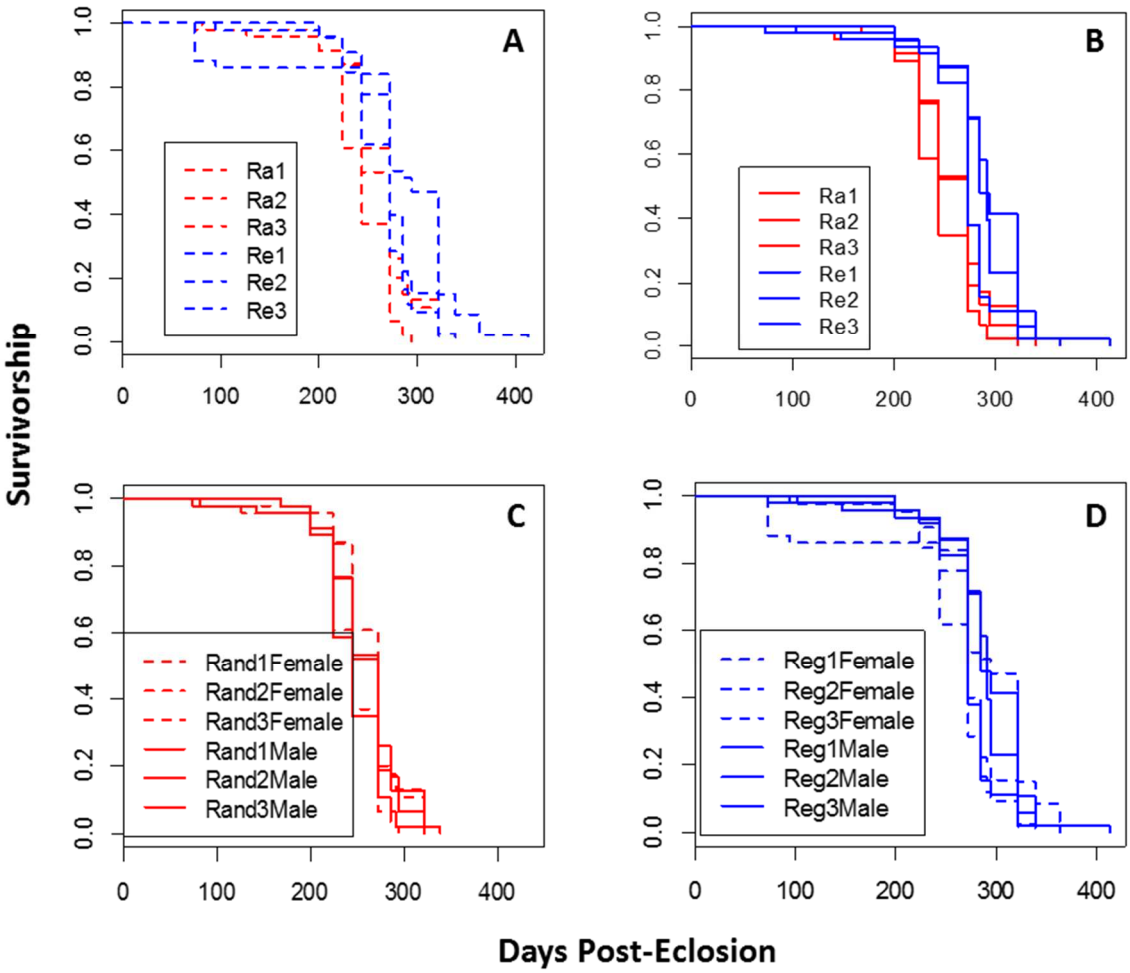

Figure 6. Age-specific survivorship against days post-eclosion, across replicates 1-3 of Random (Ra) and Regular (Re) feeding regimes, when starved, for: (A) Random vs Regular focal females; (B) Random vs Regular focal males, (C) Random females vs males, (D) Regular females vs males. Starvation (agar-only diet), began 6hours post-eclosion. Moisture was provided via agar plugs.

3.4.6 Survival on Low Protein Food

There was no significant difference in focal female survival between the Random and Regular regimes when held on low protein food (nested coxme: z=0.14, p=0.890; median lifespan=33days, 27days, respectively; Figure 7A; Table S5). There was also no significant difference in male survival on low protein food between the Random and Regular regimes (nested coxme: z=0.17, p=0.860; median lifespan=39days, for both regimes; Figure 7B; Table S6).

Focal males survival lived significantly longer than focal females from the Random regime when held on low protein food (nested coxme: z=3.72, p=0.009; median lifespan=33days, 39days, respectively; Figure 7C). This was a reversal to the direction of SDL observed for the Random regime on standard food (Figure 3C).

Regular focal males also lived significantly longer than Random focal females when held on low protein food (nested coxme: z=2.45, p=0.0424; median lifespan=27days, 39days, respectively; Figure 7D). The extent of the sex difference in lifespan was reduced on the Regular in comparison to the Random regime.

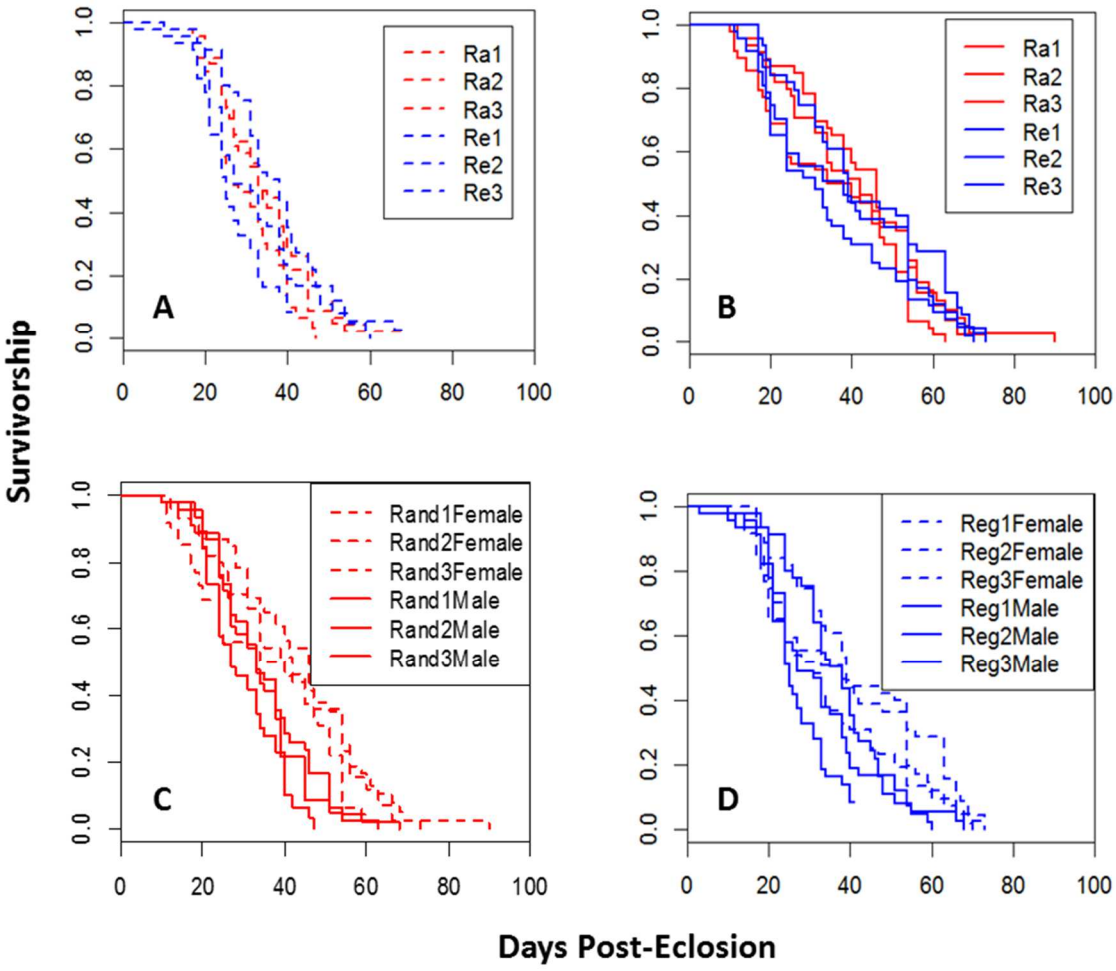

Figure 7. Age-specific survivorship against days post-eclosion, across replicates 1-3 of Random (Ra) and Regular (Re) feeding regimes, held on low protein food (20% SYA), for: (A) Random vs Regular focal females; (B) Random vs Regular focal males, (C) Random females vs males, (D) Regular females vs males.

3.4.7 Survival on High Protein Food

There was also no significant difference in focal female survival on high protein food between the Random and Regular regimes (nested coxme: z=0.38, p=0.70; median lifespan=70days, 67days, respectively; Figure 8A; Table S7). Males from the Regular regime lived significantly longer than males from the Random regime on high protein food (nested coxme: z=3.34, p=0.0155; median lifespan=54days, 51days, respectively; Figure 8B; Table S8) consistent with observations on the standard and starvation diets.

There were highly significant sex differences in survival within the Random feeding regime on high protein food. Random females lived significantly longer than Random males (nested coxme: z=10.6, p<0.001; median lifespan=70days, 51days, respectively; Figure 8C). Regular females also lived significantly longer than Regular males on high protein food (nested coxme: z=4.77, p=0.003; median lifespan=67days, 54days, respectively; Figure 8D). However, the extent of this SDL was reduced when compared with the Random feeding regime.

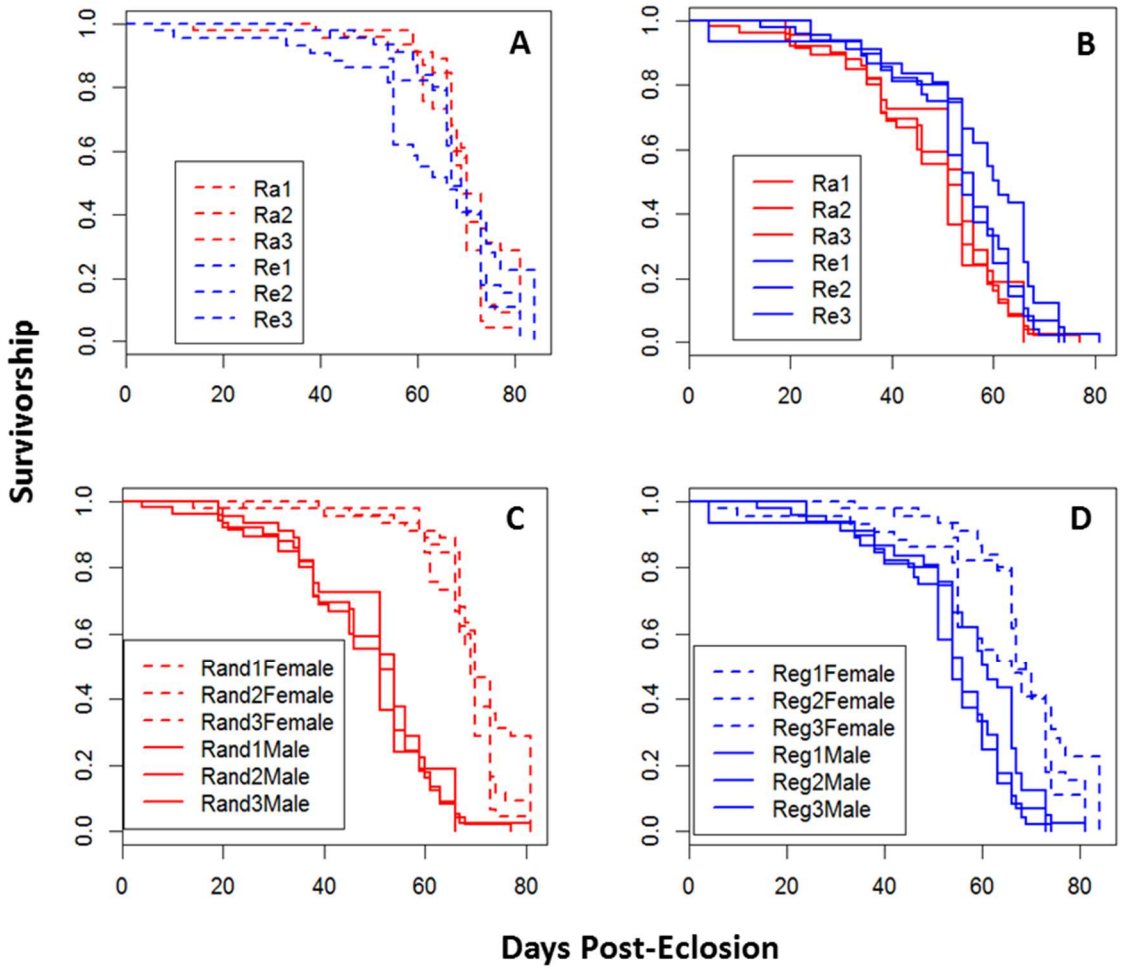

Figure 8. Age-specific survivorship against days post-eclosion, across replicates 1-3 of Random(Ra) and Regular (Re) feeding regimes, held on high protein food (120% SYA), for: (A) Random vs Regular focal females; (B) Random vs Regular focal males, (C) Random females vs males, (D) Regular females vs males.

3.4.8 Focal Female Reproduction on Low Protein Food

There was no significant difference in focal female egg or offspring production between the regimes on low protein food over the lifetime (glmer: $z=0.822$, $p=0.411$, Figure 9A; $z=1.11$, $p=0.267$, Figure 9B; respectively). Egg production and offspring production both significantly decreased with age (glmer: $z=42.33$, $p<0.001$; Figure 9A; glmer: $z=25.48$, $p<0.001$; Figure 9B; respectively). Egg and offspring production fell to zero by 10 days post-eclosion, with the exception of one replicate of the Regular regime. The sharp decline in offspring production after 3 days post-eclosion was likely due to the depletion of sperm stores (females were only once-mated) and also due to the associated drop in egg production on the poor quality diet.

There was no significant difference in the egg to adult viability between Regular and Random females over time on low protein food (glmer: t=0.299, d.f.=5, p=0.753; Figure 9C) and egg to adult viability also changed significantly over time (glmer: t=6.867 , d.f.=5 , p<0.001). The wide error bars for egg to adult viability at day 10 (Figure 9C) reflected the small sample sizes at this stage.

There was no significant difference in early (day 3) egg counts between Random and Regular females (Two Sample t-test: t = 1.399, df = 4, p = 0.235; Figure 9A inset) or in early offspring counts (Two Sample t-test: t = 0.783, df = 4, p = 0.477; Figure 9B inset) on low protein food. Egg to adult viability also did not differ between Random and Regular regimes (GLM: z=0.918, d.f.= 5, p=0.359; Figure 9C inset).

Total lifetime egg or offspring production also did not differ significantly between Random and Regular females, on low protein food (Two Sample t-test: t = 1.162, df = 4, p = 0.310; mean=606, 519; t = 0.674, df = 4, p = 0.537; mean= 368, 332; respectively).

3.4.9 Focal Male Initial Reproduction on Low Protein Food

There was no significant difference in initial (day 3) focal male egg production or offspring production, between the Random and Regular regimes, when held on low protein food (Two Sample t-test: t = 1.175, df = 4, p-value = 0.305; mean egg count = 46, 40, respectively; t = 0.768, df = 4, p = 0.485; mean offspring count = 36, 33, respectively). Initial focal male egg to adult viability also did not differ significantly between the Random and Regular regimes on low protein food (GLM: z=0.563, d.f.=5, p=0.574; mean=0.788, 0.828, respectively).

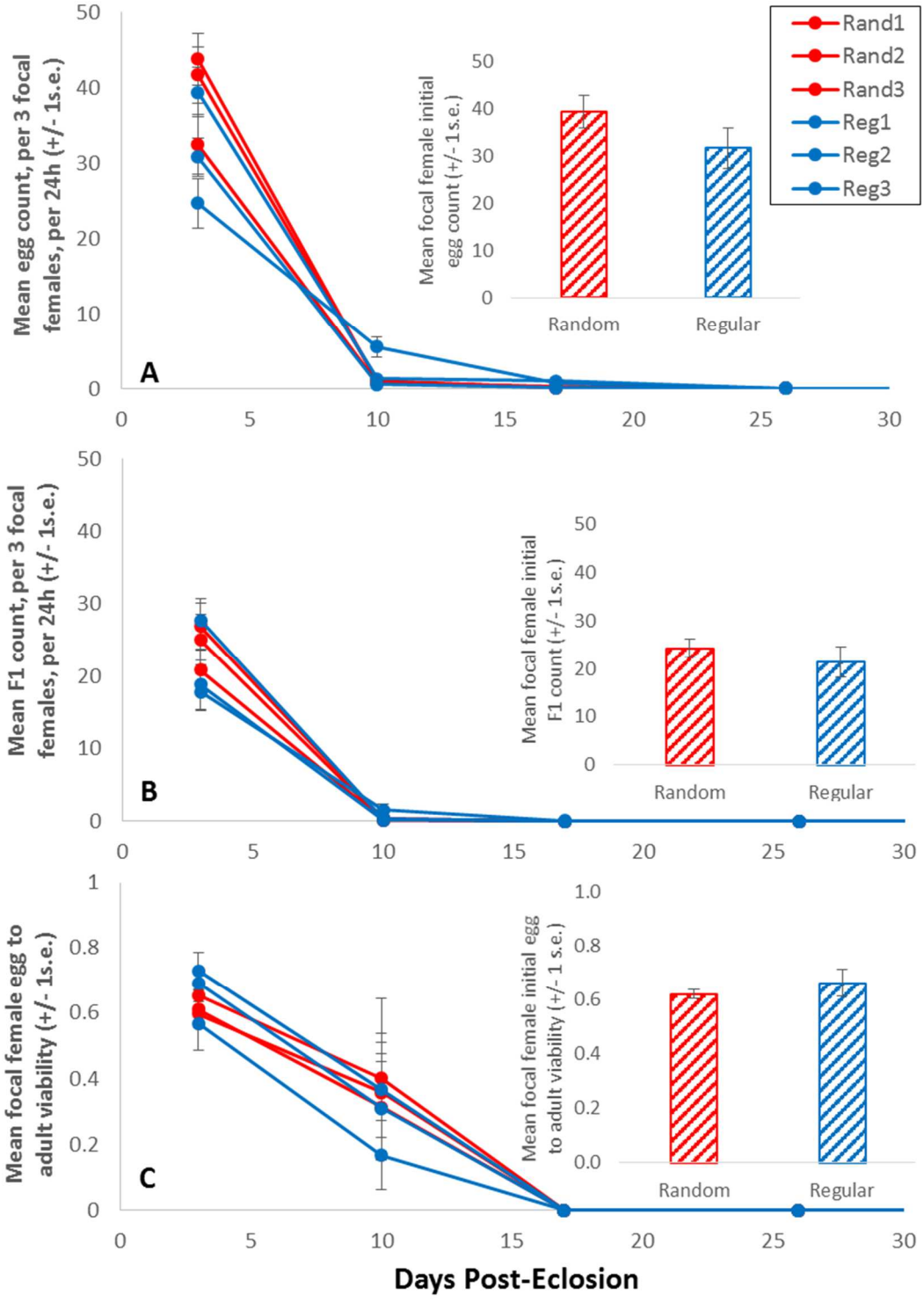

Figure 9. Mean focal female egg production (A), F1 offspring production, (B) and egg to F1 offspring viability (C), per 3 females, per 24h, against days post-eclosion, for replicates 1-3 each of the Rand and Reg feeding regimes, held on low protein food (20% SYA). Mean number of offspring that emerged from the 24h egg lay vials (A), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (B). Egg to adult viability is defined as the mean proportion of eggs laid by groups of 3 females during 24h which eclosed as adults (C). Data displayed up until 30 days

post-eclosion, for clarity, as all counts were zero after that timepoint. Insets for (A), (B) and (C) show mean initial (day 3) egg, offspring and egg to adult viability values respectively. All error bars display +/- 1 standard error.

3.4.10 Focal Female Reproduction on High Protein Food

Random females had significantly greater egg production than Regular females, on high protein food, over lifetime (glmer: $z=2.49$, $p=0.0127$, Figure 10A), though there was no significant difference in focal female offspring production between the regimes on high protein food (glmer: $z=0.269$, $p=0.788$, Figure 10B). There was also no significant difference in focal egg to adult viability between regimes (glmer: $t=0.301$, d.f.=5 $p=0.739$, Figure 10C). Egg production, offspring production and egg to adult viability all significantly decreased with age across both regimes (glmer: $z=92.75$, $p<0.001$, Figure 10A; glmer: $z=89.51$, $p<0.001$, Figure 10B; glmer: $t= 31.050$, d.f.=5, $p<0.001$, Figure 10C; respectively) on high protein food.

There was no significant difference in focal female initial (day3) egg counts, offspring counts or initial egg to adult viability, between the Random and Regular regimes held on high protein food (Two Sample t-test: $t = 0.773$, $df = 4$, $p = 0.483$; mean =57, 48 eggs, respectively, Figure 10A inset; $t = 0.362$, $df = 4$, $p = 0.736$; mean=47, 43 offspring, respectively, Figure 10B inset; GLM: $t = 0.630$, d.f. = 5, $p = 0.563$; mean viability = 0.839, 0.735, respectively, Figure 10C inset).

Total lifetime egg and total lifetime offspring production also did not differ significantly between Random and Regular females on high protein food (Two Sample t-test: $t = 1.613$, $df = 4$, $p = 0.182$; mean= 4049, 3123; $t = 0.458$, $df = 4$, $p = 0.671$; mean= 1542, 1386; respectively).

3.4.11 Focal Male Initial Reproduction on High Protein Food

There was no significant difference in initial (day 3) focal male egg production or offspring production, between the Random and Regular regimes when held on high protein food (Two Sample t-test: $t = 1.440$, d.f. = 4, $p = 0.223$; mean egg count = 60, 46, respectively; $t = 1.270$, d.f. = 4, $p = 0.273$; mean offspring count = 53, 40, respectively). Initial focal male egg to adult viability also did not differ significantly between the Random and Regular regimes on high protein food (GLM: $z=0.170$, d.f. = 5, $p=0.865$; mean = 0.862, 0.861, respectively).

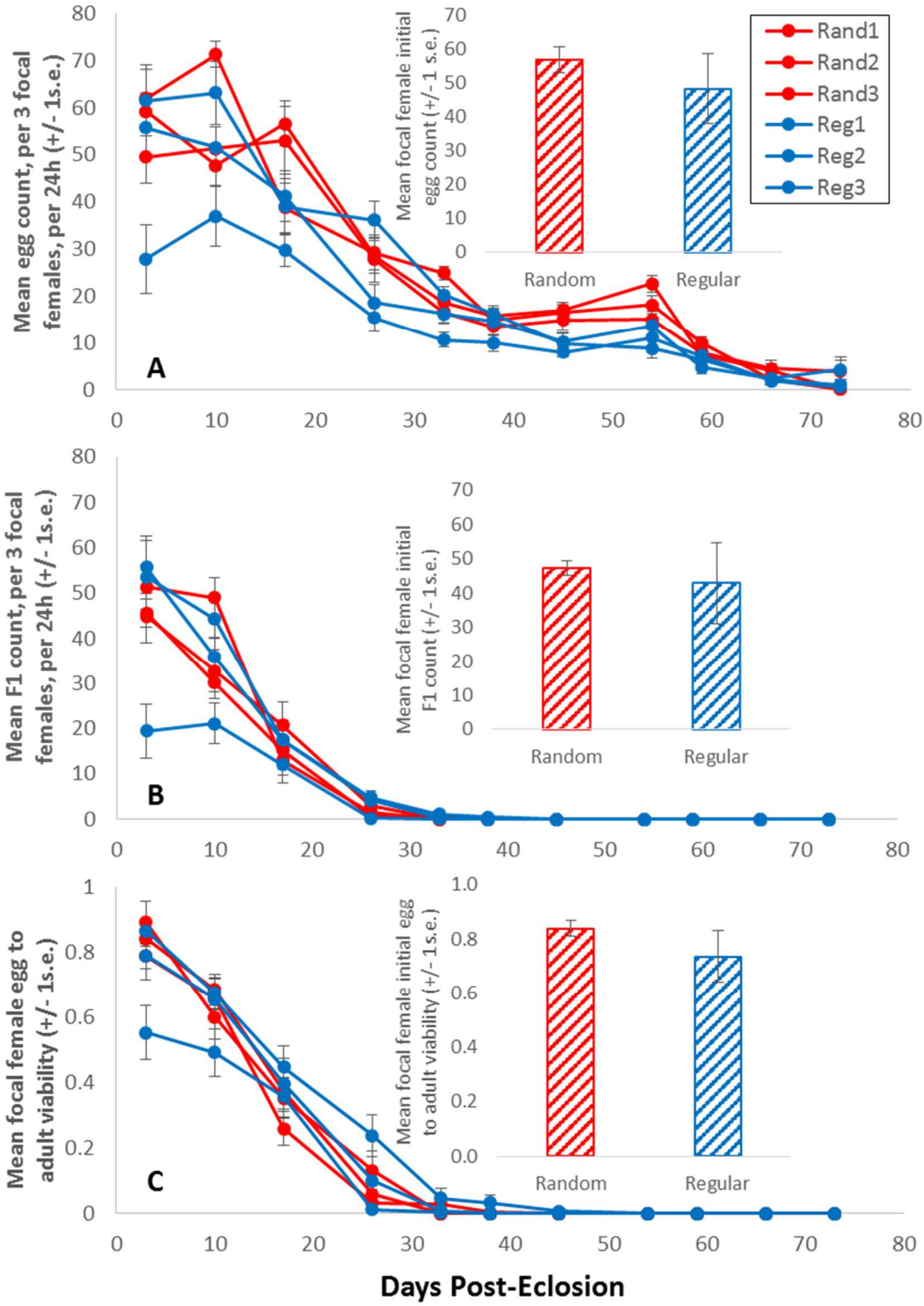

Figure 10. Mean focal female egg production (A), F1 offspring production, (B) and egg to F1 offspring viability (C), per 3 females, per 24h, against days post-eclosion, for replicates 1-3 each of the Rand and Reg feeding regimes, held on high protein food (120% SYA). Mean number of offspring that emerged from the 24h egg lay vials (A), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (B). Egg to adult viability is defined as the mean proportion of

eggs laid by groups of 3 females during 24h which eclosed as adults (C). Insets for (A), (B) and (C) show mean initial (day 3) egg, offspring and egg to adult viability values respectively. All error bars display +/- 1 standard error.

3.4.12 Focal Female Fitness and Focal Male Initial Fitness, on Low Protein Food

There was no significant difference in focal female potential fitness (calculated from egg counts) or in focal female realised fitness (calculated from offspring counts) between the Random and Regular regimes when held on low protein food (Two Sample t-test: t = 1.395, df = 4, p = 0.236, Figure 11A; t = 0.896, df = 4, p = 0.421, Figure 11B; respectively).

There was also no significant difference in the point estimates of focal male initial (day 3) fitness between Random and Regular regimes on low protein food (potential fitness: Two Sample t-test, t = 1.164, df = 4, p = 0.309; mean = 1.040, 0.999, respectively; realised fitness: Two Sample t-test, t = 0.790, df = 4, p = 0.474; mean = 0.964, 0.932, respectively).

3.4.13 Focal Female Fitness and Focal Male Initial Fitness, on High Protein Food

Focal female and focal fitness patterns on the high protein diet matched those on the low protein diet. There was no significant difference in focal female potential or realised fitness between the Random and Regular regimes when held on high protein food (Two Sample t-test: t = 0.835, df = 4, p = 0.451, Figure 12A; t = 0.566, df = 4, p = 0.602, Figure 12B; respectively).

There was also no significant difference in the point estimates of focal male initial (day 3) fitness, between Random and Regular regimes on low protein food (potential fitness: Two Sample t-test, t = 1.506, df = 4, p = 0.207; mean = 1.128, 1.035, respectively; realised fitness: Two Sample t-test, t = 1.345, df = 4, p = 0.250; mean = 1.085, 0.990, respectively).

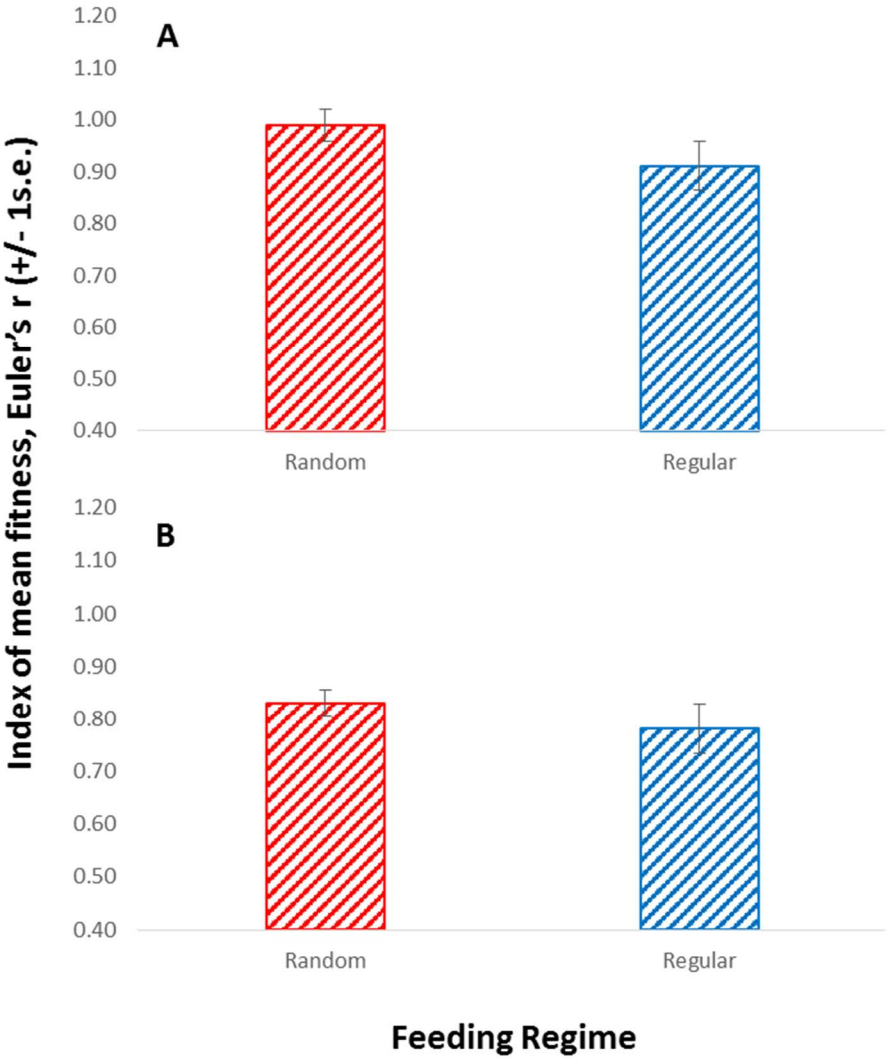

Figure 11. Index of mean fitness (+/- 1s.e.) for focal females from Random and Regular regimes, held on low protein food (20% SYA) calculated as Euler's r using age-specific egg counts (A) or age-specific offspring counts (B). Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3); n=45 individuals per line.

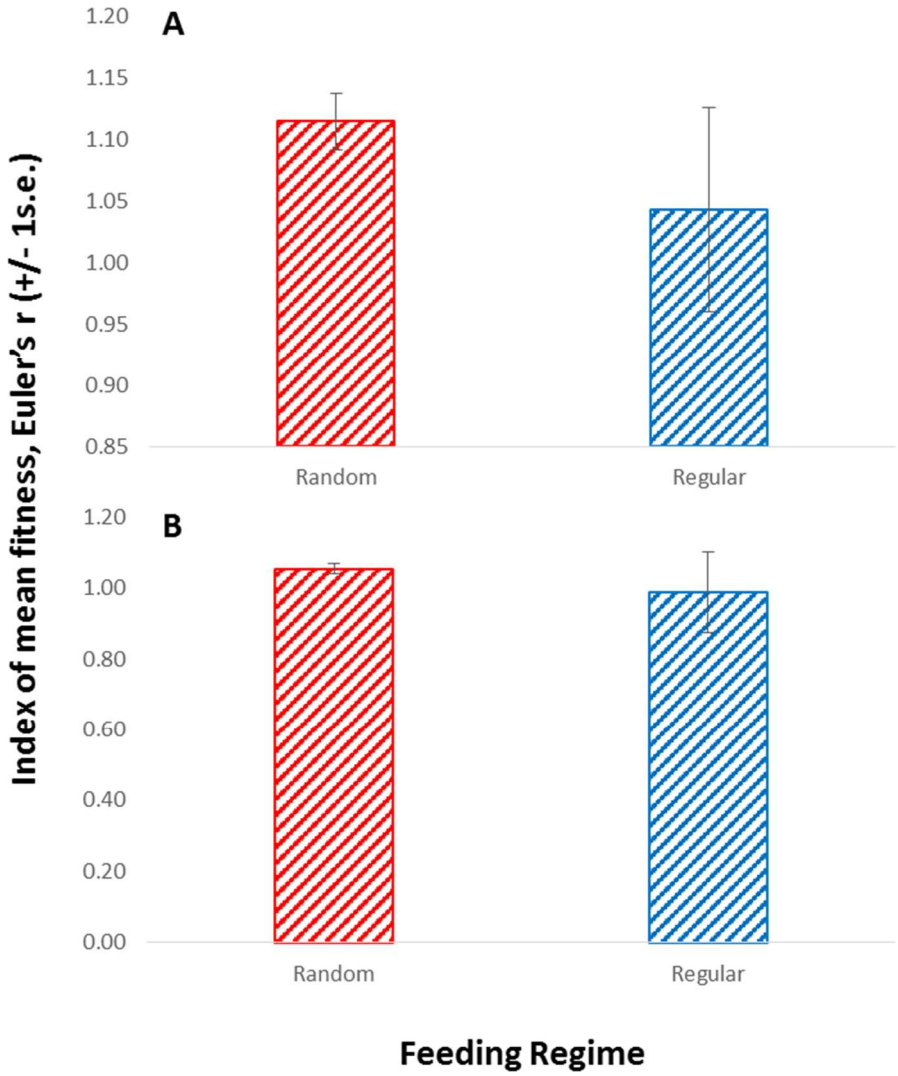

Figure 12. Index of mean fitness (+/- 1s.e.) for focal females from Random and Regular regimes, held on high protein food (120% SYA) calculated as Euler's r using age-specific egg counts (A) or age-specific offspring counts (B). Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3); n=45 individuals per line.

3.5 Discussion

Nutritional mismatches between evolved ('ancestral') and proximate ('modern') diets or feeding regimes can be detrimental to fitness. The Thrifty Genotype (TG) hypothesis predicts the theoretical costs of mismatches between an evolutionary history of unpredictable cycles of feast and famine, and a modern diet of *ad libitum* feeding on diets of consistently increased nutritional content (Neel, 1962; Prentice et al., 2005). Yet little is known about whether evolved feeding regimes can ameliorate the costs of nutritional mismatches by enhancing resilience and plasticity to poor quality or to novel proximate nutritional environments. Here I addressed these questions by directly manipulating evolved feeding regime and measuring the life history consequences across several mismatched proximate diets, in both sexes simultaneously, using the fruit fly, *Drosophila melanogaster*.

I found that the manipulation of evolved feeding regime and proximate diet, led to evolved sex differences in lifespan. Patterns of sexual dimorphism for lifespan were influenced by both evolved feeding regime, proximate diet and their interaction. Differences in female and male lifespan are widely documented across many species (e.g. Promislow, 1992; Moore & Wilson, 2002; Liker & Szekely, 2005; Clutton-Brock & Isvaran, 2007). Much less is known about the possible nutritional factors underlying the patterns of this sexual dimorphism for lifespan (SDL).

Manipulation of evolved feeding regime changed the extent of SDL. SDL was enhanced in the Random regime, when compared with the Regular regime, for all proximate diets except the starvation regime, in which SDL was not observed in either regimes. For the standard and high protein diets, enhanced SDL was driven by the reduction in Random male lifespan relative to Regular male lifespan as female lifespan did not differ between regimes. For the low protein diet, enhanced SDL was driven by the marginal (N.S.) increase in Random male lifespan relative to Regular male lifespan and the marginal (N.S.) decrease in Random female lifespan relative to Regular female lifespan. The lifespan effects of evolutionary manipulation on feeding regime were therefore a predominantly male-specific phenomenon. Similarly, Wit et al. (2015) also found male but not female lifespan, in *Drosophila,* to be correlated with environmental variation.

Proximate diet (of either standard, low protein or high protein food) changed the direction of sexual dimorphism patterns for lifespan, independent of regime. Females lived longer than males on standard food and high protein food, as is widely documented for *Drosophila* on standard (SYA) food (e.g. Magwere et al., 2004). However, males lived longer than females on low protein food, akin to *Drosophila* on a standard diet as in a study by Wit et al. (2015). Protein is particularly important for females to maintain egg production, metabolism and growth, perhaps explaining

the reduction in female lifespan below male lifespan on low protein. Together, these results suggest that the direction of SDL was condition-dependent.

Previous work in *D. melanogaster* has shown that changes in the direction of sex differences in lifespan can arise from diet manipulation within a single generation. Magwere *et al.* (2004) found that graded dietary restriction of both sucrose and protein content simultaneously, over the lifetime, led to sex differences in lifespan, peak survival and baseline mortality rates. The direction of sexual dimorphism for lifespan across proximate diets in my work here followed a similar pattern in terms of relative magnitude and direction to the direction of sex differences in lifespan across the 3 most equivalent levels of dietary restriction (20%, 100% and 120% dietary restriction treatments) in the Magwere *et al.* (2004) study. Whilst there were inevitably differences between the studies, arising in part from the different dietary compositions used, general lifespan patterns were in agreement. Importantly, in my study I was able to determine the effect of protein content alone, separate from sucrose.

I found no sex differences in lifespan on the starvation diet. Perhaps the agar-only starvation diet in my study, which consisted of the absence of food from 6h post-eclosion, with moisture provision only, was sufficiently harsh to drastically reduce lifespan in both sexes concurrently. Whilst I predicted flies from the Random evolutionary history would have been more resilient to the starvation regime, in line with Thrifty Genotype predictions (Neel, 1962), this did not translate into increased lifespan and in fact Random males lived shorter than Regular males, contrary to predictions.

An aim of this study was to address two conflicting theories about the life history consequences of an evolutionary history of an unpredictably fluctuating (Random) feeding regime, when compared to the predictable (Regular) feeding regime. I proposed that the Random evolved feeding regime would select for increased plasticity and resilience to novel or low quality proximate diets, to ameliorate the costs of mismatched nutrition predicted by the TG hypothesis (Neel, 1962; Prentice et al., 2005). Contrary to predictions, the Random lines did not show increased resilience or plasticity to novel environments or starvation, in terms of increased survival, when compared with Regular lines. In fact, Random male lifespan was significantly lower than Regular male lifespan on all proximate diets except for the low protein diet where there was no difference in male survival. Similarly, there was also no regime effect on female lifespan.

It is possible that the predicted increased resilience and plasticity of Random lines was manifested in another life history or physiological trait. For example, Random females had significantly higher age-specific egg production than Regular females. This evolved upregulation of fecundity would

be expected for Random lines, on high quality (high protein) diets, and could be beneficial in advance of a subsequent 'famine'. There was also an indication of increased fitness and lifetime reproductive output for Random females for all diets except for starvation, although these trends were non-significant. However, contrary to the TG hypothesis that a switch from the Random history (which approximated feast-famine cycles) to a consistently good quality (high protein) diet would be costly (Neel, 1962; Prentice et al., 2005), there was no reduction in Random female fitness relative to Regular females on the high protein diet. Only Random male survival was reduced relative to Regular males, as observed for all proximate diets in the experiment.

Together, these results suggests that the life history responses observed do not generally fit with the original TG predictions and that if the Random history had selected for increased resilience and plasticity, this was manifested in a trait other than those assayed in the current experiment. Further work to determine the fat composition of individuals derived from both feeding regime histories on standard diets could reveal possible physiological differences in line with TG predictions.

No life history trade-offs between survival and initial reproduction were observed for either evolved feeding regime on either diet, contrary to Zwaan et al. (1995) and Flatt (2011). This could be explained by the absence of a significant difference in initial reproduction between regimes for either sex, even though survival varied. This suggests that survival differences between regimes and between sexes were not driven by trade-offs with reproduction, even if resources were limited. This is contrary to life history theory which predicts survival differences may arise from re-allocation of limited resources across trade-offs between soma and gametes (Trivers, 1972; Bonduriansky et al., 2008; reviewed by Magwere et al., 2004). However, my results are consistent with Adler et al. (2013) who also found survival differences in the absence of trade-offs with reproduction.

Fitness did not differ significantly between regimes, hence reduced survival was not compensated for by increased fitness, as suggested by Maklakov & Lummaa (2013). Even though Random lines had a smaller body size than Regular lines for both sexes (Perry et al., unpubl.) this did not lead to decreased fecundity. Furthermore, although Random males had reduced survival compared to Regular males, evolved feeding regime did not influence female survival. This suggests that body size differences were not the most significant factor influencing the lifespan differences for males and were not associated with reproductive output.

The lack of significant differences in fitness and reproduction between evolved feeding regimes may, in part, be explained by the greater variation in these traits between regime lines within the

Regular regime than between regime lines on the Random regime. The Random evolutionary history seemed to select for a more consistent life history response than the Regular regime. Experimental evolution studies in the laboratory can be vulnerable to the effects of inbreeding, due to reduction in effective population size (as discussed in Wit et al., 2015). We reduced the potential for inbreeding through maintenance at large population sizes. Also, we used 3 replicate experimentally evolved fly populations, for each regime, to distinguish between possible life history changes arising from drift, as opposed to life history changes arising from selection. As there was generally less variation in survival patterns and in reproduction patterns between the Random lines, than between the Regular lines, this supports the conclusion that evolved responses between regimes arose from responses to selection rather than drift.

In summary, I conducted an empirical test of the life history consequences of manipulation of evolutionary feeding regime across a series of mismatched proximate diets, to test predictions arising from the Thrifty Genotype hypothesis. Contrary to predictions, I showed that evolution under a random, unpredictable feeding regime did not significantly enhance resilience or plasticity to starvation or novel environments, in terms of survival, reproduction or fitness. Furthermore, I found no survival, reproduction or fitness costs specific to a mismatch between the evolved Random feeding regime and a high protein proximate diet, as would be predicted by the TG hypothesis. In fact, female egg production was elevated for Random lines, over Regular lines, on high protein food. Instead, interesting patterns of SDL were revealed. Evolved feeding regime altered the extent of SDL and proximate diet altered the direction of SDL, driven largely by male-specific effects and in the absence of life history trade-offs. Nutritional mismatches between evolved and proximate nutrition therefore appeared most important in driving the evolution of distinct patterns of lifespan differences between the sexes.

The results of this study offered the opportunity for a further investigation of the fitness consequences of enhanced versus reduced SDL between the evolved feeding regimes in both sexes simultaneously. It was thought that this could further elucidate the fitness consequences of nutritional mismatches and the potential for amelioration of mismatches due to the nature of the evolved feeding regime. This approach was pursued in the research described in Chapter 4.

Novel perspectives could also arise from the physiological comparison of individuals, of both sexes, from both evolved feeding regimes via measurement of fat composition, to determine whether enhanced resilience or plasticity was reflected in an alternative trait. Individuals from Random lines would be expected to have increased fat deposition, over Regular lines, in line with Thrifty Genotype predictions (Neel, 1962; Prentice et al., 2005). Random flies evolved a smaller

body size than Regular flies (Perry et al., unpub.), but it is not yet known whether this is reflected in lipid content. Fat composition (determined using methods outlined in Ballard et al., 2008) could be measured in females and males from both regimes at a standard age (e.g. 10 days post-eclosion) on the common garden diet and across the low protein, high protein and starvation diets. This would determine any interaction between regime and proximate diet on fat composition.

3.6 References

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3.7 Supplementary Material

Baseline Focal Female Median Lifespan on Standard (SYA) Food

Table S1. Average focal female survival for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand1	Rand2	Rand3	Reg1	Reg2	Reg3
Median lifespan (days)	64	68	63	58	62	63
Interquartile range (days)	15	11	12	20	11	13

Baseline Focal Male Median Lifespan on Standard (SYA) Food

Table S2. Average focal male survival for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand1	Rand2	Rand3	Reg1	Reg2	Reg3
Median lifespan (days)	42	47	40	47	57	58
Interquartile range (days)	33	22	31	31	29	22

Focal Female Median Lifespan on Starvation Diet

Table S3. Average focal female starvation survival for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand 1	Rand 2	Rand 3	Reg 1	Reg 2	Reg 3
Median lifespan (hours)	**244**	**272**	**272**	**294**	**272**	**272**
Interquartile range (hours)	**48**	**38**	**28**	**50**	**41**	**13**

Focal Male Median Lifespan on Starvation Diet

Table S4. Average focal male starvation survival for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand 1	Rand 2	Rand 3	Reg 1	Reg 2	Reg 3
Median lifespan (hours)	244	272	272	285	291	272
Interquartile range (hours)	48	38	28	50	22	13

Focal Female Median Lifespan on Low Protein Food

Table S5. Average focal female survival on low protein food (20% SYA), for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand 1	Rand 2	Rand 3	Reg 1	Reg 2	Reg 3
Median lifespan (days)	33	27	33	27	24	34
Interquartile range (days)	14	13	18	18	8	14

Focal Male Median Lifespan on Low Protein Food

Table S6. Average focal male survival on low protein food (20% SYA), for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand 1	Rand 2	Rand 3	Reg 1	Reg 2	Reg 3
Median lifespan (days)	46	34	39	39	36	31
Interquartile range (days)	23	32	28	26	43	26

Focal Female Median Lifespan on High Protein Food

Table S7. Average focal female survival on high protein food (120% SYA), for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand 1	Rand 2	Rand 3	Reg 1	Reg 2	Reg 3
Median lifespan (days)	69	70	70	60	69	67
Interquartile range (days)	6	6	18	19	7	8

Focal Male Median Lifespan on High Protein Food

Table S8. Average focal male survival on high protein food (120% SYA), for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand 1	Rand 2	Rand 3	Reg 1	Reg 2	Reg 3
Median lifespan (days)	51	51	54	54	60	54
Interquartile range (days)	21	18	16	10	14	13

Chapter 4: Evolutionary manipulation of feeding regime alters sexual dimorphism for lifespan and sex-specific fitness in the fruit fly, *Drosophila melanogaster*

4.1 Abstract

Sexual dimorphism for lifespan (SDL) is a widespread, but poorly understood, phenomenon. Little is known about the causes of sex differences in lifespan or the consequences of SDL for sex-specific fitness. I tested the hypothesis here that increased SDL allows both females and males to achieve greater sex-specific fitness and hence that the expression of SDL minimises sexual conflict. I used replicated selection lines of the fruit fly, *Drosophila melanogaster*, which had been maintained for over 360 generations on either unpredictable ('Random') or predictable ('Regular') feeding regimes. Previous work described in Chapter 2 showed that this evolutionary manipulation of feeding regime led to enhanced SDL in Random lines in comparison to the Regular lines. This provided a valuable system in which to test the sex-specific fitness consequences of SDL. I found that enhanced SDL was achieved by specific changes to the life history of males from the Random lines. The key changes were increased initial reproductive output and reduced survival in Random in comparison to regular males. This was associated with significantly increased fitness in males from the Random lines. In contrast, female fitness was not significantly different between the Random and Regular females (that experienced enhanced and reduced SDL, respectively). Hence increased SDL was associated with a resolution of sexual conflict in males and a stable state in females. Between-regime differences in SDL were not associated with differences in developmental traits. Overall the results showed that the expression of enhanced SDL, resulting from experimental evolution of feeding regimes, was associated with male-specific changes to life history leading to increased fitness. A co-authored manuscript (Elizabeth Duxbury, Tracey Chapman & Wayne Rostant), based on the contents of this thesis chapter and combined with the baseline life history assay from thesis chapter 3, has been accepted for publication by *Proceedings of the Royal Society B: Biological Sciences* (Appendix).

4.2 Introduction

4.2.1 Sexual dimorphism for lifespan, sexual conflict and sex-specific fitness

Females and males often differ in their evolutionary interests (Parker, 1979). This arises from the differing reproductive roles of the sexes, which can occur in many different reproductive traits, such as mating frequency, fertilisation success and lifespan differences (e.g. Dean et al., 2007). Sexual conflict can be manifested within or between genes (intra versus inter locus sexual conflict, respectively, e.g. Rice & Holland, 1997; Bonduriansky & Chenoweth, 2009). When a different allele of a focal locus is favoured in each sex, selection arising from sexual conflict may act in opposite directions in males and females (sexually antagonistic selection). The potential for sex-specific phenotypic divergence may be constrained by the shared genome of the sexes, leading to intralocus sexual conflict (as reviewed by Chapman et al., 2003; Chapman, 2006). This may place an evolutionary constraint on sex-specific adaptation (Delph et al., 2004; Poissant et al., 2010) for traits whose expression is an emergent property of the interactions between the sexes (e.g. mating frequency) or for traits for which there is an underlying genetic correlation between the sexes (e.g. body size, locomotory activity, e.g. Long & Rice, 2007, but see also Fuchikawa & Okawa, 2013).

It has been proposed that sexual conflict may influence the evolution of sexual dimorphism (SD), such that the expression of SD may act to relax evolutionary constraints on the sexes imposed by the effects of their shared genome (reviewed by Cox & Calsbeek, 2009). SD is widely documented in life history, behavioural and morphological traits, as well as in gonad and gamete development (Fairbairn, 2013). The nature of SD can be highly species-specific or diet-dependent. For example, the direction of SD for adult body size reverses between two species of dung fly and between different larval environments (Ding & Blanckenhorn, 2002).

Sex differences in lifespan are particularly widespread across the majority of animal taxa (Promislow, 1992; Moore & Wilson, 2002; Liker & Szekely, 2005; Clutton-Brock & Isvaran, 2007). However, the causes and consequences of this sexual dimorphism for lifespan (SDL), and the possible factors that affect the extent of SDL, are still poorly understood (e.g. Regan & Partridge, 2013). One leading hypothesis is that enhanced SDL could be a mechanism to resolve sexual conflict and allow females and males to achieve increased sex-specific fitness (as reviewed by Cox & Calsbeek, 2009).

Broad differences in the extent of SDL across animal species have been associated with variation in mating systems (e.g. Liker & Szekely, 2005; Clutton-Brock & Isvaran, 2007). In these studies,

SDL is elevated in promiscuous systems, but reduced under monogamy. Promiscuity leads to costs for male lifespan from intensified male-male competition, and a shorter effective breeding period than for females, so is proposed to reduce the selection on increased longevity in males compared to females, hence increasing SDL (Trivers, 1972; Clutton-Brock & Isvaran, 2007). Sex-specific reductions in longevity may be further explained by sex-specific patterns of extrinsic mortality, ageing onset and ageing rate, over lifetime (e.g. Trivers, 1972; Clutton-Brock & Isvaran, 2007).

Within species, SDL extent can also be altered by diet and may show a complex relationship with increasing dietary restriction (DR). For example, SDL in *Drosophila melanogaste*r is maximised by a 60% reduction in the standard dietary yeast and sugar content (the DR level which optimised female lifespan) and SDL is minimised or absent at extreme food concentrations (below 30%, or above 130% of the standard dietary yeast and sugar content) (Magwere et al., 2004). There is also emerging empirical evidence for the role of male-specific hormones in reducing male lifespan below that of females, thus enhancing SDL (Trivers, 1985; Min et al., 2012). Experimental removal of male hormones increased male lifespan, relative to intact males, such that the sexes no longer differed in their lifespan and hence SDL was lost (Min et al., 2012).

More recently, it has been found that in mixed sex populations, the production of pheromones by one sex can directly reduce the lifespan of the other, via interaction with insulin signalling pathways, in both flies and worms (Gendron et al., 2013; Maures et al., 2013). Exposure to female pheromones reduced male lifespan in *Drosophila*, even in the absence of mating (Gendron et al., 2013). These pheromonal effects are akin to the post-mating reduction in female lifespan induced by receipt of the male seminal fluid protein, sex peptide, reported in *Drosophila* (e.g. Wigby & Chapman, 2005). These responses provide further support that the interaction between the sexes can influence the lifespan of one or both sexes, influencing the extent of SDL.

4.2.2 Sex-specific life history trade-offs, evolved feeding regimes and development

Sex differences in lifespan within species can arise from the adaptive, sex-specific optimisation of trade-offs of lifespan with reproductive, mating or developmental traits, leading to sex-specific life history strategies (e.g. Trivers, 1972; Bonduriansky et al., 2008, reviewed by Maklakov & Lummaa, 2013). Trade-offs can arise both from the genetic coupling of traits, such as via antagonistic pleiotropy (Williams, 1957) and from the physiological allocation of limited resources and energy across life history components (e.g. Reznick, 2010). For example, a trade-off between extended lifespan and reduced early life reproduction has been documented empirically in *Drosophila* (e.g. Zwaan et al., 1995; Flatt, 2011).

Despite numerous theoretical predictions surrounding life history trade-offs, relatively little is currently known about the sex-specific impact of reproductive costs on survival trajectories in both sexes simultaneously (reviewed by Lemaitre *et al.*, 2015). Little is also known about the effect of direct manipulation of SDL on parameters of fitness.

My previous research results highlighted that evolutionary manipulations of feeding regimes were associated with the expression of SDL (Chapter 3). Replicated experimental evolution for >360 generations (over 15 years) under divergent dietary regimes in the fruit fly, *D. melanogaster* was linked with enhanced SDL. Selection lines that had been maintained on a 'random', unpredictable feeding regime exhibited enhanced SDL in comparison to control lines fed according to a 'regular' feeding regime, as measured in once-mated flies tested on a common garden standard diet. Individuals from the Random regime were also significantly smaller in both sexes in comparison to the Regular regime (Perry et al., unpub.).

Developmental traits can also impact on lifespan and may be influenced by dietary manipulations or by nutritional evolutionary history. Experimental evolution of *Drosophila melanogaster* under chronic larval malnutrition (diluted food) for 112 generations selected for a reduction in the 'critical size' of larvae required for the progression to, and initiation of, metamorphosis (Vijendravarma et al., 2012). Presumably, if more larvae could progress to the puparium stage for metamorphosis, this could lead to a higher egg to puparium viability and a reduced time period between egg and puparium stages. However, the reduction in critical size of larvae which Vijendravarma et al. (2012) observed, may have arisen as an artefact of selection on shorter development time used in the experimental evolution rearing regime of the study (selecting individuals which developed faster to contribute to the next generation), rather than as a direct result of manipulation of evolutionary larval diet. Contrastingly, Edgar (2006) found no effect of *Drosophila* nutrition on the puparium-adult development. Together, this suggests that the potential effect of evolutionary manipulation of nutrition on developmental traits and hence their potential influence on SDL, is not yet clear.

To date, there have been no direct empirical tests of the age-specific fitness consequences associated with enhanced versus reduced SDL in both sexes. This knowledge gap has partly arisen from the lack of an appropriate empirical system in which to test these predictions.

The differing extent of SDL between Random and Regular lines provided an opportunity to test hypotheses regarding the fitness consequences and adaptive value associated with enhanced/reduced SDL, respectively. I conducted an investigation of the evolved life history responses of reproductively active individuals of both sexes simultaneously, from Regular and

Random feeding regime lines, in a common garden environment. I measured developmental parameters, fitness parameters (age-specific survival, age-specific reproductive output) and mating frequency.

I tested the hypothesis that enhanced SDL could be a mechanism to resolve sexual conflict and allow females and males to achieve increased sex-specific fitness. I predicted that there would be evidence of adaptive sex-specific optimisation of life history trade-offs (Maklakov & Lummaa, 2013), correlated with the intermittent nutritional stress imposed by the Random feeding regime. My approach allowed elucidation of the sex-specific fitness consequences of SDL and investigation of evolved life history responses arising from evolutionary manipulation of feeding regime.

4.3 Materials and Methods

Experimental individuals were the second generation of offspring (F2) originating from eggs laid by grandparents (P1) derived from the 3 replicated populations of Regular and Random feeding regime cages (Chapter 3, Figure 1). Two generations of rearing under standard conditions were conducted to minimise maternal effects. Eggs were collected from females (P1) by introducing a single yeasted red grape juice agar plate into each of the 3 Regular and 3 Random cages, for 24h. First instar larvae were transferred to SYA vials at controlled density of 150 larvae/vial (as Chapter 3). Adult flies (F1 generation) were allowed to emerge and freely mate in their larval vials for 24h and then tipped (without CO_2 anaesthesia) onto fresh SYA bottles for another 12-24h of free mating. This ensured all F1 individuals were sexually mature (aged between 12h and 48h). 400 F1 females from each of the 6 experimental lines were then transferred into a mini-cage with yeasted purple agar plate, using light CO_2 anaesthesia, and allowed to egg-lay for 6h (after first allowing laying onto a separate and later discarded preparation plate for 24h to encourage egg laying). The short egg laying window allowed for precise measurement of subsequent developmental timings.

4.3.1 Developmental assay

First instar F2 larvae (n=3000, per mini-cage purple agar plate) were transferred to 20 SYA vials, at a density of 150 larvae/vial. The exact time of placing larvae in the vials was recorded, for later calculation of development time parameters. Adults emerging from half of the larval vials (n=10) were used to record developmental parameters. Numbers of puparia were recorded up to 3 times per day (from day 5 to day 7 of development) and the numbers of adults recorded up to

twice per day (from day 9 to day 13 of development). This enabled calculation of developmental timings and developmental viability between the first instar larval, puparium and adult stages.

4.3.2 Life history assay

Adults emerging from the half of the F2 larval vials were collected as the F2 generation 'focal' flies for the adult fitness experiment. Sample sizes of 51 adults/sex/line were used for the survival assay and for weekly matings. A subset of 45 adults/sex/line were used to assess weekly reproductive output.

Virgin wild-type (WT) Dahomey flies of both sexes (n=480/sex) derived from standard density cultures (150 larvae per vial) were generated each week for mating with the focal flies in the experiments. Emerging WT flies were collected as virgins and held in single sex groups of 10 per SYA vial until they were introduced to the focal flies.

Initial matings between virgin focal flies and virgin WT flies were set up 3 days post-eclosion (to ensure sexual maturity). Using light CO_2 anaesthesia, 3 focal adults were placed with 3 standard WT adults of the opposite sex per vial for 24h. Multiple individuals were housed together to introduce biologically-relevant male-male competition. The mating schedule in the male and the female experiments was therefore identical. Assays of mating behaviour were recorded and mated/non-mated status noted every 20mins for the final 3h of each 24h mating period. This allowed indices of the proportion of each sex that mated, to be determined.

After mating, focal females and focal males were transferred to single sex vials of standard food (SYA) at a density of 3 flies/vial, under light CO_2 anaesthesia. WT females and WT males were discarded after mating. Initial egg counts for both focal sexes were made from the 24h mating period. A 24h egg laying period did not result in egg overcrowding (Chapter 3) and hence allowed the estimation of potential reproductive output. Egg vials were retained to determine egg-adult viability and frozen 13 days after egg laying, for later counting of number of offspring.

For the first 2 weeks of the experiment, twice weekly matings of focal females and males with WT mates (standard 3-day-old virgin WTs) were conducted, and twice weekly egg counts and offspring counts recorded, to assess early reproductive output. Weekly matings and reproductive output counts were then performed for the remainder of the experiment. All matings followed the same protocol as the initial mating.

Every 2-3 days (Monday, Wednesday, Friday) food vials were exchanged and the groupings of 3 focal flies per vial were shuffled, to randomise the positioning of focals in vials with fewer than 3 flies (due to mortalities or censors). The focal sexes were housed in single sex vials throughout

the experiment (except during weekly matings with WT adults). Focal female and focal male mortalities were checked daily and Kaplan Meier survivorship curves were plotted.

4.3.3 Statistical analyses

All statistical analyses were performed in R version 3.2.1 (R Core Development Team, 2015).

Development time and developmental viability

Developmental viability was expressed as proportion data and analysed using a generalised linear model (GLM), with quasi-binomial errors, to account for overdispersion. Sex ratio of emerged adults was compared to a 1:1 sex ratio using a Pearson's Chi Squared test. Development time data were tested for normality using the Shapiro Wilk test and for equality of variances using the Levene's test, separately for each treatment level. Differences in development between regimes were analysed using a two sample t-test, as the normality and equality of variances assumptions were met. A regime-sex interaction effect on development time was tested for using a GLM with normal errors.

Survival analysis

Survival analyses were performed using nested, mixed effects Cox Proportional Hazards regression analysis on age-specific mortality data, separately for focal females and focal males. A mixed effects Cox model was fitted using the 'coxme' function from the 'coxme' package. Diet Type (Random, Ra or Regular, Re) was fitted as a fixed effect and Line (replicate cage: Ra1, Ra2, Ra3, Re1, Re2, Re3) nested within Diet Type, as a random effect. Likelihood ratio tests (anova) showed that for all data, the nested coxme model had greater explanatory power and better model fit than either the simple Cox PH model, or a non-nested coxme model.

Sex-specific survival differences were tested for by combining the female and male datasets and fitting 'coxme' models. Sex was fitted as a fixed effect and a new term, 'NewLine' (the unique cage identifier: Re1F, Re2F, Re3F, Re1M, Re2M, Re3M) nested within Sex, as a random effect. Again, the nested coxme model had greater explanatory power than either the simple Cox PH model or a non-nested coxme model.

All age-specific mortality data were first tested for the proportional hazards (PH) assumption of Cox analysis, using both graphical and analytical tests. The majority of data satisfied the PH assumption. Parametric survival analysis was performed for the two datasets with the largest potential violation of the PH assumption and the results compared with the mixed effects Cox 'coxme' analysis, to find best model fit. A maximum likelihood approach was used to compare 11

different parametric models and find the best model fit (adapted from Archer et al., 2015). Linear mixed effects models were used to analyse lifespan data. Parametric survival analysis returned the same results as the mixed effects Cox model, both matching the degree of significance, when a highly significant survival difference had been found and matching the non-significant difference in survival found in the other dataset. This justified the use of 'coxme' analysis on all survival data.

Age-specific reproduction analysis

Age-specific egg count and offspring count data were analysed using generalised linear mixed effects models, separately for each sex, using the 'glmer' function from the 'lme4' package in R. Experimental line (Random 1, Random 2, Random 3, Regular 1, Regular 2, Regular 3) and the number of days post-eclosion were fitted as categorical random effects and feeding regime (Regular or Random) was fitted as a fixed effect. No individual-level random effect was included in the model, as individuals were not uniquely identifiable from this experiment (measures were taken from randomised groupings of 3 individuals, at each time point).

The data were overdispersed in all cases. To account for this, an observation-level random effect was added to each 'glmer' model and a maximum likelihood model comparison was used to determine best model fit.

Egg to adult viability was calculated as the proportion of eggs laid by groups of 3 focal females that hatched as viable offspring, at each timepoint. Proportion data was arcsine transformed, to normalise and then analysed with a glmer, with Gaussian errors, from the 'lme4' package (same output as lmer).

Initial egg and offspring counts (from 3 days post-eclosion) were also analysed separately, for both sexes, using the same approach as for development time data, to determine whether differences in fitness indices were associated with differences in initial reproduction counts (as the fitness index, Euler's r, is weighted towards early reproduction) and for comparison with the initial egg and offspring counts from Random and Regular individuals assayed in Chapter 2 (also at 3 days post-eclosion).

Lifetime reproduction analysis

An index of total lifetime egg production and an index of total lifetime offspring production was calculated separately for each sex and each treatment population by summing egg or offspring counts, respectively, across the lifetime. Mean and standard errors for total lifetime reproduction values, for each feeding regime (Random and Regular) and each sex, were determined.

Differences in total lifetime egg or offspring production between regimes were analysed identically to development time data.

Female and male fitness analysis

Female and male fitness indices were calculated as the intrinsic rate of population growth (the Malthusian parameter, Euler's r), using the Euler equation (Gotelli, 2001; Wigby & Chapman, 2005), separately for each treatment line. The Euler equation calculates an index of fitness from age-specific survivorship and age-specific reproduction values and is weighted towards early life reproduction. Age-specific egg counts (per 24h) were used to calculate 'potential fitness' and age-specific offspring counts (per 24h) were used to calculate 'realised fitness'. Offspring counts and egg counts were halved, to account for the genetic contribution of one parent (the mother or father, respectively) to the offspring generation. Fitness data was analysed identically to development time data.

Mating data analysis

An index of the proportion of individuals that mated from each treatment line population was calculated separately for each focal sex. For each weekly mating day (n=10), the total number of matings recorded each 20 minutes, over the 3h mating observation, were summed, to give the total number mated per 3h mating, for each line and each focal sex. The total number of matings recorded over lifetime (across all weekly matings) for each focal sex and line were then calculated, and expressed as a proportion of the sum of total number of pairs surviving at each weekly mating over lifetime.

Indices of mean proportion mated over lifetime per treatment line were analysed, separately for each sex, using a generalised linear model with binomial errors. Overdispersion was accounted for by using quasi-binomial errors. A maximal GLM model including regime, sex and their interaction was fitted. Stepwise removal of the most non-significant model terms from the maximal model and likelihood ratio tests were used to test for significance of model terms and derive the minimal adequate model.

For all life history parameters analysed, the Levene's test was used to test for significant differences between regimes, in the variation between regime lines. In all cases, no significant difference was found, satisfying the equal variance assumption of the statistical tests used.

4.4 Results

4.4.1 Developmental Viability

There was no significant difference in developmental viability between Random and Regular feeding regimes, for overall first instar larva (L1) to adult (GLM: t = 0.702, p = 0.485) (Figure 1A), for L1 to puparium (GLM: t = 1.249, p = 0.214) (Figure 1B) or puparium to adult (GLM: t = 1.416, p = 0.162) (Figure 1C). There was also no significant departure from a 1:1 adult sex ratio (Pearson's Chi Squared test: X-squared = 775.75, df = 754, p-value = 0.284; Table S1). There was no significant difference between the sexes or between the regimes in the number of adults emerged (GLM: 'sexes' t = 0.405, p = 0.686; 'regimes' t = 0.483, p = 0.630).

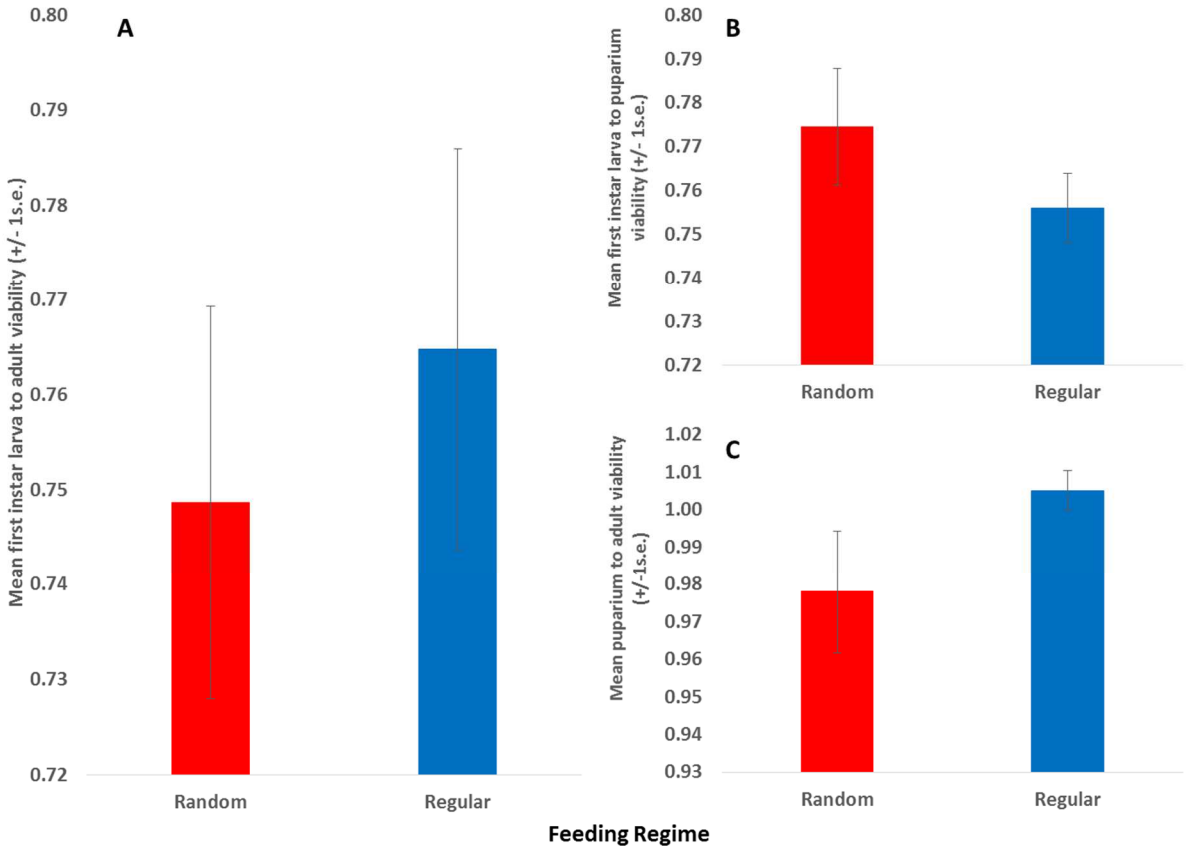

Figure 1. Mean developmental viability (+/- 1 s.e.) for focal adults from Random and Regular feeding regimes, developing on standard food, at first instar larva to adult (A), first instar larva to puparium (B) and puparium to adult (C) developmental stages.

4.4.2 Development Time

There was also no significant difference in development time between focal adults from Random and Regular feeding regimes, for overall L1 to adult development time (Two Sample t-test: t = 0.292, df = 4, p = 0.785) (Figure 2A), for L1 to puparium (Two Sample t-test: t = 0.426, df = 4, p = 0.692) (Figure 2B) or puparium to adult (Two Sample t-test: t = 0.243, df = 4, p = 0.820) (Figure 2C).

Female L1 to adult development time was significantly shorter than male L1 to adult development time, for both the Random regime (Two Sample t-test: t=3.332, df=4, p=0.0291) and the Regular regime (t=7.496, df = 4, p = 0.00170). There was no significant regime effect on the sex differences in development time (GLM: t=0.344, p=0.740) (Figure 3).

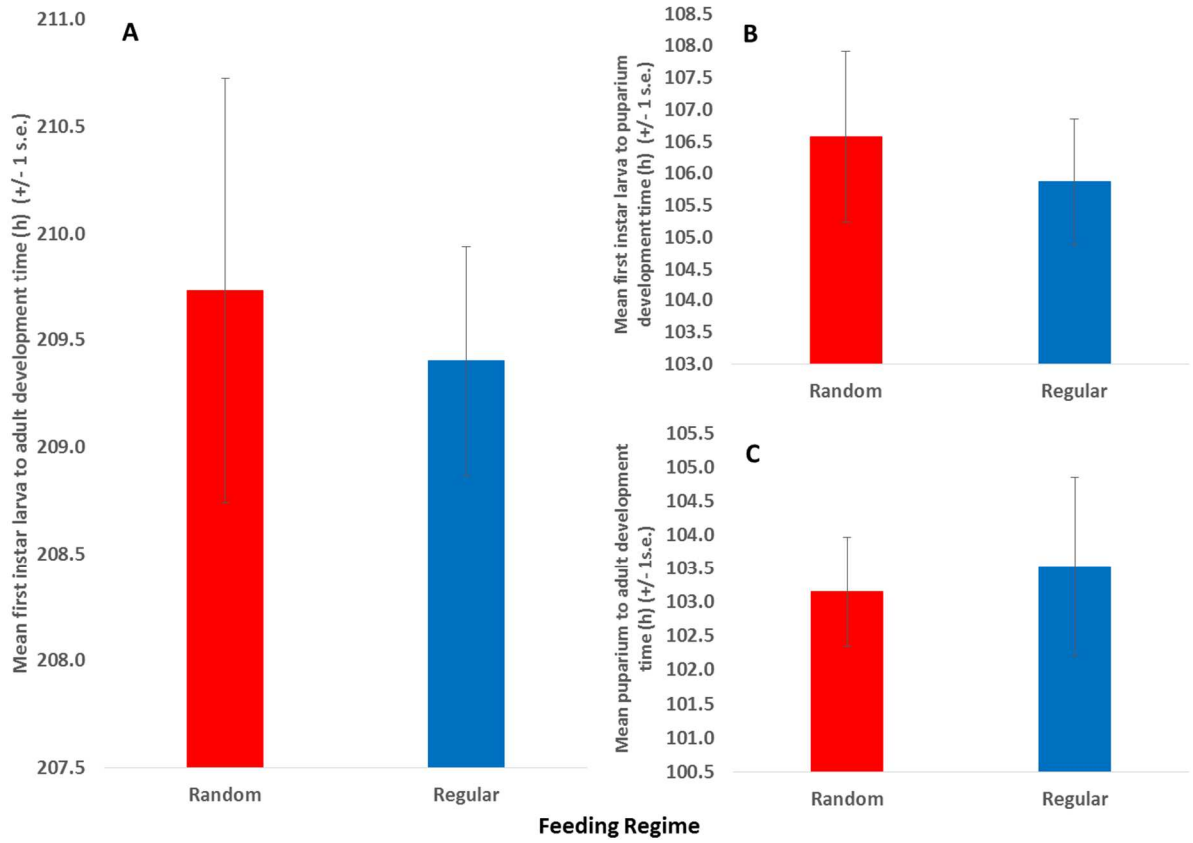

Figure 2. Mean development times (+/- 1 s.e.) for focal adults from Random and Regular feeding regimes, developing on standard food, at first instar larva to adult (A), first instar larva to puparium (B) and puparium to adult (C) developmental stages.

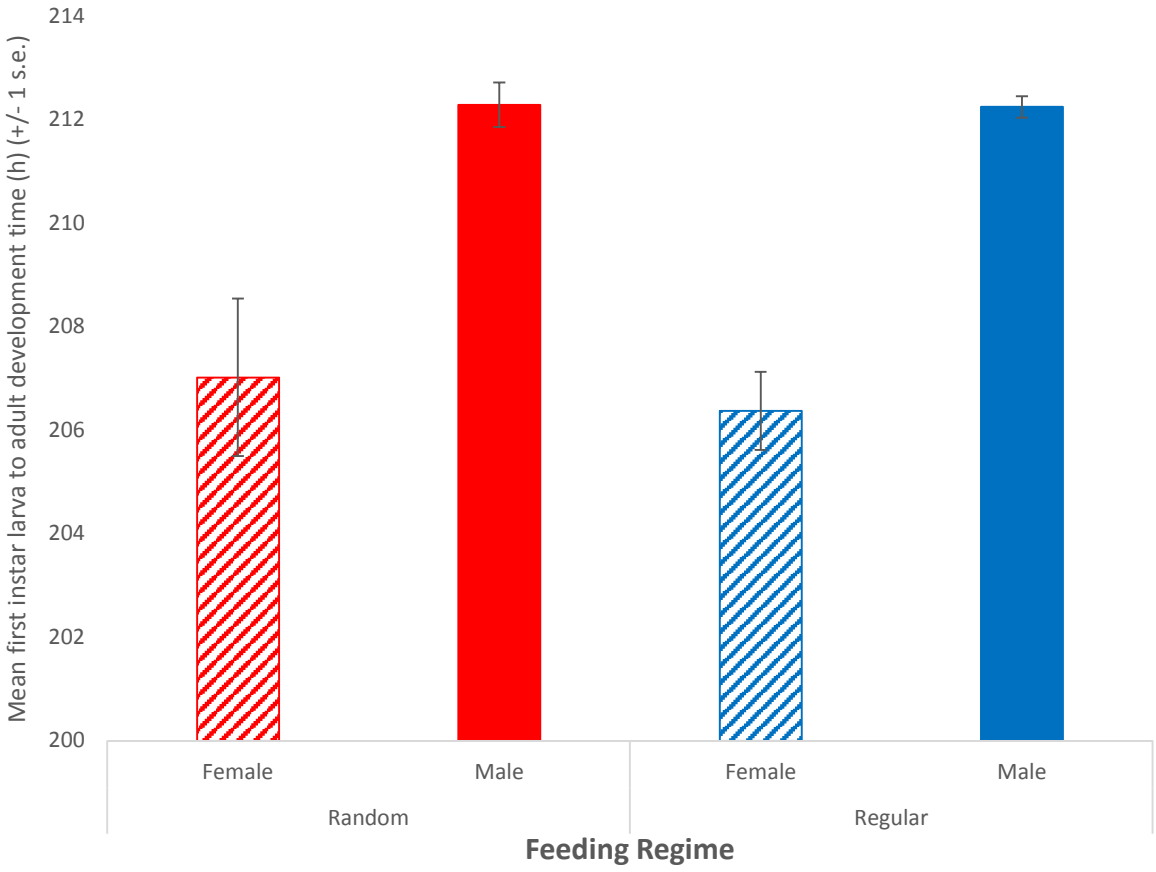

Figure 3. Mean first instar larva to adult development time (+/- 1 s.e.) for focal females and focal males from Random and Regular feeding regimes.

4.4.3 Survival

There was no significant difference in focal female survival between the Regular and Random regimes (nested coxme: z=1.31, p=0.19; median lifespan=58days, 60days, respectively; Figure 4A; Table S2). Regular focal males lived significantly longer than Random males (nested coxme: z=2.39, p=0.017; median lifespan=51days, 47days, respectively; Figure 4B; Table S3).

There were highly significant sex differences in survival within the Random feeding regime. Random focal females lived significantly longer than Random focal males (nested coxme: z=4.42, p<0.001; median lifespan=60days, 47days, respectively) (Figure 4C). The extent of this pronounced sex difference in survival was reduced in the Regular feeding regime, but there was a significant difference between Regular female and male survival (nested coxme: z=4.56, p=0.0476, median lifespan=58days, 51days, respectively) (Figure 4D).

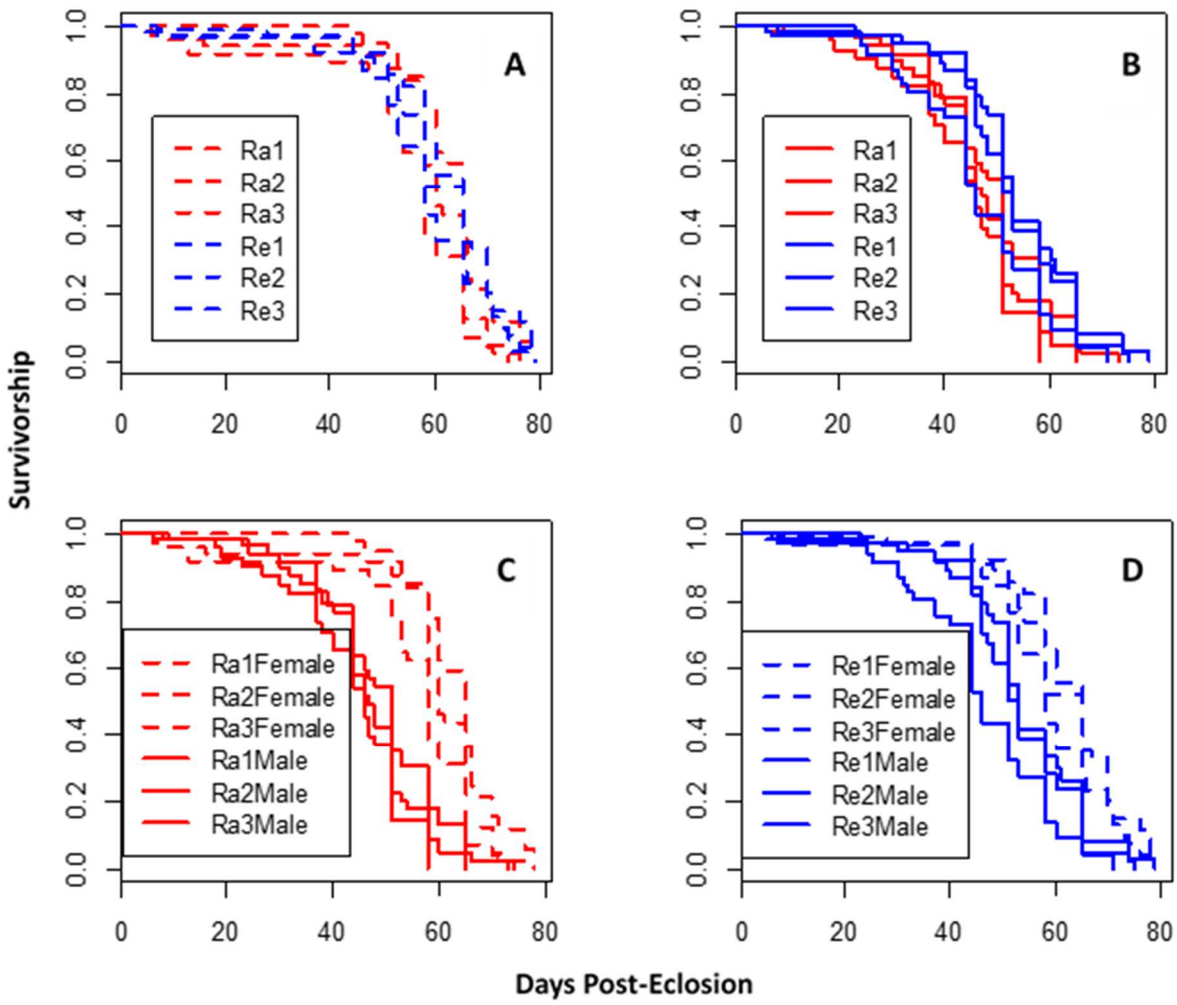

Figure 4. Age-specific survivorship against days post-eclosion, across replicates 1-3 of Random(Ra) and Regular (Re) feeding regimes, for: (A) Random vs Regular focal females; (B) Random vs Regular focal males, (C) Random females vs males, (D) Regular females vs males.

4.4.4 Focal Female Age-Specific Reproduction

There was no significant difference in focal female egg or offspring production between the Random and Regular regimes over the lifetime (glmer: $z=0.284$, $p=0.776$, Figure 5A; glmer: $z=0.183$, $p=0.855$, Figure 5B; respectively). Egg production and offspring production both significantly decreased with age across both regimes (glmer: $z=71.8$, $p<0.001$; Figure 5A; glmer: $z=71.6$, $p<0.001$; Figure 5B; respectively).

There was no significant difference in the focal female egg to adult viability between Random and Regular lines over time (glmer: $t=0.626$, d.f.=5, $p=0.480$; Figure 5C) and egg to adult viability also changed significantly over time (glmer: $t=10.191$, d.f.=5, $p<0.001$).

For comparability with the day 3 egg and offspring counts taken in Chapter 2 and due to the importance of early egg counts in weighting the estimate of fitness, Euler's r, I tested for differences in the early (day 3), counts between regimes, for both egg and offspring data. There was no significant difference in early egg counts between Random and Regular females (Two Sample t-test: $t =1.570$, $df = 4$, $p= 0.192$; mean=64, 74, respectively; Figure 5A inset), or in early offspring counts (Two Sample t-test: $t = 0.898$, $df = 4$, $p = 0.420$; mean=54, 61, respectively; Figure 5B inset).

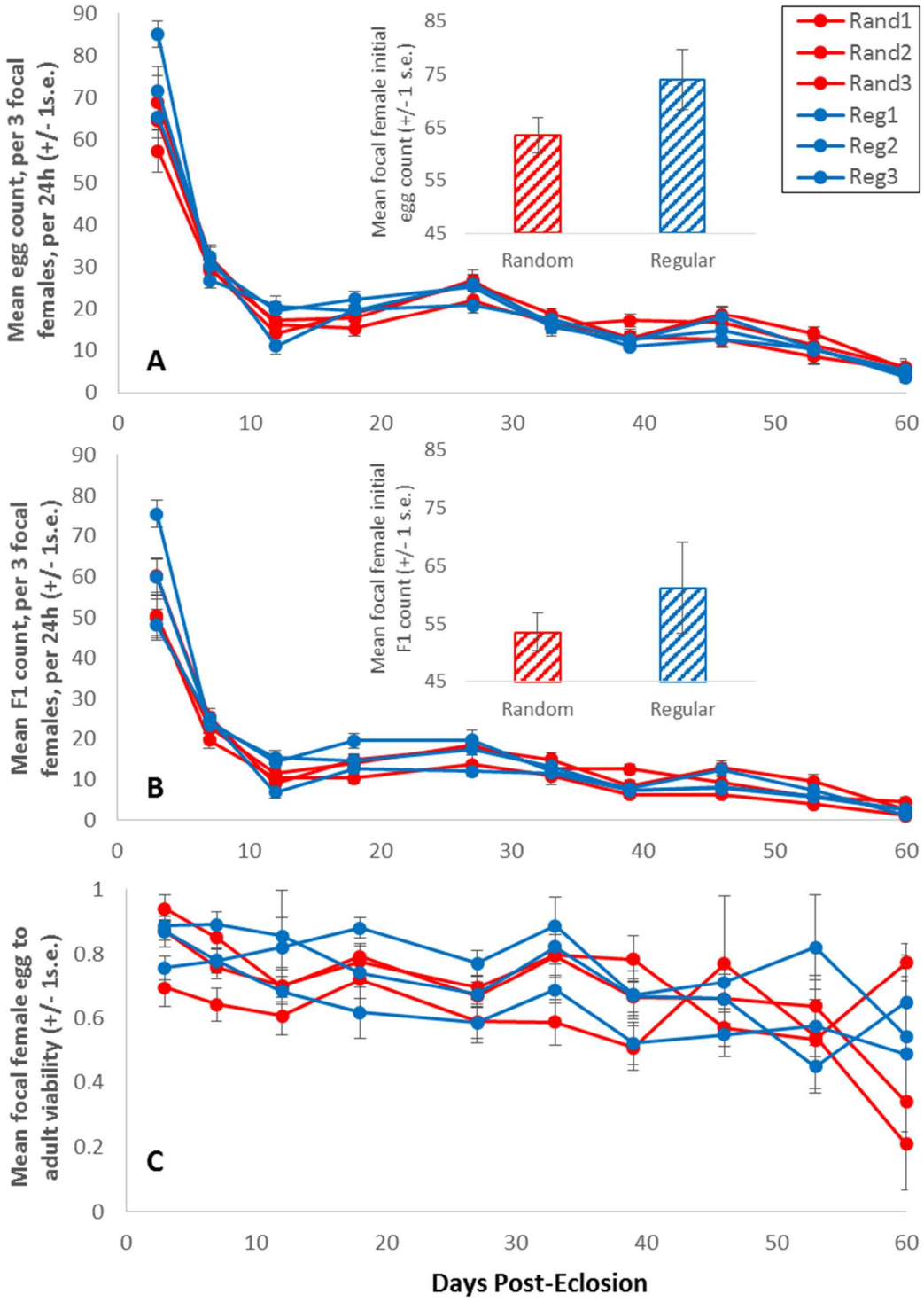

Figure 5. Mean focal female egg production (A), F1 offspring, (B) and egg to F1 offspring viability (C), per 3 females, per 24h, against days post-eclosion, for replicates 1-3 each of the Rand and Reg feeding regimes. Mean number of offspring that emerged from the 24h egg lay vials (A), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (B). Egg to adult viability is defined as the mean proportion of eggs laid by groups of 3 females during 24h which

eclosed as adults (C). Insets for (A) and (B) show mean initial (day 3) egg and offspring counts, respectively. All error bars display +/- 1 standard error.

4.4.5 Focal Male Age-Specific Reproduction

There was also no significant difference in focal male egg or offspring production between the Random and Regular regimes, over the lifetime (glmer: $z=1.089$, $p=0.276$, Figure 6A; glmer: $z=0.966$, $p=0.334$, Figure 6B; respectively). Egg production and offspring production both significantly changed with age, across both regimes (glmer: $z=39.1$, $p<0.001$, Figure 6A; glmer: $z=65.7$, $p<0.001$, Figure 6B; respectively). There was an unexpected peak in egg production at day 33 for all but one of the treatment lines.

There was also no significant difference in the focal male egg to adult viability between Random and Regular lines over the lifetime (glmer: $t=0.347$, $d.f.=5$, $p=0.700$; Figure 6C) and a significant decrease in male egg to adult viability with age (glmer: $t=19.808$, $d.f.=5$, $p<0.001$).

Early, day 3, offspring counts, however, were significantly higher for Random males than Regular males (Two Sample t-test: $t=4.286$, $df=4$, $p= 0.0128$; mean=66, 57, respectively; Figure 6B inset), though there was no significant difference between egg counts for Regular and Random males at day 3 (Two Sample t-test: $t = 2.336$, $df = 4$, $p = 0.0797$; mean= 70, 62, respectively; Figure 6A inset).

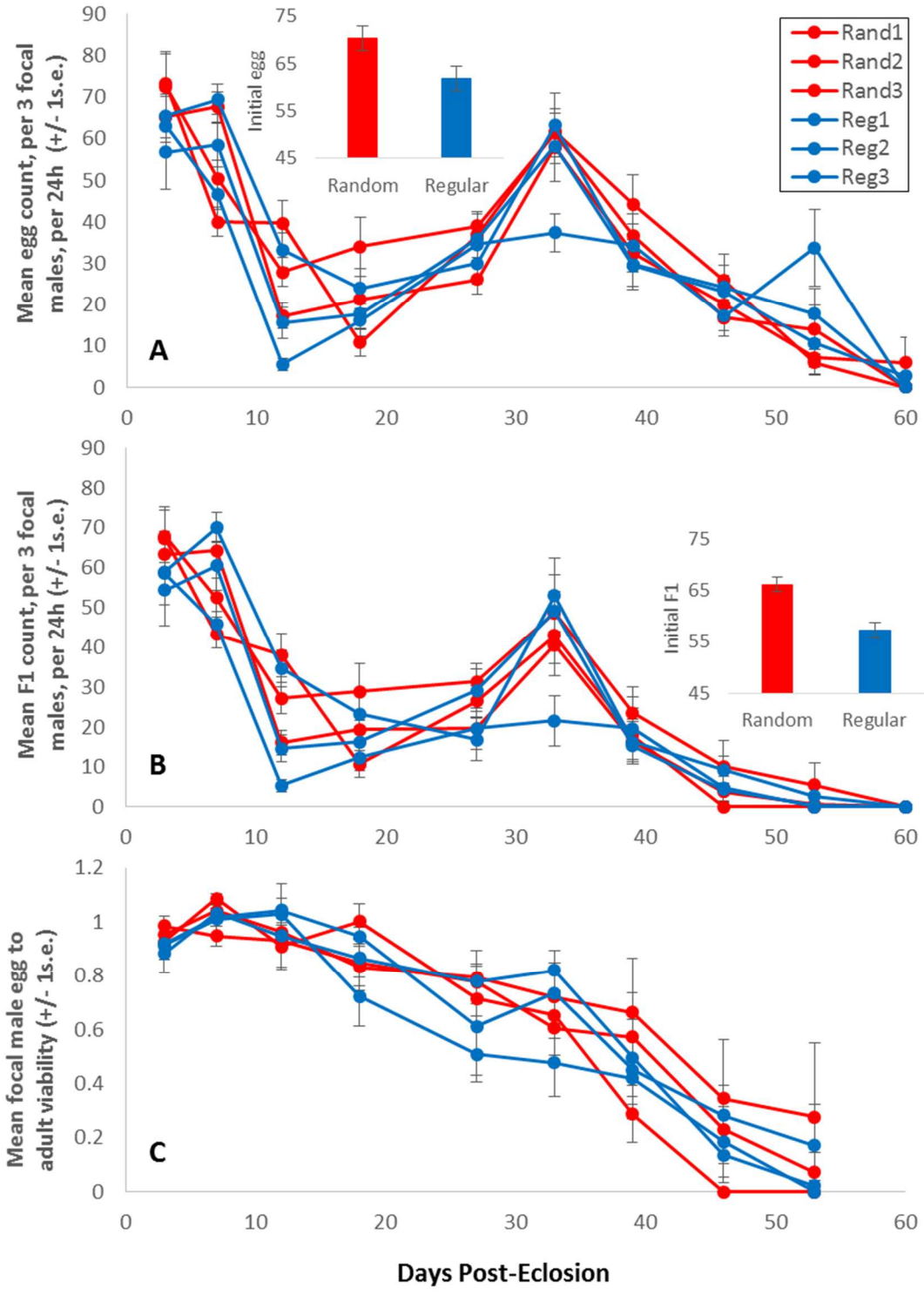

Figure 6. Mean focal male egg production (A), F1 offspring, (B) and egg to adult viability (C), per 3 males, per 24h, against days post-eclosion, for replicates 1-3 each from the Rand and Reg feeding regimes. Mean number of offspring that emerged from the 24h egg lay vials (A), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (B). Egg to adult viability is defined as the mean proportion of eggs laid by groups of 3 WT females that had been mated to the focal males, during 24h, which eclosed as adults (C). Data shown for the period where n>5 for

each treatment line. Insets for (A) and (B) show mean focal male initial (day 3) egg and offspring counts, respectively. All error bars display +/- 1 standard error.

4.4.6 Focal Female and Male Lifetime Reproduction

There was no significant difference in total lifetime egg production between Random and Regular females (Two Sample t-test: t=0.626, d.f.=4, p=0.565; mean=3090, 3236, respectively), or between Random and Regular males (Two Sample t-test: t=0.890, d.f.=4, p=0.424; mean=4349, 4019, respectively). There was also no significant difference in total lifetime offspring production between Random and Regular females (Two Sample t-test: t=0.498, d.f.=4, p=0.644; mean=2277, 2442, respectively), or between Random and Regular males (Two Sample t-test: t=0.820, d.f.=4, p=0.458; mean=3554, 3242, respectively).

4.4.7 Focal Female and Focal Male Fitness

There was no significant difference in female or male potential fitness (calculated from egg counts) between Random and Regular regimes (two sample t-test: t = -1.511, df = 4, p-value = 0.205; t = 2.264, df = 4, p-value = 0.086; respectively) (Figure 7A).

Whilst there was also no significant difference in female realised fitness (calculated from offspring counts) between Random and Regular regimes (two sample t-test: t = 0.806, df = 4, p-value = 0.465); there was a significant difference in male realised fitness between the feeding regimes (two sample t-test: t = 4.323, df = 4, p-value = 0.0124) (Figure 7B). Random males showed a significant increase in fitness compared to Regular males, perhaps driven by the significant increase in early life Random male offspring production over Regular males (as analysed above; Figure 6B).

Random female realised fitness was significantly lower than Random male realised fitness (two sample t-test: t = 3.434, df = 4, p = 0.0264), but there was no significant difference in realised fitness between Regular females and Regular males (two sample t-test: t = 0.293, d.f. = 4, p = 0.784). Using a combined model, there was a significant sex-regime interaction effect on potential fitness (glm: t = 2.454, p-value = 0.0397), indicating that sex differences in potential fitness were influenced by evolved feeding regime and that fitness differences between the regimes were different for the sexes (Figure 7A). Realised fitness data did not show this significant interaction term (glm: t = 1.742, p-value = 0.120).

Sex-specific fitness values were not directly comparable between males and females, however, as the sexes differ in their reproductive potential (and optimal mating frequency) and the weekly reproductive output of each sex was estimated differently, in this experiment, so inter-sex fitness comparisons of fitness may have be confounded. A standardisation approach to making sex-specific fitness values comparable, adapted from Brommer et al. (2011), was tested, which involved dividing age-specific reproductive counts, by the day 3 count for that sex and that treatment line (a timepoint where the mating regimes of the sexes was equivalent and to maintain treatment line integrity) and then calculating fitness using these standardised values (see Supplementary Material). This standardisation approach altered the patterns of inter-sex and inter-regime fitness comparisons (Figure S1), largely arising from the disproportionate effect of early (day 3) reproduction on male fitness. This could be expected given that the Euler's r fitness estimate is weighted by early reproduction. These results suggested that it was appropriate to conduct fitness comparisons on the unstandardized data between regimes, for each sex separately.

4.4.8 Focal Female and Focal Male Mating Frequency

A significantly greater proportion of Regular males than Random males mated, during the 3hour observations of weekly matings, over their lifetimes (glm: $z = 2.122$, $p = 0.0338$); but there was no difference in the mean proportion of focal females that mated during weekly mating observations, over lifetime, between feeding regimes (glm: $t = 0.096$, $p = 0.928$) (Figure 8). A significantly greater proportion of focal males than focal females mated (glm: $t=5.454$, $p<0.001$), but there was no significant regime x sex interaction effect on the proportion mated (glm: $t = 0.838$, $p = 0.426$) (Figure 8).

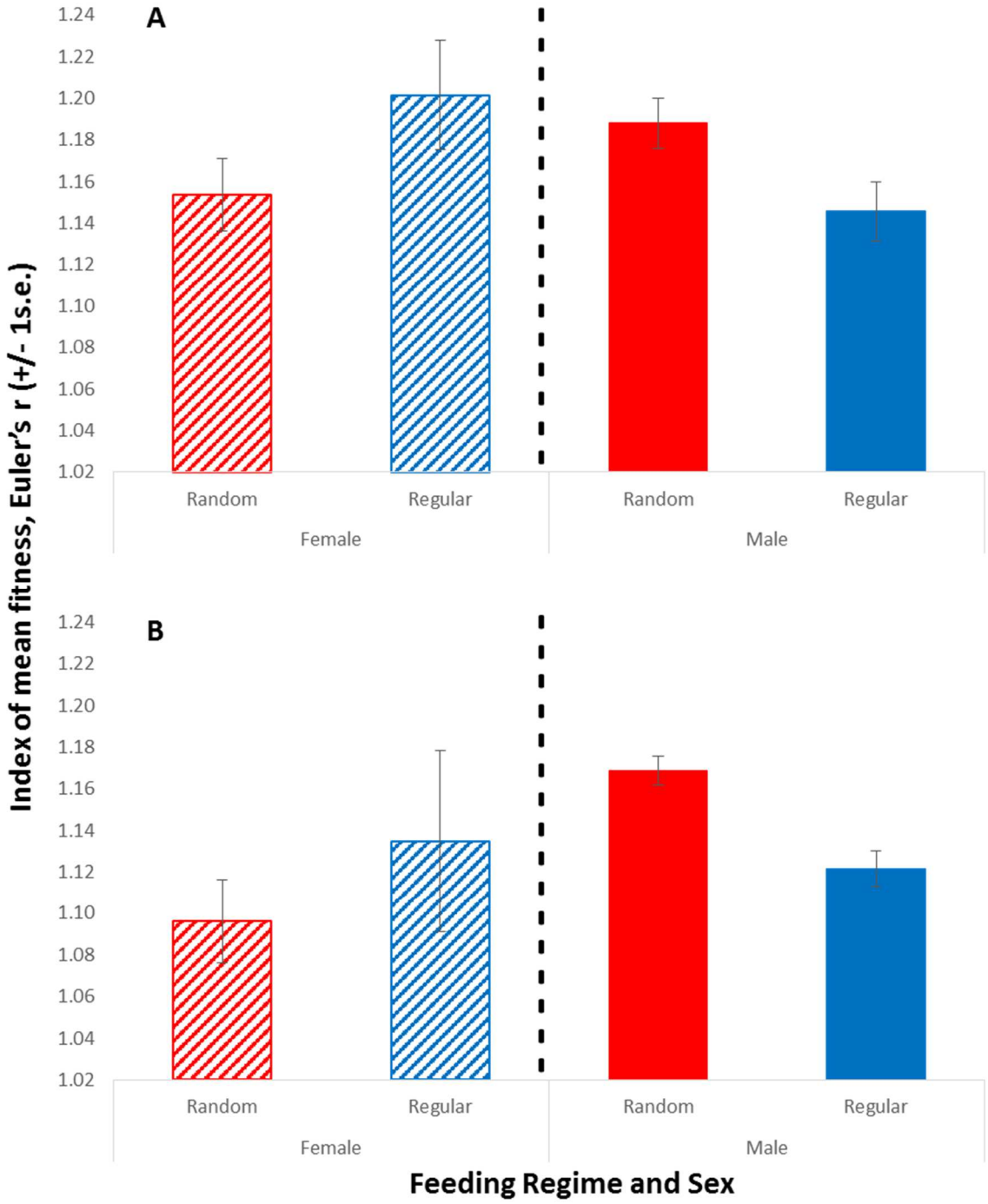

Figure 7. Index of mean fitness (+/- 1s.e.) for focal females and males from Random and Regular regimes, calculated as Euler's r using age-specific egg counts (A) or age-specific offspring counts (B). Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3); n=45 individuals per line. Hatched bars indicate females and solid bars indicate males.

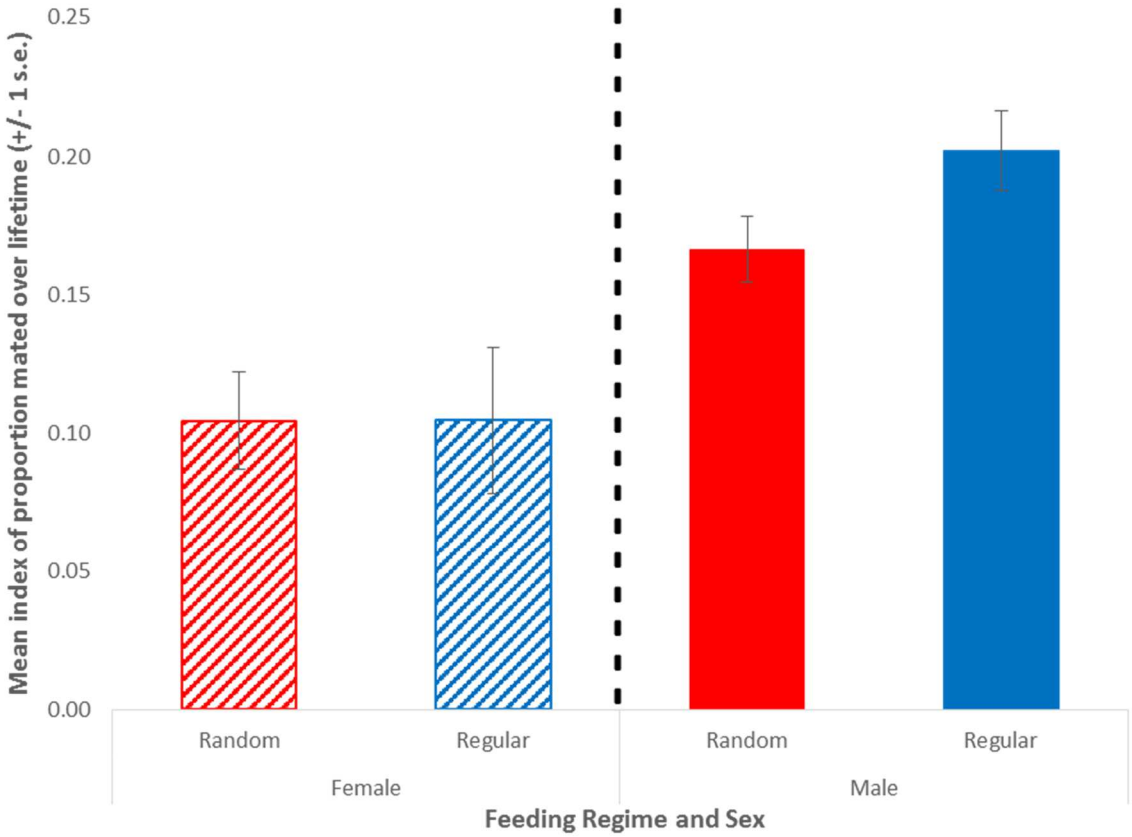

Figure 8. Index of mean proportion mated for Random and Regular feeding regime lines for each sex, over lifetime. Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3), during the 3h observations of weekly matings, across lifetime. Hatched bars indicate females and solid bars indicate males.

4.5 Discussion

Differences in female and male lifespan are widely documented across many species (e.g. Promislow, 1992; Moore & Wilson, 2002; Liker & Szekely, 2005; Clutton-Brock & Isvaran, 2007). Much less is known about possible factors that can influence the extent of this sexual dimorphism for lifespan (SDL) within species or the sex-specific fitness implications of SDL. Here I addressed these questions by directly manipulating feeding regimes and measuring the life history consequences of enhanced SDL in both sexes simultaneously.

I found that evolutionary manipulation of feeding regime altered the extent of SDL in *D. melanogaster*. SDL was enhanced in the Random regime, when compared with the Regular regime. Importantly, this enhanced SDL was driven by the reduction in Random male lifespan relative to Regular male lifespan, as female lifespan did not differ between regimes. For both

regimes, females lived longer than males, as is widely documented for *Drosophila* on standard (SYA) food (e.g. Magwere et al., 2004, but see also Wit et al. (2015)).

Enhanced SDL was associated with increased fitness of Random males over Regular male fitness (treated as the baseline level of fitness), as predicted; but there was no significant difference in female fitness between regimes, contrary to predictions that enhanced SDL would lead to increased fitness for both sexes. Random males appeared able to compensate for a reduced lifespan, with an increased fitness through an early burst of reproductive output. This finding is consistent with theory that fitness and lifespan are not always positively correlated (e.g. Maklakov & Lummaa, 2013) and that this relationship instead depends upon the scheduling of reproduction within the life history.

Random males achieved higher fitness, despite a reduced lifespan, by allocating resources into increased early reproductive output (progeny production), which increased their fitness (Euler's r index). This suggests a possible life history trade-off between early reproduction and lifespan, in concordance with previous empirical work in *Drosophila* (e.g. Zwaan et al., 1995; Flatt, 2011). Increased early productivity was achieved, even though Random males mated less frequently than Regular males over their lifetime. Random males may therefore have had a greater reproductive investment per mating than Regular males, assuming that a higher proportion of the population mating during weekly 3 hour periods over lifetime was a reliable indicator of individual mating frequency, as was predicted. The increased mating frequency was not apparently of sufficient magnitude to lead to a cost to lifespan.

The reduced lifespan of Random males compared with Regular males was not associated with any between-regime differences in developmental viability or timing. This was contrary to what would be expected if there had been a reduction in larval 'critical size' following selection on larval malnutrition (Vijendravarma et al., 2012) in the Random lines. However, my results are consistent with Edgar (2006) who also found no effect of nutrition on puparium to adult development. Random males did have a smaller body size than Regular males (Perry et al., unpub.), which may have been associated with the reduced lifespan found in Random males here. Body size alone, however, is unlikely to explain the lifespan differences observed, as Random females were also smaller than Regular females (Perry et al., unpub.), yet females did not differ in lifespan. Early reproduction therefore showed a stronger association than did body size with the lifespan differences between regimes.

The overall patterns of survival and reproduction in the reproductively active individuals, in my study, matched the results with once-mated individuals from the Regular and Random lines

(Chapter 2). The nature of the sex difference in lifespan differed slightly, just crossing the 5% significance threshold (p=0.0476) for weekly-mated individuals here but not for the once-mated Regular individuals in Chapter 2 (p=0.440). Small differences between the experiments could have arisen both from mating regime differences and between-experiment variability. Importantly, both experiments were consistent in finding enhanced SDL in the Random regime when compared with the Regular regime.

Together these results suggest that evolutionary manipulation of feeding regime resulted in a specific change to male life history strategy. Random males may maximise their fitness by allocating fewer resources into somatic maintenance, body size assurance and survival and instead investing more into early reproduction. Regular males, in contrast, may allocate more resources into body size assurance and survival and less into early reproduction, resulting in lower fitness than Random males.

Life history strategy alteration in response to selection on feeding regime appeared to be male-specific. An interaction between sex and the life history trade-off between lifespan and reproduction was also observed by Adler et al. (2013) in dietary restricted neriid flies, *Telostylinus angusticollis*. In this species the trade-off was present in females and absent in males, in a reversal to the sex-specific patterns I observed. The results suggest that selection for environmental manipulations can lead to lifespan extension, even in the absence of an adaptive re-allocation of resources away from reproduction, in favour of somatic maintenance and survival.

Sex-specific life history trade-offs over investment into soma (survival and maintenance) and gametes (reproduction) are often posited as evolutionary explanations for sexual dimorphism for lifespan (e.g. Maklakov & Lummaa, 2013). That is, investment into survival and maintenance, may come at a cost of reduced reproductive investment, or the reverse, and there may be differential sex-specific optimisation of energy investment and allocation (Trivers, 1972; Bonduriansky et al., 2008; Reznick, 2010). My work therefore provides empirical evidence to support the existence of sex-specific life history trade-offs, which I found were present in males and absent in females. My study also demonstrates the male-specific impact of reproductive costs (increased early reproductive output) on survival (for the Random regime). This is a theoretical area on which little empirical work has previously been conducted (Maklakov & Lummaa, 2013).

A life history strategy that favours early reproduction over later survival, despite a reduced body size, as I observed in males, could be adaptive following an evolutionary history of unpredictable (Random) food availability. If Random individuals had an increased ability to readily capitalise on resources when they became available (during ‘feast’ periods) and convert them into increased

early productivity, then this would allow them to achieve increased fitness, particularly in an environment when food supply was unpredictable. Experimental evolution of *Drosophila* under high extrinsic mortality (90% mortality induced twice per week) also led to a similar life history strategy of reduced body size, increased early fecundity and reduced lifespan, when compared with lines selected for low extrinsic mortality (10% induced mortality, twice per week) (Stearns et al., 2000). This supports the assertion that the life history responses observed in Random males could be part of an adaptive, male-specific life history strategy, after evolving under an unpredictable feeding regime of periodically limited resources.

Females, in contrast, maintained their lifespan, reproductive output and mating frequency at the same level across both feeding regimes, and so, unlike males, did not appear to evolve an altered life history strategy in response to feeding regime manipulation. It is possible that the widely documented increased body size of females over males, in *Drosophila*, which was also recorded for the feeding regime lines (Perry et al., unpub.), may have provided increased resources to secure lifespan and early reproduction at the same level for females from both regimes and without a life history trade-off. The body size difference was greater between the sexes than between the regimes, so may have been sufficient to explain more of the inter-sex lifespan variation, than had been possible for inter-regime variation in male lifespan. Evidence is still equivocal, however, about a positive association between SDL and body size between species (Toigo & Gallard, 2003). Sex-specific lifespan patterns could be the result of different selection pressures acting on the sexes (Bonduriansky et al., 2008; Rogell et al., 2014). As there was no sex bias in adult emergence, there was no evidence of a stronger developmental selection filter for either sex, suggesting that sex-specific selection pressures, if present, were more likely to have acted on adults.

Sexual conflict appeared to be at least partly resolved under enhanced SDL. Some authors argue that sexual dimorphism (SD) can only ever offer a partial resolution of sexual conflict, as the sexes are still restrained from reaching optimal fitness by the majority of their shared genomes (Cox & Calsbeek, 2009; Maklakov & Lummaa, 2013). This arises from the observation that little empirical evidence exists for the presence of “modifier” genes that could control the sex-specific gene expression required for SD, as their evolution is thought to be slow (Lande, 1980; Fairbairn & Roff, 2006). Here, sexual conflict resolution was defined as the increased fitness of both sexes under enhanced SDL, when compared with reduced SDL. SDL was proposed to relax the genetic constraint on lifespan between the sexes, allowing both sexes to achieve higher fitness.

I found that sexual conflict was resolved for males by the degree of enhanced SDL present in Random over Regular lines as fitness was increased for males but not for females. However, female fitness was maintained at a constant level for both enhanced and reduced SDL. This life history strategy could be beneficial, rather than costly, for females, if they had already achieved optimal fitness, in the absence of enhanced SDL. In this sense, sexual conflict may have been partially resolved by allowing each sex to either achieve (males), or maintain (females) their optimal life history strategy and fitness, under enhanced SDL (Bonduriansky et al., 2008; Rogell et al., 2014). The sexes may have differed in their absolute values of fitness optima, but have achieved the optimum for their respective sex, under enhanced SDL.

The increased male fitness associated with enhanced SDL may not only have been attributed to the direct effect of relaxed constraints on fitness, but could also be explained by the effects of evolutionary manipulation of feeding regime directly. Random male fitness may have increased (relative to Regular male fitness) due to the direct effect of rearing history on the parameters of fitness (age-specific survival and production), rather than just being due to the greater lifespan differences between the sexes on the Random regime. It is possible that a combination of both enhanced SDL and direct correlations of the fitness parameters with evolved feeding regime, led to increased male fitness.

Inter-sex comparisons of fitness and mating frequency for each regime, were not directly comparable, as the sexes differ in their mating schedules and reproductive potential. Males experience a lower cost from mating and reproduction. They were observed to mate more than females and to stimulate a larger reproductive output in the young (day 3) WT virgin females to which they were mated, in comparison to the reproductive output of focal females. Consequently, males would be expected to have higher 'absolute' fitness than females, independent of experimental treatment (feeding regime). When fitness was standardised for each sex, against the early (day 3) reproductive output (for each regime respectively) the patterns of fitness changed. This suggested that the disproportionate effect of early reproduction on male fitness influenced inter-sex comparisons of standardised fitness. This result was a function of the correlation between early reproduction and fitness for males. Hence I concluded that between-regime fitness comparisons for each sex separately, using non-standardised values, were most appropriate.

Experimental evolution studies in the laboratory can be vulnerable to the effects of inbreeding, due to reduction in effective population size (as discussed in Wit et al., 2015). We reduced the potential for inbreeding through maintenance at large population sizes. Also, we used 3 replicate

experimentally evolved fly populations, for each regime, to distinguish between possible life history changes arising from drift, as opposed to life history changes arising from selection. Survival and reproduction patterns were broadly consistent between the 3 replicate populations for each regime, supporting the conclusion that evolved responses between regimes arose from selection, rather than drift.

Evolved changes in the extent of sexual dimorphism for lifespan may also have been influenced by additional factors such as changes in mating frequency or even mating system. For example, SDL is enhanced under promiscuity and reduced under monogamy (e.g. Liker & Szekely, 2005; Clutton-Brock & Isvaran, 2007). I found that evolved increases in mating frequency in Regular males were not associated with enhanced SDL. This is contrary to the direction that would have been expected from the association of SDL patterns with broad scale mating systems. Instead, differences in male mating frequency may have been associated with evolutionary feeding history, directly, rather than with the extent of SDL. Female mating frequency did not differ between regimes, or between enhanced versus reduced SDL. Further direct tests of the relationship between mating frequency and SDL extent would be required to distinguish between the effects of SDL and correlations with evolved feeding regime.

In summary, I conducted an empirical test of the sex-specific consequences of sexual dimorphism for lifespan (SDL), in both sexes of the same species simultaneously, in response to the direct manipulation of evolutionary feeding regime. I showed that evolutionary feeding regime manipulation enhanced the extent of SDL, under a random, unpredictable feeding regime; in comparison to the reduced SDL on a regular feeding regime. Enhanced SDL allowed greater male-specific fitness, arising from increased early male reproduction and associated with decreased lifetime mating frequency, but in the absence of regime effects on development. Female fitness, in contrast, did not differ between regimes. Sexual conflict was therefore partially resolved through increased or maintained fitness levels under SDL enhancement.

This study offers an opportunity for an investigation of the mechanistic basis of SDL (Chapter 4) and for further investigation of the association between SDL, sexual conflict and fitness. Novel perspectives could arise from directly manipulating the degree of sexual conflict experienced and measuring the extent of lifespan dimorphism for the sexes when sexual conflict is increased or reduced. Theory predicts that SDL reduces sexual conflict, but it is currently uncertain how manipulation of sexual conflict could influence the degree of SDL observed. Assessment of life history consequences of manipulating sexual conflict, and specifically, the patterns of optimal fitness under reduced sexual conflict would be useful for comparison with the sex-specific fitness

patterns under enhanced SDL. Further, directly manipulating SDL extent, independent of evolved feeding regime and measuring the sex-specific fitness consequences, would distinguish directly between the fitness effects of enhanced SDL and of evolutionary manipulation of feeding regime. The degree of genetic variation for SDL could be assayed in *Drosophila melanogaster* Genetic Reference Panel (DGRP), fully sequenced genetic isolines (Mackay et al., 2012) and then corresponding sex-specific fitness determined, to address this question.

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4.7 Supplementary Material

Sex Ratio of Emerged Adults in Development Assay

Table S1. Pearson's Chi Squared test analysis of sex ratio of emerged adults, separately for each treatment line (Ra1, Ra2, Ra3, Re1, Re2, Re3). Analysis performed on actual counts of females and males for each treatment line and compared with a probability of 0.5 for each sex.

Treatment Line	Pearson's Chi Squared Statistics		
	X-squared	Df	p-value
Ra1	52.5	48	0.304
Ra2	70.0	64	0.283
Ra3	80.0	72	0.242
Re1	70.0	63	0.254
Re2	40.5	35	0.241
Re3	45.0	42	0.348

Focal Female Median Lifespan

Table S2. Average focal female survival for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand1	Rand2	Rand3	Reg1	Reg2	Reg3
Median lifespan (days)	**60**	**65**	**58**	**58**	**65**	**58**
Interquartile range (days)	**7**	**8**	**14**	**13**	**12**	**12**

Focal Male Survival

Table S3. Average focal male survival for each experimental line (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3)

	Rand1	Rand2	Rand3	Reg1	Reg2	Reg3
Median lifespan (days)	**47**	**46**	**51**	**46**	**53**	**51**
Interquartile range (days)	**7**	**14**	**14**	**19**	**12**	**14**

Standardised Focal Female and Male Fitness (Euler's r)

There was no significant difference in either female or male potential fitness (calculated from standardised egg counts) between Random and Regular regimes (two sample t-test: t = 1.242, df = 4, p = 0.282; t = -0.627, df = 4, p-value = 0.565; respectively) (Figure 1A). Random females showed a non-significant increase in potential fitness compared to Regular females, whereas Random males showed a non-significant decrease in fitness compared to Regular males.

Standardised potential fitness was significantly higher in males than females, for both Random and Regular regimes (two sample t-test: t = -4.604, df = 4, p = 0.01; t = -4.136, df = 4, p-value = 0.0144; respectively) (Figure 1). Using a combined model, there was no significant interaction between sex and regime on potential fitness (glm: t = 1.162, p-value = 0.279), indicating evolved feeding regime did not significantly change the potential fitness differences between the sexes (Figure 1A).

There was also no significant difference in either female or male realised fitness (calculated from standardised offspring counts) between Random and Regular regimes (two sample t-test: t = 0.459, df = 4, p = 0.670; t = -0.522, df = 4, p = 0.630; respectively) (Figure 1B). Random females showed a non-significant increase in realised fitness compared to Regular females, whereas Random males showed a non-significant decrease in fitness compared to Regular males (Figure 1B).

Standardised realised fitness was significantly higher in males than females, for both Random and Regular regimes (two sample t-test: t = -6.418, df = 4, p = 0.003; t = -2.966, df = 4, p = 0.0413; respectively) (Figure 1B). Using a combined model, there was no significant interaction between sex and regime on realised fitness (glm: t = 0.665, p-value = 0.525), indicating evolved feeding regime did not significantly change the fitness differences between the sexes.

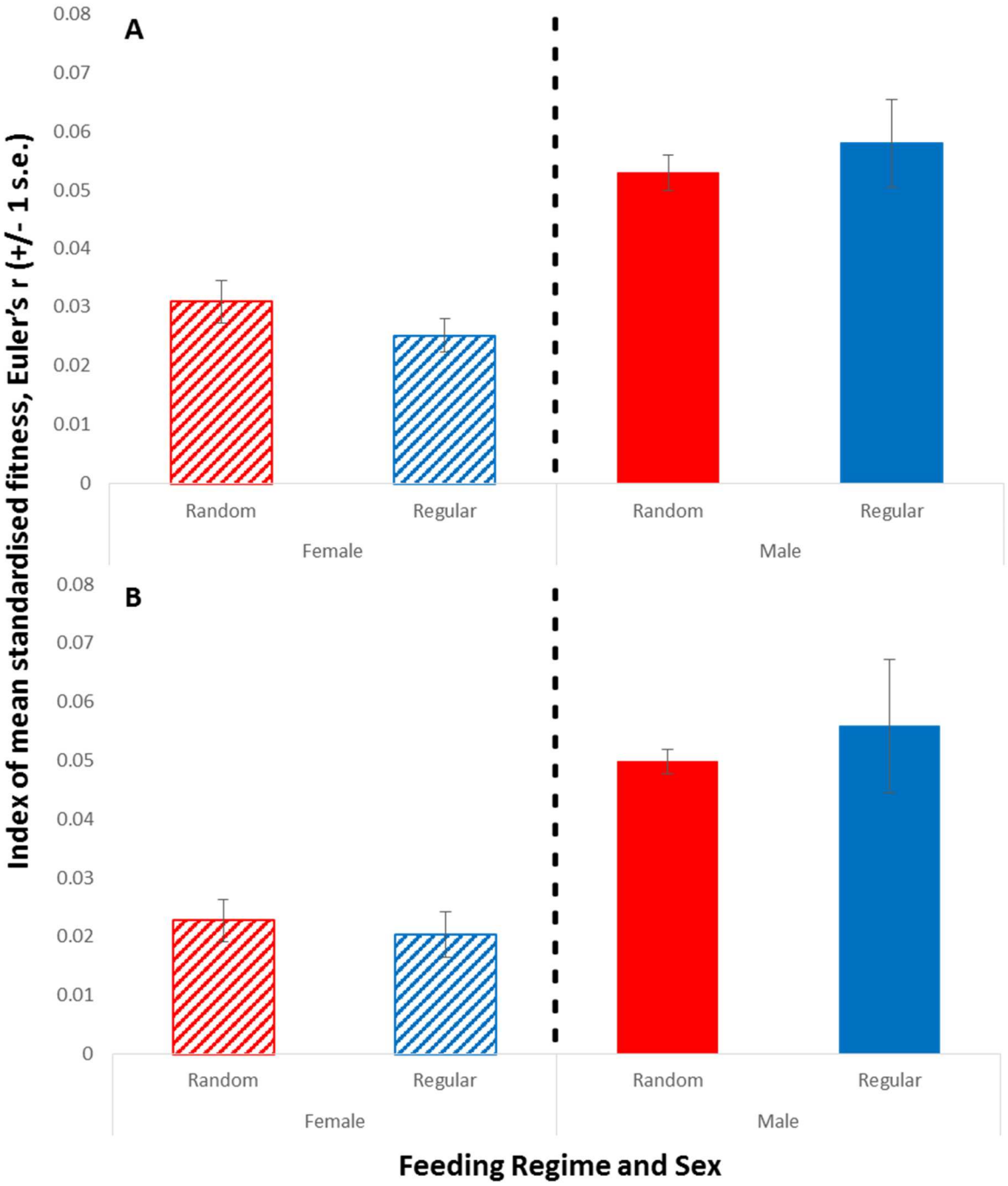


Figure S1. Index of mean standardised fitness (+/- 1s.e.) for focal females and males from Random and Regular regimes, calculated as Euler's r using standardised age-specific egg counts (A) or standardised age-specific offspring counts (B). Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3); n=45 individuals per line. Hatched bars indicate females and solid bars indicate males.

Chapter 5: Transcriptome-wide response to evolutionary manipulation of feeding regime in the fruit fly, *Drosophila melanogaster*

5.1 Abstract

Long-term (evolutionary) nutritional manipulation of feeding regime resulted in the increased expression of sexual dimorphism for lifespan (SDL) via changes to male life history (Chapter 4). These changes were accompanied by evolved shifts in sex-specific fitness patterns that resulted in decreased sexual conflict. Here I used transcriptomics to gain insight into the mechanisms underlying the evolved differences between the lines and into the differences associated with the expression of increased SDL. I profiled gene expression differences using RNA-sequencing of mRNAs (mRNA-seq) on flies sampled from the experiment described in Chapter 4, at 10 days post-eclosion. The RNA from populations of male and female head/thorax and abdomen body parts were profiled. The RNA-seq data were subjected to a set of rigorous bioinformatics quality checks. The resulting pattern of differential expression showed functional enrichment in genes associated with nutrition, lifespan, post-mating responses, regulation and epigenetic modification. The data show that signatures of evolved differences in experimental evolution experiments can successfully be captured using RNA-seq. These results contribute to our growing knowledge on the genetic and transcriptomic basis of life history responses to nutritional selection.

5.2 Introduction

Nutritional feeding regimes that persist over evolutionary time can have pronounced life history consequences for the individuals that experience them. Nutritional mismatches between ancestral and modern diets are predicted by evolutionary theory to carry fitness costs (Thrifty Genotype hypothesis; Neel, 1962; Prentice et al., 2005). However, the nature of the mismatch may reduce or eliminate these costs under some conditions (Chapter 3). Furthermore, artificial selection under different nutritional regimes can lead to the evolution of fixed life history strategies that are adapted to the prevailing environment (Stearns, 1992). There are few studies of the sex-specific responses to long-term nutritional selection, or of the underlying mechanistic basis of these life history responses.

I previously found that evolutionary manipulation of feeding regime altered the extent of sexual dimorphism for lifespan (SDL) and resulted in sex-specific patterns of fitness (Chapter 4).

Replicated selection lines of *D. melanogaster*, reared for over 360 generations on an unpredictable ('Random') feeding regime, showed elevated SDL in comparison to control lines reared on a predictable ('Regular') feeding regime, when both were assayed on a common garden diet.

The increased SDL in the Random lines was associated with male-specific life history changes (Chapter 4). Random males had reduced survival, but increased early reproductive output and higher overall fitness in comparison to Regular males. There were no female fitness difference between Random and Regular regimes. Therefore, enhanced SDL was associated a partial resolution of sexual conflict (Cox & Calsbeek, 2009).

The mechanistic underpinnings of these pronounced sex-specific life history responses that arose from the evolutionary manipulation of feeding regime are unknown. In this chapter I addressed this gap in knowledge by describing the transcriptomic (gene expression) differences between the nutritional selection lines. I did this to gain insight into the transcriptomic basis underlying life history responses to evolutionary manipulations of nutrition, into the expression of SDL and into sex-specific fitness.

Recently, studies have begun to investigate gene expression changes associated with sexual dimorphism in general, or associated with single-sex patterns of lifespan and ageing. For example, Perry et al. (2014) investigated possible candidates associated with sex-biased gene expression, during *D. melanogaster* development. Transcriptional changes have also been identified in females across lifetime, during normal ageing, under caloric restriction, or after mating, in single sex comparisons (Pletcher *et al.*, 2002; Zhou et al., 2014a). However, no transcriptional studies have yet combined the themes of nutritional manipulation, sexual dimorphism, lifespan and ageing. Hence we lack an understanding of the mechanistic basis of SDL. This is particularly relevant given that interactions between sex and age can influence the transcriptional response to selection in flies, as reported in a study that selected on postponed senescence (Wilson et al., 2013). This highlights the potential for the existence of sex-specific transcriptional patterns of ageing.

A large body of experimental work in *Drosophila* and other model species has quantified the life history responses to manipulation (in knockout and knockdown studies) of candidate genes in the insulin (IIS) and TOR signalling pathways. These nutrient-sensing pathways have an important role in mediating the response of lifespan to nutrition (particularly dietary restriction). Some work has been conducted on the interaction of these gene manipulations with diet or sex. For example, in *Drosophila*, the IIS/TOR signalling network includes many candidate genes empirically linked with

effects on female lifespan (e.g. *chico, PTEN, FOXO* and *Ink*; see: Teleman, 2010; Partridge et al., 2011; Suppl. Mat. Figure S1). Gene knockouts in the insulin-signalling pathway can also produce sex-specific effects (as reviewed by Magwere et al., 2004), suggesting that insulin-signalling is likely to be involved in sex-specific responses to diet and may be relevant to nutrition-associated SDL.

Many candidate genes linked with lifespan often show antagonistic pleiotropy (AP) or opposing beneficial and then deleterious life history effects at different points across the lifespan (Williams, 1957). An example would be a single gene that increased early life fecundity but incurred survival costs later in life. The trade-off between benefits and costs across lifetime can be condition-dependent and based on the nutritional environment. Therefore, it is possible that single genes could simultaneously influence several life history responses to nutritional manipulation.

In addition to the genetic coupling and trade-off of lifespan with other life history traits via AP (Williams, 1957), sex differences in lifespan may also trade-off with other life history traits, such as reproduction, mating or development, due to allocation of limited resources across different life history traits (e.g. Trivers, 1972; Bonduriansky et al., 2008; Reznick, 2010; reviewed by Maklakov & Lummaa, 2013). Candidate genes underlying life history traits which trade-off with the trait of interest (such as lifespan), may therefore also be differentially expressed between experimental treatments that alter lifespan.

Other candidate genes or pathways that could be associated with the extent of SDL include those related to hormone signalling (Trivers, 1985; Min et al., 2012), pheromone production (Gendron et al., 2013; Maures et al., 2013) or the sex peptide pathway (Wigby & Chapman, 2005). In these studies, the lifespan of one or both sexes was influenced by the interaction between the sexes. Hence the extent of SDL could be affected by gene expression changes in these pathways.

Gene expression quantification

Most sexual dimorphism arises from differential gene regulation rather than differences in the sequence of coding genes. Therefore, analyses of gene expression profiles are a useful and relevant tool to aid in quantifying and determining the mechanistic basis of sex-specific life history differences.

A powerful method now widely used for gene expression quantification is RNA-sequencing (RNA-seq). RNA-seq has transformed the field of transcriptomics (Wang et al., 2009; Ozsolak et al., 2011), by enabling increased resolution for the identification and quantification of RNA across cells, tissues and whole organisms. Unlike traditional microarray techniques, which require pre-

defined probes, RNA-seq generates a greater diversity of reads, enabling the quantification of both known and unknown RNA transcripts for differential expression analyses. RNA-seq also generates discrete distributions of abundances for expressed transcripts, providing more information on expression levels than the continuous distributions of light intensities produced from microarray probes (t Hoen et al., 2008; Agarwal et al., 2010). Furthermore, mRNA-seq can provide information on the expression level of alternative splice variants (Trapnell et al., 2012), on variation in the expression levels across transcripts (e.g. Dillies et al., 2013). In addition, sRNA-seq can provide a finer scale identification of classes of non-coding small RNAs (Pais et al., 2011; Stocks et al., 2012; Studholme, 2012). To summarise, in comparison to microarrays, RNA-seq provides increased power and accuracy in quantifying differential gene expression, via its ability to identify an increased extent and range of gene expression and to determine expression profiles across whole transcripts.

Like all sequencing technologies, RNA-seq is subject to biases (Hansen et al., 2010; Zheng et al., 2011). These can arise from ligation (Sorefan et al., 2012), random hexamer priming (Hansen et al., 2010), differences in transcript length (Roberts et al., 2011) and variable GC content (Risso et al., 2011). To determine the extent of these biases and any differences in the accuracy of gene expression quantification between experimental samples, thorough quality checking is conducted, during and after sequencing (see Materials and Methods). Quality checking also enables some bias to be accounted for, so improving the reliability of biological inference from the analysis of RNA-seq data.

Another potential cofound inherent in the comparison of gene expression levels between experimental treatment categories can arise from tissue allometry. This refers to the scaling relationships between particular tissues (such as the gonad) and the proportion of a total sample (from which RNA is extracted) that the tissue represents (Harrison et al., 2015). If a particular tissue represents a higher proportion of the total sample in some treatment lines than others (i.e. non-isometric scaling), this can lead to apparent gene expression differences between treatments, for which there was no differential expression. Instead, the confounding gene expression differences have arisen from an increase in tissue size, rather than an increase in gene expression per cell. Tissue allometry is particularly highlighted in the literature for birds and mammals (Harrison et al. 2015), and generally for organisms with large body size. To date, less attention has been drawn to this issue in insects such as *Drosophila.*

To reduce the likelihood of tissue allometry confounding the interpretation of differential expression analysis, RNA can be extracted and sequenced from divisions of the whole organism,

such as body parts, tissues or even single cells (Sandberg, 2014; Harrison et al., 2015; Parker et al., 2015). Furthermore, it has been shown that while some extent of tissue-scaling relationships are ubiquitous and can influence between-experiment comparisons, normally only large differences in tissue allometry would alter the detection of differential expression, at a standard 2-fold threshold (Montgomery & Mank, 2016). This can be partly accounted for in bioinformatics analyses.

Here I used the power of RNA-seq to describe the transcriptional basis of sex-specific life history responses to long-term nutritional selection. This is a novel study because, although differential gene expression is expected to underlie sexual dimorphism, the genome-wide responses to altered SDL, or to evolutionary manipulation of nutrition, have not yet been measured.

To tackle this question, I used mRNA-seq to investigate the transcriptional changes associated with the evolutionary manipulation of feeding regime, with SDL and divergence in sex-specific fitness responses (Chapter 4). I used the same replicated evolved feeding lines of *D. melanogaster* (Chapters 3 & 4) and predicted signatures of differential gene expression. I reasoned that sex-specific life history responses to nutritional selection could result in changes in gene expression in either coding genes or in regulatory elements that influence gene expression, such as, 5' or 3' UTRs. I predicted that genes or regulatory elements linked with nutrient-sensing pathways (insulin-signalling, TOR), reproduction, ageing, fat production and energy storage (metabolism) would be differentially expressed. I also predicted that the Random lines would exhibit increased sex-biased gene expression, as a consequence of the enhanced SDL found in these lines, in comparison to the Regular lines (Chapter 4). I expected more differential expression in males between the Random and Regular regimes than in females because of the male-specific life history changes observed (Chapter 4).

5.3 Materials and Methods

A new bioinformatics framework with high sensitivity to detect subtle patterns of gene expression was used. The use of RNA-seq also enabled the detection of unknown (un-annotated) transcripts (Marioni et al., 2008; Sultan et al., 2008). The analysis of sequence data followed a rigorous bioinformatics pipeline (Suppl. Mat. Figure S2). A thorough set of quality checks were employed followed by a novel subsampling method for normalisation, before identification of differential expression (Mohorianu et al., in review). The newer methods I employed can provide comparable

or enhanced results in comparison to alternative approaches (Anders et al., 2010; Love et al., 2014; Zhou et al., 2014b).

Sample preparation

The random and regular males and females used for the transcriptional analysis were sampled from the life history experiment described in Chapter 4. The Random and Regular regimes were derived from replicated populations of *Drosophila melanogaster* (wild type Dahomey), maintained in population cages, at 25°C, 50% humidity and 12:12 light dark cycle, on standard yeast agar (SYA) food. Populations were reared under an evolutionary history of food supplied at regular or random intervals (Chapter 4). There were 3 independent replicate population cages for each of the random and regular feeding regimes.

For the life history experiment (Chapter 4), eggs were collected from the cages and reared through 2 generations in a common garden environment (to minimise maternal effects), using a standardised larval density of 150 larvae/SYA vial (as Data Chapter 3). Virgin ('focal') adults were collected upon eclosion (n=30 flies/sex/treatment line), using ice anaesthesia, and mated to standard Dahomey wild type (WT) individuals for 24h at 3 days post-eclosion, then housed in single sex vials (3 flies/vial), on a common garden diet of SYA. Focal adults were mated again, 8 days post-eclosion, as in the initial mating. Focal female and focal male mortalities were checked daily and Kaplan-Meier survivorship curves were plotted.

For the transcriptomics analysis, a sample size of 30 focal individuals/sex/treatment line were flash frozen in liquid nitrogen (in individual Eppendorf tubes), at 10 days post-eclosion (just prior to the divergence of survivorship trajectories between treatments). All samples were flash frozen within 45 minutes of each other at the same time of day (3pm) to control for circadian effects on gene expression. Samples were stored at -80°C, to preserve the RNA.

Each RNA sample contained 30 pooled flies. Flies were divided into separate head/thorax (HT) and abdomen (A) body parts, on dry ice, prior to extraction. The fully factorial design comprised 24 RNA samples of male (M) and female (F) samples from the 3 biological replicates of random (Ra) or regular (Re) feeding regimes, divided into HT and A body parts. Separate body parts were profiled to avoid excessive loss of tissue-specific signal due to swamping.

RNA extraction

Total RNA was extracted from each of the 24 pooled samples, using a miRVana (Ambion) kit, following the manufacturer's instructions (Life Technologies). RNA quality and quantity was assessed using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific). Samples were

also quality controlled, prior to sequencing, by the sequencing provider (BaseClear). All yields were above 100ng/µl in 100ul of RNA storage solution (Life Technologies, AM7000) at absorbance λ = 260. Samples sent for sequencing all had at least 5µg of total RNA, with a minimal volume of 20µl per sample.

mRNA sequencing

RNA libraries were prepared for sequencing using standard Illumina HiSeq2500 and rapid run protocols (BaseClear provider). Directional, single-end sequencing was employed. The 24 RNA samples were run across 6 sequencing lanes, at 4 samples/lane, to yield an expected >30M reads/sample. Indexes were allocated to samples (Suppl. Mat. Table 1). Due to low read numbers, two samples were subsequently re-sequenced. The sequencing method was based on poly(A) selection, to preferentially select only mRNA transcripts and to minimise or exclude all other types of non-coding (nc)RNAs including: small nuclear (sn)RNAs, small nucleolar (sno)RNAs, micro (mi)RNAs, miscellaneous (misc)RNAs, ribosomal (r)RNAs and transfer (t)RNAs.

Bioinformatics analysis

All analyses were done using custom made Perl (Strawberry Perl ver. 5.24.0.1) and R (ver. 3.2.2) scripts.

Quality check

Sequencing data were analysed using a rigorous set of quality check (QC) procedures (DeLuca et al., 2012; Wang et al., 2012) and new approaches (Mohorianu et al., in review), following a thorough bioinformatics pipeline (Suppl. Mat. Figure S2). QC involved assessment of: (i) sequencing depth (read number), the number of unique reads and the complexity (i.e. the ratio of non-redundant to redundant reads; Mohorianu et al., 2011), (ii) nucleotide composition and strand bias, (iii) genome matching and gene (annotation) matching reads (e.g. mRNAs, t/rRNAs, miRNAs, UTRs, introns, intergenic regions), determined using the PatMaN (Pattern Matching in Nucleotide databases; Prufer et al., 2008), and (iv) correlation and Jaccard similarity indices on the original and normalised data (Mohorianu et al., 2011). These checks determined sample quality and identified any sample outliers.

Subsampling normalisation

Gene expression levels were defined as the algebraic sum of abundances of incident reads to each transcript (Mortazavi et al., 2008; Finotello et al., 2014). A novel method of normalisation was used, which combined subsampling (Efron & Tibshirani, 1993) with bootstrap evaluation of the

subsamples (Mohorianu et al., in review), to equalise between-sample variation in read numbers and complexities (ratio of unique to non-unique numbers of reads) and to minimise bias in the detection of differential expression. Normalisation of expression levels minimised technical variation in the data, arising from differences in sequencing depths. This enabled the detection of subtle differential expression between treatments (Mohorianu et al., in review).

Each sample was first subsampled incrementally to investigate the consistency of the subsamples (i.e. whether the subsamples preserved the pattern of the original data, to identify any over-sampled reads, or high levels of noise). If the proportion of redundant, genome matching reads was unchanged after subsampling, for each sample, then technical variation was minimal and we concluded that the subsampling was appropriate. Subsamples were consistent if (i) the proportion of redundant genome matching reads in the original versus subsamples remained constant, and (ii) the average point-to-point correlation of expression across all transcripts remained > 95%. Any outstanding fine-scale variation remaining in the data was then minimised using localised quantile normalisation (Bolstad et al., 2003). For each sex, body part and feeding regime treatment gene expression data were visually inspected, pre- and post-normalisation. The frequency distributions of transcript abundances were represented as box plots, and MA plots showed any differences in gene expression between replicates or treatments as a function of transcript abundance

Hierarchical differential expression

A hierarchical approach was then used to identify where the biggest differences in gene expression lay and to partition the analysis on this basis, i.e. first by body part (HT versus A), then by sex and finally by feeding regime (Random versus Regular). The order of the hierarchy was based on comparing the amplitude of differential expression found in the data, arising from the experimental design (Mohorianu et al., in review). Hence, the largest expression level differences were found between body parts, then sexes and finally between regimes.

The hierarchical differential expression also allowed 'leaky' genes to be filtered out from the data. 'Leaky' genes are those which were expressed at low abundances in the opposite body part to which they normally function in and so represent a 'shadow' of the much higher expression abundances in the 'correct' body part. The division of flies into HT and A body parts, prior to RNA extraction is never 100% efficient, so the high sensitivity of RNA-seq can detect and measure this small amount of tissue leakiness.

Replicate to replicate differential expression was then determined on the non-'leaky' genes, followed by treatment differential expression. A stringent threshold of 1.4 offset fold change

(OFC), with an offset of 20, determined empirically, was used to detect differential expression. The offset was determined from the threshold between noise (for low abundance reads) and signal, and was used to avoid false positives. The efficiency of poly(A) selection during library preparation was not consistent, resulting in varying proportions of ncRNAs, between samples. To minimise bias from this source, all reads mapping to ncRNAs were excluded from the normalization and subsequently from the differential expression analyses.

Functional description of differentially expressed (DE) transcripts

Functional analysis consisted of a description of the list of genes showing DE, based on maximal, non-overlapping confidence intervals.(i.e. intervals created on the minimum and maximum expression levels between the 2 or 3 biological replicates for each treatment).

5.4 Results

Quality checks

Distributions of quality scores for each position on a read (total read length = 50 nucleotides; Suppl. Mat. Figure S3) were narrow and of high quality, indicating good quality scores for all positions for all samples. The slightly lower quality scores for the first 4 nucleotides of reads is a known characteristic of Illumina HiSeq2500 adapters. The proportion of accepted reads, which contained only assigned bases was also high (>99%).

(i) Sequencing depth and complexity

The sequencing depth is defined as the total number of (redundant) reads per sample and per sequencing lane. Samples had sufficient coverage for 4 out of 6 of the sequencing lanes. The distribution of reads across samples in these lanes was close to a random uniform distribution (equal number of reads per sample; X^2 test, results not shown), indicating equal loading of samples to these lanes (Suppl. Mat. Table S2). For the 2 lanes containing samples with insufficient sequencing depth, 2 samples were selected for re-sequencing, also based on complexity scores (as below).

Complexities (i.e. the ratio of non-redundant to redundant reads) were determined (Suppl. Mat. Table S3). Four samples had high complexity values, which were not consistent with those for the other biological replicates. Two samples from these were re-sequenced (Re3FHT, Ra1MHT), resulting in complexity values with considerably improved comparability. These were then included in the differential expression analyses. The other two outliers were excluded (Re1FA,

Re3MA). Complexity values varied between treatments within the expected range for *D. melanogaster* (from 0.20 to 0.29).

(ii) Nucleotide composition and strand bias

Nucleotide composition was represented and evaluated using sequence logos, in which the information content was the log2 probability of occurrence of each nucleotide base at each position (Schneider et al., 1986). These were done across all reads (separately for each sample, Suppl. Mat. Figure S4), for nucleotide distributions for each sample (Suppl. Mat. Figure S5) and for nucleotide distributions for all samples combined (Suppl. Mat. Figure S6).

The expected nucleotide proportions of each nucleotide in *D. melanogaster* are: A/T=0.28 and G/C=0.22. For individual samples (Figure S4, S5) and for all samples combined (Figure S6), we observed some patterns that matched expected nucleotide compositions but also some unexpected features. There was the expected preference for T and C. However, the compositions of G and A consistently differed from the expectation across reads. There was a cyclical preference for G, every 4nt across all samples (Figure S6), the explanation for which is currently unclear.

The presence of variation at the beginning of the reads corresponded to ligation bias, which is usually visible for the first 10-14nt of reads (Figures S4, S5, S6). There was no pronounced pattern in nucleotide composition for the final 38nt positions. Unusual nucleotide compositions could be accounted for by the nature of directional library preparation during sequencing. However, these biases were observed across all samples (tight box plots on Figure S6) and are not expected to have affected interpretation of results. From this assessment, all samples were deemed suitable for subsequent analyses.

(iii) Genome matching and gene matching reads

The matching of the mRNA-seq reads was conducted against the *D. melanogaster* genome v6.09 and the related annotations, full length, allowing 0 mis-matches and 0 gaps. The proportions of genome (transcriptome) and annotations matching reads were assessed to check the usable number of reads, and hence, the reliability of the data. A high proportion of reads matched to the genome (mean % of reads matching to genome: redundant=75%, non-redundant=60%; Table S3), indicating a high sequencing accuracy, across samples. This was also indicated by the proportion of redundant reads being higher than the proportion of non-redundant reads, for all samples (Table S3). The proportions of genome and protein coding genes (mRNA) matching reads were

very similar (mean % of reads matching to the genome versus mRNA: redundant=75%, 75%; non-redundant=60%, 59%; respectively; Table S3, S4), which indicated a high efficiency of poly(A) selection during library preparation, across samples. The proportion of reads matching to most ncRNAs was low, suggesting minimal contamination. The proportion of (redundant) reads matching to rRNA varied from 2-9%, suggesting it could confound the analysis of differential expression, if not filtered out. We observed that rRNAs had been removed with variable efficiency during the poly(A) selection process of library preparation (which preferentially selects for mRNA).

(iv) Jaccard similarity index and point-to-point Pearson Correlation Coefficient (PCC)

Point-to-point Pearson Correlation Coefficients (PCC) and the Jaccard similarity indices were used to make pairwise comparisons between samples and to compare data pre- and post-subsampling normalisation, hence determining the degree of technical variation in the data. The Jaccard similarity index is used as an unbiased quantification of the similarity between samples, enabling determination of whether replicates are more similar than treatments. It is calculated from the number of genes found at the intersection of the 2 samples compared, divided by the number of genes found in at least one of the samples. Comparisons are made between the top 2000, top 1000 or top 500 most abundant genes in each sample, to avoid inclusion of low abundance mRNA expression levels.

Jaccard similarity analysis consisted of 5 main stages. Pre-normalisation, raw reads, samples with the reads of low sequence complexity (containing only 1 nucleotide base for >70% of their sequence) were removed and reads matching to all classes of non-mRNA (including rRNA) were filtered out, for all samples. Subsampling normalisation (at 30M) was first performed on the original dataset (containing all reads), for selected samples. Subsampling normalisation (at 26M) was then performed on the samples without any rRNA incident reads. Next, a localized quantile normalisation was performed on pairs of Ra/Re samples (except for samples Re1FA and Re3MA, which had been excluded, as described earlier), for excluding subtle variation in gene expression.

Jaccard similarity analyses of the filtered data, both before and after subsampling normalisation, showed that there was greater replicate-to-replicate differential expression than treatment to treatment differential expression (data not shown). Pre- and post-normalisation Jaccard similarity indexes varied, for each of the rankings of most abundant genes, suggesting there was some degree of technical variation in the data.

Point-to-point Pearson Correlation Coefficients were calculated via pairwise comparisons of samples, at each nucleotide position across the transcript length, after subsampling normalisation had been conducted at 26M and post-filtering out of reads matching. Pairwise comparisons of the p2pPCC between replicates of the same treatment were made and plotted against the log2 average abundance of each gene (Figure S7). All plots showed the same general trend. As expected, as the overall abundance increased, there was a rapid increase in similarity in expression profiles for every gene between the 2 replicates compared. A PCC value of 0.5 (indicated by a blue line) approximately indicated the noise level, (an average normalized abundance of approximately 16) below which most variation was seen in expression profiles between replicates. Therefore, this value of 16 indicated that an offset fold change (OFC) of 20 would be appropriate for calculating DE and filtering out noise. Average point-to-point correlation was above 95% (upper blue lines, Figure S7) for an average log2 abundance of around 50.

(v) Distributions of abundances before and after normalisation

Pre- subsampling normalisation, the distributions of abundances of transcripts (post-filtering out of non-mRNAs and post-removal of reads with low sequence complexities), between biological replicates for each treatment and between treatments differed considerably (Figure S8). The sexes, in particular, had very different distributions of abundances. This was characterised by narrower boxplots (less variation in transcript abundance) for males than females, and male abdomen samples had clusters of high abundance genes, which were absent from females. Furthermore, there was an interaction between sex and body part. There was a greater difference in the abundance plots between head/thorax versus abdomen body parts in males than in females (Figure S8, top).

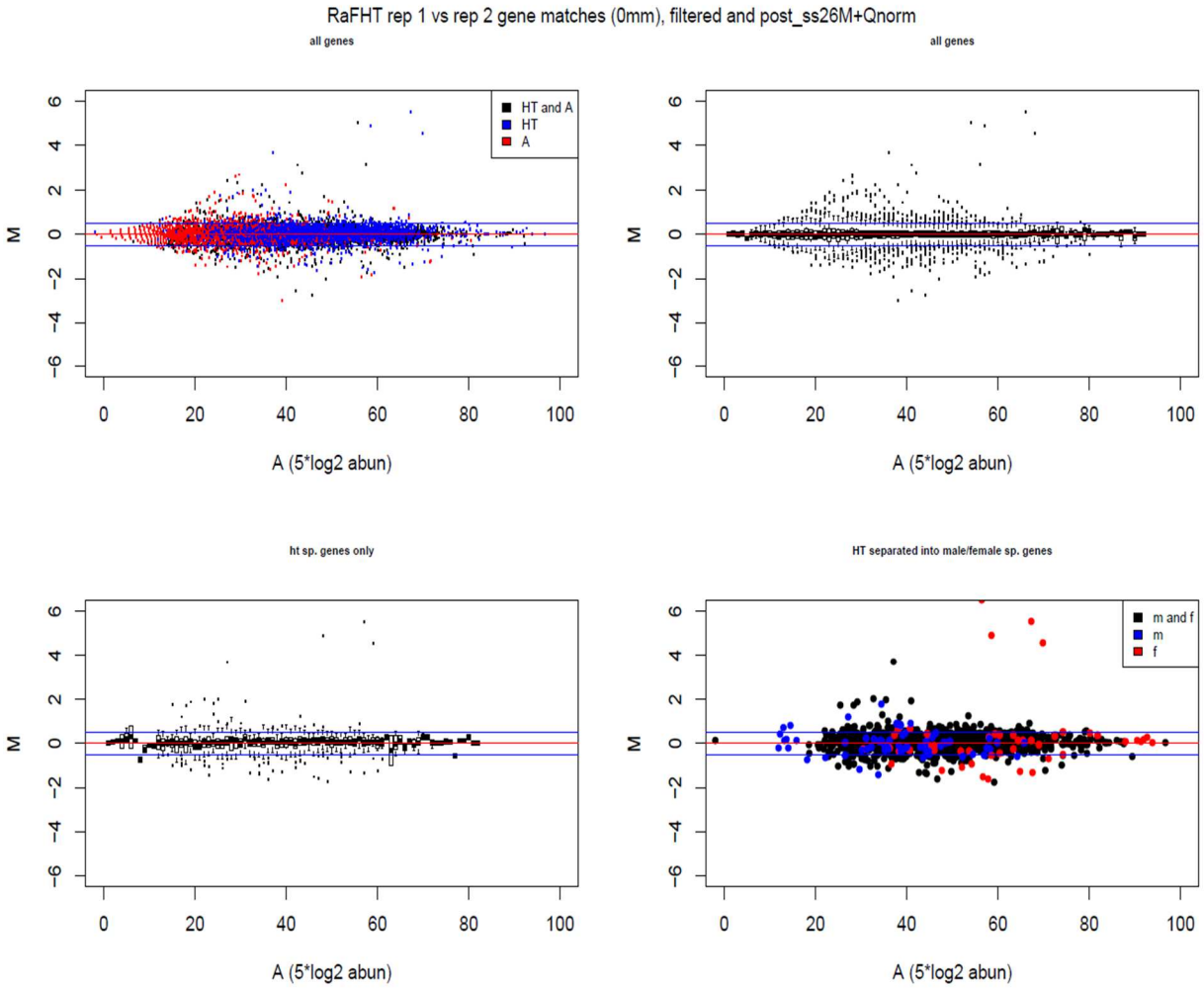
Post- subsampling normalisation, distributions of abundances between biological replicates within each treatment were almost identical (Figure S8, bottom). Pre-normalisation differences between the sexes and the sex x body part interaction effect were still present. The total number of genes pre- and post-subsampling normalisation was similar (pre-normalisation: 15046 genes, post-normalisation: 14988 genes). Therefore, to avoid bias arising from different distributions of abundances, separate quantile normalisations were performed for each sex and for each body part. Pairwise comparisons between the feeding regime treatments were then performed post-quantile normalisation, for each sex and for each body part.

Hierarchical differential expression

MA plots

Post-normalisation, replicate to replicate comparisons of differential expression were conducted (Figure 1A & Figure 1B). Median differential expression centred on the zero line, indicating that the normalisation had been efficient (no average differential expression between biological replicates). Technical variation between replicates had been filtered out and therefore all biological replicates were included in differential expression analyses (except the previously excluded samples). The leaky genes (those mainly expressed in the other body part), were at low abundance, as expected. Differential expression between replicates (deviation from the line M=0; Figures 1A & 1B) was reduced after the leaky genes were filtered out. Patterns of differential expression differed to a greater extent between the sexes for abdomen samples, than for head/thorax samples. The MA plots indicated that hierarchical differential expression required division both into body parts and into sexes, prior to differential expression analysis. MA plots for all samples were similar to the respective plots in Figure 1A and Figure 1B. Differential expression (DE) between biological replicates could mask differential expression between treatments. Therefore the low between-replicate DE observed aided in the subsequent analyses below.

A


RaFHT rep 1 vs rep 2 gene matches (0mm), filtered and post_ss26M+Qnorm
all genes
HT and A
HT
A
M
A (5*log2 abun)
all genes
M
A (5*log2 abun)
ht sp. genes only
M
A (5*log2 abun)
HT separated into male/female sp. genes
m and f
m
f
M
A (5*log2 abun)

B

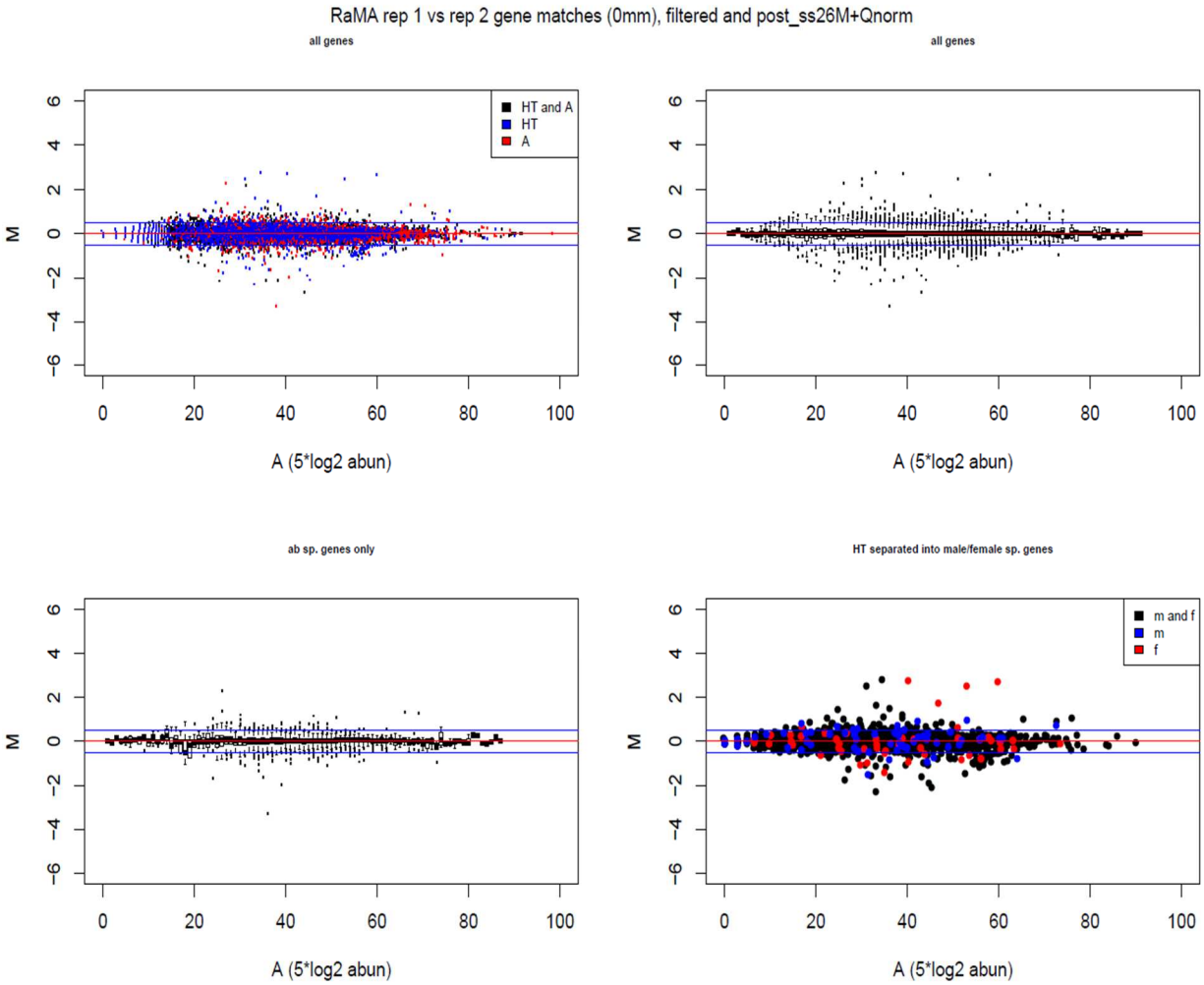


Figure 1. MA plots showing the rep-rep DE (M), with log2 (OFC), against the average abundance (A, on log2 scale), for: (A) a female HT sample (top 4 plot panel, RaFHT) and (B) a male A sample (bottom 4 plot panel, RaMA). All samples (except the 2 excluded samples) were normalised, using the subsampling method, at 26M reads and then subjected to localized quantile normalisation, to remove fine-scale variation. On each MA plot, M (Y axis) is the log2 ratio of abundance (with offset fold change of 20), as an indicator of the degree of differential expression between the 2 samples, and A (X axis) is $\log_2$ average abundance of the 2 samples compared. For each 4 panel plot ((A) and (B)): the top left figure represents the scatter MA plot for all genes expressed in the samples (red=abdomen-specific, A; blue=head/thorax-specific, HT; black=present in both body parts). The top right figure represents the same data as in A but as standard boxplots (for clarity) for all genes, indicating the median and the interquartile range (IQR). A median on the 0 line indicated successful normalisation. The bottom left figure indicates only genes expressed in the body part of interest and the bottom right figure indicates genes only expressed in the body part of interest, separated by sex (male-specific, female-specific and expressed in both sexes). Deviation from the 0 line of differential expression (M) indicate the presence of replicate-replicate differential expression. The numbers of outliers was reduced when leaky genes were removed, indicating

that replicate-replicate differential expression was reduced. There was a reduced separation between the sexes for head/thorax samples and a more pronounced separation between the sexes for abdomen samples.

Structure of hierarchical differential expression

Reads matching to genes were first partitioned by body part (head/thorax versus abdomen versus both), then by sex (male versus female versus both) and finally by feeding regime (Random versus Regular), according to the numbers of genes differentially expressed at each level and the magnitude of the differential expression (according to Figure 2).

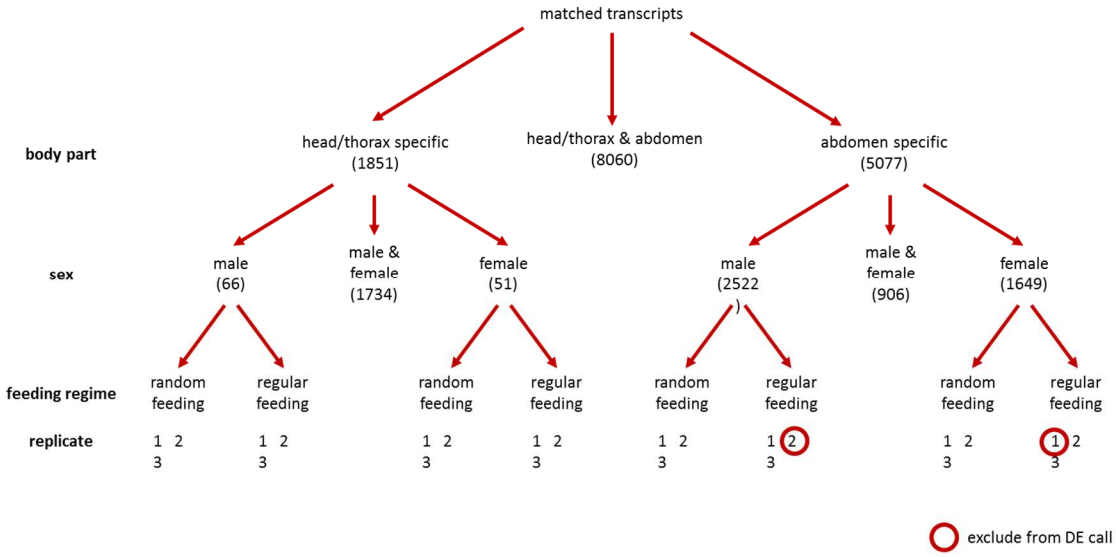


Figure 2. Hierarchy of differential expression. Matched transcripts were separated first by body part, then by sex and finally by feeding regime. Biological replicates 1-3 were nested within each feeding regime. Numbers of genes assigned to each category are indicated in brackets. Two samples (circled) were excluded from differential expression analyses due to low sequencing depths and complexities (as described previously).

Differentially expressed genes

Numbers and functions of DE genes

Genes that were differentially expressed between replicates and between treatments were annotated using FlyBase (http://flybase.org/) and Fly Atlas (Chintapalli et al., 2007) to determine

the functional groups which showed enrichment, for the DE genes, using the AmiGO2 gene ontology database (http://amigo.geneontology.org/amigo)

Replicate-replicate DE

Biological replicate-to-replicate (rep-rep) differential expression (DE) was calculated after removal of reads of low sequence complexity, followed by filtering to remove reads incident not incident to mRNA. Subsampling normalisation was then carried out at 26M reads, followed by localized quantile normalisation (carried out on samples as 4 groups: Ra/ReMHT, Ra/ReFHT, Ra/ReMA and Ra/ReFA), to remove fine-scale technical variation. Samples Re3MA and Re1FA were excluded from the analysis, as discussed previously. Differential expression was called with an offset fold change of 2 and an offset 20. Differential expression between biological replicates was determined separately for genes expressed specifically in the head/thorax (HT), specifically in the abdomen (A) and on genes expressed in both body parts (Table 1).

Much of the differential expression between biological replicates was attributable to heat shock and immune response genes. This is a common feature observed in experimental individuals in response to handling and likely represents uncontrolled variation between treatments.

Treatment	HT-specific/ AB-specific/ both	Functional enrichment of DE genes (p value range obtained from the AmiGO enrichment)
RaMHT	**HT**	Response to heat and bacteria, pheromone binding , nutrient reservoir, protein synthesis (<0.001-0.01)
ReMHT	**HT**	Nutrient reservoir activity, protein synthesis, pheromone binding (<0.001,0.002-0.003)
RaFHT	**HT**	Response to heat and bacteria, pheromone binding, metabolism (<0.001-0.040, 0.023)
ReFHT	**HT**	Response to heat and bacteria, nutrient reservoir activity, pheromone binding, protein synthesis and degradation, metabolism (<0.001-0.030)
RaMA	**A**	Unclassified
ReMA	**A**	Unclassified
RaFA	**A**	Starch degradation, cell structure, starch and lipid degradation (<0.001-0.048)
ReFA	**A**	Cuticle development and body morphogenesis, metabolism, cell structure, homeostasis, starch degradation (<0.001-0.016)
RaMHT	**both**	Unclassified
ReMHT	**both**	Unclassified, response to stress, heat shock and bacteria, protein degradation (<0.001-0.004)
RaFHT	**both**	Neural function and protein degradation (0.002-0.010)
ReFHT	**both**	No DE, protein degradation (<0.001)
RaMA	**both**	Unclassified
ReMA	**both**	Unclassified
RaFA	**both**	Unclassified, protein degradation (0.002)
ReFA	**both**	Cell structure, heat shock, cuticle development, body morphogenesis, protein metabolism (<0.001-0.03, 0.038)

Table 1. Functional enrichment analysis on the functions of genes differentially expressed (DE) between biological replicates for each treatment, after partitioning into body part specific categories

(head/thorax, HT specific; abdomen, A specific; or expressed in both body parts). Treatments consisted of samples from Random (Ra) and Regular (Re) feeding regimes, for both sexes (male, M and female, F), divided into HT and A body-parts. Functions assigned using the AmiGO2 gene ontology database (as described in the Materials and Methods). Functions and p-values in black indicate those above a standard 2fold threshold of DE and functions and p-values in red arise from the annotation of all genes differentially expressed between biological replicates (regardless of magnitude of DE).

Treatment DE

Identification of differential expression between feeding regime treatments (Random versus Regular) was conducted according to the hierarchical design. The magnitude of differential expression between regime treatments was small and subtle (in comparison to rep-rep DE), so differentially expressed genes were those showing non-overlapping maximal confidence intervals. Most DE between treatments was below the 2fold (log2(OFC) <1), or 1.4fold (log2(OFC) <0.5) threshold often used to classify DE. The functions identified in the differential expression between regime treatments are listed below (Table 2).

In the head/thorax body part, only minimal numbers of genes were differentially expressed between Random and Regular treatments (numbers of genes upregulated in Random, Ra relative to Regular, Re regime: females= 1, males=6; downregulated in Ra relative to Re: females=2, males=0). In the abdomen body part, larger numbers of genes were upregulated than downregulated in Random relative to Regular females ('Ra vs. Re female': upregulated=171 genes, downregulated=65 genes); but the reverse was true for males ('Ra vs. Re male': upregulated=149 genes, downregulated=499 genes).

Samples compared	**HT-specific/ AB-specific**	**M-specific/ F-specific/ both (MF)**	**Upreg. or downreg. in Ra versus Re**	**Functional enrichment of DE genes (p value range)**
RaMHT, ReMHT	HT	M	U	Unclassified
RaMHT, ReMHT	HT	M	D	No DE
RaMHT, ReMHT	HT	MF	U	Regulation of muscle contraction, respiration (0.026- 0.032).
RaMHT, ReMHT	HT	MF	D	Response to temperature, cell-signalling (<0.001-0.031).
RaFHT, ReFHT	HT	F	U	Unclassified
RaFHT, ReFHT	HT	F	D	Unclassified
RaFHT, ReFHT	HT	MF	U	Vision and metabolism (0.034-0.046)
RaFHT, ReFHT	HT	MF	D	Sensory perception of sound, homeostasis, cell structure (0.003-0.040)
RaMA, ReMA	AB	M	U	Post-mating behaviour, lipase activity, antioxidant activity, cellular regulation (0.002-0.014)
RaMA, ReMA	AB	M	D	Regulation of: gene expression, protein phosphatase activity, cellular processes, development; metabolism, cell division, cell-signalling, response to chemical stimuli, development (of egg, larvae, pupae, organs), oogenesis (<0.001-0.049)
RaMA, ReMA	AB	MF	U	Protein degradation (0.035)
RaMA, ReMA	AB	MF	D	Unclassified
RaFA, ReFA	AB	F	U	snRNA metabolic process, histone methylation, transcriptional repression, regulation of protein metabolism, female reproduction

				and oogenesis, cell division (mitosis), DNA repair (<0.001-0.047)
RaFA, ReFA	AB	F	D	Nucleic acid binding (0.002)
RaFA, ReFA	AB	MF	U	Protein degradation (0.010-0.049)
RaFA, ReFA	AB	MF	D	Unclassified

Table 2. Functional enrichment analysis of genes differentially expressed (DE) between feeding regime treatments (Random, Ra and Regular, Re), after partitioning by body part (head/thorax, HT specific; abdomen , A specific; expressed in both body parts) and then by sex. Differential expression was determined for all genes from non-overlapping maximal confidence intervals, Functions and p-values were assigned using the AmiGO2 tool against the gene ontology (GO) database (as described in the Materials and Methods). Direction of DE (either up or downregulation) is highlighted for ease of reference.

5.5 Discussion

The evolutionary manipulation of feeding regime can lead to pronounced life history consequences. Replicated selection lines evolved under an unpredictable (Random) feeding regime exhibited a greater extent of sexual dimorphism for lifespan (SDL), than lines evolved under a regime of predictable (Regular) feeding (Chapter 4). Increased SDL was driven by male-specific life history changes, leading to the partial resolution of sexual conflict (Chapter 4). However, little was known about the gene expression (transcriptomic) changes associated with the manipulation of evolved feeding regime, or with SDL. I used RNA-seq to compare gene expression profiles between Random and Regular lines, and to test the prediction that enhanced SDL was associated with increased sex-specific gene expression (e.g. Pointer et al., 2013).

Functional analysis of genes differentially expressed (DE) between Random and Regular feeding regime treatments revealed enrichment in a diverse range of biological functions, including those relevant to the contexts of nutrition, lifespan, reproduction and ageing. I found that genes involved in male post-mating responses (coding for seminal fluid and accessory gland proteins), were upregulated in Random lines over Regular lines. This would be expected as male post-mating responses can influence the egg laying behaviour of their female mates (e.g. Wigby & Chapman, 2005) and we observed greater (early) reproductive output, in the female mates of Random males than Regular males (Chapter 4). In contrast, genes involved in cell division, metabolism and development were downregulated in Random males, which is in agreement with their reduced body size in comparison to Regular males (Chapter 4).

Furthermore, there was a positive association between the direction of expression of specific candidate genes, and previously observed patterns of male lifespan (Chapter 4). For example, the insulin-like peptide 5 (ILP5), a component of the insulin/TOR nutrient-sensing pathways and a determinant of *Drosophila* lifespan (e.g. Teleman, 2010; Partridge et al., 2011; Suppl. Mat. Figure S1), was downregulated in shorter-lived Random males, in comparison to Regular males. Other relevant candidates downregulated in Random males, included methuselah-like 8, which is associated with adult lifespan and responses to starvation, and Indy2 (I'm not dead yet 2), which is associated with the regulation of adult lifespan, obtained from FlyBase annotations. The downregulation, in Random males, of these relevant candidates, known to be involved in dietary and/or lifespan responses, therefore reflected the Random male-specific reduction in lifespan observed in response to the unpredictable evolved feeding regime (Chapter 4).

But DE was not only restricted to coding genes. In addition, we also found expression changes in regulatory elements, between the feeding regime treatments, linked with key biological

processes, including protein metabolism, cell division and development. This suggests that small non-coding RNAs, such as microRNAs, may have been involved in regulating the gene expression patterns observed and could therefore be associated with the nutritionally-induced patterns of SDL.

Surprisingly, there was functional enrichment for histone modification and methylation patterns, which is normally characteristic of epigenetic regulation of gene expression. Epigenetic patterns were upregulated in Random females, in comparison to Regular females. This is interesting as I detected no survival, reproductive or developmental differences between females from Random and Regular feeding regimes (Chapter 4), suggesting other (perhaps metabolic or physiological) differences existed in females between the regimes, which require further investigation. Random females also showed upregulation of genes related to reproduction and DNA repair (maintenance), supporting the absence of a life history trade-off between survival and reproduction found in Random females (Chapter 4).

Together, these results suggest that the life history differences observed between regimes, could have arisen from the combination of differential expression in coding genes, regulatory elements (such as small RNAs) and processes associated with epigenetic modification. Epigenetic changes have previously been implicated in the cross-generational effects of nutrition (e.g. Heard & Martienssen, 2014) and could also be involved in the promotion of nutritionally-induced phenotypic or life history plasticity and thus have relevance in the context of long-term nutritional selection and altered nutrition.

Most sexual dimorphism (SD) is achieved via sex-specific gene expression, to circumvent the constraints placed on the sexes by their shared genome (e.g. Cox & Calsbeek, 2009; Griffin et al., 2013; Perry et al., 2014). However, it was less certain if a positive association existed between the degree of SDL expressed and the degree of sex-specific gene expression. One study provided the first evidence that sexual dimorphism, in the context of the extent of male secondary characteristics expressed, could be linked with sexually dimorphic transcriptomes (Pointer et al., 2013), but far less is known about SDL. In my work, it was difficult to fully interpret sex-specific patterns of gene expression, as the function of all sex-specific genes in the head/thorax body part were unclassified (or there was no differential expression). In the abdomen, however, females had a larger range of functional classes and a greater number of upregulated genes in the Random than the Regular regime; whereas for males, more genes, with a greater range of functions, were downregulated in the Random than the Regular regime. In both cases this could suggest that there was greater sex-specific regulation of expression levels in the Random than the

Regular regime, but not greater absolute expression levels necessarily (particularly as Random males only upregulated around a quarter of the number of genes, than Regular males did). Further investigation would be required to determine the magnitude of sex-specific expression between the regimes.

Despite the increased magnitude of differential expression between replicate lines (biological replicates), within feeding regime treatments, than between the Random and Regular treatments, the functions of replicate-replicate DE were of less biological relevance. For example, the functions of rep-rep DE genes were mainly arose from differential responses to experimental handling (heat shock and immune response genes) so are likely to be less important to the feeding regime-associated life history patterns we observed.

Overall, the powerful technique of RNA-seq. successfully detected subtle signatures of differential expression between regime treatments, demonstrating its suitability for experimental evolution experiments. Furthermore, the rigorous bioinformatics pipeline of quality checks (Mohorianu et al., in review; Suppl. Mat. Figure 2), demonstrated that the data was of high quality, successfully identified samples for re-sequencing and outliers, efficiently filtered out spurious reads, and minimised bias from the sequencing process, so improving the reliability of biological conclusions.

Together, my results add to the emerging discipline of the transcriptomic basis of nutritionally-associated sexual dimorphism for lifespan, on which there has so far been little study. Single-sex transcriptional responses to calorie restriction or post-mating, have been previously investigated (Pletcher *et al.*, 2002; Zhou et al., 2014a). There is also an indication that sex-specific ageing patterns can be detected via transcriptomics (Wilson et al., 2013). However we combined these aspects, to contribute to the poorly studied field of sex-specific responses to nutritional selection and the associated patterns of gene expression. We revealed patterns of gene expression associated with the pronounced sex-specific life history responses to evolutionary manipulation of feeding regime that were previously unknown. This has wider importance for understanding the mechanistic basis of SDL expression, sex-specific fitness and important implications for sexual conflict (Maklakov & Lummaa, 2013).

This study now offers the potential for the further detailed investigation of the differential gene expression patterns between the evolved feeding regime treatments and their associated life history patterns. An important first step would be the qPCR validation of candidate genes associated with the insulin- and TOR-signalling pathways (or with other relevant functions),

identified in the functional enrichment analysis. Novel insights into the extent of sex-specific gene expression, associated with enhanced or reduced SDL, could arise from the analysis or the magnitude of differential expression between the sexes, for each feeding regime. Finally, further investigation of regulatory changes, via the specific extraction and selective sequencing of small RNAs, from all samples, and also further investigation of epigenetic changes; could then reveal a comprehensive view about the mechanistic basis of the life history responses associated with evolutionary manipulation of feeding regime.

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5.7 Supplementary Material

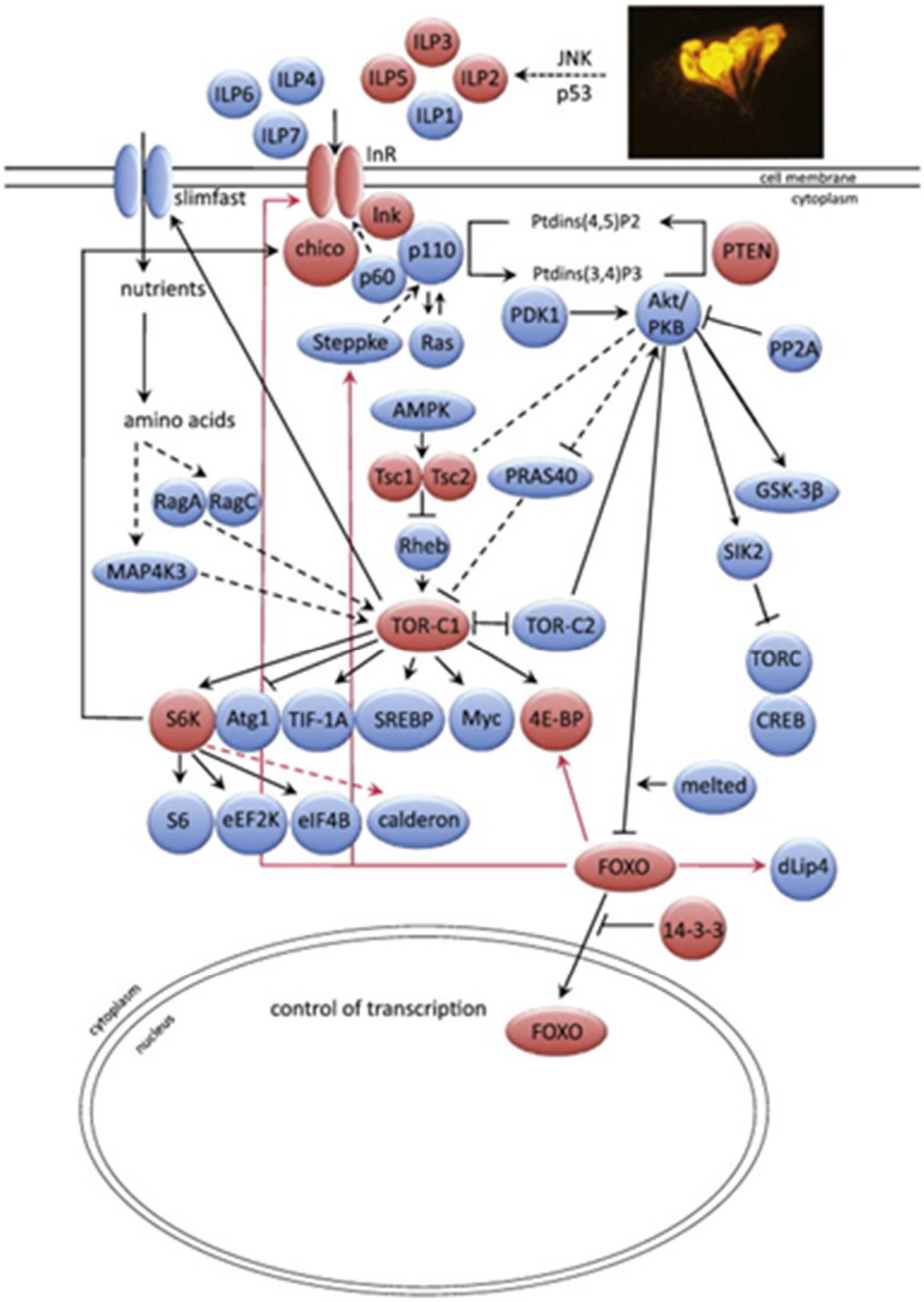

Figure S1. The insulin/TOR (IIS/TOR) signalling network in *Drosophila* (adapted from Teleman, 2010; Partridge et al., 2011). Ovals represent genes. Red ovals represent genes found empirically to be also associated with *Drosophila* lifespan. Arrows indicate direction of activation and red arrows indicate that activation involves transcriptional regulation.

Pipeline for bioinformatics analysis of RNA-seq data

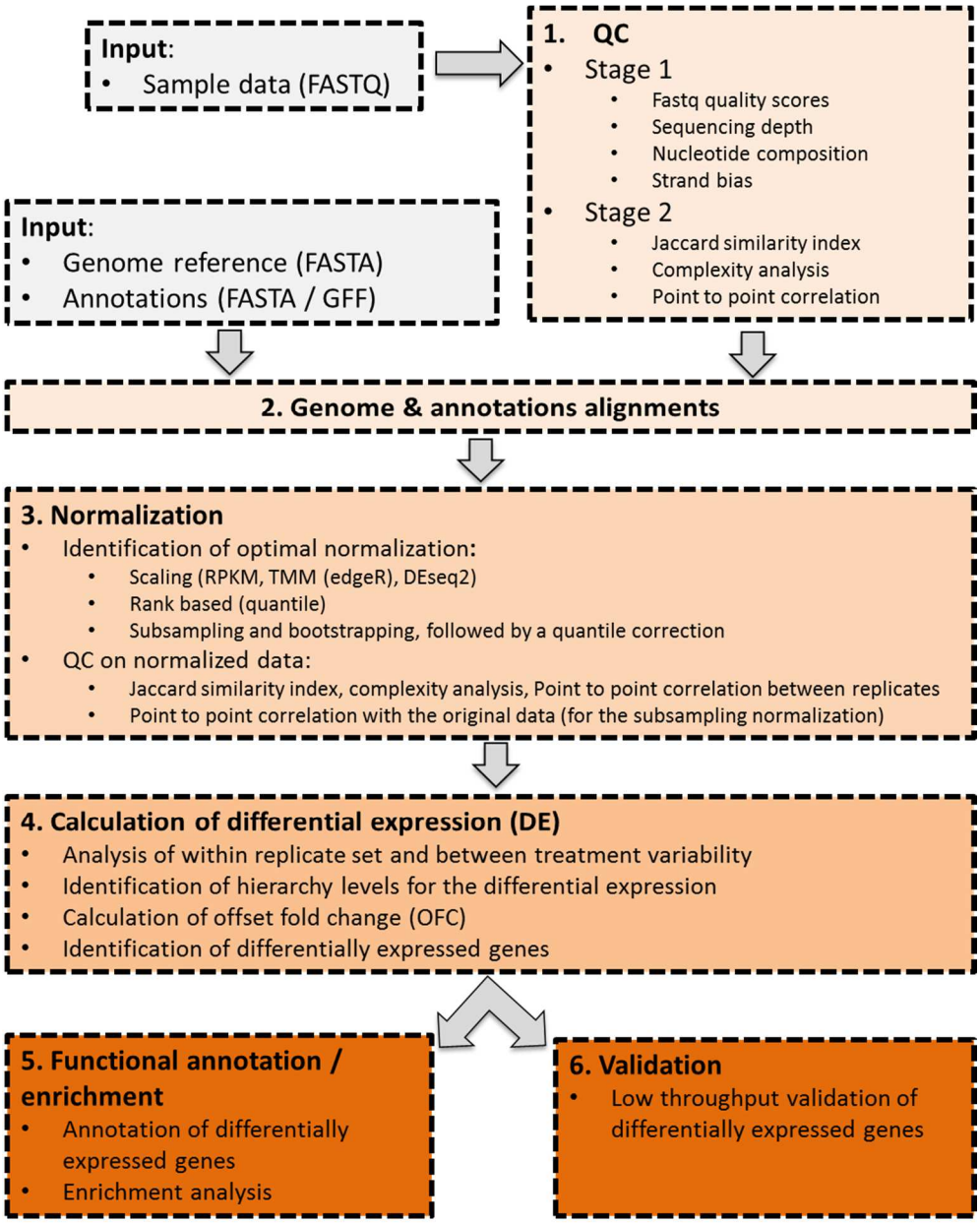

Figure S2. Pipeline for the bioinformatics analysis of RNAseq data (as Mohorianu et al., in review.)

Step 6 (validation) will be conducted in further work.

Sample ID	Index	Feeding regime	Sex	Body part	Biological replicate
Ra1FHT	18 (GCGGAC)	Random	Female	Head/thorax	1
Ra2FHT	18 (GCGGAC)	Random	Female	Head/thorax	2
Ra3FHT	18 (GCGGAC)	Random	Female	Head/thorax	3
Re1FHT	19 (TTTCAC)	Regular	Female	Head/thorax	1
Re2FHT	19 (TTTCAC)	Regular	Female	Head/thorax	2
Re3FHT	19 (TTTCAC)	Regular	Female	Head/thorax	3
Ra1FA	21 (CGAAAC)	Random	Female	Abdomen	1
Ra2FA	21 (CGAAAC)	Random	Female	Abdomen	2
Ra3FA	21 (CGAAAC)	Random	Female	Abdomen	3
Re1FA	20 (GGCCAC)	Regular	Female	Abdomen	1
Re2FA	20 (GGCCAC)	Regular	Female	Abdomen	2
Re3FA	20 (GGCCAC)	Regular	Female	Abdomen	3
Ra1MHT	18 (GCGGAC)	Random	Male	Head/thorax	1
Ra2MHT	18 (GCGGAC)	Random	Male	Head/thorax	2
Ra3MHT	18 (GCGGAC)	Random	Male	Head/thorax	3
Re1MHT	19 (TTTCAC)	Regular	Male	Head/thorax	1
Re2MHT	19 (TTTCAC)	Regular	Male	Head/thorax	2
Re3MHT	19 (TTTCAC)	Regular	Male	Head/thorax	3
Ra1MA	21 (CGAAAC)	Random	Male	Abdomen	1
Ra2MA	21 (CGAAAC)	Random	Male	Abdomen	2
Ra3MA	21 (CGAAAC)	Random	Male	Abdomen	3
Re1MA	20 (GGCCAC)	Regular	Male	Abdomen	1
Re2MA	20 (GGCCAC)	Regular	Male	Abdomen	2
Re3MA	20 (GGCCAC)	Regular	Male	Abdomen	3

Table S1. Experimental design and indexes used for sequencing. Indexes show that reliable de-multiplexing is possible.

Sample ID	Sequencing depth (total read number)	Proportion of lane read total
Ra1FHT	32,643,949	0.18
Re2MHT	31,545,357	0.18
Re3FA	53,374,545	0.30
Ra1MA	60,467,949	0.34
Ra2MHT	39,257,134	0.23
Re1FHT	34,443,704	0.20
Re2MA	39,963,501	0.23
Ra3FA	58,523,239	0.34
Ra3FHT	40,402,570	0.22
Re1MHT	43,452,216	0.24
Re2FA	57,724,894	0.32
Ra3MA	38,647,806	0.21
Ra3MHT	29,412,424	0.19
Re2FHT	41,477,377	0.27
Re1MA	39,822,525	0.26
Ra1FA	41,453,184	0.27
Ra2FHT	28,984,466	0.26
Re3MHT	27,839,495	0.25
Re1FA	24,632,087	0.22
Ra2MA	30,102,196	0.27
Ra1MHT	21,217,563	0.22
Re3FHT	17,897,836	0.19
Re3MA	23,535,444	0.25
Ra2FA	32,380,685	0.34

Table S2. Sequencing depth (total number of redundant reads) across samples and lanes. Horizontal lines indicate the 4 samples allocated to each sequencing lane. Proportion of reads allocated to each sample within a lane are calculated from total number of reads for the respective lane. Samples are derived from the 3 biological replicates for each feeding regime (Random, Ra; Regular, Re), of each sex (F, M) and for each body part (head/thorax, HT; abdomen, A).

	Raw (total) reads			Genome matching reads (Dme v. 6.09)				
Sample ID	**Redundant (R)**	**Non-redundant (NR)**	**Complexity (NR/R)**	**Redundant (R)**	**Non-redundant (NR)**	**% R**	**% NR**	**Complexity (NR/R)**
Ra1FHT	32,508,462	8,191,065	0.25	24,481,356	4,998,048	75	61	0.20
Ra2FHT	28,803,754	7,383,506	0.26	21,653,078	4,536,138	75	61	0.21
Ra3FHT	40,278,286	9,290,771	0.23	30,134,723	5,447,341	75	59	0.18
Re1FHT	34,315,196	8,416,012	0.25	25,705,932	4,999,186	75	59	0.19
Re2FHT	41,370,925	9,308,888	0.23	31,136,789	5,595,022	75	60	0.18
Re3FHT	17,820,077	5,298,861	0.30	13,293,911	3,257,657	75	61	0.25
Re3FHT	35,293,039	8,652,032	0.25	26,024,852	4,834,006	74	56	0.19
Ra1FA	41,346,625	9,748,731	0.24	31,437,860	5,889,608	76	60	0.19
Ra2FA	32,239,035	8,506,289	0.26	24,280,078	5,099,512	75	60	0.21
Ra3FA	58,304,200	12,253,241	0.21	44,126,534	6,977,253	76	57	0.16
Re1FA	24,477,936	7,382,842	0.30	18,599,047	4,689,735	76	64	0.25
Re2FA	57,547,266	11,430,629	0.20	43,671,428	6,558,382	76	57	0.15
Re3FA	53,153,050	11,927,776	0.22	40,408,656	7,018,700	76	59	0.17
Ra1MHT	21,125,711	6,764,016	0.32	15,859,044	4,234,706	75	63	0.27
Ra1MHT	35,375,123	9,790,209	0.28	26,247,122	5,664,266	74	58	0.22
Ra2MHT	39,111,986	10,032,667	0.26	29,313,139	5,960,174	75	59	0.20
Ra3MHT	29,336,642	8,410,074	0.29	22,019,226	5,189,930	75	62	0.24
Re1MHT	43,316,821	10,355,802	0.24	32,535,920	6,103,765	75	59	0.19
Re2MHT	31,410,613	8,533,021	0.27	23,686,357	5,251,373	75	62	0.22
Re3MHT	27,663,650	7,865,475	0.28	20,826,948	4,899,707	75	62	0.24
Ra1MA	60,211,475	12,957,666	0.22	44,901,996	7,526,623	75	58	0.17
Ra2MA	29,913,513	8,146,604	0.27	22,364,510	5,019,208	75	62	0.22
Ra3MA	38,527,116	9,647,776	0.25	28,571,005	5,652,458	74	59	0.20
Re1MA	39,720,925	9,960,546	0.25	29,543,882	5,999,263	74	60	0.20
Re2MA	39,815,822	10,088,830	0.25	29,650,458	5,986,068	74	59	0.20
Re3MA	23,433,499	7,205,712	0.31	17,346,211	4,431,199	74	61	0.26

Table S3. Complexities and % genome matching, for sequenced and re-sequenced samples, derived from non-redundant, unique (NR), and redundant (R) reads. Numbers of redundant and non-redundant reads are shown. Samples are arranged by treatments as in Table S1. Samples in red were highlighted for exclusion, due to high complexity values that deviated from the other replicates for their respective treatments. Two of the four samples highlighted for exclusion were re-sequenced and new complexities determined (purple). Numbers and percentages of redundant and of non-redundant reads matching to the *D. melanogaster* genome (version 6.09) were determined. Total numbers of (raw) reads indicated the

quality of sequencing, and were acquired prior to the removal of low complexity reads and filtering. Numbers of genome matching reads indicated the quality of the RNA extraction.

mRNA matching reads						rRNA matching reads				
Sample ID	**Redundant (R)**	**Non-redundant (NR)**	**% R**	**% NR**	**Complexity (NR/R)**	**Redundant (R)**	**Non-redundant (NR)**	**% R**	**% NR**	**Complexity (NR/R)**
Ra1FHT	24,389,162	4,942,097	75	60	0.20	1,592,522	5,418	4.9	0.1	0.003
Ra2FHT	21,589,461	4,493,264	75	61	0.21	1,031,117	4,723	3.6	0.1	0.005
Ra3FHT	30,045,761	5,390,200	75	58	0.18	1,534,925	4,900	3.8	0.1	0.003
Re1FHT	25,625,358	4,947,820	75	59	0.19	1,419,995	4,674	4.1	0.1	0.003
Re2FHT	31,046,564	5,537,368	75	59	0.18	1,412,724	4,935	3.4	0.1	0.003
Re3FHT	13,256,598	3,231,315	74	61	0.24	800,778	4,693	4.5	0.0	0.006
Re3FHT	25,950,631	4,786,403	74	55	0.18	1,634,557	5,761	9.2	0.1	0.004
Ra1FA	31,365,443	5,846,796	76	60	0.19	1,519,743	4,978	3.7	0.1	0.003
Ra2FA	24,223,300	5,062,589	75	60	0.21	1,017,190	4,391	3.2	0.1	0.004
Ra3FA	44,033,318	6,921,706	76	56	0.16	1,820,729	5,732	3.1	0. 0	0.003
Re1FA	18,560,407	4,663,091	76	63	0.25	792,784	4,325	3.2	0.1	0.005
Re2FA	43,571,504	6,501,088	76	57	0.15	2,162,558	6,999	3.8	0.1	0.003
Re3FA	40,323,255	6,967,721	76	58	0.17	1,807,000	5,196	3.4	0.0	0.003
Ra1MHT	15,782,662	4,183,871	75	62	0.27	1,162,409	4,383	5.5	0.0	0.004
Ra1MHT	26,120,479	5,587,061	74	57	0.21	1,986,117	5,068	9.4	0.1	0.003
Ra2MHT	29,196,681	5,887,358	75	59	0.20	1,672,282	4,782	4.3	0.0	0.003
Ra3MHT	21,927,328	5,128,203	75	61	0.23	1,387,982	4,494	4.7	0.1	0.003
Re1MHT	32,400,413	6,023,409	75	58	0.19	2,442,068	5,514	5.6	0.1	0.002
Re2MHT	23,599,581	5,196,606	75	61	0.22	1,411,371	4,979	4.5	0.1	0.004

Re3MHT	20,744,011	4,843,374	75	62	0.23	1,166,169	4,530	4.2	0.1	0.004
Ra1MA	44,686,546	7,389,416	74	57	0.17	1,350,052	5,954	2.2	0.0	0.004
Ra2MA	22,258,760	4,943,952	74	61	0.22	813,326	4,358	2.7	0.1	0.005
Ra3MA	28,428,574	5,556,072	74	58	0.20	1,133,507	5,550	2.9	0.1	0.005
Re1MA	29,408,054	5,905,198	74	59	0.20	979,119	5,123	2.5	0.1	0.005
Re2MA	29,509,161	5,891,220	74	58	0.20	1,099,022	5,420	2.8	0.1	0.005
Re3MA	17,261,265	4,369,071	74	61	0.25	580,394	4,246	2.5	0.1	0.007

Table S4. Numbers of redundant (R) and non-redundant, unique (NR) reads and proportions matching to annotated *D. melanogaster* mRNAs and rRNAs. Samples in red were highlighted for exclusion, due to high complexity values that deviated from the other replicates for their respective treatments. Two of the four samples highlighted for exclusion were re-sequenced and new complexities determined (purple). Numbers and percentages of redundant and of non-redundant reads matching to genes and rRNA in *D. melanogaster* (version 6.09), were determined. rRNA was drawn from annotation classes of miscRNA, ncRNA and pseudogenes. Reads matching to genes (mRNA), included rRNA (prior to filtering).

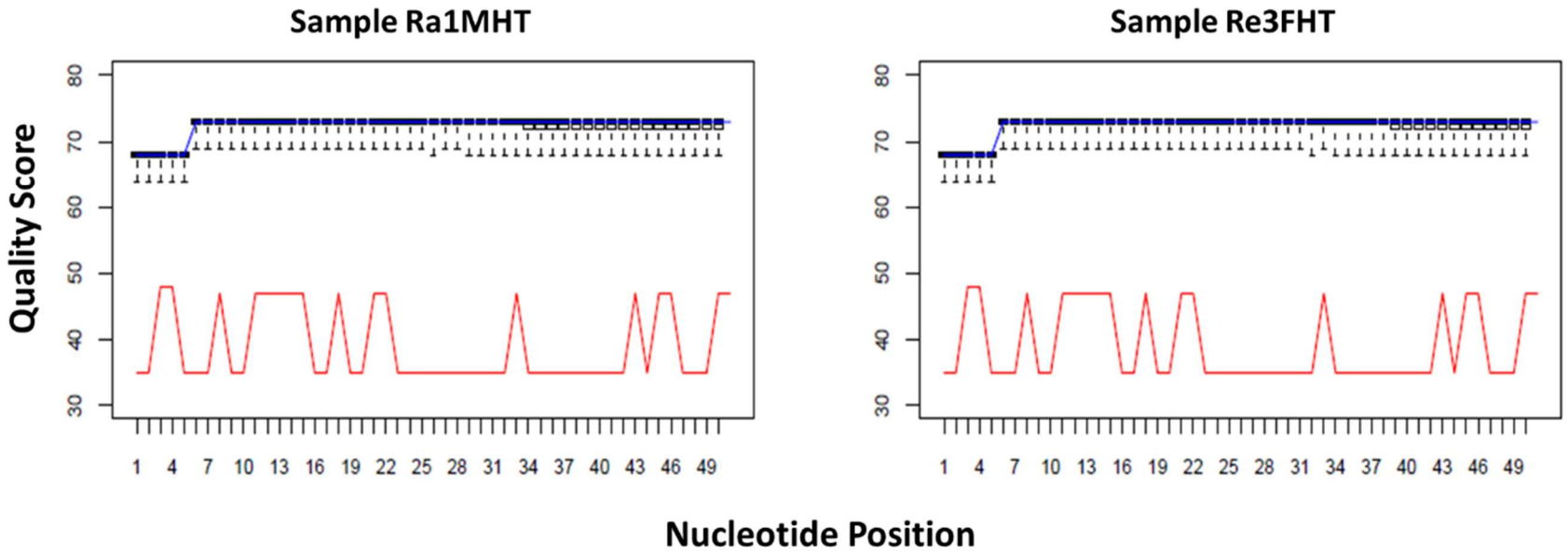

Figure S3. Distribution of quality scores along 51 nucleotide reads for the two re-sequenced samples (Ra1MHT, Re3FHT). Boxplots indicate the interquartile range and whiskers extend to the top and bottom 5% of quality scores at each read position. Blue lines show the maximum and red lines show the minimum quality scores. Quality scores above 60 indicate high sequencing accuracy and scores below 30 indicate low accuracy. Distribution plots for all samples followed an almost identical pattern to these examples.

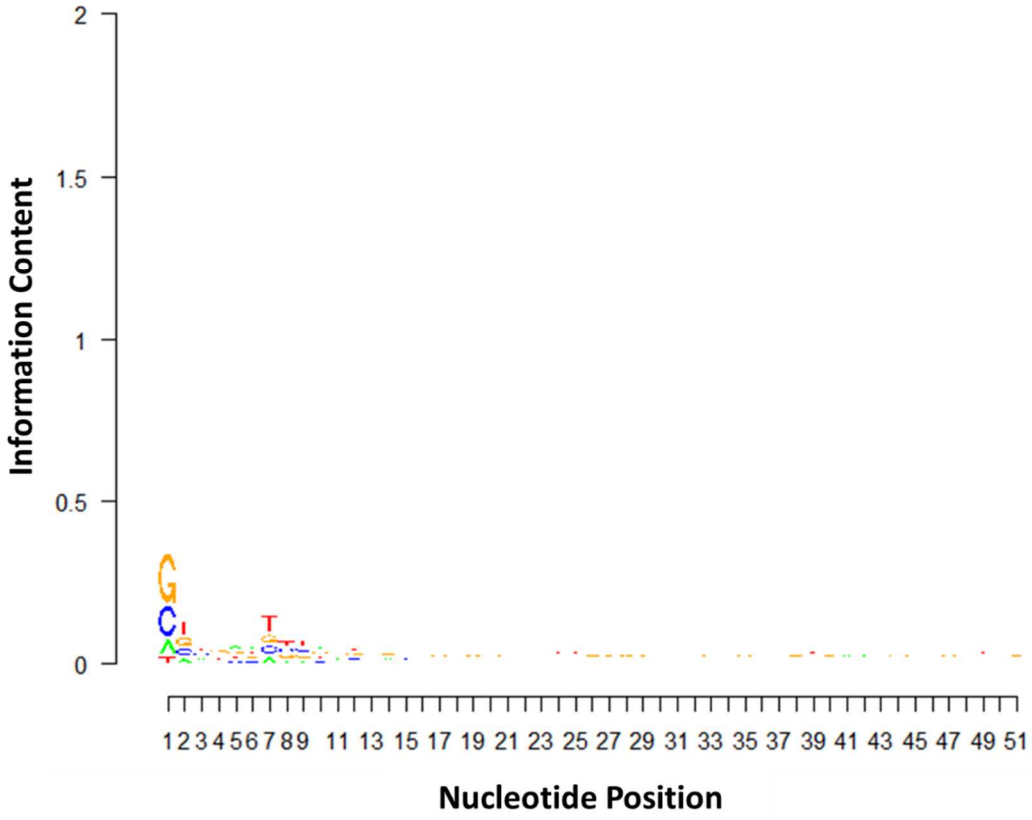

Figure S4. Sequence logo of nucleotide composition across nucleotide positions for all reads of one sample. Information content (log2 probability of occurrence) is a measure of deviation away from an equal proportion of each of the 4 nucleotide bases (G, C, A and T) at each nucleotide position (1-51). An information content of 0 indicates all nucleotides have an equal, 25%, probability of occurrence and a content of 2 indicates all reads have been assigned the same nucleotide base at that read position. All samples followed a similar pattern to this example.

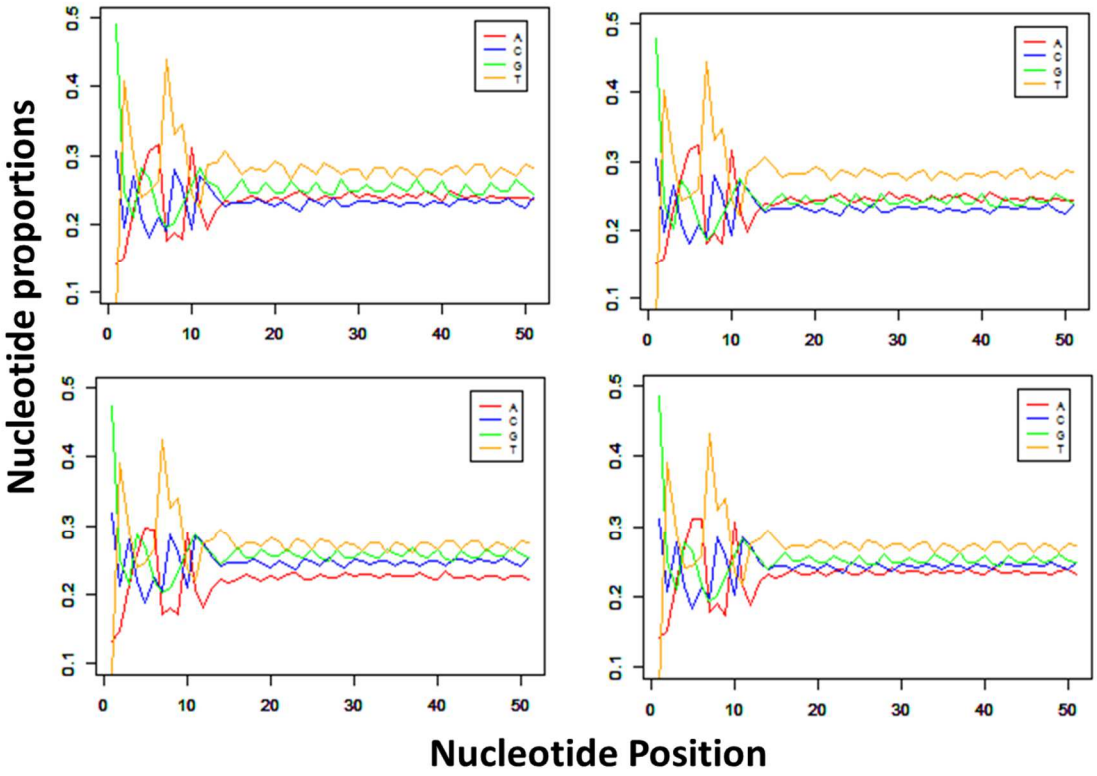

Figure S5. Nucleotide composition for all reads at each nucleotide position (1-51) for 4 samples. Line plots representing the proportions of each nucleotide base: A (red), C (blue), G (green) and T (orange), across all reads, at each nucleotide position. Example samples displayed from left to right, top to bottom are Ra1FHT, Re2MHT, Re3FA and Ra1MA. All samples followed similar patterns to each other, showing some expected ligation bias over the initial ~12-14 nt, which is not expected to have affected subsequent quantification of differential expression. There was little bias after the initial positions. Expected nucleotide compositions for each nucleotide base in *D. melanogaster* are: A/T=0.28 and G/C=0.22 (for further details on comparison to expected patterns, see text).

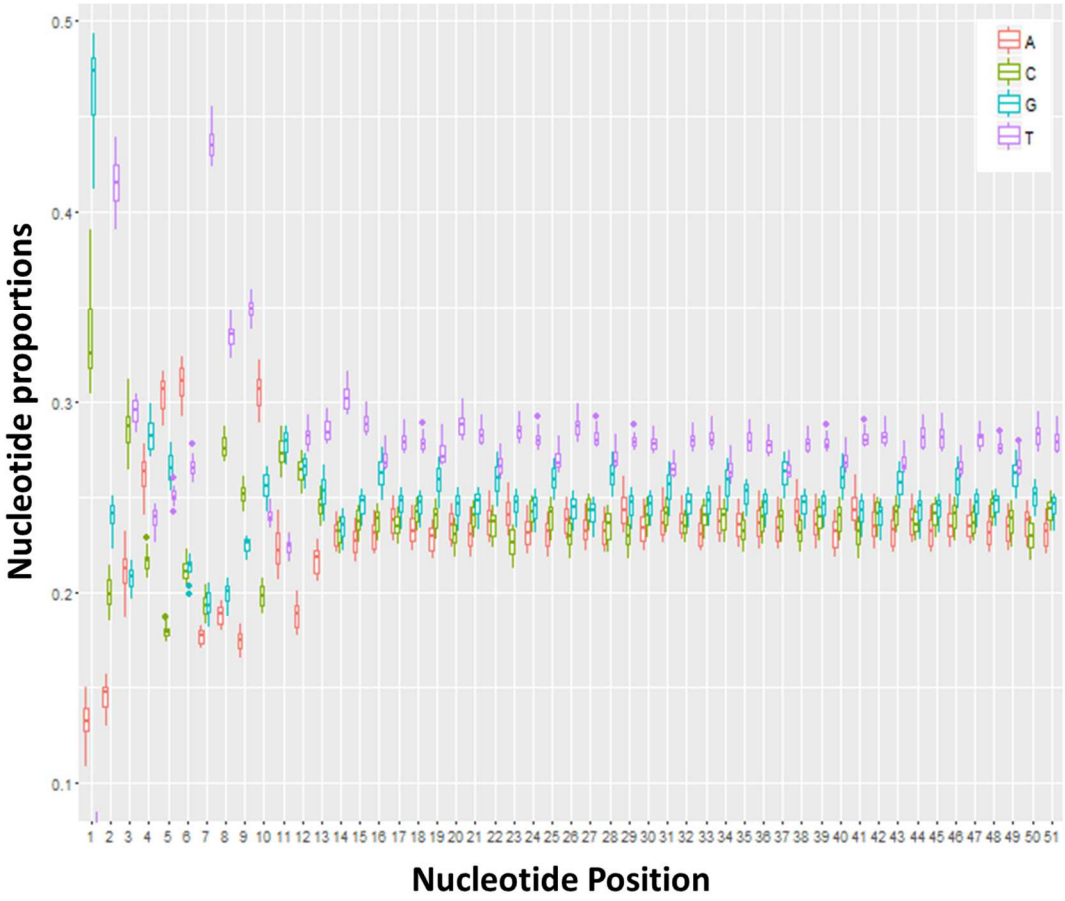

Figure S6. Nucleotide composition for all samples combined, across nucleotide positions (1-51). Proportions of each nucleotide base (A, C, G and T) are expressed on the y axis, against nucleotide position across the read (x axis). Box plots indicate the medians and interquartile ranges, and whiskers extend to the top and bottom 5% of the data, between all samples, at each nucleotide position.

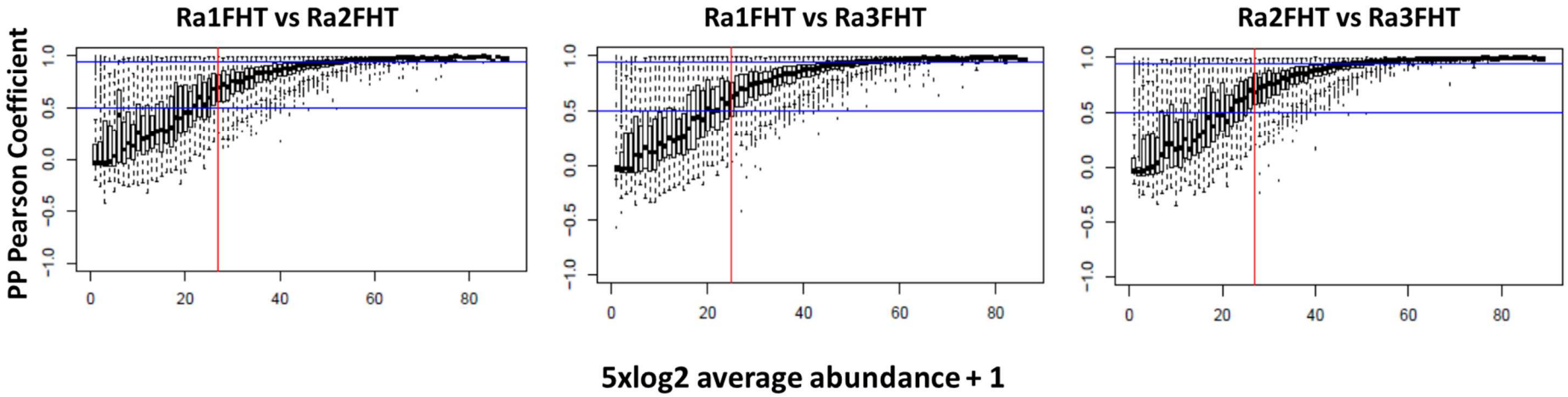

Figure S7. Distribution of point to point Pearson Correlation Coefficient (PCC) against abundance (log2 scale). The point to point PCC indicates the similarity of the expression profiles between pairwise comparisons of biological replicates (illustrated for treatment RaFHT, all treatments followed similar PCC patterns). The transformed average abundances of each gene are shown, after subsampling normalisation at 26M, filtering out of reads matching to non-mRNA (using 0 mismatches) and removing spurious low complexity reads. Box plots indicate the medians and interquartile ranges and whiskers extend to the top and bottom 5% of PCC, for genes, at each average abundance. The blue lines at PCC value of 0.5 indicates the noise threshold level, below which most replicate-replicate variation occurred, for low abundance genes, to the left of the red line (non-transformed abundance of 20, corresponding to the offset fold change of 20 used for calculating differential expression). Average point-to-point correlation was above 0.95 (upper blue lines), for an average log2 transformed abundance of around 50. Low replicability (low PCC) was observed at low abundances, mainly due to the presence of few spurious incident reads and replicability increased with increasing abundance.

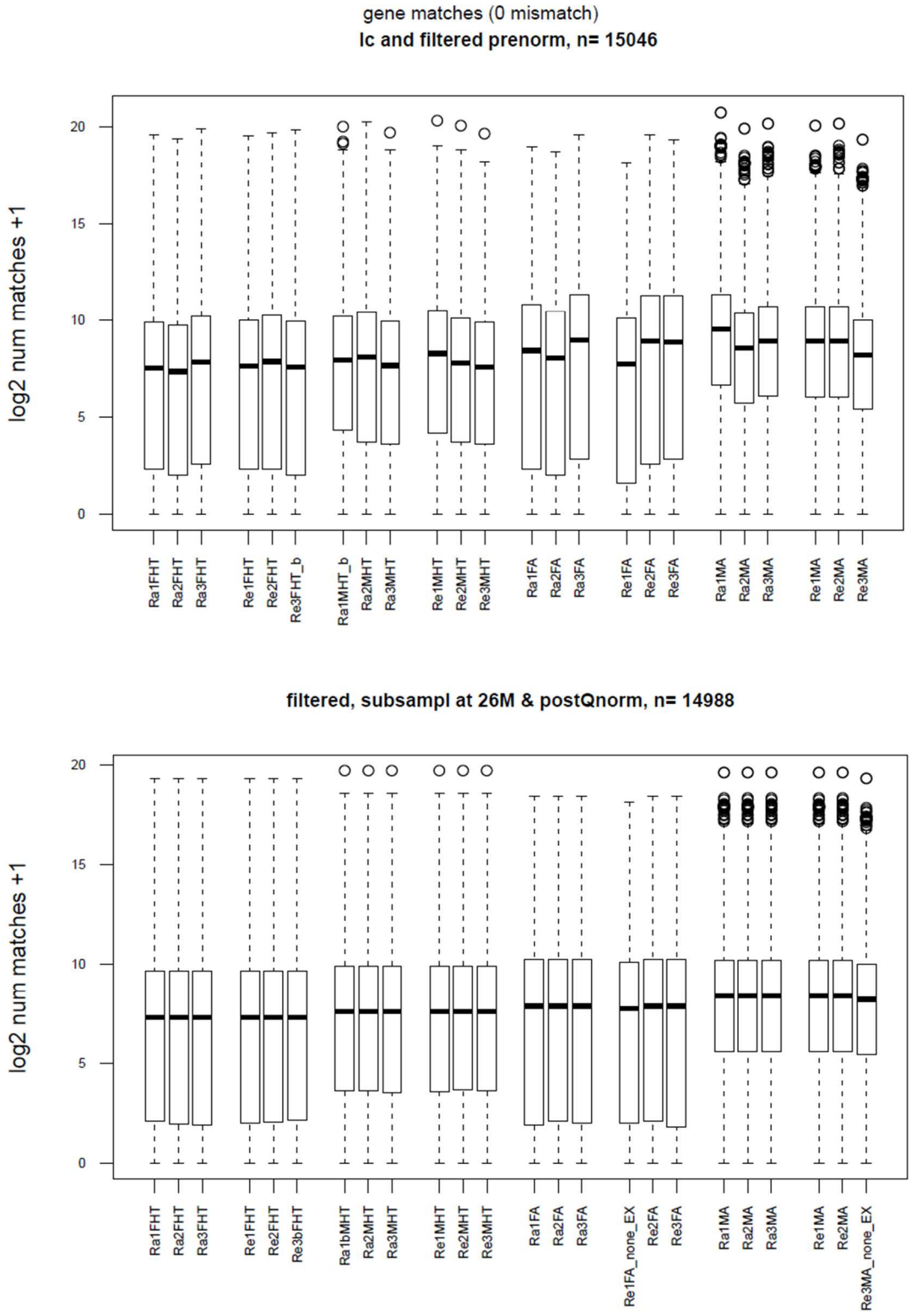

Figure S8. Distribution of transcript abundances pre- (top plot) and post-normalisation (bottom plot). Low sequence (lc) complexity reads, that contained more than 70% of their sequence assigned to a single nucleotide base, were removed. Reads were filtered, to remove those matching to non-mRNA and only reads matching (with 0 mismatches) to genes, were included. The total number of genes before

subsampling was 15406 and the number of quantified genes after normalisation was 14988, so the loss of genes was minimal. Samples marked with ‘EX’ were the excluded from the analysis, as explained previously, based on Tables S2 and S3. Quantile normalization was localised on pairs of Ra (Random)/Re (Regular) feeding regime samples, within sex (male, M; female, F) and within body part (head/thorax, HT; abdomen, A).

Chapter 6: Life history consequences of elevated activity levels in the fruit fly, *Drosophila melanogaster*

6.1 Abstract

Elevated metabolic rate is widely considered to be costly to lifespan. Several studies have shown that increased activity levels (exercise) can increase resting metabolic rate, (energy expenditure while awake but inactive). However, little empirical work has been conducted to test the life history consequences of direct manipulation of activity levels, in a tractable model system. I addressed this omission by developing a technique to consistently and significantly elevate activity levels in the fruit fly, *Drosophila melanogaster* across the lifetime. Exercise was elevated in a quantifiable manner and in a way that did not interfere with sleep. I tested the effect of elevated activity on age-specific survival and reproduction in females within a single generation on a standard food diet. Two replicates of the experiment were conducted. The results showed that there was no detectable effect of elevated activity levels on female survival, reproduction or fitness in comparison to stationary controls. Overall the results showed that elevated activity levels were not always costly to fitness, concluding that the effects of sex and of activity intensity must be considered when determining the life history consequences of activity manipulation.

6.2 Introduction

Unprecedented, long-term changes in modern human lifestyles in westernised countries, such as increasing levels of inactivity and shifts towards more sedentary lifestyles, have raised concerns over the health costs of a greater energy intake than expenditure (e.g. Mokdad et al., 2000; Speakman & Selman, 2003). Energy expenditure while at rest is defined as resting metabolic rate, and across several animal studies, is normally elevated after short-term and long-term increases in activity levels (e.g. Speakman & Selman, 2003). However there has been little work to directly and quantifiably test the consequences of increased activity levels, on life history, in an experimental context. The association between activity and lifespan or ageing is the subject of several complementary and conflicting theories, explored below.

Live fast, die young

There is a long history of “live fast, die young” (rate of living) theories that propose that increased metabolic rates and energy expenditure, such as those associated with increased locomotor activity, should lead to physiological costs and reduced lifespan via physiological and life history trade-offs (Pearl, 1928). These “live fast die young” trade-offs are often associated with early life performance (such as metabolism, early mating and reproduction), at the expense of reduced longevity (e.g. Travers et al., 2015), akin to the life history patterns arising from genes that show antagonistic pleiotropy (Williams, 1957).

Condition dependence

In contrast, theory that is centred on the concept of condition-dependence, proposes that there should be a positive association between metabolic rate and lifespan. Hence high condition individuals would have a high metabolic rate and vice versa. There is some empirical support for this idea. For example, a study in the Glanville fritillary butterfly (*Melitaea cinxia*) identified a positive association between peak metabolic rate and lifespan. This suggested that individuals with a higher metabolic rate were in better condition and more likely to possess adaptations associated with long lifespan (Niitepold & Hanski, 2013). The study also highlighted that the association could have an underlying molecular basis. A polymorphism in the *Pgi* (*Phosphoglucose isomerase*) gene was found to be associated with both metabolic rate and lifespan (Niitepold & Hanski, 2013).

Condition-dependent positive associations between metabolic rate (or activity levels) and lifespan could also be the result of co-evolved adaptations related to both traits. It is also known that increased activity levels can lead to an increased resting metabolic rate (e.g. Speakman & Selman, 2003). However, as the Niitepold & Hanski (2013) study did not directly manipulate activity levels to determine the effect on lifespan, but instead selected individuals that naturally differed in their activity levels, it is possible that both traits arose from co-evolved adaptations or condition. Several confounding factors could therefore have influenced the association between metabolic rate and lifespan, in part coevolving alongside the association. To tease apart these effects, activity manipulation needs to be conducted within a single generation, to reducing any potential confounding effects influencing lifespan.

Variance of activity levels with age, as a biomarker for ageing

In addition to general positive or negative associations of activity or metabolic rate over the whole lifespan, a growing body of literature is now focusing on how activity levels may vary over an individual's lifetime (e.g. Koh et al., 2006; Koudounas *et.al.*, 2012). In this context, activity is often treated as a covariate or a response variable in the analyses, to determine, for example, whether certain activity patterns could act as a signature, or biomarker, of biological ageing (Koudounas *et.al.*, 2012). Many studies measure the levels of "spontaneous locomotor activity" (SLA) (e.g. Le Bourg et al., 1984; Koudounas et al., 2012) in the absence of external perturbations (such as those which could be induced by experimental manipulation).

Not all studies have found consistent patterns of age-related activity variance within a single species, or indeed any variation in mean activity levels (measured as SLA) over lifetime in either sex (e.g. in the fruit fly, *Drosophila* melanogaster, Le Bourg et al., 1984). This is surprising given that sleep and activity patterns are governed, in part, by the circadian rhythm and would be expected to be closely associated with age (Koh et al., 2006). It is likely that consistent patterns were perhaps obscured by between-individual and between-population variation in SLA, or perhaps also due to the insufficient frequency of activity observations (e.g. Le Bourg et al., 1984).

Other factors affecting activity levels

Genetic correlations between SLA levels and lifespan could be one explanation for between-individual, between population and between-study variation in patterns of activity level variance with age. For example, Fernandez et al. (1999) found activity levels to vary across age, sex and genetic line in *D. melanogaster*. This highlighted the potentially important role of genetic background and sexual dimorphism in lifespan and activity patterns and suggested that in this context, activity could be used as a biomarker for ageing. Direct genetic manipulation experiments have also indicated an underlying genetic basis associated with activity and lifespan. For example, in *D. melanogaster*, the absence of the mitochondrial heat shock protein, Hsp22, led to a 40% reduction in lifespan and a 30% reduction in locomotor activity, in comparison to those individuals expressing Hsp22 at normal levels (Morrow et al., 2004). However, genetic correlations between SLA and fecundity or SLA and lifespan are not always found (e.g. Le Bourg et al., 1984).

Age-related activity patterns can also be influenced by life history or environmental factors. For example, in *Drosophila melanogaster*, mating reduces female daytime quiescence (inactivity) by 70% during the middle of the light (active) cycle following transfer of the seminal fluid protein, sex

peptide (SP) from males (Issac et.al., 2010). This highlights that mating environment can be an important determinant of activity levels and hence have a direct or indirect influence on lifespan.

Many dietary manipulation studies consider that the effects of locomotor or feeding activity could influence the life history consequences, or ageing patterns observed. Even in a human context, the importance of both diet and activity (exercise) are frequently considered simultaneously, in the context of healthy ageing. However, surprisingly little empirical work has been done to address the role of altered activity patterns on ageing trajectories and life history traits, via the direct manipulation of activity levels. This omission was tackled here.

Manipulation of activity levels

A good and reliable method of manipulating activity levels to result in consistent and sustained differences is key to any direct study of the consequences of variation in activity on lifespan. One study used activity manipulation in mice with pre-symptomatic Huntingdon's Disease, by introducing voluntary exercise with a running wheel, although no effect on lifespan was found (Potter et.al., 2010). The study was complicated by the inability to directly quantify or control the level of use of the running wheel. Hence levels of activity and lifespan patterns could not be directly correlated. It is also possible that in this system, enhanced activity levels were not necessarily associated with increased lifespan or increased fecundity, despite the expected positive correlations arising from individuals in good condition (e.g. as asserted by Le Bourg et al., 1984)

Measurement of activity levels

To quantify the effect of direct manipulation of activity levels on lifespan, a robust measurement of locomotor activity levels was required. In *Drosophila,* activity level measurement is commonly conducted via continuous recording of the number of crosses of an infrared light beam per 1-5 minute interval, with a Trikinetics *Drosophila* Activity Monitor (as in Koudounas *et.al.*, 2012). Quantitative measures such as total activity, average activity and total sleep during night and day can then be determined from this set up.

However, several studies have highlighted complications of measuring female activity levels using this method. Female reproductive activity, such as egg laying, could interfere with the measurement of activity levels (as if females are moving around on the food, then they are not crossing infrared beam of light). This explains why studies of female locomotor activity are often

lacking from the literature, and why several previous studies have assayed only males (Chiu et.al., 2010; Koudounas et.al., 2012). This is problematic, as locomotor patterns can be sex-specific (e.g. Fernandez et al., 1999; Issac et al., 2010) and hence not necessarily general. For example, male but not female *D. melanogaster* have an increased period of inactivity (sleep) around midday and early afternoon, on 12:12 light:dark cycles, which is around 2.5 times the length of that observed in females (Issac et al., 2010). Therefore, further studies in females or in both sexes simultaneously are required to capture the fitness consequences of natural variation in activity patterns.

Knowledge gap

There has been little empirical focus to date on the direct effects of activity on lifespan in *Drosophila* or in the healthy individuals of other species. Little work has directly manipulated activity levels, accurately or quantified elevated activity levels and measured the resulting life history consequences, within the same study. Furthermore, many studies highlight the need for more empirical work in this area (e.g. Le Bourg et al., 1984) and especially for females.

I aimed to address this omission by directly manipulating and quantifying activity levels and then measuring the life history consequences of elevated activity levels in female *D. melanogaster*. Tracking over a single generation avoided the potential confounds of co-evolved, condition-dependent adaptations (as highlighted by Niitepold & Hanski, 2013). Females were used, to gain an initial indication of possible associations and to enable a logistically more straightforward quantification of reproductive output. I also aimed to address the lack of females used in locomotor studies (Chiu *et.al.,* 2010; Koudounas *et.al.*, 2012).

I first designed an experimental method that consistently and robustly elevated activity levels across the lifespan in comparison to stationary controls with normal levels of activity. Activity levels were quantified via direct observation in frequent spot samples of behaviour, to avoid difficulties associated with the indirect interpretation of behavioural patterns obtained from Trikinetics equipment (e.g. Koudounas *et.al.*, 2012). I then measured survival and reproduction responses to the elevated versus normal activity level treatments.

I predicted that increased activity levels would reduce lifespan, in line with the general theoretical assumption that a high metabolic rate and energy expenditure is associated with the physiological cost of a reduced lifespan via life-history trade-offs. I also predicted that females with increased activity levels would have reduced reproductive output as a result of the allocation of less of their limited time budget to egg laying.

6.3 Materials and Methods

6.3.1 Pilot manipulation of activity levels

Pilot work was first conducted to design a method of manipulating the activity of female fruit flies (*Drosophila melanogaster*) that consistently elevated activity levels during the active light phase each day and across their lifespan. The aim was not to interfere with the inactive (sleep) phase (dark cycle). Activity levels were defined as the proportion of individuals moving in spot samples of behaviour. A higher level of activity referred to more time spent moving (more individuals moving). Methods tested for inducing higher activity included a tilting plate (high and low tilting speeds were compared), a rocker and roller (Stuart Digital Rocker & Roller SRT6D: 6 roller version: simultaneously rotated and tilted the vials, several speeds tested) and varying container size used to house flies. I predicted that activity would be increased in a large relative to a small container as individuals had to travel further to reach the food.

The only manipulation which consistently and robustly elevated activity levels, when compared with controls was the high speed (70rpm) tilting plate (pilot data not shown, patterns as Figure S2). The tilting mechanism exploited the natural negative geotactic behaviour of flies (to climb or fly upwards), in order to increase activity levels.

6.3.2 Experimental individuals

Female fruit flies used for activity and lifespan assays were derived from a large population cage of wild type (WT) Dahomey (Dah) *Drosophila melanogaster*, reared on standard (SYA) food and maintained at 25°C, 50% relative humidity and a 12:12h light:dark cycle. Eggs were collected on a yeasted red grape juice agar oviposition plate (for 15h). First instar larvae (n=1100) were transferred to SYA vials at a controlled density of 100 larvae/vials. Upon eclosion, adults were maintained in larval vials for 2 days, to allow all females to be mated, before collecting the mated females (n=64) for the experimental set-up, which was achieved using light CO_2 anaesthesia. All females were therefore age-matched (to within 24hours) to control for any age-associated differences in sleep/activity patterns (Koh *et.al.,* 2006).

6.3.3 Manipulation of activity levels for life history assay

Two activity level treatments were established: ‘enhanced activity’ versus ‘controls’. Mated females were randomly allocated to treatments (n=32 females/treatment) and individually-housed in vials containing standard (SYA) food. ‘Enhanced activity’ vials were taped to the tilting plate in a standard vertical orientation. ‘Control’ vials were taped to paper on the nearby bench

(no external locomotor source), in an identical orientation to the vials on the plate (Suppl. Mat. Figure S1). The activity of controls was therefore akin to the "spontaneous locomotor activity" measured in other studies (e.g. Le Bourg et al., 1984; Koudounas et al., 2012).

The tilting plate was switched on for 2 separate 4h periods (periods (9:30am-1:30pm; 2:00pm-6:00pm, inclusive) during the light cycle of the 12h light: 12h dark cycle lighting regime. This timing avoided disruption of sleep during dark periods, minimised disruption of feeding immediately preceding lights on or lights off and minimised habituation to motion. Increased activity is believed to accompany 'dawn' (just before and just after lights on) and 'dusk' (around lights off) (as Chiu *et.al.*, 2010). Vials were undisturbed during the period (1:30pm-2:00pm) that the plates were switched off as flies are reported to be inactive during this period (reviewed by Issac et al., 2010).

Vials were set-up and the tilting plate was switched on at 4 days post-eclosion. The first activity measurements were recorded at 5 days post-eclosion, so that the initial disturbance of set-up did not confound the recording of activity levels. Activity treatments were maintained for the entire lifespan of the females.

6.3.4 Measurement of activity levels

Activity levels were measured via spot sampling of all individuals every 20 minutes over 2 x 3hour periods when the tilting plate was switched on (10:00am-1:00pm, 2:30-5:30pm; inclusive). This was done across 7-8 observation days during the first month of adult life. This allowed some of the variation in activity levels with age to be captured (Le Bourg, 1987). The first sampling time was 30minutes after the plates were switched on at 9:30pm, to minimise confounding effects from initial perturbations (from the plate switch on). During each spot sampling period, the number of individuals engaged in four discrete behavioural categories was recorded (from a single scan of all vials). The categories were: motionless on food (including feeding), motionless on wall (including grooming), walking and flying. Categories were determined from pilot observation of the most common forms of behaviour exhibited by individually stored females. The proportion of individuals moving (walking or flying) was determined for each time point on each observation day.

6.3.5 Life history assay

Using the same females from the activity assays, I also recorded daily mortalities, to determine age-specific survival. Weekly egg counts were also taken from the once-mated females from both activity treatments. Egg vials were saved for offspring emergence and the recording of offspring

counts. From these weekly egg and offspring counts, I determined the age-specific reproductive responses to consistently increased activity levels, versus controls. SYA food vials were changed 4 times per week, prior to the period when the tilting plate was switched on (before 9:30am).

Two identical replicates of the activity and life history assay were independently conducted, to check for repeatability. The only difference between the time replicates was the tilting plate used to enhance activity levels. For the first time replicate, an older tilting plate was used until 12 days post-eclosion (including the first 3 activity observation days). This was switched to a newer tilting plate for the remainder of the first replicate (due to technical problems with the older plate). The new plate was used for the entirety of the second replicate. Both tilting plates were set to 70rpm and all other factors were equal.

Many studies of locomotor activity exclude females, as their egg laying behaviour makes it difficult to monitor activity levels. However, direct observation of flies here meant that activity levels could still be recorded, even if the females did not cross the centre of the vial. This allowed me address the relative knowledge gap on female locomotor activity (in fruit flies).

6.3.6 Statistical analyses

All statistical analyses were performed in R version 3.2.1 (R Core Development Team, 2015).

Activity Analysis

The proportion of individuals moving (walking or flying) at each timepoint, for each observation day, was calculated for both activity treatments ('enhanced activity' versus 'control'), for the 2 time replicates of the experiment. An index of mean proportion moving per observation day was calculated across timepoints per day, for each activity treatment and for each time replicate. Data from each time replicate of the experiment was analysed separately. Differences between the activity treatments in the proportion moving across observation days, was analysed using generalised linear mixed effects models ('glmer' function from the 'lme4' package) with binomial errors. Activity treatment was fitted as a fixed effect and days post-eclosion as a random factor. The data were overdispersed in all cases. To account for this, an observation level random effect was added to each 'glmer' model (the log-normal Poisson distribution). Model comparison was conducted with 'anova' likelihood ratio tests.

Survival Analysis

Survival analyses were performed using Cox Proportional Hazards regression analysis, on age-specific mortality data, separately for each time replicate of the experiment. All age-specific mortality data satisfied the proportional hazards assumption of Cox analysis, for both graphical and analytical tests. A Cox model was fitted using the 'coxph' function from the 'survival' package. Individuals that were lost or died during experimental manipulation, were treated as censors in the Cox model. Activity treatment ('enhanced activity' versus 'control') was fitted as a categorical factor. Model comparison was conducted as before.

Age-Specific Reproduction Analysis

Age-specific egg counts and offspring counts were analysed using generalised linear mixed effects models ('glmer' function from the 'lme4' package), to account for the temporal pseudoreplication arising from taking repeated counts from the same individuals over time. The experimental replicates were analysed separately. Poisson error structure was used for count data. Egg count or offspring count was the integer response variable. Activity treatment was fitted as categorical fixed effect. The number of days post-eclosion each count was taken was fitted as a continuous random effect and a unique identifier assigned to each individual was also fitted as a random effect.

Overdispersion was accounted for by adding an observation level random effect to each 'glmer' model. Maximum likelihood model comparison showed that this provided best model fit and accounted for zero-inflation in the dataset. Model reduction was conducted from a maximal model, to result in the minimal model containing only the significant terms.

Egg to adult viability was calculated as the proportion of eggs laid that hatched as viable offspring at each timepoint. Proportion data were arcsine transformed and then analysed with a glmer, with Gaussian errors from the 'lme4' package (same output as lmer).

Lifetime Reproduction Analysis

Lifetime offspring production, also termed lifetime reproductive success (LRS) was used as a measure of fitness. Indices of total lifetime egg production and total lifetime offspring production were calculated separately for each individual from each activity treatment population and each time replicate by summing weekly 24h egg or offspring counts, respectively, across the lifetime. Lifetime egg and offspring production data were non-normal in most cases, so the non-parametric Mann-Whitney U test was used to compare median egg and offspring production values between activity treatment populations for each time replicate. For data that did satisfy the normality

assumption, the results from a Student's two sample t-test matched those from the non-parametric Mann Whitney U test, in terms of degree of non-significance, so the non-parametric tests were reported in all cases, for comparability between analyses. The non-parametric tests were also more conservative, so reduced the likelihood of type 1 errors.

6.4 Results

Female Activity Levels

The 'enhanced activity' treatment led to significantly greater proportion of females observed moving across days, in comparison to the stationary 'controls'. This was found in both the first (glmer: $z=3.293$, $p<0.001$; Figure 1A) and the second (glmer: $z=7.951$, $p<0.001$; Figure 1B) replicate experiments. Increased activity levels were also consistent across all time points within each observation day (for example, see Suppl. Mat. Figure S2). The only exception was the negligible effect of the 'enhanced activity' treatment on movement, between 5 and 12 days post-eclosion, in the first replicate (Figure 1A) when an older tilting plate was used. This plate was switched to a newer tilting plate after 12 days post-eclosion, for the first replicate and used for the entirety of the second. It was observed that the newer tilting plate had a spontaneous judder every few minutes, which may have helped prevent habituation to motion.

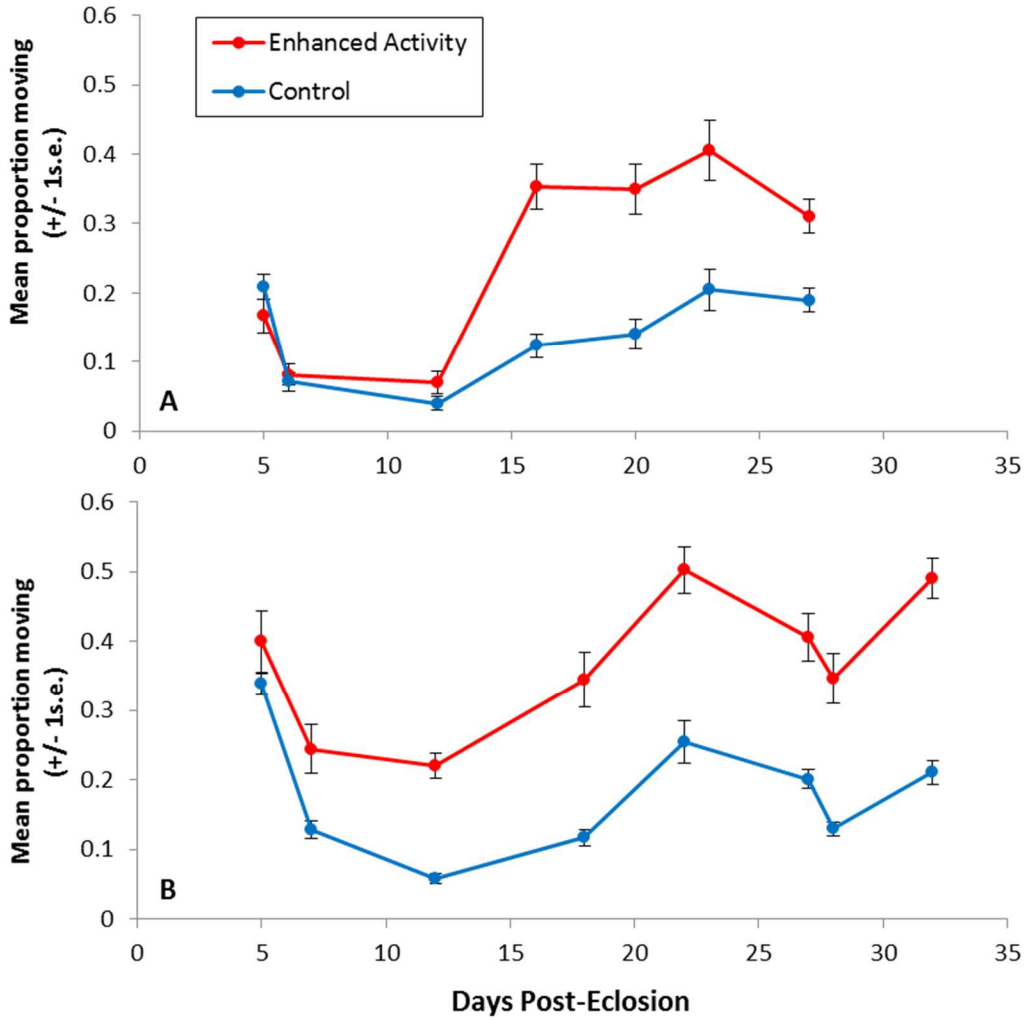

Figure 1. Mean proportion of females moving, against days post-eclosion, across activity level treatments (enhanced activity, control), for: (A) first replicate and (B) second replicate. Means were calculated across time points, for each observation day. Movement was classified as walking or flying. The tilting plate used to enhance activity levels was switched from an older plate to a newer plate, after 12 days post-eclosion for the first time replicate (A) and the same new tilting plate was used for the second time replicate (B). Initial n=32 individually-housed females/activity treatment/time replicate. Time replicates ran independently and concurrent to the respective life history assay (same flies used to monitor activity levels and life history parameters).

Female Survival

There was no significant difference in female survival, between the 'enhanced activity' and 'control' treatments, for either the first (coxph: z=0.433, p=0.665; median lifespan = 65days, 67days, respectively; Figure 2A), or the second replicate (coxph: z=0.009, p=0.993; median lifespan = 69days, 67days, respectively; Figure 2B). Consistently raised activity levels therefore had no significant effect on female survival, relative to stationary controls.

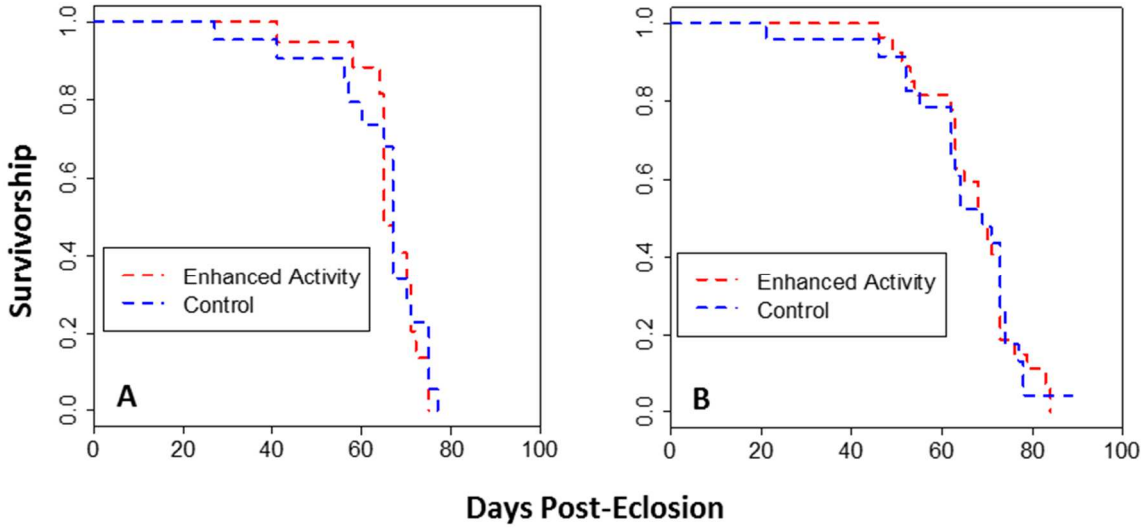

Figure 2. Age-specific survivorship against days post-eclosion, across activity level treatments (enhanced activity, control), for: (A) first time replicate and (B) second time replicate. Activity levels were enhanced using a tilting plate and compared to stationary controls. Time replicates were conducted independently. Initial n=32 individually-housed females/treatment/time replicate.

Female Reproduction

There was no significant effect of activity treatment on female age-specific egg or offspring production, over the lifetime, for either the first (glmer: z=0.178, p=0.859, Figure 3A; z=0.146, p=0.884, Figure 3B) or the second replicate (glmer: z=0.992, p=0.321, Figure 4A; z=0.911, p=0.362, Figure 4B). Egg production and offspring production both significantly declined with age for both activity treatments ('first replicate' glmer: z=8.532, p<0.001, Figure 3A; z=7.658, p<0.001, Figure 3B; 'second replicate' glmer: z=10.049, p<0.001, Figure 4A; z=12.042, p<0.001, Figure 4B).

Egg to adult viability was also not significantly affected by activity treatment ('first replicate' glmer: t=1.061, d.f.=1, p=0.287, Figure 3C; 'second replicate' t=0.722, d.f.=1, p=0.475, Figure 4C) and declined significantly with age ('first replicate' glmer: t=9.57, d.f.=1, p<0.001; 'second replicate' t=15.73, d.f.=1, p<0.001).

Total lifetime egg production and total lifetime offspring production did not differ significantly between the 'enhanced activity' and the 'control' treatments, for either the first (Mann-Whitney U test: 'eggs', W=558.5, p=0.536; median=24, 24, respectively; 'offspring', W=606.5, p=0.199; median=10, 2, respectively), or the second replicate (M-W U test: 'eggs', W=451, p=0.417; median=56, 67, respectively; 'offspring', W=475.5, p=0.629; median=21, 23, respectively).

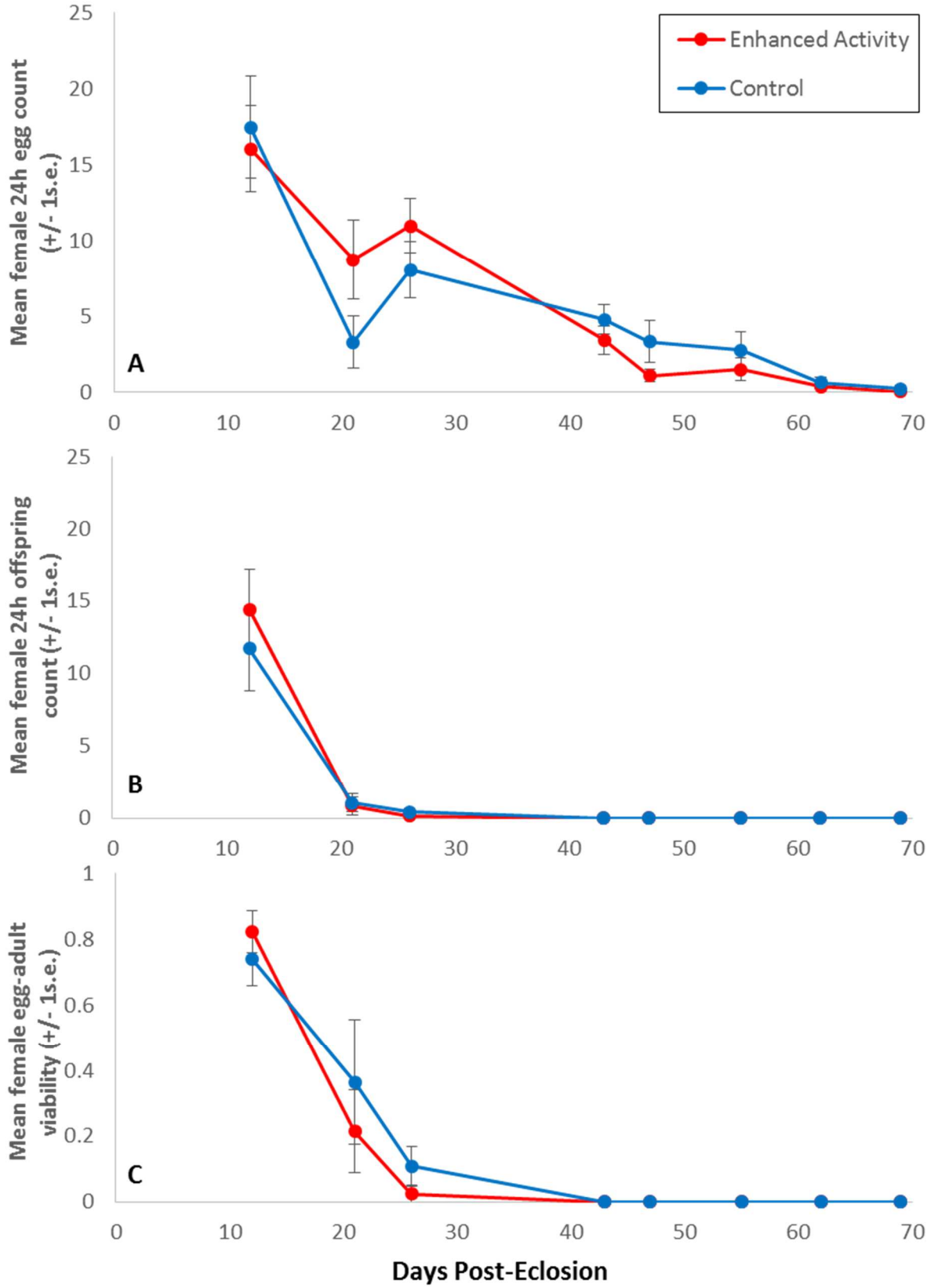

Figure 3. Mean focal female egg production (A), offspring production (B) and egg to adult viability (C), per female, per 24h against days post-eclosion, for the two activity level treatments (enhanced activity, control), at the first time replicate. Initial n=32 individually-housed females/treatment. Egg to adult

viability is defined as the proportion of eggs which eclosed as adults (C). Error bars display +/- 1 standard error.

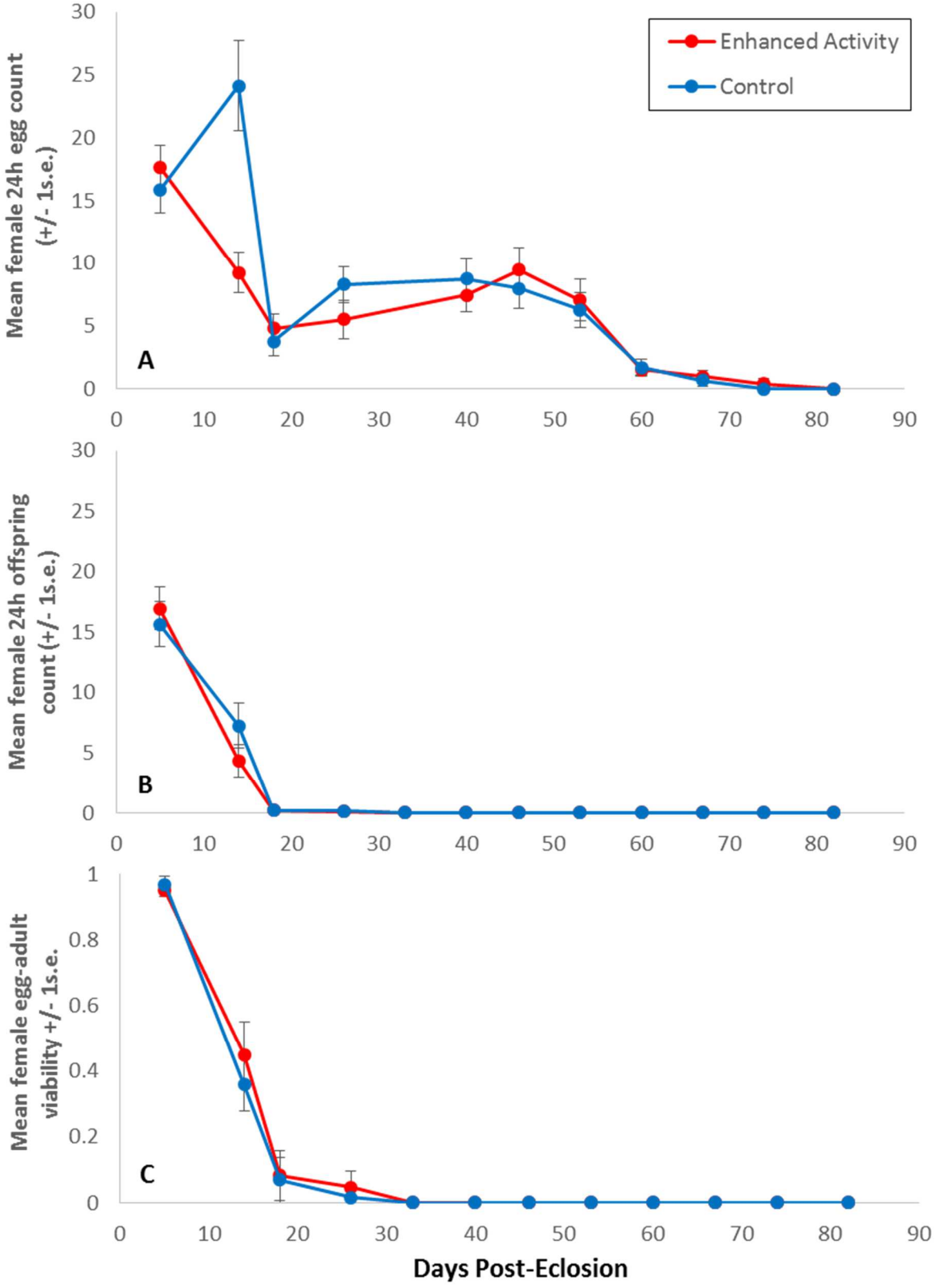

Figure 4. Mean focal female egg production (A), offspring production (B) and egg to adult viability (C), per female, per 24h against days post-eclosion, for the two activity level treatments (enhanced activity, control), at the second time replicate. Initial n=32 individually-housed females/treatment. Egg to adult

viability is defined as the proportion of eggs that eclosed as adults (C). Error bars display +/- 1 standard error.

6.5 Discussion

High metabolic rate and energy expenditure are widely regarded to be costly to lifespan and fitness, as predicted by "live fast, die young" life history theories (Pearl, 1928). Conflicting theories suggest the importance of condition-dependence in determining whether the association between activity levels or metabolic rate and lifespan will be positive or negative (Niitepold & Hanski, 2013). However, there has been a considerable lack of empirical studies that directly and quantifiably manipulate activity levels and directly measure the age-specific consequences on lifespan and reproduction, particularly in females. Here I addressed this omission by directly elevating activity levels, quantifying the degree of activity elevation and measuring the consequences for female survival and reproduction, using the fruit fly, *Drosophila melanogaster*.

I found that direct, consistent elevation of activity levels over the entire lifetime had no effect on female survival, age-specific reproduction, lifetime reproduction or fitness. This result was independently replicated across two identical time replicates of the experiment. The absence of survival and reproductive responses to consistently elevated activity was contrary to predictions that increased activity levels would be associated with reduced lifespan.

Predictions had been derived from "live fast, die young" life history theory that proposes a negative association between metabolic rate (or energy expenditure) and lifespan (Pearl, 1928). It was therefore assumed that elevated activity levels would lead to a sufficient increase in metabolic rate or energy expenditure to have negative consequences for lifespan (but see Vaanholt et al., 2009). It is possible however, that although activity levels were consistently and significantly elevated compared with controls, the extent of activity elevation was below the threshold for which life history costs would be manifested. This is akin to the idea that increased activity (or exercise) can be beneficial or neutral to fitness, up until levels where high intensity activity can be detrimental.

Furthermore, I found that increased movement was not correlated with reduced egg laying, as had been predicted. This either suggests that increased activity did not reduce the time allocated to egg laying, or that most egg laying occurred during the period when the tilting plate was switched off. It is known that most feeding occurs during the period just surrounding lights on and lights off, when the tilting plate was also switched off (Chiu *et.al.*, 2010; Issac et al., 2010). Hence,

it is possible that female egg laying also peaked around this time, as both factors are correlated. This could explain the absence of reproductive effects from elevated activity outside of the period of peak egg laying.

Two other studies which also attempted to determine the lifespan effects of directly manipulating activity levels in mice, also found no lifespan effects of elevated activity (Potter et.al., 2010; Vaanholt et al., 2010). The studies supplied exercise wheels to either healthy mice, or those with pre-symptomatic Huntingdon's disease and measured their lifespan, compared with controls. It is uncertain the degree of activity level elevation experienced by individuals in the Potter et al. (2010) study, or whether the effect of carrying the disease may have confounded lifespan patterns. It is clear that more work is required on the lifespan effects of direct manipulation of activity regime, in healthy individuals, to determine the universality of conclusions.

The lack of life history consequences arising from variation in exercise could potentially be explained by sex-specific effects of elevated activity. Variation in activity levels with age have previously shown sex-specific patterns in *Drosophila* (e.g. Fernandez et al., 1999; Issac et al., 2010). It possible that lifespan consequences of elevated activity levels may have been absent in females but present in males. This is thought to be unlikely, however, given the degree of non-significance between activity level treatments observed for females. My study successfully addressed the knowledge gap on females (as highlighted by Chiu *et.al.,* 2010; Koudounas *et.al.*, 2012), by directly studying them here.

I also found fluctuation of activity levels with age, during the first month of lifetime (approximately one third to one half of total lifespan), for which I observed patterns of activity (e.g. Koh et al., 2006; Koudounas *et.al.*, 2012). I also found a decline in activity levels with age, as expected (e.g. Koh et al., 2006; Koudounas *et.al.*, 2012), but see Le Bourg et al. (1984). In my study, activity levels might act as a biomarker for ageing (as Koudounas *et.al.*, 2012).

In summary, I conducted an empirical test of the age-specific life history consequences of consistently elevated activity levels, in female fruit flies. I showed that activity levels could be effectively and consistently elevated and directly quantified, via direct observation over frequent spot sampling periods. Contrary to predictions, I showed that consistently elevating activity levels over lifetime did not lead to costs for lifespan, reproduction or fitness, when compared to controls, for the extent of activity elevation applied; but there was a decline in activity with age, as predicted. Together, the results suggest that the association between activity and lifespan could be dependent on context (e.g. sex and activity level used).

This study now offers the opportunity for an extended investigation of both sexes simultaneously, to determine whether life history consequences of activity manipulation are sex-specific. Novel perspectives could also arise from using a range of levels of activity manipulation (e.g. greater tilting speeds), to determine the effects of a higher intensity activity regime. This could allow potential identification of thresholds or tipping points in the association between activity and lifespan (whilst in the context of biological reality). It is possible that energy expenditure on activity could be costly to other life history components. Further study of the interaction between proximate diet manipulation and activity manipulation and the life history consequences of this interaction could reveal important insights. Finally, testing the effects of activity manipulation on individuals of both sexes from the Random and Regular lines, could reveal the implications of nutritional evolutionary history, on the life history consequences of altered activity levels.

6.6 References

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6.7 Supplementary Material

Experimental Set-Up for Activity Manipulation via Tilting Plate

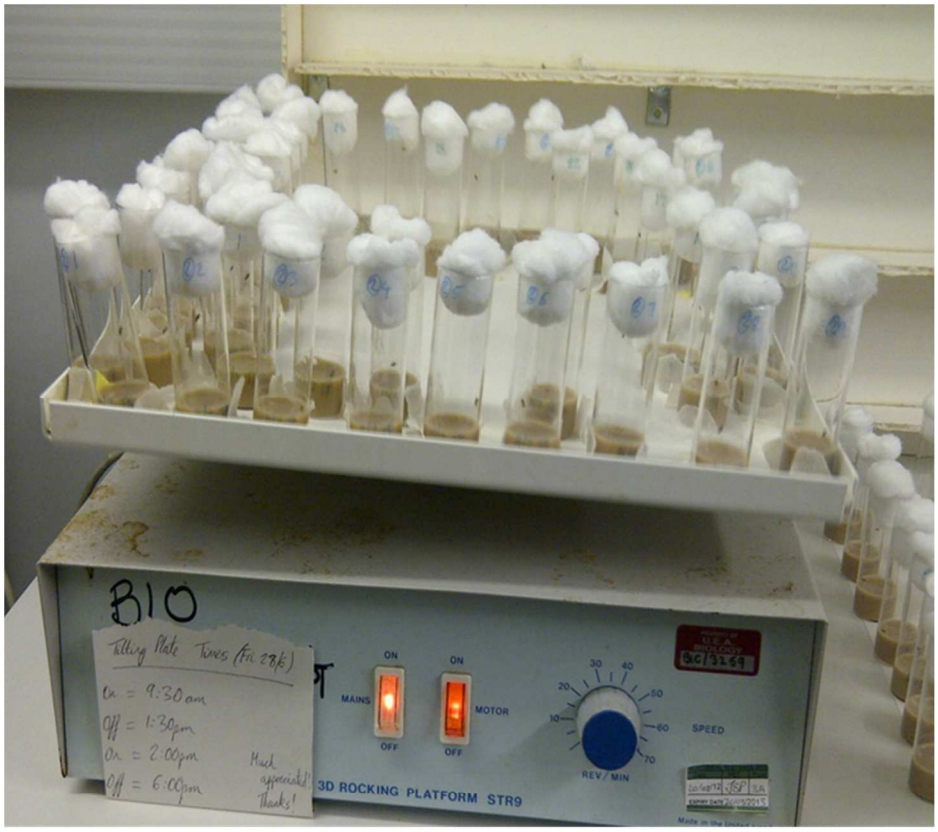

Figure S1. Experimental set-up of vials for the 'enhanced activity' (above) and the 'control' (below) treatments. Female flies were individually-housed in SYA vials. Tilting plate used to enhance activity levels was set at 70rpm.

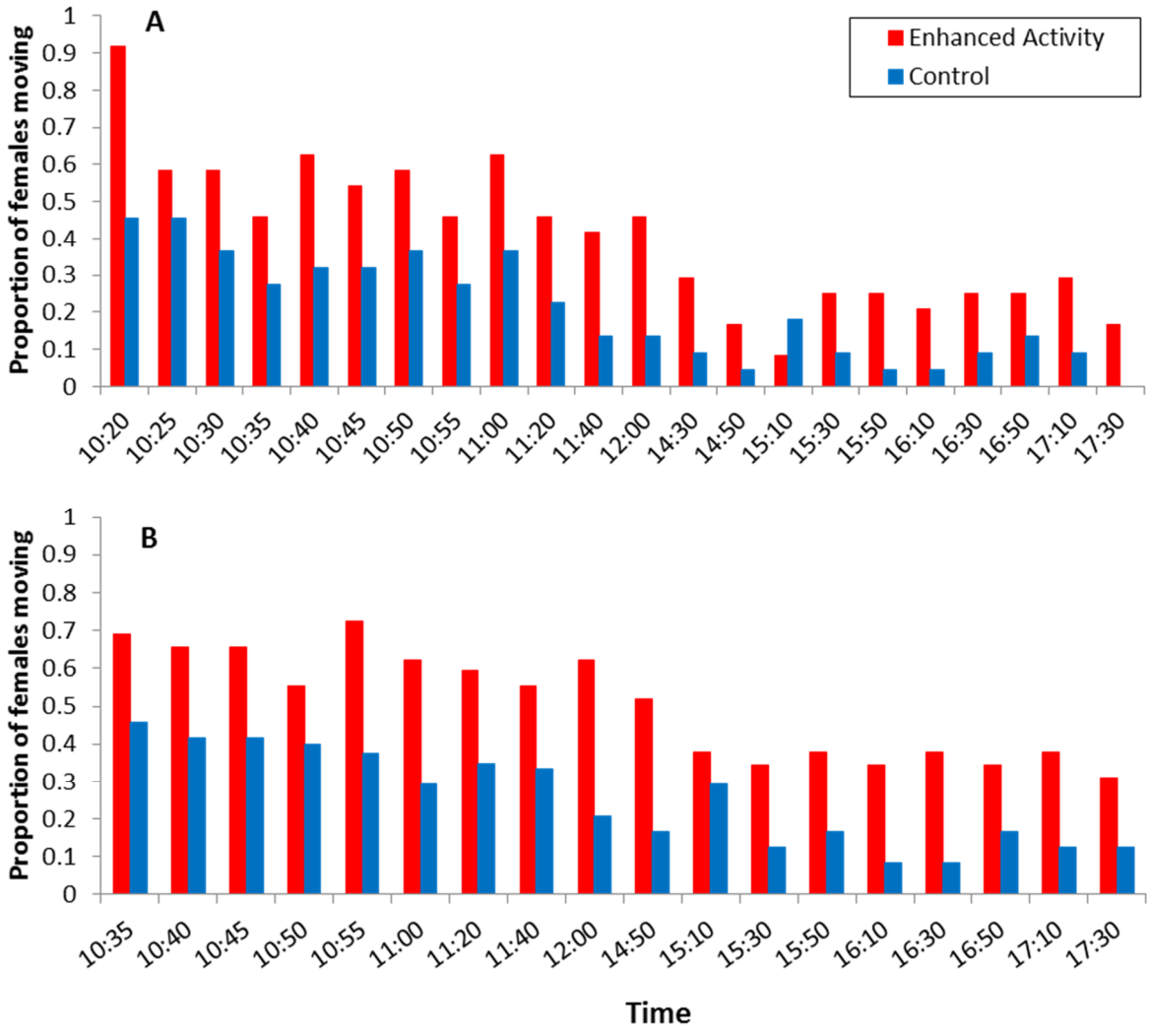

Figure S2: Proportion of females moving, against time points on a single day, across activity level treatments (enhanced activity, control), for: (A) first time replicate and (B) second time replicate. Data shown for (A) at 23 days post-eclosion and for (B) at 22 days post-eclosion, for female age comparability between time replicates. Movement was classified as walking or flying. The newer tilting plate was used to enhance activity levels for all data displayed here. The tilting plate was switched off 1:30pm-2pm and activity levels were not monitored for the natural inactive period around this time.

Chapter 7: General Discussion

Altered nutrition and lifestyles are widely regarded to carry fitness costs. Dietary manipulation, in particular, has been the subject of a broad body of literature (e.g. Chapman & Partridge, 1996; Maroso, 2005; Tatar, 2007; Barnes et al., 2008; Fontana et al., 2010). Across a diverse range of taxa, empirical work has identified possible short-term impacts of diet, on patterns of lifespan, ageing, reproduction and disease susceptibility, within a single generation (Chapman et al., 1994; Chapman & Partridge, 1996; Mair et al., 2004; Skorupa et al., 2008; Barnes et al., 2008; Gese et al., 2016). Far less is known empirically on the life history effects of long-term (evolutionary) nutritional manipulation, or of single-generational or cross-generational nutritional mismatches (switches). The research described in this thesis addressed these knowledge gaps and provided one of the first empirical tests of evolutionary theory concerning nutritional mismatches (the Thrifty Phenotype and Thrifty Genotype hypotheses). Contrary to widespread consensus, I provided strong empirical evidence for the context-dependent reduction of fitness costs predicted from altered (mismatched) nutritional environments (Chapters 2-4). Dietary studies sometimes assert that exercise (activity levels) may also influence life history, yet little has been done to directly manipulate activity levels in healthy (disease-free) individuals and then measure fitness (Potter et.al., 2010; Vaanholt et al., 2010). My results demonstrated a novel example where the direct manipulation of activity levels did not affect female fitness (Chapter 6).

Mismatches between the diet experienced during development (normally the maternal diet) and that consumed during adult life were expected to result in fitness costs, according to the Thrifty Phenotype hypothesis (Hales & Barker, 1992; Hales et al., 1997). I showed that these predicted costs were not obligatory, and were not universal between the sexes, across life history components, or under particular mating regimes (Chapter 2). Importantly, the nature of the mismatch (that is, the particular protein content of the developmental versus the adult diet), determined the presence or absence of fitness costs (Chapter 2). I found that fitness costs in adulthood, arising from mismatched nutrition, could be reduced by particular developmental diets (Chapter 2). This is in line with theory concerning developmental, phenotypic and life history plasticity (Sultan, 2003; Bateson et al., 2004, reviewed by Flatt & Schmidt, 2009, e.g. Stearns, 1992; Pigliucci, 2001). Interestingly, *D. melanogaster* is not believed to exhibit compensatory (catch-up) growth after a poor developmental diet, unlike that predicted or observed in other species (e.g. Metcalfe & Monaghan, 2001; Ozanne & Hales, 2004; Innes & Metcalfe, 2008). However, it is likely that other individual-level physiological changes may have occurred during development, in combination with population-level selection filter effects from the harsh early

stress. These changes better predisposed, or selected for, individuals who would be more resilient to the mismatch and so did not suffer fitness costs. Similarly, a high protein developmental diet may have better predisposed individuals for a later poor quality (low protein) mismatched adult diet, enabling enhanced fitness (survival and reproduction) over those which did not have the good start. This is a good example of the 'silver spoon' effects reported in other studies in different contexts, for where a good start leads to increased resilience to later harsh conditions (e.g. Bateson et al., 2004; Hopwood et al., 2014).

Fitness costs were also expected to have arisen from mismatches between evolved and proximate ('modern') nutrition, as predicted by the Thrifty Genotype hypothesis (Neel, 1962; Prentice et al., 2005). Again, I found that costs were not ubiquitous and could be ameliorated by long-term evolution under a particular nutritional selection regime (Chapter 3). Specifically, evolution under an unpredictable ('Random') feeding regime (that approximated periods of feast and famine), for over 360 generations, led to the evolution of distinct life history patterns on mismatched proximate ('modern') diets of low and high protein food, in comparison to populations with an evolutionary history of predictable ('Regular') feeding (Chapter 3). This interaction between long-term nutritional selection and the fitness consequences of a nutritional mismatch has not, to the best of my knowledge, been previously investigated and was a novel test of Thrifty Genotype theory.

For both short-term (single generational) and long-term (evolutionary) nutritional mismatches, it was inferred that the predicted benefits of particular developmental diets or evolved feeding regimes may have been manifested in adult physiology, but this has yet to be tested. It would be interesting to determine whether the enhanced resilience and plasticity predicted in Random over Regular lines (by the Thrifty Genotype hypothesis; Neel, 1962; Prentice et al., 2005), or following development on a low versus high protein diet (as predicted by developmental viability selection filter theory), was reflected in increased fat deposition even in the absence of an increased body size. It is possible that Random individuals had a higher lipid content, despite their smaller body size, in comparison to Regular individuals, in response to their unpredictable feeding history. Fat deposition could be measured as adult body lipid content (as Ballard et al., 2008), in adults of both sexes that had developed under high and low protein diets and also in adults from the Random and Regular evolved feeding regimes held on the proximate diets used in Chapter 2. This would provide further information on the physiological consequences of proximate and evolutionary manipulation of nutrition.

Surprising patterns emerged from the evolutionary manipulation of feeding regime. That is, evolution under the Random feeding regime enhanced the extent of sexual dimorphism for lifespan (SDL) expressed, in comparison to the Regular regime, when assayed on a common garden, standard diet (Chapters 3 & 4). I predicted that the expression of enhanced SDL could allow relaxation of the constraints on the sexes for phenotypic divergence, imposed by their shared genome, minimising sexual conflict and leading to increased sex-specific fitness for both sexes, in line with theory (Cox & Calsbeek, 2009). This hypothesis was partially upheld. Enhanced SDL in Random lines in comparison to Regular lines was driven by a male-specific decrease in survival, but an increase in early male-specific reproduction (Chapter 4). This led to increased male fitness under enhanced SDL (Chapter 4). Female survival and reproductive output did not differ significantly between the evolved feeding regimes, so female-specific fitness was maintained at a constant level between the associated altered extents of SDL (Chapter 4).

My results demonstrated a partial resolution of sexual conflict, associated with the enhanced extent of SDL expressed under a Random, unpredictable feeding regime (Chapter 4). Sexual conflict resolution is a contentious field (Cox & Calsbeek, 2009). It is unknown whether a full resolution of sexual conflict is possible, due to the constraints on the sexes to achieve optimal phenotypic divergence for maximal fitness, imposed by their shared genome. My results therefore contribute to the significant knowledge gap on factors which can alter the degree of sexual conflict present and suggest that at least partial conflict resolution is possible.

Despite the shared genome of the sexes, most sexual dimorphism is achieved via differential gene expression patterns between the sexes (e.g. Cox & Calsbeek, 2009; Griffin et al., 2013; Perry et al., 2014). It follows that an enhanced extent of sexual dimorphism for lifespan, would be expected to be associated with a greater extent of sex-specific gene expression patterns, in comparison to populations where SDL was either reduced or absent. Furthermore, as the altered extent of SDL had been associated with evolved manipulation of nutrition, I expected candidates linked with diet, lifespan and ageing (such as those involved in insulin- and TOR-signalling pathways, (e.g. Teleman, 2010; Partridge et al., 2011)) could be linked with sex-specific gene expression patterns. In particular, male-specific changes in gene expression were predicted to have arisen, as male-specific shifts in life history had been observed. Indeed, we found differential gene expression between the sexes and between the feeding regimes in a diverse range of functions, associated with nutrition, lifespan, post-mating responses, regulation and epigenetic modification (Chapter 5). These candidate genes identified will be validated via qPCR. The functions of candidate genes will also be further examined to identify potential ‘thrifty gene’ candidates that are upregulated in Random lines and have functions related to increased fat deposition, feeding and metabolism.

To complement the initial functional analysis of differentially expressed genes between the sexes and regimes (Chapter 5), it would be relevant to investigate the magnitude of sex-specific gene expression between Random and Regular lines. This would allow us to directly test the expectation that enhanced SDL in Random over Regular lines was achieved via increased sex-specific gene expression, in line with theory (e.g. Cox & Calsbeek, 2009; Griffin et al., 2013; Perry et al., 2014). Sex-specific analysis of gene expression data generated on each sex from Random and Regular lines would address this question. Consideration of sex-specific expression profiles would provide a more direct test of the genomic changes underlying selection.

Furthermore, it would also be informative to examine the functional relationships between upregulated and downregulated genes in a particular sex, to assess if they are indicative of the presence or absence of life history trade-offs observed in males versus females, respectively. It would be predicted, for example, that males from the Random lines may have shown a concomitant upregulation of genes related to reproduction and downregulation of genes related to lifespan, in line with the life history responses observed. Females, in contrast, would not be expected to show this trade-off at the gene expression level as females from both regimes showed no evidence of a significant lifespan-reproduction trade-off.

Interestingly, not only did we find gene expression changes in genes relevant to feeding, lifespan, ageing and reproduction, but also expression changes in regulatory elements and the regulation of key biological processes themselves (Chapter 5). This suggests that small non-coding RNAs, such as microRNAs, may have been involved in regulating the gene expression patterns observed. More work is required to investigate this exciting area. For example, selective sequencing of small RNAs and testing for upregulation in treatments in which there was a corresponding signature of gene expression regulation, could provide novel insights.

Furthermore, I found differential gene expression signatures associated with histone modification and methylation patterns (Chapter 5), suggesting the interesting involvement of possible epigenetic changes, in the life history patterns observed (Chapter 5). Epigenetic effects have been previously linked with the cross-generational implications of nutrition (e.g. Heard & Martienssen, 2014). Epigenetic marks can be passed from parent to offspring, modifying gene expression, but without altering the underlying DNA sequence (e.g. Heard & Martienssen, 2014). There is evidence that parental diet (and even grandparental diet) can leave epigenetic marks on the genome (genomic imprinting) and can have cross-generational influences on offspring (e.g. Heard & Martienssen, 2014). These could complement the physiological changes observed and further

investigation of the presence of methylation patterns and histone modification could provide insight into the possible role of epigenetics in the life history responses to evolved nutrition.

In conjunction with the role of altered nutrition in producing pronounced life history responses, another component of altered lifestyles, activity level, is often associated with lifespan and health. Despite this frequent association between activity levels, lifespan and ageing (e.g. see several studies which use activity levels as a biomarker for ageing: Koudounas *et.al.*, 2012) there have been few direct tests of the effects on lifespan, reproduction and fitness, particularly in females (Potter et.al., 2010 Vaanholt et al., 2010). I directly addressed this omission by demonstrating a method (the use of a high speed tilting plate) which consistently, robustly and quantifiably elevated female activity levels relative to stationary controls (Chapter 6). However, surprisingly, I found no difference in the lifespan, reproduction or fitness of females between the activity level treatments (Chapter 6). This was contrary to 'live fast die young' theory (Pearl, 1928) and also contrary to the predicted positive association between metabolic rate (which can be linked with activity levels, see Speakman & Selman, 2003) and lifespan (Niitepold & Hanski, 2013). I concluded that effects of activity levels on lifespan may have been context-dependent and further work on a finer scale range of activity levels and on both sexes, perhaps even across a range of diets, would allow the association between altered activity levels and life history, to be investigated in more depth.

In conclusion, the work presented in this thesis has wider importance for understanding the mechanistic basis of SDL expression, sex-specific fitness and important implications for sexual conflict (Maklakov & Lummaa, 2013). Overall, my results make a novel contribution to the study of nutritional mismatches and long-term nutritional selection. I also highlight the importance of simultaneously studying both sexes and several age-specific components of life history, in different proximate environments, to fully elucidate the fitness consequences of nutritional manipulation.

Future work could usefully build on the gene expression differences found between evolved feeding regimes in which there was enhanced or reduced SDL. To further elucidate the possible genetic basis of sexual dimorphism for lifespan (SDL) and to explain the differing capacity for sexual dimorphism to evolve (between the regime lines), it would be useful to investigate the genetic architecture of SDL and to further investigate candidate genes and chromosomal regions involved. The genetic architecture of a trait includes the number of genes that explain variation in that trait and their relative effect sizes on the phenotype. This kind of analysis can also provide information on whether the candidate loci are linked, which may influence the evolution of that

particular trait of interest. Finally, information on genetic architecture allows determination of the degree of intersexual genetic correlation versus sex-biased gene expression for a particular trait. It is possible that sex-specific genetic architecture may be required for the evolution of SDL and so it would be interesting to identify genomic regions associated with (partial) sexual conflict resolution.

To investigate the possible genetic architecture of SDL and the degree of natural genetic variation which exists for SDL, a genetic screen could be performed across many genetically distinct and fully sequenced genetic isolines, such as the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al., 2012) or the *Drosophila* Synthetic Population Resource (DSPR) lines (e.g. Branco et al., 2016). These *Drosophila* genetic isolines are a natural source of genetic variation and have sequenced genomes, facilitating the identification of candidate genes, genomic regions and genetic architecture related to the phenotypic trait measured (e.g. sex-specific lifespan and fitness) (Branco et al., 2016).

A genetic screen of this nature would also identify naturally occurring alleles (genetic determinants) associated with SDL from the naturally occurring genetic variation between the lines in the panel. It would enable identification of segments of the genome with loci that are associated with the trait of interest, which could then be narrowed down using QTL analysis, to identify SNP markers. The functions of candidate regions could then be identified. Although it is known that there is considerable variation in longevity between DGRP lines (Ivanov et al., 2015), the degree of genetic variation in SDL in these lines has yet to be investigated. It is certainly possible that SDL, like lifespan itself, will also be a multifactorial trait.

One limitation of the genetic screening approach is that identified alleles may have a smaller effect size on the phenotype than loss of function mutations, so a combination of the genetic screen with testing the phenotypic effect of loss of function mutants (via knocking out single candidates identified in Chapter 5, in individuals from the Random and Regular lines) could provide a more comprehensive approach to determining the genetic basis of SDL. Information on the possible genetic architecture and genetic basis of SDL from DGRP or DSPR lines could then inform findings from the Random and Regular evolved feeding regime lines. Furthermore, this genetic screening approach could also provide information on the generality of observed life history consequences of nutritional manipulation (such as the altered extent of SDL), across a range of genetic backgrounds.

My work also sets the stage for further phenotypic investigation of the relationship between SDL, sexual conflict, sex-specific fitness. It would be interesting to now test how directly manipulating

the degree of sexual conflict, influences the extent of SDL expressed. The extent of sexual conflict could be manipulated by experimentally evolving populations under different sex ratios (e.g. female biased, male biased and equal sex ratio lines) and then measuring the degree of SDL under enhanced versus reduced sexual conflict. This would allow determination of fitness parameters under different sexual conflict scenarios, to reveal optimal fitness when sexual conflict is reduced.

Another extension to phenotypic work on the Random and Regular evolved feeding regime lines, would be to test the life history responses of individuals of both sexes, from both regimes, to a greater range of proximate diets. This could include a finer-scale range of protein concentrations, within biologically-relevant limits (e.g. Chapman et al., 1994, Chapman & Partridge, 1996; Magwere et al., 2004; Fricke et al., 2010) as recommended by Partridge et al. (2005) and by nutritional geometry approaches (Simpson & Raubenheimer, 2007; Archer et al., 2009). Furthermore, the inclusion of several more concentrations and perhaps also independent manipulation of different dietary nutrients (e.g. dietary sugar content), would provide more information on the generality and extent of the life history consequences of nutritional manipulation.

Although detailed information existed for the timing of feeds over evolutionary history, for both Random and Regular feeding regimes, little is known so far of the impact of these feeding regimes on the within-cage population dynamics. In addition, the precise degree of feast and famine experienced by individuals within the cage populations is also unknown. To answer these questions it would be useful to be able to census cage populations at different points through the feast-famine feeding cycles, to attempt to quantify the extent of the selection pressure exerted on individuals in the Random versus Regular cages, in terms of population crashes and physiological responses to starvation. It would also be interesting to monitor the moisture and nutritional content of the food at various stages of the feeding cycle, to assess potential nutritional stress during the selection regimes. Finally, inspection of food bottles from both Random and Regular cages would be interesting to determine differences in larval density and hence whether larval malnutrition is a factor influencing evolved life history responses.

The increased fitness of Random males under conditions where SDL was enhanced relative to Regular males was determined by measuring reproductive output with standard WT females. This suggested that Random males were either better at enhancing the fecundity of these females (direct fitness benefits), were less harmful to them, or both. To examine this further, it would be interesting to measure focal male fitness after competition with WT males. This would test the possibility that random males are less harmful to their female mates but also less competitive in

fertilisations.

Together, these future experiments could elucidate the mechanistic basis of life history responses to nutritional manipulation in greater detail and potentially reveal general explanations for the intriguing life history patterns observed following nutritional mismatches.

7.1 References

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Chapter 8: Appendix

Manuscript accepted for publication by *Proceedings of the Royal Society B: Biological Sciences*, based on the contents of thesis Chapter 4 and the baseline life history assay from thesis Chapter 3, co-authored by Elizabeth Duxbury, Wayne Rostant & Tracey Chapman

Evolutionary manipulation of feeding regime alters sexual dimorphism for lifespan and reduces sexual conflict in *Drosophila melanogaster.*

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Running header: Lifespan sex dimorphism and conflict.

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Abstract

Sexual dimorphism for lifespan (SDL) is widespread, but poorly understood. A leading hypothesis, which we test here, is that strong SDL can reduce sexual conflict, by allowing each sex to maximise its sex-specific fitness. We used replicated experimental evolution lines of the fruit fly, *Drosophila melanogaster*, which had been maintained for over 360 generations on either unpredictable 'Random' or predictable 'Regular' feeding regimes. This evolutionary manipulation of feeding regime led to robust, enhanced SDL in Random over control, Regular lines. Enhanced SDL was associated with a significant increase in the fitness of focal males, tested with wild type females. This was due to sex-specific changes to male life history, manifested as increased early reproductive output and reduced survival. In contrast, focal female fitness, tested with wild type males, did not differ across regimes. Hence increased SDL was associated with a reduction in sexual conflict, which increased male fitness and maintained fitness in females. Differences in SDL were not associated with developmental time or developmental survival. Overall, the results showed that the expression of enhanced SDL, resulting from experimental evolution of feeding regimes, was associated with male-specific changes in life history, leading to increased fitness and reduced sexual conflict.

1. Introduction

In the more than half a century since the major tenets of the evolutionary theory of ageing were formulated [1-3] a huge body of supporting empirical evidence has been gathered [4-9]. However, despite this, we still have surprisingly little understanding of the striking, and seemingly universal, sexual dimorphism in lifespan (SDL). Such differences are widespread across animal taxa [10-14] and are often associated with variation in mating systems [13, 14]. This suggests an explanation relating to sexual selection and associated differential risks of extrinsic mortality [11, 15]. For example, SDL is reported as elevated in promiscuous systems, but reduced under monogamy. Promiscuity leads to increased survival costs for males from intensified male-male competition and a shorter effective breeding period than for females. This is proposed to reduce selection for mechanisms that increase longevity in males compared to females, hence increasing SDL [14, 16]. Other explanations for sex-specific variation in lifespan across species include the so-called 'mother's curse' associated with the effects of female-only purging of mitochondrial mutations [17] and the differential sensitivity of males versus females to the effects of mutations that accumulate on the sex chromosomes (the 'unguarded X' (or indeed Z) hypothesis [18]). These hypotheses have gained some empirical support [19, 20]. However, it is noted that there is a general paucity of experimental work in this area [21].

Within species, significant variation in the magnitude of SDL expressed is best explained by the degree of sexual selection and conflict [11, 15]. Hence, factors such as nutrition, which affect the expression of sexual characters, can also be important in the determination of SDL. For example, within species, the extent of SDL can show marked plasticity in response to proximate factors such as diet. In *Drosophila melanogaster* SDL is maximised by a 60% reduction in the standard dietary yeast and sugar content and minimised or absent at extreme food concentrations (<30%, or >130% of the standard dietary yeast and sugar content) [22]. Male-specific hormones can also reduce male lifespan below that of females, thus enhancing SDL [18, 23]. The production of pheromones by one sex can also directly reduce the lifespan of the other via interaction with insulin signalling pathways in both flies and worms [24, 25]. Exposure to female pheromones reduced male lifespan in *Drosophila*, even in the absence of mating [24]. These findings support the idea that the interaction between the sexes via sexual selection and sexual conflict exert significant influences on the lifespan of one or both sexes, thus altering the magnitude of SDL [11, 15].

Sex-specific variation in longevity may result from sex-specific patterns of extrinsic mortality, ageing onset and ageing rate, over lifetime [14, 16]. The causes of such differences are

thought to result from the expression of sex-specific life histories [21] and hence differential sex-specific optimisation of energy investment or allocation [15, 16, 26]. SDL may arise from the sex-specific optimisation of trade-offs of lifespan with reproductive, mating or developmental traits, leading to sex-specific life history strategies [15, 16, 21]. Hence, underpinning the expression of SDL are differences in the magnitude of reproductive costs [27] and associated sex-specific trade offs. These may often differ substantially between males and females. However, despite numerous theoretical predictions surrounding life history trade-offs, relatively little is currently known about the sex-specific impact of reproductive costs on survival trajectories in both sexes [28].

Ultimately, the causes and consequences of SDL are still poorly understood [11, 15, 20, 29]. One leading hypothesis, which we test here, is that enhanced SDL could be a mechanism by which sexual conflict is reduced, by allowing females and males to express sex-specific life histories and hence increase their sex-specific fitness [11, 15, 30]. It is known that genetic correlations constrain the sexes from reaching their optimal lifespan [31] and that selection on the optimal lifespan in one sex increases fitness of that sex but reduces fitness of the other [32]. However, there are as yet no direct empirical tests of the age-specific fitness consequences associated with enhanced versus reduced SDL in both sexes. This knowledge gap has partly arisen from the lack of an appropriate empirical system in which to test these predictions. We address this omission by using lines of *Drosophila melanogaster* fruit flies subjected to replicated experimental evolution for >360 generations (over 15 years) under divergent random and regular feeding regimes. In these evolutionary regimes, food is provided either regularly each week (‘Regular’) or randomly within a 28 day cycle (‘Random’). The same absolute quantity of diet is provided to each regime, but Random regime lines experience periods of nutritional stress and surfeit. The Random lines have evolved enhanced SDL in relation to controls (see below) offering an ideal opportunity to test for associated differences in sex-specific fitness.

We used the Random and Regular feeding lines to test the prediction that increased SDL, as expressed by Random in comparison to Regular lines, is associated with decreased sexual conflict through adoption of sex-specific life histories that lead to higher fitness for males and females. The overarching rationale was that the Random lines, in which there was greater SDL, would show increased sex-specific fitness in comparison to lines in which SDL was reduced. We conducted separate experiments to measure the lifespan and fitness of focal females and males from the Random and Regular lines held with non-focal standard wild type individuals.

2. Methods

(a) Flies and culturing.

Experimental individuals were the second generation of offspring (F2) originating from eggs laid by grandparents (P1) derived from the 3 replicated populations of Regular and Random feeding regime cages (electronic supplementary material, figure S1). Two generations of rearing under standard conditions were conducted to minimise maternal effects. First instar larvae were transferred to Sugar Yeast Agar (SYA) vials (15g agar, 50 sugar, 100g yeast, 30 ml Nipagin (10% w/v solution and 3ml propionic acid per L) at controlled density of 150 larvae/vial. Adults (F1 generation) were allowed to emerge and freely mate in their larval vials for 24h and then tipped into fresh SYA bottles for another 12-24h of free mating. This ensured all F1s were sexually mature and aged between 12h and 48h. 400 F1 females from each of the 6 experimental lines were then transferred into a mini-cage with yeasted purple agar plate and allowed to egg-lay for 6h. The short egg laying window allowed for precise measurement of subsequent developmental timings.

(b) Life history assay.

Adults emerging from F2 larval vials were collected as the F2 generation 'focal' flies for the adult fitness experiment. Sample sizes of 51 adults/sex/line were used for the survival assay and for weekly matings. A subset of 45 adults/sex/line was used to assess weekly reproductive output. Virgin wild-type (WT) Dahomey flies of both sexes (n=480/sex) derived from standard density cultures (150 larvae per vial) were generated each week for mating with the focal females and focal males in the experiments. WT flies were collected as virgins and held in single sex groups of 10 per SYA vial until they were introduced to the focal flies. Initial matings between virgin focal flies and virgin WT flies were set up 3 days post-eclosion. Using light CO_2 anaesthesia, 3 focal adults were placed with 3 standard WT adults of the opposite sex per vial for 24h. Multiple individuals were housed together to introduce biologically-relevant male-male competition. The mating schedule in the male and the female experiments was identical. Assays of mating behaviour were recorded every 20mins for the final 3h of each 24h mating period. This allowed indices of the proportion of each sex that mated to be determined.

After initial matings focal females and males were transferred to single sex vials containing SYA medium at a density of 3 flies/vial, under light CO_2 anaesthesia. Initial egg counts for both focal sexes were made from this 24h mating period. Egg vials were retained to determine egg-adult viability and frozen 13 days after egg laying, for later counting of number of offspring.

For the first 2 weeks of the experiment, twice weekly matings of focal females and males with WT mates (standard 3-day-old virgin WTs) were conducted, and twice weekly egg counts and offspring counts recorded, to assess early reproductive output. Weekly matings and reproductive output counts were then performed for the remainder of the experiment. All matings followed the same protocol as the initial mating.

Every 2-3 days food vials were exchanged and the groupings of 3 focal flies per vial were shuffled, to randomise the positioning of focals in vials with fewer than 3 flies (due to mortalities or censors). The focal sexes were housed in single sex vials throughout the experiment (except during weekly matings with WT adults). Focal female and focal male mortalities were checked daily.

(c) Statistical analyses.

All statistical analyses were performed in R version 3.2.1 [33] using the base 'stats' package, except where otherwise stated.

Development time and developmental viability. Developmental viability was expressed as proportion data and analysed using a generalised linear model (GLM), with quasi-binomial errors, to account for overdispersion. Development time data were tested for normality using the Shapiro Wilk test and for equality of variances using the Levene's test, separately for each treatment level. Differences in development time between regimes were analysed using a two sample t-test, as the normality and equality of variances assumptions were met. A focal-sex $\times$ feeding regime interaction effect on development time was tested for using a GLM with normal errors.

Survival. Survival analyses were performed using mixed effects Cox Proportional Hazards regression on age-specific mortality data. Prior to analyses, the data were tested for potential violation of the proportional hazards (PH) assumption using both graphical and analytical tests. As a further test, parametric survival analysis was performed for a subset of the data with the largest potential PH violation as follows. A maximum likelihood approach, implemented in the 'bbmle' [34] package, was used to compare 11 different parametric models and find the best model fit (adapted from [35]). Subsequent parametric survival analysis of the returned comparable results to the mixed effects Cox model. This, coupled with the finding that the data satisfied the PH assumption, justified the use of the semi-parametric Cox PH method for all the main survival analyses, implemented using the 'coxme' package [36]. The models were specified to test for the effects of the two fixed explanatory factors of interest, namely sex and feeding regime. We split

the dataset in order to calculate the relevant hazard ratios (HR) for each sex and regime, where HR indicates the risk of death for 2 treatments relative to each other (e.g. if one group died at twice the rate per unit time as another, the HR would be 2). However, in a combined model, we utilized the entire dataset to include an interaction term to directly test for the effect of evolutionary feeding regime on SDL. Each model included a random effect of cage, which was tested against a simpler model without this term via Likelihood Ratio Test (LRT). In all models, dropping the random effect resulted in a worse model fit and justified the retention of this term. In the first two models we analysed within-sex effects of feeding regime on survival. Here, age-specific mortality was modelled as a response to a single, fixed factor, namely feeding regime, and a random effect of line nested within feeding regime. The second two models analysed the effect of evolutionary feeding regimes on the differences in survival between the two focal sexes, i.e. SDL. In these, age-specific mortality was modelled as a response to a single fixed factor, sex, and a random effect of line nested within sex. The final combined model included age-specific mortality as a response to focal sex and feeding regime as fixed main factors as well as a fixed focal sex x feeding regime interaction and a random effect of line nested within feeding regime.

Age-specific reproduction. Age-specific egg count and offspring count data were analysed with generalised linear mixed effects models (GLMMs), separately for each sex, using the 'glmer' function from the 'lme4' package in R [37]. Experimental replicate and the number of days post-eclosion were fitted as categorical random effects and feeding regime (Regular or Random) as a fixed effect. No individual-level random effect was included in the model, as individuals were not uniquely identifiable from this experiment (measures were taken from randomised groupings of 3 individuals, at each time point). The data were overdispersed in all cases. To account for this, an observation-level random effect was added to each GLMM and a maximum likelihood model comparison was used to determine best model fit. Egg to adult viability was calculated as the proportion of eggs laid by groups of 3 focal females that hatched as viable offspring, at each time point. Proportion data were arcsine transformed to normalise and then analysed with a linear mixed model (LMM). Initial egg and offspring counts (from 3 days post-eclosion) were also analysed separately, for both sexes, using the same approach as for development time data, to determine whether differences in fitness indices were associated with differences in initial reproduction counts (as the fitness index, Euler's r, is weighted towards early reproduction: for description of fitness calculation, see below).

Lifetime reproduction. An index of total lifetime egg production and an index of total lifetime offspring production was calculated separately for each sex and each treatment population by summing egg or offspring counts, respectively, across the lifetime. Mean and standard errors for

total lifetime reproduction values, for each feeding regime (Random and Regular) and each sex, were determined. Differences in total lifetime egg or offspring production between regimes were analysed identically to development time data.

Female and male fitness. Female and male fitness indices were calculated as the intrinsic rate of population growth (the Malthusian parameter, Euler's r), using the Euler equation [38, 39], separately for each treatment line. The Euler equation calculates an index of fitness from age-specific survivorship and age-specific reproduction values and is weighted towards early life reproduction and is directly related to the lambda fitness metric [40, 41]. Age-specific egg counts (per 24h) were used to calculate 'potential fitness' and age-specific offspring counts (per 24h) were used to calculate 'realised fitness'. Offspring counts and egg counts were halved, to account for the genetic contribution of one parent (the mother or father, respectively) to the offspring generation. Fitness data were analysed identically to the development time data.

Mating frequency. An index of the proportion of individuals that mated from each treatment line population was calculated separately for each focal sex. For each weekly mating day (n=10), the total number of matings recorded each 20 minutes, over the 3h mating observation, were summed, to give the total number mated per 3h mating, for each line and each focal sex. The total number of matings recorded over lifetime (across all weekly matings) for each focal sex and line were then calculated, and expressed as a proportion of the sum of total number of pairs surviving at each weekly mating over lifetime. Indices of mean proportion mated over lifetime per treatment line were analysed, separately for each sex, using a GLM with binomial errors. Overdispersion was accounted for by using quasi-binomial errors. A maximal GLM model including regime, sex and their interaction was fitted. Stepwise removal of non-significant model terms from the maximal model, and likelihood ratio tests, were used to test for significance of model terms and to derive the minimal adequate model.

3. Results

We hypothesised, based on the proximate responses of SDL to diet [22], that SDL would change in these lines. Data from an initial pilot experiment conducted with once-mated females and males were consistent with this idea and showed that lines maintained on a random, unpredictable feeding regime had evolved significantly enhanced SDL in comparison to control lines fed according to a regular feeding regime (electronic supplementary material, figure S2). We then used these lines to test the prediction that, in fully reproductive flies, the expression of enhanced SDL would be associated with increased sex-specific fitness and hence a reduction in sexual conflict. We measured the survival and reproductive successes of focal males and focal females,

separately, from the Random and Regular lines. To maintain reproductive activity throughout life, all flies were given 24h exposure to wild type individuals of the opposite sex every 7 days. We indicate directionality to differences in lifespan, where appropriate, on the basis of comparisons to the Regular regimes, which replicate the standard cage culture conditions.

(a) Lifespan and SDL.

We predicted the existence of adaptive sex-specific optimisation of life history trade-offs [21] correlated with the intermittent nutritional stress imposed by the Random feeding regime. The results supported the predictions. Consistent with the pilot data (electronic supplementary material, figure S2), we saw significantly enhanced SDL associated with a specific change to the life history of the Random males. There was no significant difference in focal female survival (median lifespan Regular=58 days, Random=60 days; coxme regression: Hazard Ratio $(HR)_{(Reg/\ Rand)}$ = 0.76, z = 1.31, p = 0.19; figure 1a; electronic supplementary material, table S1). However, male survival was significantly greater for Regular (median = 51 days) in comparison to Random males (median = 47 days; coxme regression: $HR_{(Reg/\ Rand)}$ = 0.61: z = 2.39, p = 0.017; figure 1b). SDL was expressed as a significant sex difference in survival within the Random regime (median female lifespan = 60 days, males = 47 days; coxme regression: $HR_{(Male/\ Female)}$ = 3.58, z = 4.42, p < 0.001; figure 1c). SDL was less marked in the Regular regime (median lifespan females = 58 days, males = 51 days; coxme regression: $HR_{(Male/\ Female)}$ = 2.12, z = 4.56, p <0.001; figure 1d). The suggested pattern of SDL showing an interaction with sex across regimes was confirmed by the combined statistical model. This revealed a significant focal sex $\times$ feeding regime interaction effect on survival (coxme regression: $HR_{(Reg\ male/\ Rand\ male)}$ = 0.68, z = 2.07, p = 0.038), which confirms significantly greater SDL in Random compared to Regular regimes.

(b) Focal female reproductive output.

There was no significant difference in focal female age-specific egg or offspring production over time (GLMMs: egg production z = 0.28, p = 0.776; offspring z = 0.18, p = 0.855; figure 2a,b) and both traits declined significantly with age across both regimes (GLMMs: eggs z = 71.8, p < 0.001; offspring z = 71.6, p < 0.001). There was also no significant difference in egg to adult viability across regime females (GLMM: t_5 = 0.63, p = 0.480; figure 2c) though again a significant effect of age (GLMM: t_5 = 10.19, p < 0.001). There were no differences in initial egg counts (two sample t-test: t_4 = 1.57, p = 0.192; mean Random = 64, Regular = 74; figure 2a inset) or offspring counts (t_4 = 0.90, p = 0.420; mean Random = 54, Regular = 61; figure 2b inset) in the focal female experiment.

(c) Focal male reproductive output.

There was also no significant overall difference in male age-specific reproductive output (GLMMs egg production: $z = 1.09$, $p = 0.276$; offspring: $z = 0.97$, $p = 0.334$; figure 3a,b) and both traits declined significantly with age (GLMMs eggs: $z = 39.1$, $p < 0.001$; offspring: $z = 65.7$, $p < 0.001$). There was no significant difference in male egg to adult viability across regimes (GLMM: $t_5 = 0.35$, $p = 0.700$; figure 3c) though again a significant decrease with age (GLMM: $t_5 = 19.81$, $p < 0.001$). However, initial offspring counts were significantly higher for random than Regular males ($t_4 = 4.29$, $p = 0.0128$; mean Random = 66, Regular = 57; figure 3b inset). There was also a non-significant trend for higher egg production in Random over Regular males ($t_4 = 2.34$, $p = 0.0797$; mean Random = 70, Regular = 62; figure 3a inset).

(d) Focal female and focal male fitness.

There was a significant difference between feeding regimes in male ($t_4 = 4.32$, $p = 0.0124$) but not female ($t_4 = 0.81$, $p = 0.465$) fitness (Table 1). Hence Random males showed a significant increase in fitness compared to Regular males, even though their lifespans were significantly shorter. This was associated with the significantly higher initial offspring production in males from the random regime (figure 2b). These results indicated that experimental evolution of feeding regimes and enhanced SDL led to sex-specific fitness differences, with males from the random regime showing significantly higher fitness.

(e) Mating frequency and developmental traits.

A significantly greater proportion of Regular than Random males mated, during the 3 hour observations of weekly matings over the lifetime. There was no difference in the mean proportion of matings observed in focal females (males GLM: $z = 2.12$, $p = 0.0338$; females GLM: $t = 0.01$, $p = 0.928$; electronic supplementary material, figure S3). There were no differences in developmental viability or developmental time across either regime (electronic supplementary material, figures S4-S6).

4. Discussion

Differences in female and male lifespan are widely documented across many species [10, 12-14]. Much less is known about the factors that influence the extent of this SDL. Here we subjected lines to evolutionary manipulation of random and regular (control) feeding regimes and found that this led to enhanced SDL in the Random regime. This was driven by a specific reduction in

Random relative to Regular male lifespan. We then measured the life history consequences of enhanced SDL in both sexes simultaneously. We tested the prediction that the existence of enhanced SDL would lead to the opportunity for constraint to be relaxed and each sex to adopt a sex-specific life history leading to higher fitness in comparison to the situation in which SDL was reduced [11,15]. In line with the prediction, enhanced SDL was associated with increased fitness of Random males as predicted under the sexual conflict theory. Random males compensated for a reduced lifespan through a significantly elevated early burst of reproductive output. Female fitness was equivalent across Random and Regular regimes, suggesting that female life history was relatively independent of changes to that of males. Hence overall the level of overall sexual conflict was reduced.

Random males achieved higher fitness, despite a significantly reduced lifespan, by allocating resources into increased early reproductive output (progeny production). This suggests a trade-off between early reproduction and lifespan [42, 43]. Increased early productivity was achieved, even though Random males mated less frequently than Regulars over their lifetime. The reduced lifespan of Random in comparison to Regular males was not associated with any between-regime differences in developmental viability or timing. Random males and females have significantly smaller body size than Regular flies (Perry, et al., unpubl.). Hence there was no straightforward relationship between body size and reproductive output or lifespan in this study. It would be interesting to probe the functional relationships further, by testing for reproductive allocation differences within the Random and Regular lines. This would allow tests of whether the life history fitness advantage of random males is associated with increased allocation of resources to reproductive tissues (testes and accessory glands) per body size. Similarly, the lack of differences in female life history across regimes would predict a lack of such divergence in reproductive allocation. Functional relationships could be further investigated through the description of sex specific gene expression profiles to examine more directly the genomic changes underlying selection.

The finding of increased fitness for the random SDL-enhanced males was necessarily based on measures of the reproductive output of wildtype females mated to them. This suggests these males are better at providing direct fitness benefits to females or less harmful to females. To examine this further, it would also be very interesting to measure focal male fitness in competition against wild type males. This would allow a test to rule out the possibility that random males are more benign but also less competitive in fertilizations.

Sex-specific life history trade-offs over investment into reproduction versus survival, as observed here, are often posited as evolutionary explanations for SDL [21]. That is, there may be differential sex-specific optimisation of energy investment and allocation [15, 16, 26]. Our work provides empirical evidence to support the existence of sex-specific life history trade-offs, which were present in males and absent in females.

A life history strategy that favours early reproduction by males over later survival, despite a reduced body size, could be adaptive following an evolutionary history of unpredictable (random) food availability [44]. If randomly fed individuals had an increased ability to readily capitalise on resources when available, then this would allow them to achieve increased fitness. Experimental evolution of *Drosophila* under high extrinsic mortality (90% mortality induced twice per week) also led to a similar life history strategy of reduced body size, increased early fecundity and reduced lifespan, when compared with lines selected for low extrinsic mortality (10% induced mortality, twice per week) [7]. However, imposing increased mortality can also have the opposite result, i.e. the evolution of increased lifespan, depending upon whether if mortality is condition-dependent rather than random [45, 46]. Hence our results suggest that mortality is random, or possibly that selection for early function is stronger than selection for stress resistance.

Females, in contrast, did not differ in lifespan, reproductive output or mating frequency and, unlike males, did not evolve an altered life history strategy in response to feeding regime manipulation. This was not due to a lack of a response in comparison to lifespan before selection, as the Regular lines essentially replicate the normal cage cultures. Nor is it attributable to a lack of raw material, as there is significant genetic variation in female lifespan (e.g. [32, 47, 48]). It is possible that there was no selection on the female life history, but given the significant body size differences we observed between regimes as an outcome of selection this seems unlikely. We suggest instead that trade-off changes expressed in males were absent in females, or that females did not respond due to the presence of inter or intralocus genetic correlations. These possibilities would be interesting to test. Sex-specific lifespan patterns could be the result of different selection pressures acting on the sexes [15, 49]. We observed no significant sex bias in adult emergence (data not shown). Hence overall there was no evidence of differential developmental selection on either sex, suggesting that sex-specific selection pressures were more likely to have acted upon adults.

Experimental evolution studies in the laboratory can be vulnerable to the effects of inbreeding, due to reduction in effective population size (as discussed in [50]). Recently an effect of inbreeding per se on the expression of male versus female lifespan has been observed [20]. We

reduced the potential for inbreeding through maintenance at large population sizes. Survival and reproduction patterns were broadly consistent between the 3 replicate populations for each regime, supporting the conclusion that evolved responses between regimes arose from selection and adaptation, rather than drift.

Sexual conflict was reduced under enhanced SDL. Some authors argue that sexual dimorphism can only ever partially resolve sexual conflict, as the sexes are constrained from reaching their optimal fitness by the majority of their shared genomes [21, 30]. This argument is derived from the observation that little empirical evidence exists for the presence of "modifier" genes that allow the sex-specific gene expression required to achieve sufficient sexual dimorphism. The evolution of such genes is also predicted to be slow [51, 52]. However, in this study we did observe a reduction of sexual conflict. This could have been through a putative relaxation of genetic constraints on shared lifespan and life histories between the sexes. The reduction of conflict came from specific shifts in male not female life history. The maintenance of female fitness under both enhanced and reduced SDL could reflect that optimal fitness was achieved even in the absence of enhanced SDL. The sexes may have differed in their absolute fitness optima, but have achieved the optimum for their respective sex, under enhanced SDL.

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Authors' contributions. TC and ED conceived the study, ED conducted the research, ED and WR analysed the data, ED and TC wrote the paper and ED, WR and TC revised the paper.

Competing interests. We declare we have no competing interests.

Data availability. The raw data are archived in the DRYAD data repository (DOI to be added).

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Figures

Figure 1. Age-specific survivorship against days post-eclosion. Shown are replicates 1-3 of Random and Regular feeding regimes: (**a**) Random vs Regular focal females; (**b**) Random vs Regular focal males, (**c**) Random females vs males, (**d**) Regular females vs males.

Figure 2. Mean focal female egg production (a), offspring (F1) production, (b) and egg to adult offspring viability (c), per 3 females, per 24h, against days post-eclosion**.** Mean number of offspring that emerged from the 24h egg lay vials (**a**), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (**b**). Egg to adult viability is defined as the mean proportion of eggs laid by groups of 3 females during 24h which eclosed as adults (**c**). Insets for (**a**) and (**b**) show mean initial (day 3) egg and offspring counts, respectively. All error bars display +/- 1 standard error.

Figure 3. Mean focal male egg production (a), offspring (F1) production, (b) and egg to adult viability (c), per 3 males, per 24h, against days post-eclosion. Mean number of offspring that emerged from the 24h egg laying vials (**a**), for each of the six weekly-mated experimental lines (Rand1, Rand2, Rand3, Reg1, Reg2, Reg3), at weekly intervals since eclosion (**b**). Egg to adult viability is defined as the mean proportion of eggs laid by groups of 3 WT females that had been mated to the focal males, during 24h, which eclosed as adults (**c**). Data are shown for the period where n>5 for each treatment line. Insets for (**a**) and (**b**) show mean focal male initial (day 3) egg and offspring counts. All error bars display +/- 1 standard error.

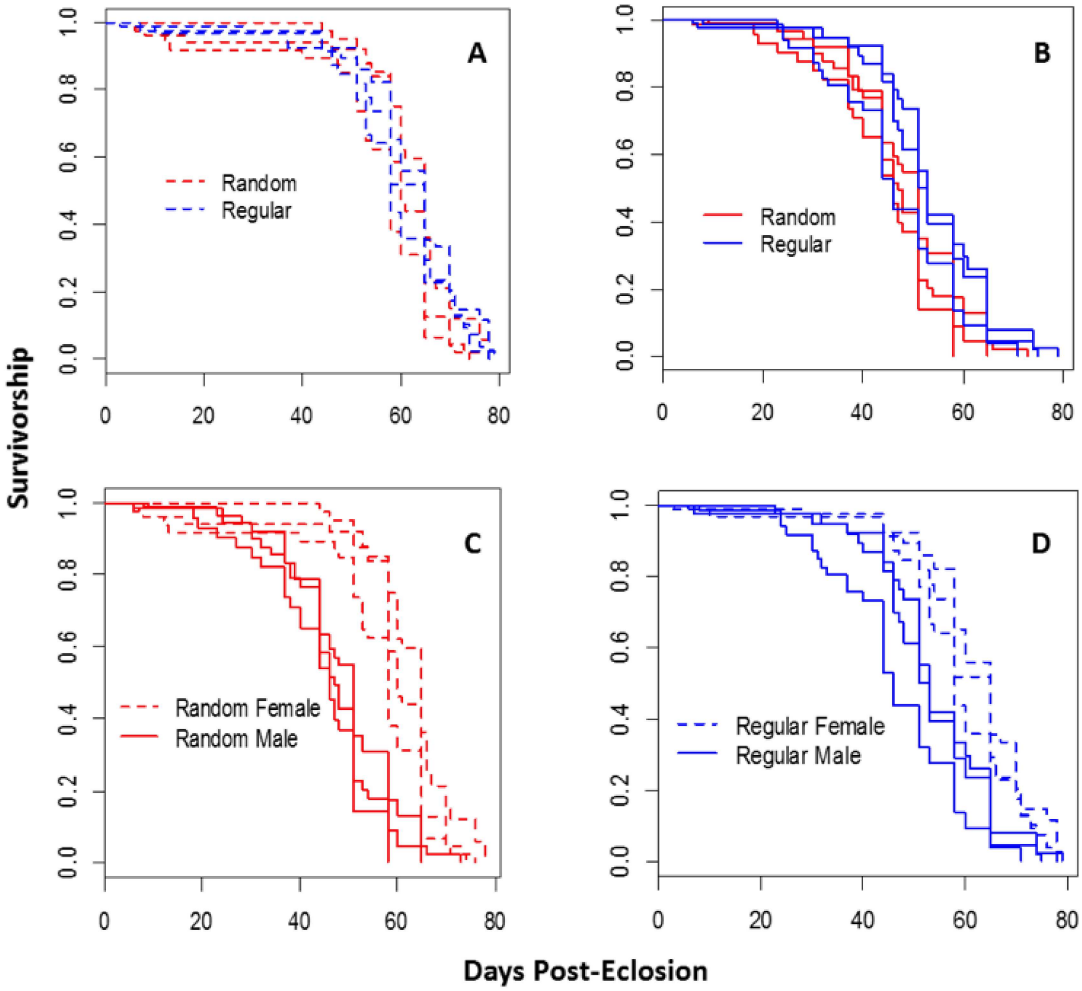
A
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Figure 1.

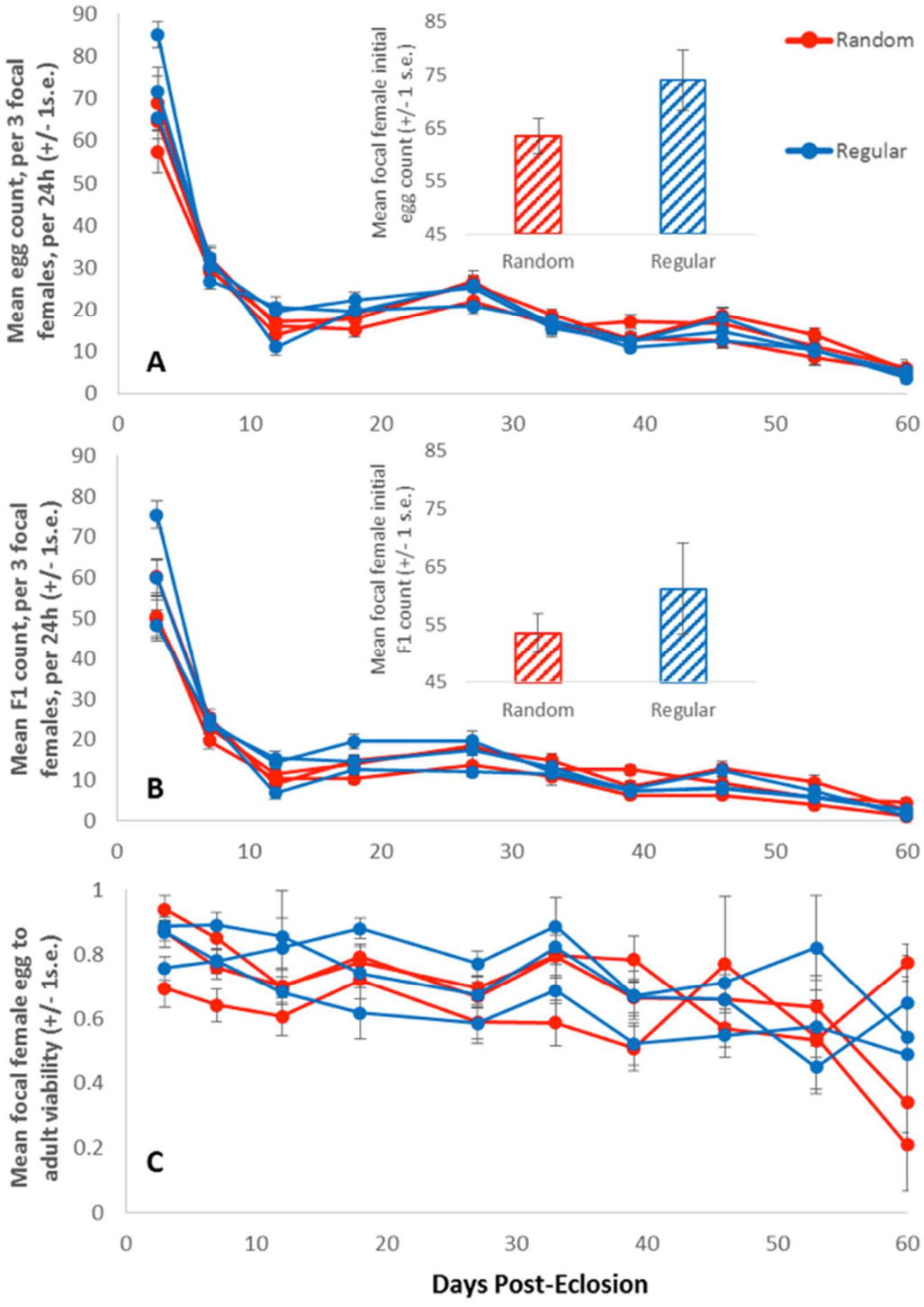
Mean egg count, per 3 focal females, per 24h (+/- 1s.e.)
Mean focal female initial egg count (+/- 1 s.e.)
Random
Regular
A
Mean F1 count, per 3 focal females, per 24h (+/- 1s.e.)
Mean focal female initial F1 count (+/- 1 s.e.)
B
Mean focal female egg to adult viability (+/- 1s.e.)
C
Days Post-Eclosion

Figure 2.

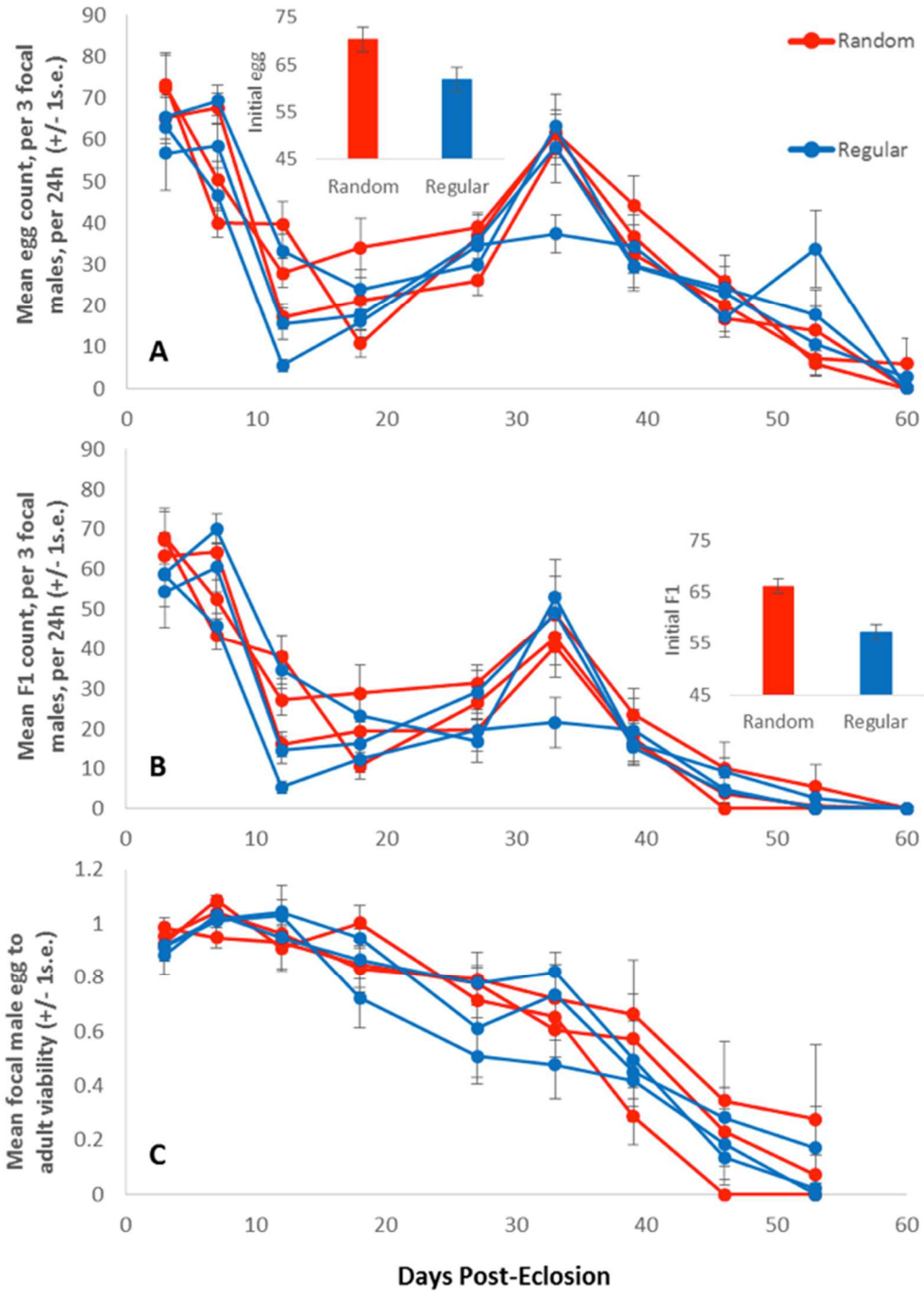
Mean egg count, per 3 focal males, per 24h (+/- 1s.e.)
Initial egg
Random
Regular
A
Mean F1 count, per 3 focal males, per 24h (+/- 1s.e.)
Initial F1
B
Mean focal male egg to adult viability (+/- 1s.e.)
C
Days Post-Eclosion

Figure 3.

Table 1. Index of mean fitness (+/- 1s.e.) for focal females and males from Random and Regular regimes, calculated as Euler's r using age-specific egg counts (a) or age-specific offspring counts (b). Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3); n=45 individuals per line.

		(a) Fitness (from egg counts)		(b) Fitness (from offspring counts)	
		Mean	**S.E.**	**Mean**	**S.E.**
Female	**Random**	1.154	0.018	1.096	0.020
	Regular	1.201	0.026	1.135	0.044
Male	**Random**	1.188	0.012	1.169	0.007
	Regular	1.146	0.014	1.122	0.008

Electronic Supplementary Material

List of ESM:

(a) Scheme of experimental design for generation of flies for main and pilot experiments

Figure S1. Experimental design for the generation of focal individuals for the experiments.

(b) Baseline pilot experiment - survival of once-mated Random and Regular males and females

Figure S2. Baseline age-specific survivorship against days post-eclosion, across replicates 1-3 of once mated Random and Regular feeding regimes, held on standard (SYA) food.

(c) Focal female and focal male mating frequency – main experiment

Figure S3. Index of mean proportion mated for Random and Regular feeding regime lines for each sex, over lifetime.

(d) Developmental viability and developmental time of the random and regular males and females - main experiment

Figure S4. Mean developmental viability (+/- 1 s.e.) for focal adults from Random and Regular feeding regimes, developing on standard food, at first instar larva to adult (a), first instar larva to puparium (b) and puparium to adult (c) developmental stages.

Figure S5. Mean development times (+/- 1 s.e.) for focal adults from Random and Regular feeding regimes, developing on standard food, at first instar larva to adult (a), first instar larva to puparium (b) and puparium to adult (c) developmental stages.

Figure S6. Mean first instar larva to adult development time (+/- 1 s.e.) for focal females and focal males from Random and Regular feeding regimes.

(e) Median survival time in days of flies - main experiment

Table S1. Median focal female and male survival in days (+ interquartile range) for Random and Regular regimes (replicates 1-3).

(a) Scheme of experimental design for generation of flies for main and pilot experiments

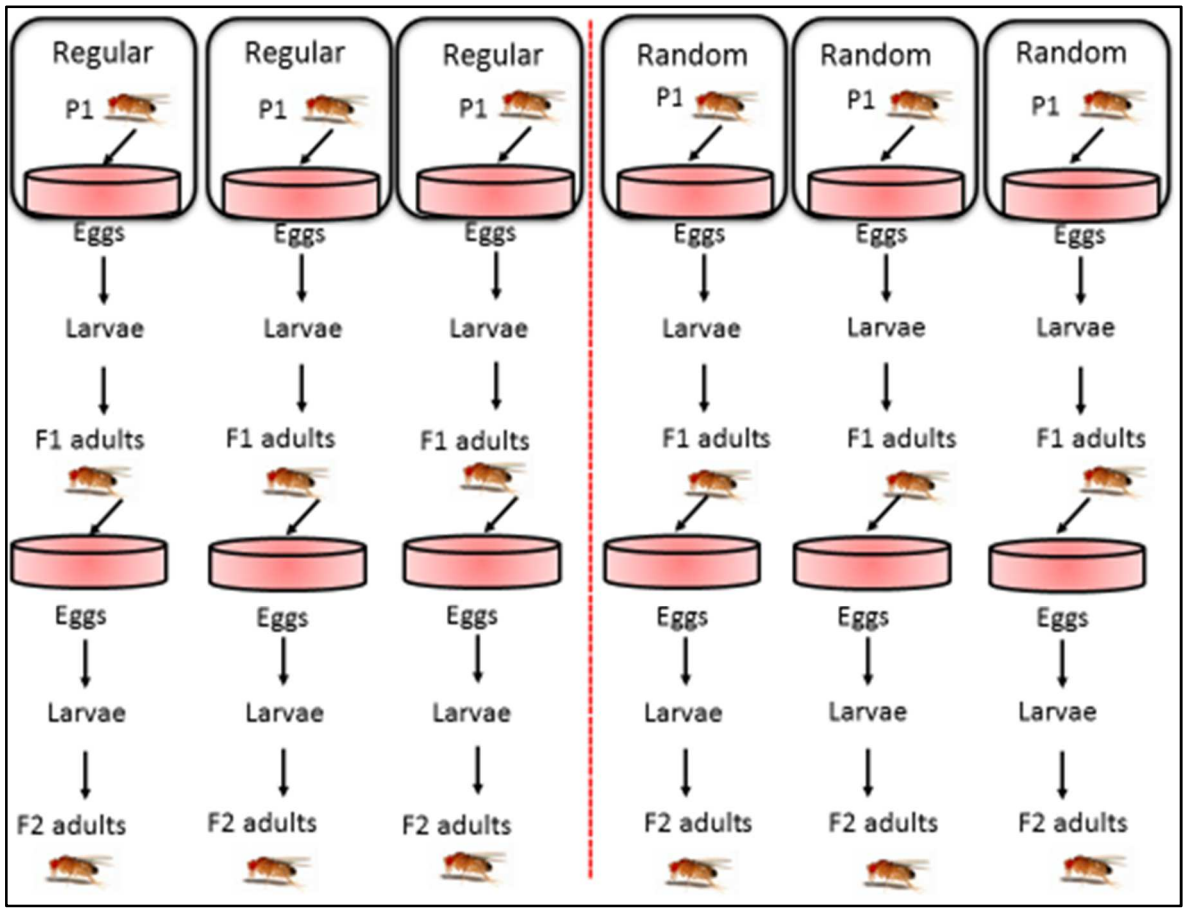

Figure S1. Experimental design for generation of focal individuals. Flies in the 'Regular' and 'Random' cages, sustained on standard yeast agar (SYA), were the grandparents of F2 flies used for experimentation. Eggs for the F1 generation were collected on red grape juice agar plates for 24h and larvae developed at a standard density of 150 larvae/vial on SYA. F1 adults were mass-mated for 36-48h, with mates from their own feeding regime line.

(b) Baseline pilot experiment - survival of Random and Regular males and females

An initial screen of survival of males and females, separately, from the Random and Regular regimes was conducted using the same methodology as in the main MS, excepting that individuals were given only a single period of mating at the beginning of their lives. Upon eclosion, matings between 12h old virgin focal flies and virgin WT flies were set-up. Under light CO_2 anaesthesia, each SYA bottle of 60 WT adults was tipped into a SYA bottle of 45 focal adults of the opposite sex, for each of the 6 experimental lines, and allowed to mate for 24h. This mass-mating set-up introduced biologically-relevant male-male competition and aimed to ensure all focal adults were mated. After mating, focal females and males were transferred to single sex vials of standard food (SYA) at a density of 3 flies/vial. Focal adults received no further matings and no further exposure to the opposite sex after the initial mating. Every 2-3 days (Monday, Wednesday, Friday) food vials were exchanged and the groupings of 3 focal flies per vial were shuffled, to randomise the positioning of focals in vials with fewer than 3 flies (due to mortalities or censors). Focal female and focal male mortalities were checked daily.

Analysis of the resulting survival of these flies revealed no significant difference in focal female survival between the Regular and Random regimes (nested coxme: $z = 0.45$, $p = 0.65$; median lifespan = 62days, 64days, respectively; figure S2; table S1). In contrast, Regular focal males lived significantly longer than Random males (nested coxme: $z = 2.50$, $p = 0.012$; median lifespan = 57days, 42days, respectively; figure S2). There were highly significant sex differences in survival within the random feeding regime. Random females lived significantly longer than Random males (nested coxme: $z = 6.74$, $p < 0.001$; median lifespan = 64days, 42days; figure S1). This pronounced sex difference in survival was absent in the Regular regime in which there was no significant difference between Regular female and male survival (nested coxme: $z = 0.78$, $p = 0.440$, median lifespan = 62days, 57days, respectively; figure S2). This was confirmed in a combined analysis of both sexes simultaneously, which revealed a significant sex x regime interaction effect on survival (coxme: $z=4.87$, $p<0.001$). This analysis shows that there was significantly greater SDL in the Random in comparison to Regular lines.

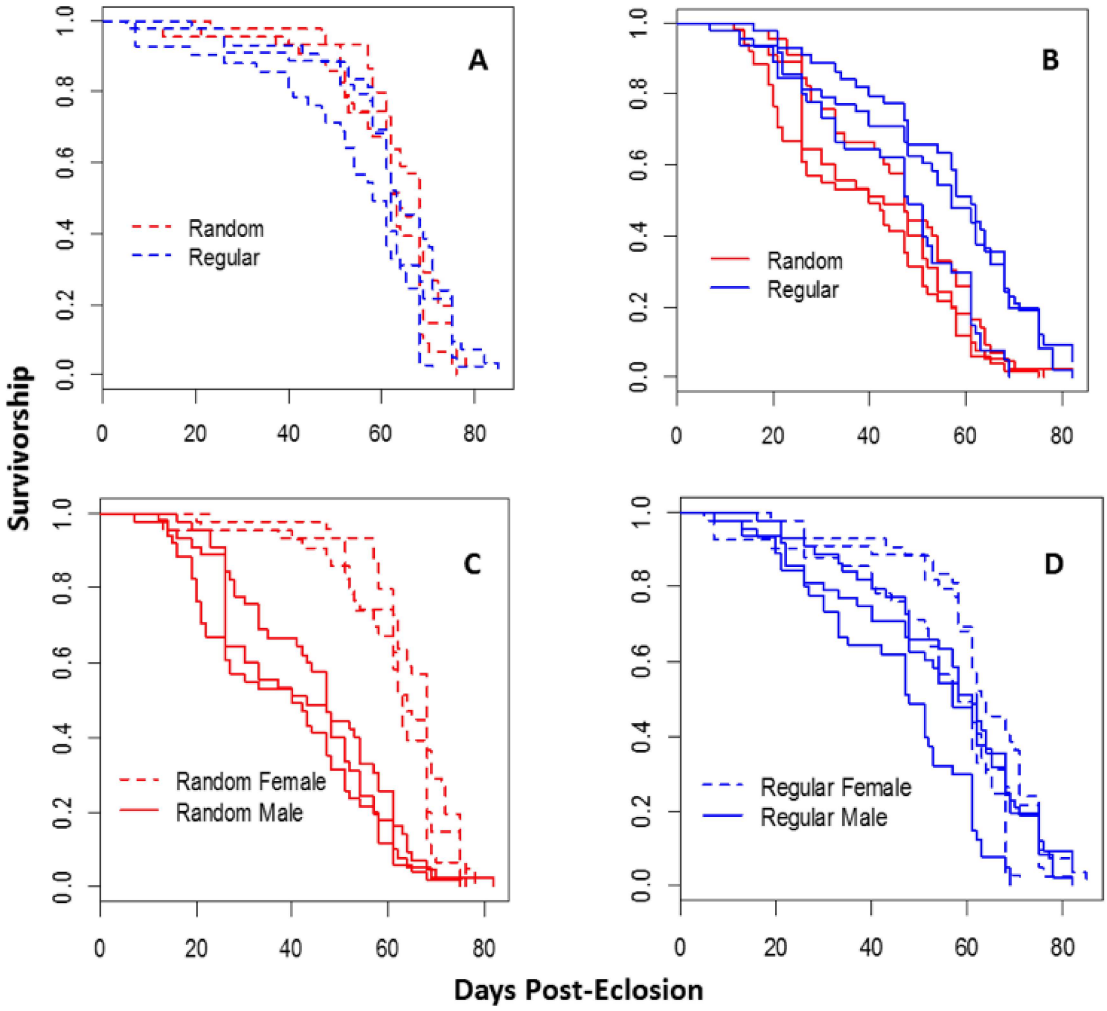

Figure S2. Baseline age-specific survivorship against days post-eclosion, across replicates 1-3 of once mated Random and Regular feeding regimes, held on standard (SYA) food. (a) Random vs Regular focal females; (b) Random vs Regular focal males, (c) Random females vs males, (d) Regular females vs males.

(c) Focal female and focal male mating frequency – main experiment

A significantly greater proportion of Regular males than Random males mated, during the 3h observations of weekly matings, over their lifetimes (GLM: $z = 2.12$, $p = 0.0338$). There was no difference in the mean proportion of focal females that mated during weekly mating observations, over lifetime, between feeding regimes (GLM: $t = 0.01$, $p=0.928$) (figure S3). A significantly greater proportion of focal males than focal females mated (GLM: $t = 5.45$, $p < 0.001$), but there was no significant regime x sex interaction effect on the proportion mated (GLM: t = 0.84, $p = 0.426$) (figure S3).

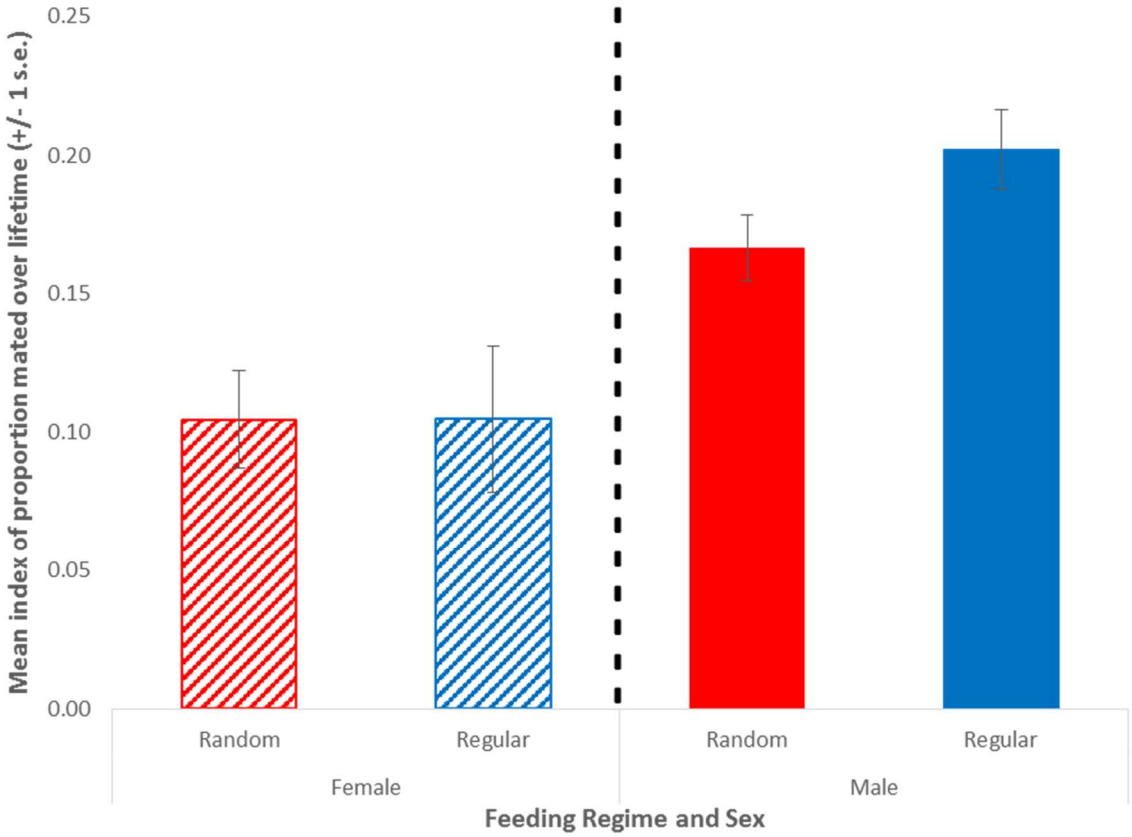

Figure S3. Index of mean proportion mated for Random and Regular feeding regime lines for each sex, over lifetime**.** Mean values for each feeding regime were calculated from the 3 lines for each regime (Random 1, Random 2, Random 3, and Regular 1, Regular 2, Regular 3), during the 3h observations of weekly matings, across lifetime. Hatched bars indicate females and solid bars indicate males.

(d) Developmental viability and developmental time of the Random and Regular males and females - main experiment

First instar F2 larvae (n = 3000 per treatment) were transferred to 20 SYA vials, at a density of 150 larvae/vial. The exact time of placing larvae in the vials was recorded, for later calculation of development time parameters. Adults emerging from half of the larval vials (n = 10) were used to record developmental parameters. Numbers of puparia were recorded up to 3 times per day (from day 5 to day 7 of development) and the numbers of adults recorded up to twice per day (from day 9 to day 13 of development). This enabled calculation of developmental timings and developmental viability between the first instar larval, puparium and adult stages.

There was no significant difference in developmental viability between Random and Regular feeding regimes, for overall first instar larva (L1) to adult (GLM: $t = 0.702$, $p = 0.485$) (figure S4a), for L1 to puparium (GLM: $t = 1.25$, $p = 0.214$) (figure S4b) or puparium to adult (GLM: $t = 1.42$, $p = 0.162$) (figure S4c). There was no significant difference between the sexes or between the regimes in the number of adults emerged (GLM: 'sexes' $t = 0.41$, $p = 0.686$; 'regimes' $t = 0.48$, $p = 0.630$).

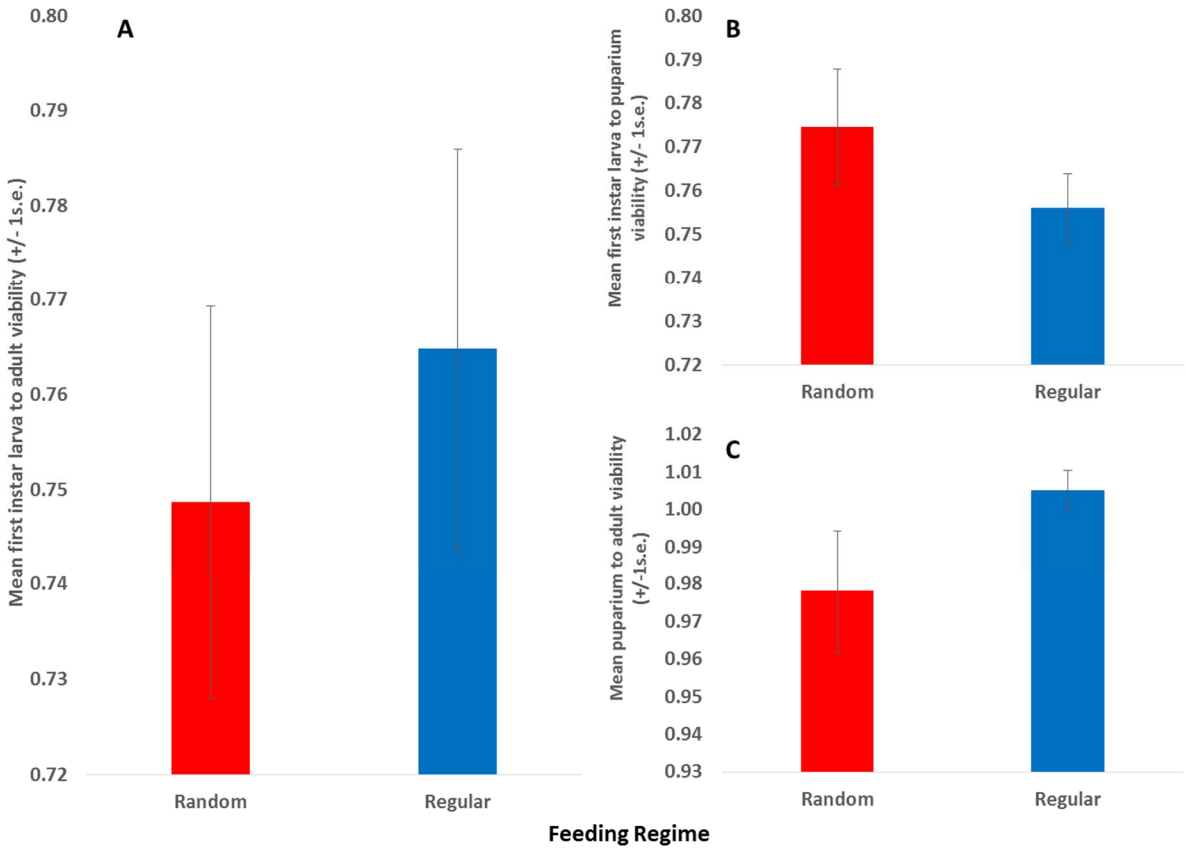

Figure S4. Mean developmental viability (+/- 1 s.e.) for focal adults from Random and Regular feeding regimes, developing on standard food, at first instar larva to adult (a), first instar larva to puparium (b) and puparium to adult (c) developmental stages.

There was also no significant difference in development time between focal adults from Random and Regular feeding regimes, for overall L1 to adult development time (two sample t-test: t_4 = 0.29, p = 0.785) (figure S5a), for L1 to puparium (t_4 = 0.43, p = 0.692) (figure S5b) or puparium to adult (t_4 = 0.24, p = 0.820) (figure S5c).

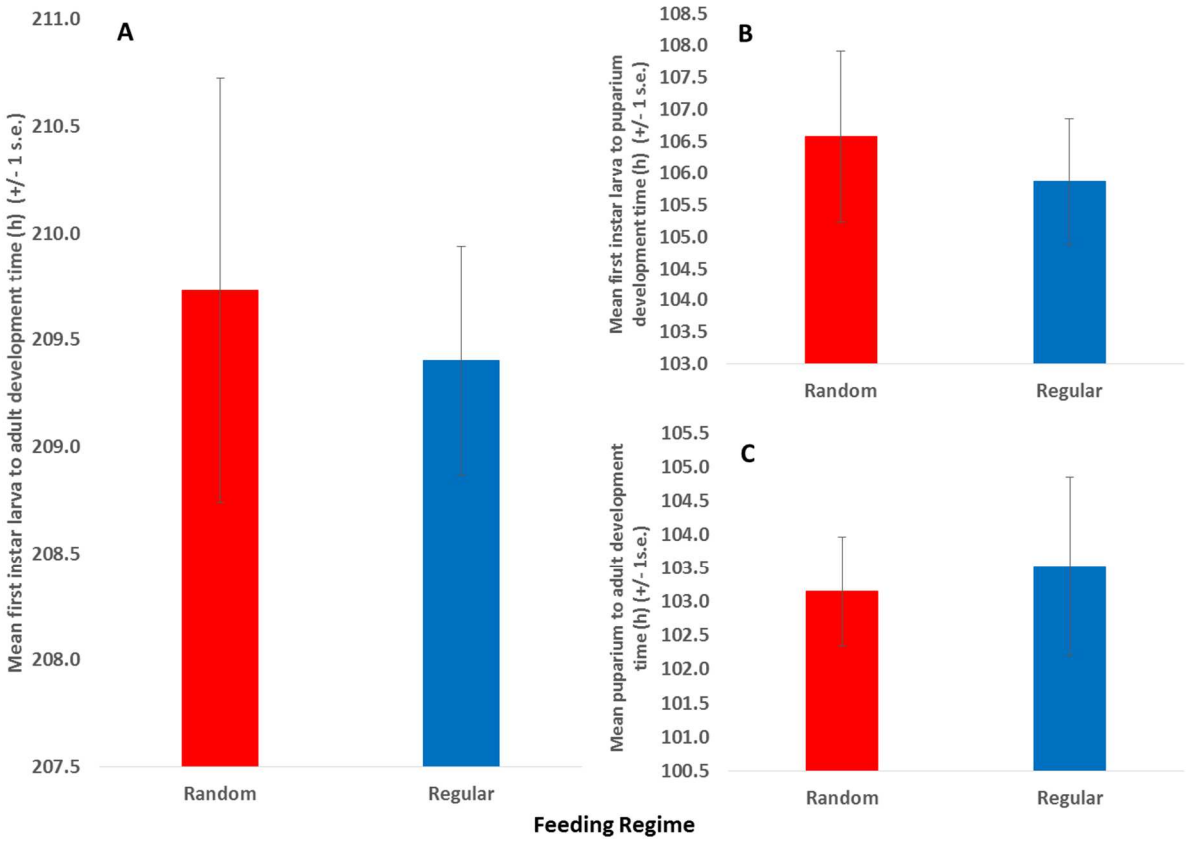

Figure S5. Mean development times (+/- 1 s.e.) for focal adults from Random and Regular feeding regimes, developing on standard food, at first instar larva to adult (a), first instar larva to puparium (b) and puparium to adult (c) developmental stages.

Female L1 to adult development time was significantly shorter than male L1 to adult development time, for both the Random regime (two sample t-test: $t_4 = 3.33$, $p = 0.0291$) and the Regular regime ($t_4 = 7.50$, $p = 0.00170$) (figure S6). There was no significant regime effect on the sex differences in development time (GLM: $t = 0.344$, $p = 0.740$) (figure S6).

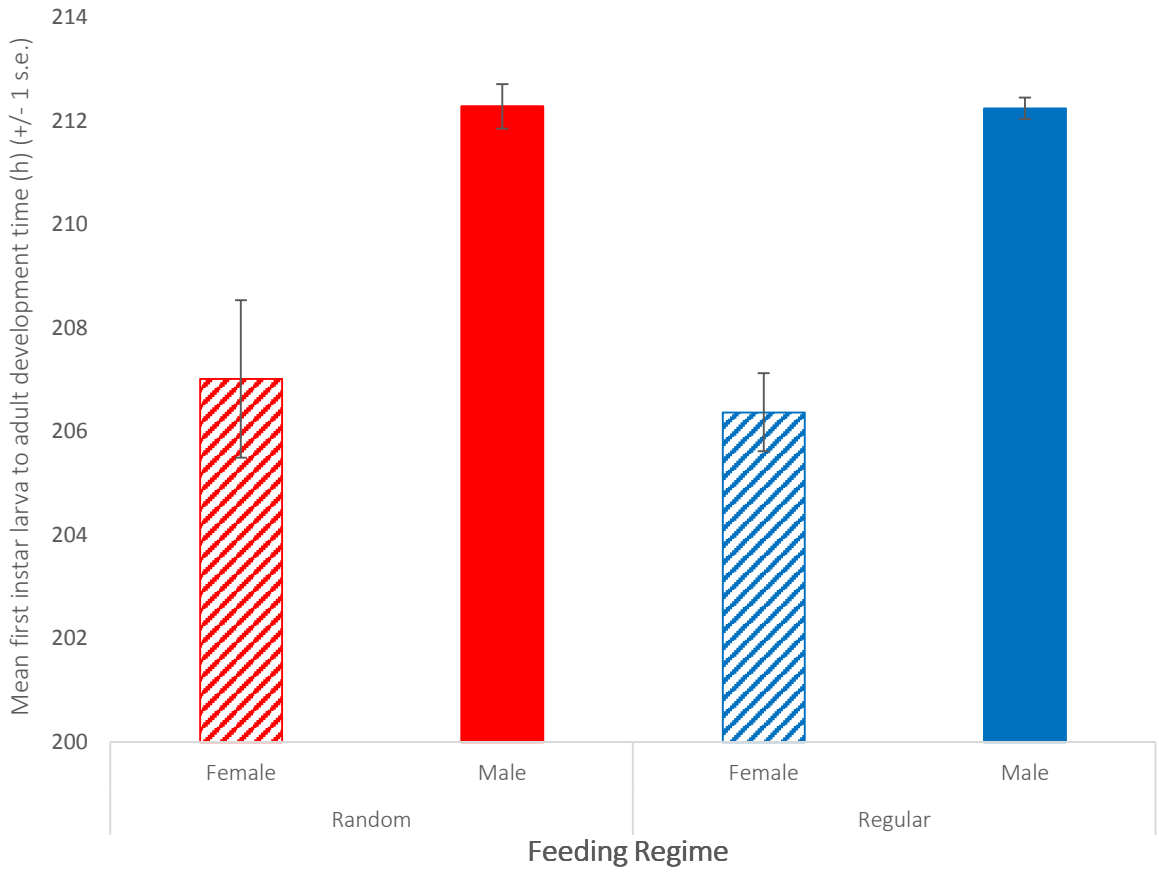

Figure S6. Mean first instar larva to adult development time (+/- 1 s.e.) for focal females and focal males from Random and Regular feeding regimes.

(e) Median survival time – main experiment

Table S1: Median focal female and male survival in days (+ interquartile range) for replicate Random (Rand 1,2,3) and Regular (Reg 1,2,3) regimes.

	Rand1	**Rand2**	**Rand3**	**Reg1**	**Reg2**	**Reg3**
Median female lifespan (interquartile range)	60 (7)	65 (8)	58 (14)	58 (13)	65 (12)	58 (12)
Median male lifespan (interquartile range)	47 (7)	46 (14)	51 (14)	46 (19)	53 (12)	51 (14)