Sex and Ageing:

A Test of Evolutionary Ideas

Doctoral Thesis by

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CERTIFICAN que Dña. Zahida Sultanova ha realizado bajo nuestra dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título "SEX AND AGEING: A TEST OF EVOLUTIONARY IDEAS", para optar al grado de Doctora en Ciencias Biológicas. Y para que así conste, en cumplimiento de la legislación vigente, así lo certificamos en Valencia, a 7 de julio de 2020.

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Table of Contents

Chapter 1. General Introduction 1

1.1. Evolution of Ageing 1

1.2. Sex-specific Ageing 6

1.2.1. Adaptive Processes 6

1.2.2. Maladaptive Processes 12

Chapter 2. Objectives 17

Chapter 3. General Materials and Methods 21

3.1. *Drosophila melanogaster* as a Model Organism 21

3.2. Experimental Populations 23

3.3. Statistical Analysis 25

Chapter 4. Social context and reproductive ageing in *Drosophila melanogaster* 27

4.1. Introduction 27

4.2. Materials & Methods 28

4.3. Results 31

4.4. Discussion 33

Chapter 5. Condition-dependent mortality, reproductive senescence and the potential for sexual conflict in *Drosophila melanogaster* 39

5.1. Introduction 39

5.2. Materials & Methods 41

5.3. Results 44

5.4. Discussion 54

Chapter 6. Male life history traits and gut microbiota in *Drosophila melanogaster* 61

6.1. Introduction 61

6.2. Materials & Methods 63

6.3. Results 71

6.4. Discussion 78

Chapter 7. The "unguarded-X" and the genetic architecture of sex-specific lifespan in *Drosophila melanogaster* 85

7.1. Introduction 85

7.2. Materials & Methods 88

7.3. Results 93

7.4. Discussion 99

Chapter 8. Genetic sex determination and sex-specific lifespan in tetrapods - evidence of a toxic Y effect 111

8.1. Introduction 111

8.2. Materials & Methods 115

8.3. Results 124

8.4. Discussion 129

Chapter 9. General Discussion 139

Chapter 10. Conclusions 147

Chapter 11. Resumen en castellano 149

References 163

Appendix 195

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Chapter 1. General Introduction

1.1. Evolution of Ageing

Ageing is the decline of an organism's biological functions with age, typically leading to a decrease in its reproductive abilities and an increase in its probability of death (Rose 1994). Such a decrease in reproduction and survival seems to be outrageously against Darwinian fitness, which probably explains why studying the evolution of ageing has captured the interest of evolutionary biologists for decades (Medawar 1952; Williams 1957; Kirkwood 1977).

The persistence of ageing despite its negative effects on fitness has its cornerstone in the idea that the strength of selection decreases with age. This idea was put forward by Fischer (1931) and Haldane (1941), and first presented in a verbal and graphical model by Peter Medawar (1946). Medawar's intuition rested on the observation that, even in the absence of intrinsic mortality, extrinsic mortality sources (e.g. predation, disease, accidents) gradually decrease the probability of individuals to survive. Therefore, Medawar predicted that, after an individual successfully develops to become an adult and starts reproducing (i.e., after sexual maturation), the strength of natural selection will gradually decrease with age, leading to a "Selection Shadow" (Figure 1.1). Hamilton was the first to formalize the idea that the strength of selection wanes across the lifetime of an individual, showing that selection gradients on mortality do actually inevitably decrease with age because the age at which a gene acts is inversely proportional to its influence on fitness (Hamilton 1966). Despite recent contention about the role of extrinsic mortality in the selection shadow (Caswell 2007; Wensink *et al.* 2017; Moorad *et al.* 2019; Day and Abrams 2020), the central tenet that selection becomes less intense with age is robust and the basis for the major theories explaining the evolution of ageing (Medawar 1952; Williams 1957; Kirkwood 1977; Rose 1994).

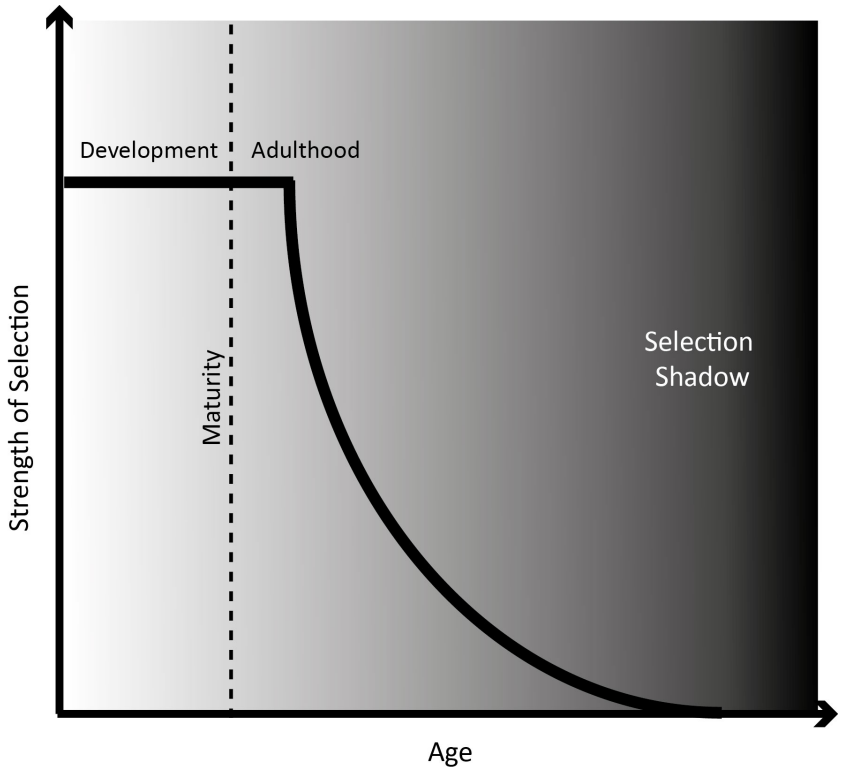

Figure 1.1. Change in the strength of selection with age. The strength of selection is expected to decline after an individual reaches adulthood (sexual maturation) and starts reproducing, leading to a "Selection Shadow" on later ages.

First, Peter Medawar proposed (1952) that ageing results from the accumulation, during evolutionary time, of negative mutations that act late in life ("mutation accumulation theory"), when natural selection is weaker (Figure 1.2a). This theory has been tested by exploring whether there is an increase in additive genetic variance in mortality rate with age, due to the accumulation of late-acting mutations. Although initial studies seemed to support this prediction by reporting an increase in additive genetic variance in mortality at later ages (Hughes and Charlesworth 1994; Charlesworth and Hughes 1996), later experiments and reanalyses have casted doubts on this conclusion (Promislow *et al.* 1996; Shaw *et al.* 1999). An extension to this theory was later proposed stating that late acting

deleterious mutations can also be maintained in the population if their early life effects are only slightly deleterious (i.e., positive pleiotropic effects, see Maklakov *et al.* 2015). The presence of these positive pleiotropic alleles is supported by mutation accumulation studies, where spontaneous mutations have been found to reduce early and late life fitness (Houle *et al.* 1997; Estes *et al.* 2005; Kimber and Chippindale 2013). To sum up, "mutation accumulation theory" predicts that, due to the selection shadow, mutations with negative effects late in life are expected to accumulate as long as they are slightly deleterious or do not have any effect on early life (i.e., selectively neutral), when selection is stronger.

Second, in 1957 George Williams posited the other theory that was to dominate evolutionary explanations of ageing, the "antagonistic pleiotropy theory". According to this theory, the evolution of ageing may ensue due to the accumulation of antagonistic pleiotropic alleles that have positive effects early in life but are deleterious late in life. As natural selection is stronger at earlier ages, mutations that increase early life fitness at the expense of late life fitness are expected to accumulate (Figure 1.2b). This theory has mainly found support in artificial selection experiments selecting for longer lifespan and testing if this causes lower early life fitness. Selection for increased late life reproduction or lifespan has indeed been found to be correlated with decreased reproduction (Rose and Charlesworth 1980; Luckinbill *et al.* 1984; Partridge *et al.* 1999; Sgrò and Partridge 1999). Yet, there are also numerous cases where lifespan increases without associated reproductive costs (reviewed in Flatt 2011), again casting doubts about the generality of this theory.

An important point here is that both theories predict an increase in mortality rate with age; however, in nature there are many examples where mortality rate plateaus at late ages (Fox *et al.* 2004; Miyo and Charlesworth 2004; Barbi *et al.*

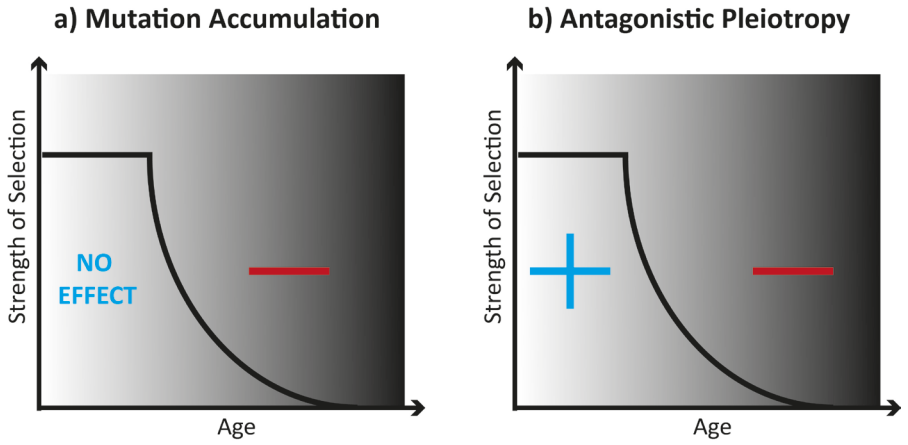

Figure 1.2. **Mutation accumulation theory vs. Antagonistic pleiotropy theory.** (a) Mutation accumulation theory suggests that ageing results from the accumulation of mutations that have no effect early in life but negative effects late in life. (b) Antagonistic pleiotropy theory suggests that ageing is caused by the accumulation of antagonistic mutations that have positive effects early in life but negative effects late in life.

2018). For example, Barbi *et al.* (2018) showed that in humans, age-specific mortality becomes constant beyond the age 105 by using high quality data from an Italian population (Barbi *et al.* 2018). Several studies have tried to marry these observations with existing theories of ageing (Mueller and Rose 1996; Pletcher and Curtsinger 1998), giving rise to two main explanations for late life mortality rates. The first one is based on selective disappearance and how it differentially affects individuals in a population depending on their robustness (a sum of factors that determine an individual's likelihood of survival). As mortality will remove less robust individuals at early ages, more robust individuals will contribute to the decreased mortality rate at late ages (Vaupel *et al.* 1979; Brooks *et al.* 1994; Chen *et al.* 2013). The second explanation is based on the fact that the strength of natural selection declines after first reproduction and becomes zero when an organism stops reproducing. In parallel with the strength of natural selection, age-specific mortality is also expected to decelerate, leading to mortality plateaus (Rauser *et al.* 2009; Mueller *et al.* 2011).

Third, in 1977 Thomas Kirkwood proposed the "disposable soma theory", which argues that as organisms have access to limited resources, they should optimize their allocation of energy between somatic maintenance and reproduction so as to maximize their fitness (Kirkwood 1977). For example, somatic maintenance requires energy as it involves costly processes such as genome repair or clearing misfolded proteins (Kirkwood 2008). Likewise, organisms spend energy for reproduction due to processes such as egg/sperm production and mating activities (Otronen 1995; Olsson *et al.* 1997; Marshall *et al.* 1999). Thus, optimizing fitness can require diverting resources from somatic maintenance to reproduction, and ageing may ensue. This differential resource allocation towards early life reproduction or development, instead of long-term somatic maintenance, will be even more beneficial in light of a decrease in selection gradients against mortality with age (Hamilton 1966; Caswell 2007; Wensink 2017). From this perspective, disposable soma theory and antagonistic pleiotropy theory are somewhat convergent in the sense that pleiotropic gene effects can modulate how to allocate energy between somatic maintenance and reproduction (Kirkwood and Rose 1991). As a matter of fact, some researchers have gone so far so as to define the disposable soma theory as a physiological mechanism of antagonistic pleiotropy theory (Maklakov and Chapman 2019).

Finally, the more modern "developmental theory of ageing" argues that senescence may result from the fact that physiological processes are optimized for early but not late ages (Maklakov and Chapman 2019). In fact, the example given by Williams in his classic paper (Williams 1957) describes an allele with beneficial effects on bone calcification during development, but deleterious effects via the calcification of arteries late in life. Actually, there is no reason why this allele could not be repressed late in life, and so this is a typical example for an allele that results in ageing due to its suboptimal expression in adulthood (de Magalhães and Church 2005; Walker 2011; De Magalhães 2012). Hence, given that it results from age-specific pleiotropic effects of alleles, developmental

theory of ageing can be considered as another mechanism of antagonistic pleiotropy theory. In contrast to the disposable soma theory, that predicts early life fitness costs (e.g. reduced fecundity) as a side effect of better somatic maintenance (e.g. higher longevity), the developmental theory of ageing predicts that increased longevity can be decoupled from these costs by age-specific optimization of gene expression. This is consistent with the identification of genes that are found to be detrimental when deactivated during development, but increase lifespan when deactivated during adulthood (Curran and Ruvkun 2007; Tacutu *et al.* 2012).

1.2. Sex-specific Ageing

One of the most complex problems in the biology of ageing lies in explaining why males and females age differently (Maklakov and Lummaa 2013). Sex-specific ageing is widely observed across the tree of life. For example, females live three times longer than males in the brown antechinus, a small marsupial, while males are twice as likely as females to survive from one year to the next in Arabian babblers, a passerine bird (Keller and Waller 2002; Clutton-Brock and Isvaran 2007). In humans, the lifespan gap is estimated to be 4.8 years - female life expectancy is 74.7 years while male life expectancy is 69.9 years (DESA 2019). Several hypotheses have been proposed to explain this phenomenon, stemming from two complementary but fundamentally distinct perspectives: adaptive and non-adaptive processes.

1.2.1. Adaptive Processes

Sex-specific ageing can be adaptive and may reflect sex differences in selective pressures and age-dependent risks of extrinsic mortality (Vinogradov 1998; Carranza and Pérez-Barbería 2007; Clutton-Brock and Isvaran 2007; Bonduriansky *et al.* 2008; Berg and Maklakov 2012; Adler and Bonduriansky 2014). Due to the evolution of anisogamy (small, cheap but numerous male

sperms vs. big, expensive but fewer female eggs) and subsequent gestation costs, females are typically the sex with higher parental investment (albeit in some species males exhibit as much or more parental investment than females –Trivers 1972– and a lower potential reproductive rate –Shuster and Wade 2003–). As a consequence, intrasexual selection is expected to be stronger in the sex with less parental investment (generally males), resulting in higher competition within that sex and, ultimately, more variation in fitness and a higher opportunity for selection (Bateman 1948, Trivers 1972). In turn, this will lead to the evolution of different reproductive strategies in the sexes (Janicke *et al.* 2016) and, inasmuch as males and females are exposed to sex-specific selection pressures, male and female life histories are expected to diverge. For example, intense intrasexual competition early in life tends to promote "live-fast, die-young" strategies in males compared to females (Trivers 1972; Promislow 1992; Kruger and Nesse 2004; Clutton-Brock and Isvaran 2007).

Sexual conflict, social context and sex-specific ageing

Sexual conflict adds another layer of complexity to sex-specific ageing theory. Firstly, differential optimization of life histories will result in intra-locus sexual conflict (IASC), which may hamper the evolution of sexually dimorphic life histories (Promislow 2003). Considering that the majority of genes are shared between males and females, alleles at the same loci are, due to diverging interests between the sexes, sometimes be selected in opposite directions in different sexes, generating IASC and constraining the evolution of sexual dimorphism. For example, IASC has been demonstrated in *Drosophila melanogaster* where Chippindale *et al.* (2001) found a negative genetic correlation for adult fitness between males and females across 40 haploid genomes (Chippindale *et al.* 2001). Secondly, inter-locus sexual conflict (IRSC), which arises due to the antagonistic interactions between phenotypic traits which are underlain by alleles at different male and female loci, can also drive sex-specific effects on longevity. For example, the seminal fluid proteins in *D. melanogaster* increase male

reproductive success at the expense of female lifetime reproductive success and lifespan (Chapman and Davies 2004; Wigby and Chapman 2005). In summary, selection for sex-specific optimal trait values is expected to cause both inter and intrasexual conflict between males and females (Maklakov and Lummaa 2013). Interestingly, recent research suggests that ageing itself can lead to sexual conflict. Given that senescence of one sex can impose significant reproductive costs to their mating partner in terms of lower fertility and offspring viability, it has been suggested (and shown in at least two species) that ageing can be a source of IRSC (Dean *et al.* 2007, 2010; Carazo *et al.* 2011). Hence, understanding the dynamic interplay between ageing and sexual conflict is an interesting and relatively unexplored avenue of research with respect to sex-specific ageing.

Similarly, we know relatively little about how social context may modulate sex-specific selective pressures and sexual conflict, and how this may affect sex-specific lifespan. For example, the sex ratio at reproduction will directly affect sexual selection and sexual conflict (Kvarnemo and Ahnesjo 1996; Kokko and Rankin 2006), and potentially sex-specific lifespan. In line with this, social context has been found to have significant effects on sex-specific lifespan and ageing in several species, including fruit flies and humans (Botev 2012; Leech *et al.* 2017). In feral fowls (*Gallus gallus)*, old males were able to sire relatively more offspring in a female-biased social context (where they have higher chance of being socially dominant), due to lower intrasexual competition in that social environment (Figure 1.3; Dean *et al.* 2010). Altogether, evidence from the literature thus suggests that social context can: (1) modulate sex-specific ageing through its effects on sexual selection and sexual conflict, and (2) modulate the intensity of selection on old ages by influencing the amount of offspring that old individuals contribute to future generations. Surprisingly, the role of social context on the evolution of sex-specific life histories has been largely overlooked.

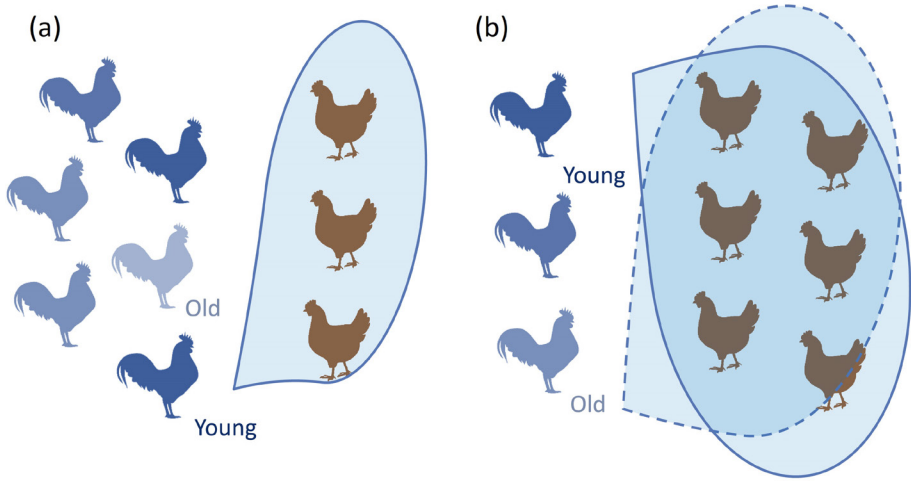

Figure 1.3. The effect of social context on the chances of young and old males to dominate a group. Rebeca Dean and colleagues demonstrated that in feral fowls: (a) young males have higher chances of being socially dominant and monopolizing females in a male-biased social context; however, (b) old and young males have similar chances of dominating the group and monopolizing females in a female-biased social context.

Extrinsic mortality and sex-specific ageing

Unsurprisingly given its relevance to explain the evolution of ageing *per se*, a priority to understand sex-specific ageing is to study extrinsic mortality effects across the sexes. The link between mortality and sex-specific life histories has received a lot of attention. In males, elevated mortalities are frequently associated with intrasexual selection, for example as a result of costly visual/vocal sexual displays (e.g. increased predation risks; Figure 1.4) and male-male combats leading to direct injury or death (Promislow 1992; Liker and Székely 2005). Intersexual selection can also cause males to die younger. In the brown widow spiders (*Latrodectus geometricus)*, males sometimes sacrifice themselves to females (i.e., sexual cannibalism) as an extreme nuptial gift that increases their reproductive success (Segoli *et al.* 2008). As an obvious consequence of this behaviour, brown widow spider females live more than three times longer than males (Mohafez 2015). Sex-specific reproductive strategies can also cause

Figure 1.4. The tragic cost of a vocal display. A male frog predated by a snake while singing courtship song in order to attract females. This is an example of how high intrasexual competition for females can make males more vulnerable to predation. Photo credits: Javier Abalos.

female-biased mortalities. For example, in the long-tailed dance fly (*Rhamphomyia longicauda*), males provide females with nuptial gifts in exchange for copulations and, therefore, females tend to exhibit high intrasexual competition for mating (Funk and Tallamy 2000). As a result, females of this species are more prone to being predated (e.g. captured by spider webs) compared to males (Gwynne and Bussière 2002). Likewise, in other species females can have lower survival than males because of the increased probability of predation during maternal care, such as in some birds (Liker and Székely 2005). In short, under natural conditions sexual selection can strongly influence which sex will survive longer to extrinsic mortality hazards.

Predicting how sex differences in extrinsic mortality translate into sex-specific intrinsic mortality (ageing) is less straightforward. High extrinsic mortality is expected to generally accelerate ageing because it will contribute decisively to

weaken natural selection with age (Haldane 1941; Medawar 1952; Williams 1957; Kirkwood 1977). However, when mortality is condition dependent, it might result in slower ageing and longer lifespan due to co-variation between survival and condition (Chen and Maklakov 2012; Maklakov *et al.* 2015; Chen *et al.* 2016). In other words, condition dependent selection for a particular trait (such as higher heat resistance) will be coupled by selection with other beneficial traits, resulting in longer lifespan (Maklakov *et al.* 2015). Irrespective of the source of mortality, inasmuch as the sexes are subject to sex-specific selection pressures, existing trade-offs are expected to be optimized in different ways in males and females (Trivers 1972; Bonduriansky *et al.* 2008; Berg and Maklakov 2012). For example, in the roundworm *Caenorhabditis remanei,* non-random extrinsic mortality causes the evolution of longer lifespan and higher female fecundity, but leads to a decline in male reproductive success, suggesting a trade-off between ageing and early reproductive success in males but not females (Chen and Maklakov 2012; Chen *et al.* 2016). Unfortunately, how random vs. condition dependent extrinsic mortality affects sex-specific life history evolution in other species remains largely unexplored.

Sex-specific life history trade-offs and their mechanisms

As a corollary, understanding the mechanisms underlying the presence of life history trade-offs across the sexes can also help us understand sex-specific selection pressures on life history. A promising research line to tap into sex-specific reproduction/survival trade-offs can be investigating the link between reproductive success and gut microbiota. Recent studies have explored the importance of gut microbiota on lifespan, ageing and female reproduction in a range of species from diverse genera such as *Drosophila* (Brummel *et al.* 2004; Ren *et al.* 2007; Clark *et al.* 2015; Gould *et al.* 2018), *Daphnia* (Sison-Mangus *et al.* 2015; Callens *et al.* 2016), *Caernohabditis* (Houthoofd *et al.* 2002; Cabreiro and Gems 2013) and humans (Tiihonen *et al.* 2010; Insenser *et al.* 2018). In contrast, we know very little about the link between reproductive success and gut

microbiota in males. If such links where to diverge between the sexes, this may allow us to better understand sex-specific ageing from both a mechanistic and functional perspective. In fruit flies at least one bacteria species has been found to affect male reproductive traits (Morimoto *et al.* 2017). Compared to the high amount of studies that have investigated the role of gut microbiota on ageing and fecundity (Wong *et al.* 2011; Clark *et al.* 2015; Clark and Walker 2018; Gould *et al.* 2018), the scarcity of studies investigating the link between male reproductive traits (competitive fitness, sperm quality etc.) and gut microbiota is surprising.

1.2.2. Maladaptive Processes

In contrast to adaptive hypotheses, maladaptive hypotheses explain sex differences in aging as maladaptive consequences of the asymmetric inheritance of different genetic components (Maklakov and Lummaa 2013).

The "mother's curse" hypothesis

The "mother's curse" hypothesis proposes that the mitochondrial genome may play a role in decreasing male lifespan, which would contribute to explain sex-specific ageing in taxa where females live longer than males (Camus *et al.* 2012). As the mitochondrial genome is maternally transmitted, mutations with deleterious effects for males can accumulate as long as they have positive, neutral, or even slightly deleterious effects for females (Charlesworth 1994; Frank and Hurst 1996; Wolff and Gemmell 2013). Consequently, maternal inheritance of mitochondrial DNA sets the scene for a sex-specific selective sieve that can lead to female-biased lifespan (Frank and Hurst 1996; Gemmell *et al.* 2004; Zeh and Zeh 2005; Dowling *et al.* 2010; Innocenti *et al.* 2011; Camus *et al.* 2012). Recent studies suggest that mitochondrial haplotype can also have sex-specific effects on lifespan in terms of cyto-nuclear interactions, where genetic variation across mitochondrial and nuclear genomes interact to shape life history outcomes (Drummond *et al.* 2019; Vaught *et al.* 2020). Although there is

substantial evidence that mitochondrial DNA can indeed affect sex-specific lifespan, it leaves many answers unexplained. For example, it does not provide an explanation for the shorter female lifespan that is observed in many taxa, such as in birds (Liker and Székely 2005).

The "unguarded-X" hypothesis

Second, the "unguarded-X" hypothesis (hereafter UXh) (Figure 1.5a) posits that sex-specific ageing may be caused by the increased expression of deleterious recessive mutations in the heterogametic sex, due to the asymmetric inheritance of the sex chromosomes (Trivers 1985). While recessive mutations in the X (or Z) chromosome will be expressed unconditionally in the heterogametic sex, the same will not happen in the homogametic sex owing to the second copy of the X (or Z) chromosome, which will "guard" against their expression. Hence, the "unguarded-X" effect generally predicts slower ageing and longer lifespan in the homogametic sex (Trivers 1985). Studies looking at the correlation between sex-specific ageing and sex determination systems have provided indirect support for this hypothesis (Pipoly *et al.* 2015; Xirocostas *et al.* 2020). Pipoly *et al.* (2015) used adult sex ratios as a proxy for sex-specific survival and found that adult sex-ratios are typically female-biased in taxa with XY sex-determination system, but male-biased in the ones with ZW sex-determination system. More recently, Xirocostas *et al.* (2020) found that the heterogametic sex tends to have higher mean/maximum lifespan across a wide taxonomic range. Finally, recent experimental evidence suggests that un-guarding the X chromosome may reduce the sex lifespan gap in *D. melanogaster* (Carazo *et al.* 2016; but see Brengdahl *et al.* 2018).

The "toxic Y" hypothesis

Finally, the more recent "toxic Y" hypothesis focuses on the role of the heteromorphic Y (or W) chromosome on sex-specific ageing (Figure 1.5b, Marais *et al.* 2018). During the evolution of sex chromosomes, recombination

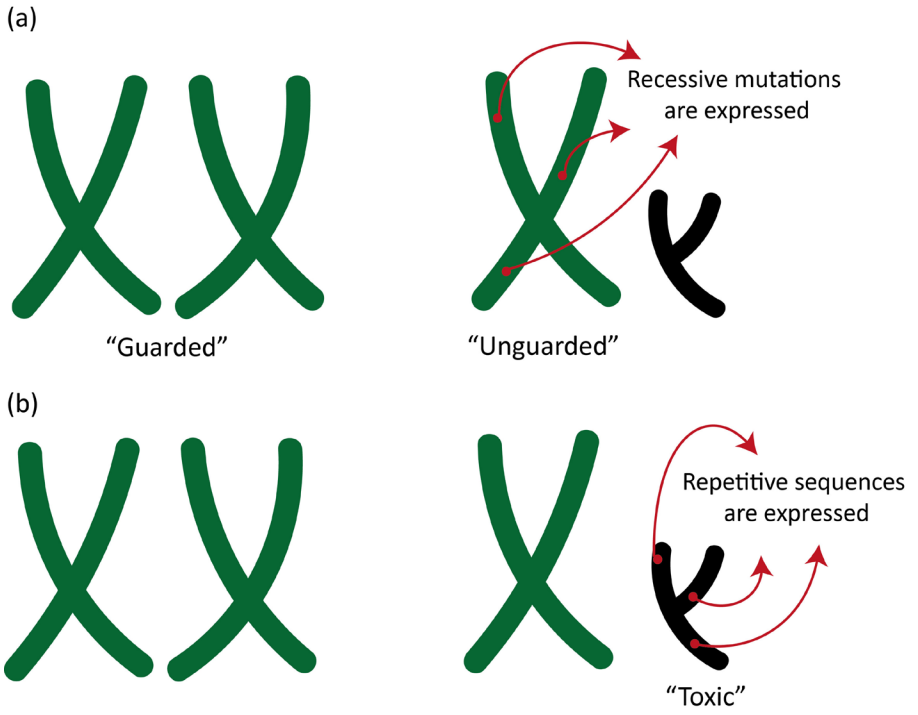

Figure 1.5. Schematic Illustrations of Unguarded-X Hypothesis and Toxic Y Hypothesis. (a) The unguarded-X hypothesis posits that heterogametic sex lives shorter because the recessive deleterious mutations in the X (or Z) chromosomes are "guarded" by the second copy of X (or Z) chromosome in the homogametic sex (on the left) but they are expressed in the heterogametic sex (on the right). (b) The toxic Y hypothesis suggests that heterogametic sex lives shorter due to the expression of deleterious repetitive sequences that are present in the Y (or W) chromosomes of the heterogametic sex (on the right).

suppression leads to the accumulation of deleterious mutations and repetitive DNA (satellite sequences and transposable elements) in the Y and W chromosomes (Bachtrog 2013; Wright *et al.* 2016). Recent evidence has shown that, in *D. melanogaster,* repetitive DNA sequences on the Y chromosome become de-repressed with age, resulting in the mis-expression of transposable elements (Brown *et al.* 2020b). In order to test how de-repression of transposable elements in the Y chromosome affects sex-specific lifespan, Brown *et al.* (2020b) generated flies with different sex chromosome karyotypes: XXY females; X0 and

XYY males in addition to wild-type karyotypes: XX females and XY males. They found a positive correlation between the de-repression of repeats and the number of Y chromosomes, and a negative correlation between average lifespan and the number of Y chromosomes (Brown *et al.* 2020b). Moreover, in another study, Brown *et al.* (Brown *et al.* 2020a) found that Y-chromosome affects heterochromatin integrity genome-wide by acting as a sink for heterochromatin machinery components and therefore diminishing the heterochromatin protection on other normally silenced repeat-rich sequences. This can further contribute to sex-specific gene expression and sexual dimorphism in life history traits, including lifespan (Brown *et al.* 2020a). Therefore, in *D. melanogaster* there is solid evidence of substantial "toxic Y" effects, where the accumulation of repetitive DNA elements can cause increased mortality of the heterogametic sex (Wright *et al.* 2016; Marais *et al.* 2018). However, the role of the "toxic Y" hypothesis in explaining broad patterns of sex differences in ageing has yet to be addressed.

Chapter 2. Objectives

The overarching aim of this thesis was to explore open questions regarding the evolution of sex-specific ageing. To this end, this thesis was organized in two parts that addressed adaptive and maladaptive questions in relation to sex-specific ageing. Adaptive questions seek to further our understanding of sex-specific selection pressures leading to sex differences in different ageing processes, while maladaptive questions focus on trying to understand how broad patterns of sex-specific ageing may have partly come about as a by-product of asymmetric inheritance between the sexes. Both types of explanations should ultimately contribute towards a comprehensive understanding of sex-specific ageing across the tree of life. These goals were achieved through five specific objectives, three on Aim 1 (Chapters 4, 5 and 6) and two on Aim 2 (Chapters 7 and 8).

Aim 1. To further our understanding of sex-specific selection pressures in relation to ageing.

Objective 1.1. To explore how the social context may influence age fitness effects in males and females (Chapter 4).

The social context can have crucial effects on sex-specific life histories for two main reasons. First, by modulating the opportunity for intra- vs. intersexual selection mechanisms, and ensuing sex-specific selective pressures. Second, by determining the relative contribution of old individuals to future generations, and hence the intensity of selection on males and females of old age. Yet, studies that explore how social context interacts with age are relatively scarce. The objective of this chapter was thus to explore if social context can modulate age effects on reproductive success in males and females in *D. melanogaster*.

Objective 1.2. To investigate the effect of condition dependent mortality on reproductive senescence of male and female cohorts (Chapter 5).

Recent research posits that condition dependent extrinsic mortality can be an important factor in the evolution of sex-specific life histories. My objective here was to explore how condition dependent mortality affects reproductive senescence in male/female cohorts of *D. melanogaster*. Here, I also aimed to address the idea that condition dependent extrinsic mortality may enhance the potential for male ageing to cause sexual conflict.

Objective 1.3. To explore how the gut microbiota may affect male life history traits (Chapter 6).

As a consequence of inherently 'live-fast, die-young' male reproductive strategies, males have often been found to trade off early reproductive success against survival. An increasing appreciation of the role that gut microbiota plays in shaping organism phenotypes has led to an emerging field in the study of ageing, but most research has focused on the influence of gut microbiota in female life history. The objective that motivated this chapter was thus to explore the link between gut microbiota, male reproductive success and ageing in *D. melanogaster*.

Aim 2. To further our understanding of how the sex determination system constrains sex-specific ageing.

Objective 2.1. To provide an experimental test of the unguarded-X hypothesis in D. melanogaster (Chapter 7).

The "unguarded-X" hypothesis focuses on the role of heteromorphic sex chromosomes (X or Z) in sex-specific aging. Recessive mutations in the X or Z chromosome will be unconditionally expressed (i.e., "unguarded") in the

heterogametic sex, but not in the homogametic sex. As a result, this may give rise to sex differences in lifespan. My objective in this chapter of my thesis was to test a fundamental prediction of the unguarded-X hypothesis: that inbreeding should depress the lifespan of the homogametic sex more than the lifespan of the heterogametic sex, for which I used *D. melanogaster*.

Objective 2.2. To use a comparative approach to test predictions from the "unguarded-X" vs. "Toxic Y" effects across vertebrates (Chapter 8).

The "unguarded-X" hypothesis has so far dominated explanations of broad sex-specific lifespan patterns. This is perhaps because it seems to fit well with the intuitive link between sex determination systems and sex lifespan gaps across taxa, such as in birds (ZW, males seem to live longer) and mammals (XY, females seem to live longer). However, both the "unguarded-X" and the more recent "toxic Y" hypotheses predict a correlation in the same direction between the sex determination system and sex-specific ageing. Predictions from these two hypotheses do diverge when it comes to the link between the relative size of the sex chromosomes and sex-specific lifespan. The unguarded-X hypothesis predicts a positive relationship between the lifespan gap (i.e., homogametic sex – heterogametic sex lifespan) and the size of the X (or Z) relative to both the Y (or W) chromosome and to the autosomes. The "toxic Y" hypothesis predicts a direct negative relationship between the size of Y (or W) chromosome and the lifespan of the heterogametic sex. My objective here was to: a) test for the existence of a direct link between sex-specific survival and the sex determination system across vertebrates, and b) explore the relationship between the size of sex chromosomes and the sex gap in lifespan in vertebrates.

Chapter 3. General Materials and Methods

3.1. *Drosophila melanogaster* as a Model Organism

In this thesis, I used the vinegar fly *D. melanogaster* as a model organism. More than a century ago, Thomas Hunt Morgan peered through a scope in his soon-to-be famous "Fly Room" at Columbia University, where bunches of ripe Bananas frequently hang prominently from the ceiling (Figure 3.1). To his surprise, the fly

Figure 3.1. An old photograph of Dr. Morgan's Fly Room, 1914. Photograph is taken from https://integrativebio.utexas.edu/about/history/the-fly-room.

that met his eyes on this particular day of 1910 had white eyes instead of the brilliant red eyes that are characteristic of this species. This event probably marked the rise of *D. melanogaster* as a model organism in biology, perhaps "the" model organism in the study of genetics and, later, evolution. Since then, *D. melanogaster* has maintained its popularity as a model organism for decades owing (apart from a historical contingency) to its easy maintenance in the laboratory, ability to produce a large number of offspring and short generation time (approximately 10 days at 25^{o}C, illustrated in Figure 3.2). It has contributed to advance research in many fields including evolutionary biology (Powell 1997),

immunology (Buchon *et al.* 2014), oncology (Vidal and Cagan 2006), neurobiology (Jeibmann and Paulus 2009) and many others (Kaun *et al.* 2011; Prüßing *et al.* 2013; Ong *et al.* 2015).

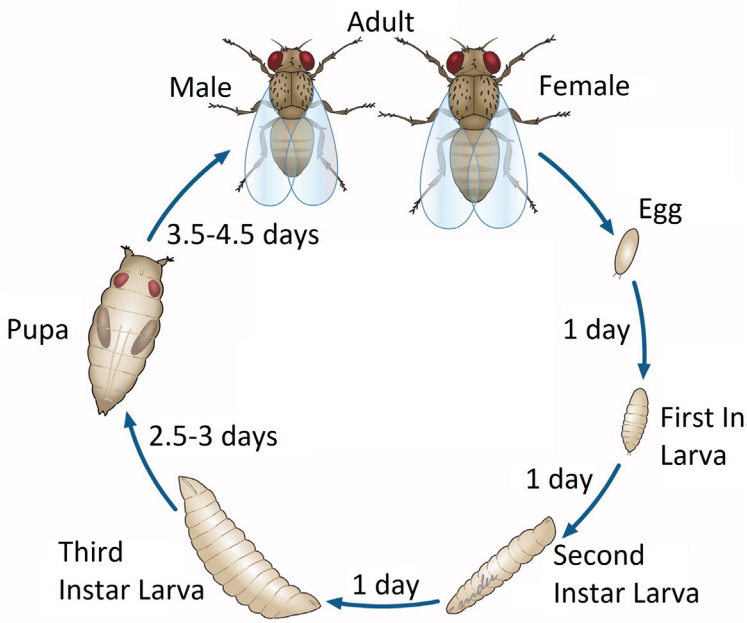

Figure 3.2. **Life cycle of *D. melanogaster*.** Illustration is modified from (Ong *et al.* 2015).

D. melanogaster is also an ideal model organism to study sex-specific life history evolution because it exhibits a sexually dimorphic lifespan, where females live longer than males (Rose *et al.* 2004). Its mating behaviour and life history traits have been very well-studied, providing the basics for more detailed research about sex-specific life history evolution. Briefly, in *D. melanogaster* males have strong intrasexual competition over mating, and females are able to re-mate with multiple males (Dow and Schilcher 1975; Pitnick 1991; Markow 2002). Intrasexual competition is mostly observed in males; however, aggression between females is also present (Ueda and Kidokoro 2002; Bath *et al.* 2017,

2018). Both males and females exhibit mate choice (Gowaty *et al.* 2003; Byrne and Rice 2006; Edward and Chapman 2012, 2013; Monier *et al.* 2018). In males, intrasexual competition is believed to be stronger than intersexual selection, while in females intersexual selection appears to be more important (Gowaty *et al.* 2003). In addition, many candidate genes involved in ageing and reproductive success in males and females have been described in this species (Parkes *et al.* 1998; Kapahi *et al.* 2004; Innocenti and Morrow 2010; Partridge *et al.* 2011; Durham *et al.* 2014). Finally, it has a low-diversity bacterial community in the gut (Wong *et al.* 2011) and its gut microbiota has been found to affect fitness and lifespan (Clark *et al.* 2015; Gould *et al.* 2018). As a result, it is an excellent organism to study the interaction between gut microbiota and sex-specific life history evolution.

3.2. Experimental Populations

We used flies from a laboratory-adapted, wild-type (wt) Dahomey stock population of *D. melanogaster* that has been maintained since 1970 with overlapping generations (Partridge and Farquhar 1983). In Chapters 4, 5 and 7, we used focal wild-type flies that hatched from eggs that we collected from these cages. In addition to wild-type flies, in the experiments where we did competitive fitness assays (Chapters 4, 6 and 7), we also used recessive mutant *sparkling poliert (spa)* flies (backcrossed into the same Dahomey genetic background). Flies homozygous for the *spa* allele exhibit a rough eye phenotype (Figure 3.3) that allows to distinguish the offspring of wt and *spa* parents in competitive fitness assays (e.g., Fricke *et al.* 2010). We note that, as seems to be usual, *spa* in our population tend to show a fitness deficit with respect to wt flies (Carazo, P. unpubl. data), but this would in no way affect the outcome of our fitness estimations, as competitors were always the same (i.e., standard *spa* flies) across different treatments. Finally, in Chapter 6, we used DGRP (*Drosophila* Genetic

Reference Panel) flies that were inbred for 20 generations and then fully sequenced (Mackay *et al.* 2012).

All flies were maintained in a 25°C room with 60% humidity under 12h:12h Light/Dark cycle in Cavanilles Institute facilities. Across the experiments, flies were fed with slightly different mediums (Table 3.1).

Figure 3.3. Wild type and *sparkling poliert* fruit flies. Photograph of a wild type female (on the right) next to a *sparkling poliert (spa)* female (on the left).

To obtain virgin flies to start up experiments, we always collected Dahomey eggs from our population cages using grape-agar filled Petri dishes with a smear of live yeast paste, which we then cultured at standardized density (Clancy and Kennington 2001). We then collected virgin adults emerging from these eggs within 7 hours of eclosion, and used them in the different assays described in each

experiment (detailed explanation in the Materials and Methods sections of the corresponding chapters).

Table 3.1. Fly food media used in different chapters. List of ingredients of the fly food media that was prepared for the experiments of each chapter.

	Food Type
Chapter 4	All flies were fed with a diet containing yeast (40 g), sugar (50 g), soya flour (10 g), corn flour (60 g), nipagin (3 g) and propionic acid (5 ml) in 1 Litres of water.
Chapter 5	All flies were fed with a diet containing yeast (40 g), sugar (50 g), soya flour (10 g), corn flour (60 g), nipagin (3 g) and propionic acid (5 ml) in 1 Litres of water.
Chapter 6	DGRP flies were fed with Bloomington Drosophila Stock Center Cornmeal Food: (https://bdsc.indiana.edu/information/recipes/bloomfood.html) *Spa* flies were fed with a diet containing yeast (40 g), sugar (50 g), soya flour (10 g), corn flour (60 g), nipagin (3 g) and propionic acid (5 ml) in 1 Litres of water.
Chapter 7	All flies were fed with a medium adapted from Lewis (1960) containing yeast (14.6 g), corn flour (72 g), soya flour (8.8 g), malt extract (72 g), molasses (20g), nipagin (2.8 g), propionic acid (5.3 ml) and phosphoric acid (0.3 ml) in 1 Litres of water.

3.3. Statistical Analysis

For most statistical analyses, we used a generalized linear regression modelling/mixed modelling approach. Prior to fitting models, we always

explored data graphically to check for heteroscedasticity and normality. We normally dealt with potential outliers by using alpha-winsorization ($\alpha = 0.05$) (Quinn and Keough 2002). After model fitting, we always ran diagnostic tests to assess model performance and check the model assumptions (i.e., absence of heteroscedasticity, normality of residuals, Winter 2013). When assumptions were not met even after standard data-transformation (e.g. log and square-root transformation), we used the non-parametric Kruskal-Wallis test (Kruskal and Wallis 1952) followed by Dunn's multiple comparisons post hoc test (Dunn 1964) whenever we found a significant effect by using the Kruskal-Wallis test. We controlled for False Discovery Rate (FDR) using the Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995). For all tests, we used an alpha value of 0.05 and all the p-values presented are two-tailed. All analyses were performed in R v. 3.3.2 (R Core Team 2016)

Chapter 4. Social context and reproductive ageing in *Drosophila melanogaster*

4.1. Introduction

Social context (e.g. sex ratio, density) has the potential to modulate age effects on reproductive success by influencing different factors such as mate encounter rate, mate choice or intrasexual competition (Kvarnemo and Ahnesjo 1996; Kokko and Rankin 2006). For example, in the feral fowl, a species with strong male-male competition over female harems, Dean *et al.* (2010) found that old males have the potential to sire a relatively higher proportion of offspring in groups with a female-biased sex ratio, compared to a male-biased sex ratio. This is due to old males having a higher chance of being socially dominant in female-biased groups, where male-male competition is low (Dean *et al.* 2010). Unfortunately, this interesting result has not been followed up by similar studies in other organisms with different mating systems, nor with respect to female age.

In this study, we used *Drosophila melanogaster* to explore how male and female age affects the reproductive success of males and females in experimental mating patches with female-biased (FB) or male-biased (MB) sex ratios. In *D. melanogaster*, males have strong intrasexual competition over mating and allocate considerable time and effort to court available females, while females are able to re-mate with multiple males (Dow and Schilcher 1975; Pitnick 1991; Markow 2002). Although intrasexual competition is mostly observed in males, aggression between females is also present, mainly when food sources are scarce (Ueda and Kidokoro 2002; Bath *et al.* 2017, 2018). Furthermore, both males and females of this species exhibit mate choice but the degree and direction of these choices can differ depending on the population of origin and the social environment (Gowaty *et al.* 2003; Byrne and Rice 2006; Edward and Chapman 2012, 2013; Monier *et al.* 2018). Based on its mating system, we predicted that

sex ratio would modulate age effects on the fitness of males and females differently. In males, intrasexual competition is believed to be stronger than intersexual selection (Gowaty *et al.* 2003). Hence, we predicted that male age would decrease reproductive success relatively more in a male-biased social context, because we expected old males to have a higher disadvantage under intense male-male competition. In contrast, in this species female intrasexual competition appears to be less important than intersexual selection (Gowaty *et al.* 2003), so we did not predict a similar outcome. Instead, *D. melanogaster* males exhibit a marked preference for young females (Cook and Cook 1975; Lüpold *et al.* 2011), so we predicted female age to decrease reproductive success more in a female-biased social context, where there is a potentially higher opportunity for males to choose young females over the old ones.

4.2. Materials & Methods

Fly maintenance

We collected Dahomey eggs from our population cages and virgin adults emerging from those eggs using the protocol described in Chapter 3 (General Materials and Methods). Then we generated old focal males and females by isolating them with excess food for 28 days prior to assays, during which time we flipped them into a new vial once a week. In contrast, young focal males and females were only kept in isolation for 3 days after their emergence and prior to assays. Young *sparkling (spa)* competitors/partners were kept in same-sex groups of 10 for 3 days after their emergence and until the beginning of assays.

Competitive fitness assays

In order to explore the effect of sex ratio and age on reproductive success, we studied the fitness of focal wt male and female flies when competing against *spa* rivals, in a factorial combination of sex ratio (i.e., male biased –4 males and 2 females– vs. female biased –4 females and 2 males–) and age (i.e., a young vs.

old focal wt male/female competing against young spa rivals for young spa mating partners). Thus, within each vial, all flies except the focal experimental fly were *spa* (Figure 4.1).

For all treatments, we allowed flies to interact and lay eggs for 2 days, after which time we discarded the males and allowed females to oviposit for 3 more days in a fresh vial. In order to control for larval density across treatments during this second period of oviposition, we separated the four females in the female-biased sex ratio treatment in two vials containing two females each. After transferring/discarding females, we incubated vials from both the first and second period of oviposition for 16 days, froze the vials, and then proceeded to count the number of *spa* and wt offspring in each vial. In order to control for the potential effects of density on the development of larvae from eggs laid during the first oviposition period, we counted the number of pupae in these vials. The density of larvae per vial (number of pupae ± SEM = 44.7 ± 1.0) was, in all cases, comfortably below the threshold for which density effects have been described in *D. melanogaster* (Miller and Thomas 1958).

Statistical analysis

To determine the effect of ageing and sex ratio on reproductive success in a way that is comparable across the two different sex ratio treatments (i.e., fixed density but which include a different amount of males and females), we standardized data. We calculated the standardized reproductive success of each focal female by subtracting the average number of offspring that belong to competitors (spa) from the observed number of offspring that belong to the focal fly (wt) for each replicate:

$$\#\text{ of offspring from focal} - \frac{\#\text{ of offspring from competitors}}{\#\text{ of competitors}}$$

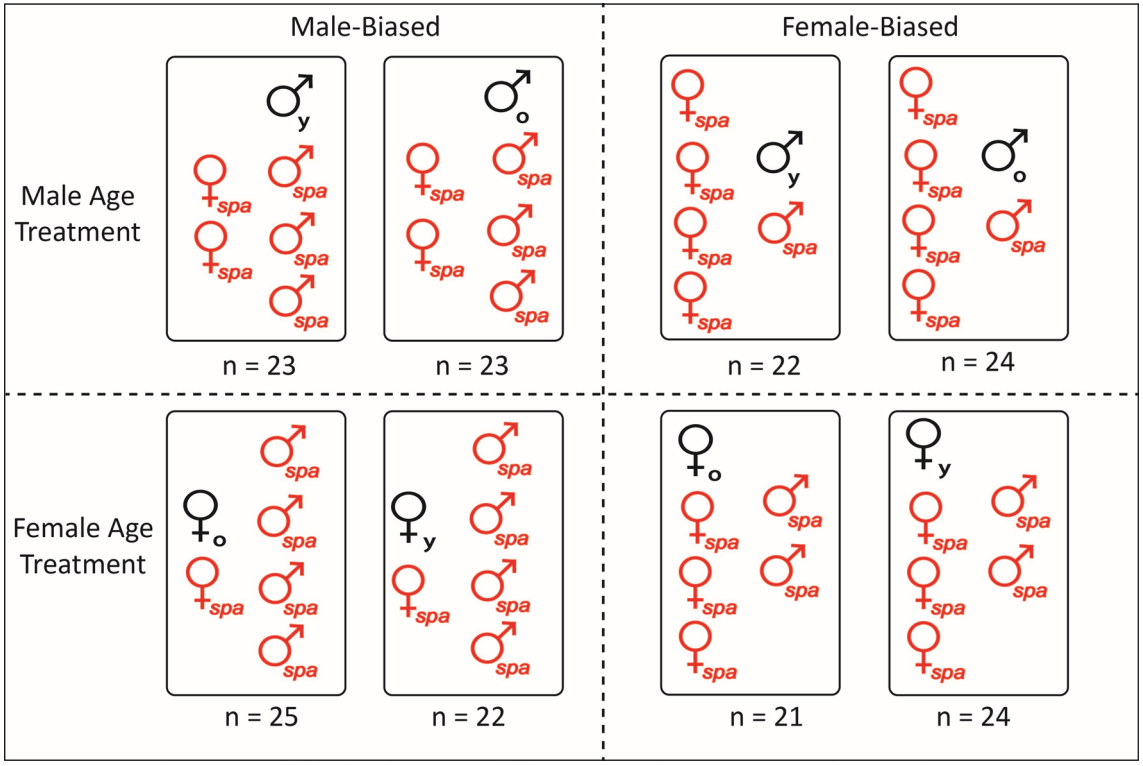

Figure 4.1. Schematic illustration of experimental design. Scheme of the different treatments implemented to measure the reproductive success of young (y) and old (o) focal flies competing with *spa* flies across male/female-biased different sex ratios.

We calculated the standardized reproductive success of each focal male using the same equation, but dividing by the number of females that were present in the corresponding mating vial (2 females in male-biased social context and 4 females in female-biased social context).

To explore the effect of age and sex ratio on reproductive success of each sex separately, we fitted a linear model including age, sex ratio, and their interactions as fixed factors. We then repeated this analysis using a restricted maximum likelihood LMMs and introducing pupae density as a random intercept effect. In order to obtain minimum adequate models, we performed backward stepwise model selection based on Likelihood Ratio Tests (LRTs).

4.3. Results

We did not find a significant age × sex ratio interaction ($F_{1,88}$ = 0.1027, p = 0.7494) or a sex ratio effect ($F_{1,89}$ = 2.1731, p = 0.144) in male reproductive success. However, we found a significant effect of age ($F_{1,89}$ = 19.2600, p < 0.001, Figure 4.2a). In the case of female reproductive success, we did not find a significant age × sex ratio interaction ($F_{1,88}$ = 0.0967, p = 0.7566) or a sex ratio effect ($F_{1,89}$ = 0.8208, p = 0.3674), but we did find a significant age effect ($F_{1,89}$ = 64.1757, P < 0.001, Figure 4.2b).

Controlling for pupae density did not qualitatively change our results. For males, there was no significant age × sex ratio interaction (χ^2 = 0.1073, df = 1, p = 0.7433) or sex ratio effect ($F_{1,89}$ = 2.1731, p = 0.144), whereas we did find a significant age effect ($F_{1,89}$ = 18.9707, p < 0.001) on male reproductive success. Similarly, for females, we did not find a significant age × sex ratio interaction (χ^2 = 0.101, df = 1, p = 0.7506) or a sex ratio effect ($F_{1,89}$ = 0.8140, p = 0.3674), but we found a significant main effect for age ($F_{1,89}$ = 64.8510, p < 0.001).

Figure 4.2. Effect of male/female age on reproductive success across different social contexts. Standard reproductive success of (a) young/old focal males in male-biased and female-biased social contexts and (b) young/old focal females in male-biased and female-biased social contexts

4.4. Discussion

In this study, we investigated the potential role that sex ratio at mating might play in modulating the fitness effects of age in *Drosophila melanogaster*. We found that both male and female age caused a decline in reproductive success but, contrary to our expectations, this effect was not modulated by sex ratio at mating (i.e., was similar in both male-biased and female-biased social context).

In the case of males, we were expecting male age to decrease the reproductive success more in a male-biased social context, where intrasexual competition is high. Like in many other organisms, in *D. melanogaster* male-male competition is expected to increase drastically in male-biased social contexts (Wang and Anderson 2010). In principle, this should lead to old males having relatively higher reproductive fitness in female-biased contexts, where male-male competition is low. Accordingly, Dean *et al.* (2010) showed that, in the feral fowl (*Gallus gallus*), the effects of age on the reproductive success of males were mitigated in female-biased (vs. male-biased) contexts. In this species, socially dominant males have privileged access to mating opportunities but females mate multiply, so sperm competition is intense (David Ligon and Zwartjes 1995; Pizzari and Birkhead 2000; Pizzari *et al.* 2002; Dean *et al.* 2010). Dean *et al.* (2010) elegantly showed that, despite old males having a lower sperm competition ability than young males, they had a relative advantage in female-biased (vs. male-biased) social groups, due to a higher possibility of being socially dominant when male-male intrasexual competition is low. The absence of similar effects in *D. melanogaster* in our study may have to do with inherent differences in the mating system of these two species.

In fruit flies, male-male competition over access to females is generally high and, in the wild, males seem to exhibit a typical resource-defense polygyny by defending pieces of decaying fruit where females feed (Markow 1988). Recent evidence suggests that male-male aggression in this context also serves a mate-

guarding function (Baxter *et al.* 2015), but flies don't live in stable social groups and hence males cannot monopolize access to females throughout their lifespan. Furthermore, lab populations like the one used in this study have been kept at high densities for thousands of generations, in conditions where mate monopolization is highly unlikely. As a result, the level of intrasexual competition in our population might not modulate age-related fitness effects as it does in feral fowls (or might do so to a lesser extent). On the other hand, intersexual competition also seems to be quite important in *D. melanogaster*, and there is good evidence that both female and male mate choice are modulated by social context (Edward and Chapman 2012, 2013; Monier *et al.* 2018). In particular, females prefer mating with young (or large) males that court more vigorously (Jagadeeshan *et al.* 2015; Rezaei *et al.* 2015), and they appear to be less choosy when sex ratios are female-biased (Monier *et al.* 2018). Hence, old males might be expected to benefit in female-biased contexts due to females being less choosy in favour of young males. At a first glance, this might make it more striking that we didn't find sex ratio to modulate age effects on male reproductive success; however, the relatively complex mating behaviour of fruit flies might be the reason behind the absence of such an effect.

For example, it is possible that our results for males are partly explained by male mate choice effects. Under female-biased sex ratios, where males are expected to be choosier, young males may benefit by choosing high quality females while old males are left to mate with low quality females. In *D. melanogaster*, ageing seems to diminish the ability of males to choose high quality females (Hu *et al.* 2014). Old males may hence fail to be choosy despite ample opportunity for male mate choice in female-biased contexts, to the benefit of young "choosy" males. An intriguing possibility is that mate-choice copying (Nöbel *et al.* 2018) may have contributed to exacerbate male age effects in the female-biased sex ratio. In *D. melanogaster*, females prefer mating with young males that court more vigorously (Jagadeeshan *et al.* 2015; Rezaei *et al.* 2015) and, in our experiment,

old and young focal males were always phenotypically distinguishable to their young rival sparkling flies (i.e., different eye-colour). Given recent findings showing that females tend to copy the mate choice of other females based on male colour cues in fruit flies (Danchin *et al.* 2018), it is possible that the inherent advantage of young males over old males due to female choice may have been exacerbated in the female-biased context, where mate-choice copying is more likely. In short, young males may hold a similar fitness advantage against old males irrespective of the sex ratio, but via different sexual selection mechanisms: via intrasexual competition and female mate choice, when the sex ratio is male-biased, and via male mate choice and female mate copying when the sex ratio is female-biased.

In the case of females, female reproductive success also decreased with age similarly in both female-biased and male-biased social contexts (Figure 4.2b). We might have expected that, in a female-biased social context with a higher opportunity for males to be choosy, males (which are all young in this case) would prefer to mate with young (vs. old) focal females, which would thus have had higher reproductive success. Several previous studies have reported the existence of both pre and post-copulatory male mate choice with respect to female age. For example, male courtship intensity decreases with female age (Cook and Cook 1975) and males allocate less sperm to old females compared to their young counterparts (Lüpold *et al.* 2011). However, being attractive to males is not always beneficial for females. Mating and male harassment are known to decrease survival and reproductive success in female *D. melanogaster* (Partridge and Fowler 1990; Chapman *et al.* 1995; Wigby and Chapman 2005). The fact that male preference for young females may have been more marked in the female-biased social context could have led males to be more harmful to these females, which in turn may have counterbalanced any benefits from male mate choice. As a matter of fact, Long *et al.* (2009) showed that male harm is preferentially directed towards intrinsically higher-fitness females and that, as a result, any

fitness advantage that could be experienced by high condition females (young females in our design) might be compensated by the costs of being attractive in a female-biased social context; at least in simple environments such as the one used in this experiment (Long *et al.* 2009; see also Yun *et al.* 2017; MacPherson *et al.* 2018). However, we think this is an unlikely explanation in our case because we used a short-term proxy of female reproductive success and male harm to females tends to curtail long-term female fecundity (Wigby and Chapman 2005). Relatively high mating costs in a male-biased social context might also contribute to explain why we did not observe an interaction between sex ratio and female age. Although the opportunity for male mate choice is lower in this context, and males might thus harm both young and old females, mating costs may be expected to be more pronounced in old females, which would tend to exacerbate age effects in a male-biased social context. Unfortunately, we currently have very little information about how social context changes intra- vs- intersexual competition in males and females, in *D. melanogaster* or other species, which means the above possibilities remain to be explored.

Studies of reproductive senescence so far have focused on understanding the effect of male and female age on reproductive success (Williams 1957; Flatt and Heyland 2011), for example by studying male/female age effects on pre-post copulatory mating abilities, mate choice, and offspring viability (Cook and Cook 1975; Dunson *et al.* 2004; Maklakov *et al.* 2009; Carazo *et al.* 2011; Lüpold *et al.* 2011; Velando *et al.* 2011; Tan *et al.* 2013). Many studies have also investigated the interaction between social context and several fitness traits such as mating duration, reproductive success, survival and lifespan (Iliadi *et al.* 2009; Bretman *et al.* 2010, 2013; Costa *et al.* 2010; Adler and Bonduriansky 2011; Zajitschek *et al.* 2013; Leech *et al.* 2017). In sharp contrast, how age effects on reproductive success may be modulated by the social context has so far been largely overlooked even though social context (such as sex ratio at mating) might

play a crucial role in modulating sex-specific age effects on reproductive success. We suggest future studies should aim to fill this gap in knowledge.

Chapter 5. Condition-dependent mortality, reproductive senescence and the potential for sexual conflict in *Drosophila melanogaster*

5.1. Introduction

Classic theories predict the evolution of ageing due to the weakening of natural selection with age, via the accumulation of negative mutations that act late in life (mutation accumulation; Medawar 1952), selection for pleiotropic alleles that have positive effects early in life but negative effects late in life (antagonistic pleiotropy; Williams 1957; Williams *et al.* 2006), and/or trade-offs between growth, reproduction and ageing (Kirkwood 1977). High extrinsic mortality can accelerate ageing because it can contribute decisively to weaken natural selection with age (Caswell 2007; Day and Abrams 2020). When mortality is condition dependent, predictions about how extrinsic mortality should affect early/late life fitness are complex (Maklakov *et al.* 2015). For example, by generating co-variation between survival and condition, non-random extrinsic mortality might result in slower ageing and longer lifespan (Chen and Maklakov 2012; Maklakov *et al.* 2015; Chen *et al.* 2016).

Under natural conditions, extrinsic mortality is frequently expected to be condition dependent (Maklakov *et al.* 2015), and the significance of condition dependent mortality to understand ageing has been studied both under natural conditions and by using experimental evolution in the laboratory (Reznick *et al.* 2004; Chen and Maklakov 2012, 2014). Reznick *et al.* (2004) found that guppies (*Poecilia reticulate*) from populations evolved under high extrinsic mortality had higher reproductive success and longer lifespan, but faster functional decline in swimming performance (Reznick *et al.* 2004). Similar findings have been reported from experimental evolution studies in a nematode, *Caenorhabditis*

remanei (Chen and Maklakov 2012; Chen *et al.* 2016). In this species, non-random extrinsic mortality caused the evolution of longer male/female lifespan and higher female fecundity, but also a sharper decline in male reproductive success, suggesting a trade-off between ageing and reproductive success in males, but not females (Chen and Maklakov 2012; Chen *et al.* 2016).

In addition to providing insight into life history evolution, studying how condition dependent extrinsic mortality affects ageing can also help us understand sexual selection and sexual conflict processes (Dean *et al.* 2010; Bonduriansky 2014). For example, it has been proposed that individuals may acquire indirect fitness benefits from choosing old mating partners if only high condition mates tend to survive to a late age (Brooks and Kemp 2001; Johnson and Gemmell 2012). In contrast, ageing is usually accompanied by a decline in reproductive abilities that can lead to direct fitness costs to individuals mating with old mates. In fact, it has been proposed and shown in at least two organisms that ageing can be a source of increased sexual conflict by increasing female mating costs (when facing old males) and hence male/female conflict over mating (Dean *et al.* 2007, 2010; Carazo *et al.* 2011). For example, in feral fowls (*Gallus gallus*), male reproductive senescence severely impacts female reproductive success so that ageing of dominant males (i.e., capable of dominating access to females in their harem) translates into sexually antagonistic payoffs for females (Dean *et al.* 2010). Similarly, in the mealworm beetle (*Tenebrio molitor*) male age imposes direct fertility costs on females, as well as lowers the quality of viable offspring (Carazo *et al.* 2011). In this species, females respond by being less receptive (and quicker to re-mate) when paired with old males, while old males invest more on female guarding than young males, reflecting increased sexual antagonism over mating. Moreover, selective disappearance can lead to cohorts with old males that have better survival abilities but higher reproductive senescence (due to survival/reproduction trade-offs). This may further intensify the age-dependent increase in male-female sexual conflict over mating by increasing the costs to

females of mating with old "surviving" males. Acknowledging the effects of condition dependent ageing may clarify whether male age can actually be a source of sexual conflict, and how important such effects may be under natural conditions, where condition dependent mortality is likely to be common.

To address the questions above, we explored the effects of male/female age on reproductive success and mating behaviour in the absence/presence of condition dependent mortality. For this purpose, we first aged cohorts of flies in the absence or presence of condition dependent extrinsic mortality for climbing-speed, a proxy of anti-predatory avoidance. This was followed by fully factorial matings with respect to age (young and old) and sex (male and female). Finally, we assessed the impact of male/female age on mating success, reproductive success (number of offspring), fecundity, egg-to-adult viability, mating duration and mating latency in both the absence and presence of condition dependent mortality.

5.2. Materials & Methods

Ageing treatments

We aged females/males in isolation with excess food for 38 days, during which time we measured the climbing speed of each experimental fly once every 8 days (starting from 3-4 days after emergence until the mating assays, a total of 5 different time points). Briefly, we introduced each fly into a graduated glass tube, gently tapped the fly to the bottom of the vial and then measured the distance it climbed in 10 seconds, which allowed us to calculate its climbing speed in cm/s (Cook-Wiens and Grotewiel 2002). For flies that climbed to the top in less than 10 seconds, the total length of the tube (12 cm) was divided by the time spent to reach the top. Climbing speed was calculated as an average of three successive measurements for each fly at each time point. At the end of this procedure, we divided "old" flies into two groups. The first group consisted of males and

females that were not exposed to condition dependent mortality. The second group included flies that were exposed to condition-dependent mortality by using a cut-off point climbing speed below which 60% of flies were considered as 'predated'. In other words, any fly for which we had measured a climbing speed below the cut-off point during the ageing treatment (at any of the 5 different climbing speed measurement time points) was predated. We maintained "young" females/males in isolation for four days before mating assays. Young females/males were not subject to simulated predation (i.e., we only had one control young treatment) because a pilot study (see Appendix) revealed that an overwhelming majority of 4 day-old flies survive simulated predation. This was confirmed in our experiment during ageing of the old treatment flies, with more than 98% of 4 day-old flies lying above the "predation" threshold. This being so, implementing "young un-predated" and "young predated" controls would have resulted in two virtually identical groups while forcing us to reduce the sample size across the rest of the treatments, for which reason we collapsed the two into a single young (i.e., 4 day old) treatment. In addition to this, a vast majority of flies (both males and females) survived simulated predation early in life (i.e., first 2-3 weeks of life), so that selective disappearance impacted flies mostly mid-to-late in their life (i.e., > 90% of males and roughly 85% of females "predated" were so after 3 weeks of age; see Figure 5.1). This means simulated predation depended greatly on both initial climbing speeds and the rate of functional senescence.

Mating assays

After ageing treatments, we mated pairs with different age combinations by putting a young/old male and a young/old female together into mating vials. Observers blind to treatments measured mating latency (time spent until copulation) and mating duration until the first mating in each vial. Behavioural observations were conducted in a 25°C room, started when the lights were on

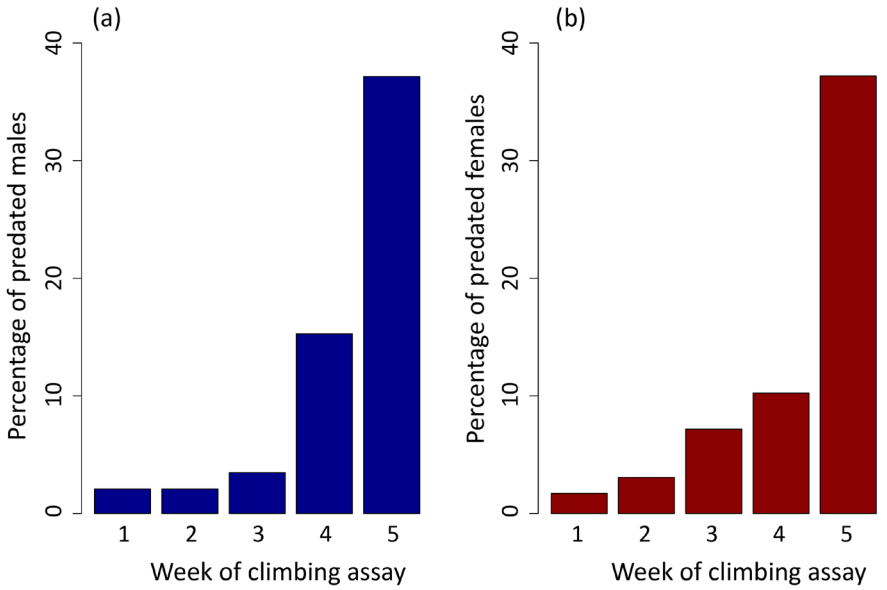

Figure 5.1. Percentage of flies predated per week. Percentage of males (a) and females (b) that were predated per week that sums up to 60% of the total number of flies in the second group of old flies.

(i.e., 10 a.m.), and lasted for 7 hours. Pairs that did not mate within these 7 hours were considered unsuccessful. After the completion of the first mating, males and unmated females were discarded, while once mated females were kept in the same mating vials where they were allowed to oviposit for 24 hours. At the end of this egg-laying period, we also discarded females and counted the number of eggs they laid during this period. Finally, we incubated vials for 16 days to allow all viable flies to emerge, froze them and then proceeded to count the number of offspring. Our sample size was of ~40 pairs for each of seven different age combinations for a total of 272 pairs (young male × young female: 40; young male × old female: 40 [no predation], old male × young female: 38 [no predation], old male × old female: 38 [no predation]; young male × old female: 39 [with predation], old male × young female: 39 [with predation], old male × old female: 38 [with predation]).

Statistical analysis

To understand how condition dependent mortality modulates the effect of male and female age on reproductive senescence, we run separate analyses for each mortality treatment (i.e., absence vs. presence of condition dependent mortality), while using the same control group (young male × young female) for both.

We used the nonparametric Kruskal–Wallis test to analyse whether there is an effect of pair age combination (young/old male × young/old female) on reproductive success and test whether there is an effect of pair age combination on fecundity and egg-to-adult viability (calculated as the proportion of the number of adults to the number of eggs). For mating duration, we fitted a Linear Model (LM) with male age, female age and their interaction as fixed factors. For mating success, we used GLMs with Binomial error distribution (successful: 1, unsuccessful: 0) with male age, female age and their interaction as fixed factors. Finally, for mating latency, we used the Kruskal–Wallis test to analyse whether pair age combination influences latency to mate in the absence and presence of condition dependent mortality.

5.3. Results

Age effects on reproductive success

The effect of pair age (i.e., young male-young female, young male-old female, old male-young female, old male-old female) was significant in both the absence and presence of condition dependent mortality (Absence: Kruskal Wallis test, $\chi^2 = 32.213$, df = 3, $p < 0.001$, Presence: Kruskal Wallis test, $\chi^2 = 29.644$, df = 3, $p < 0.001$). Briefly, old females had lower reproductive success than young ones both in the presence and absence of condition dependent mortality (Table 5.1). Conversely, old males had lower reproductive success only in the presence of condition dependent mortality (Figure 5.2 & Table 5.1).

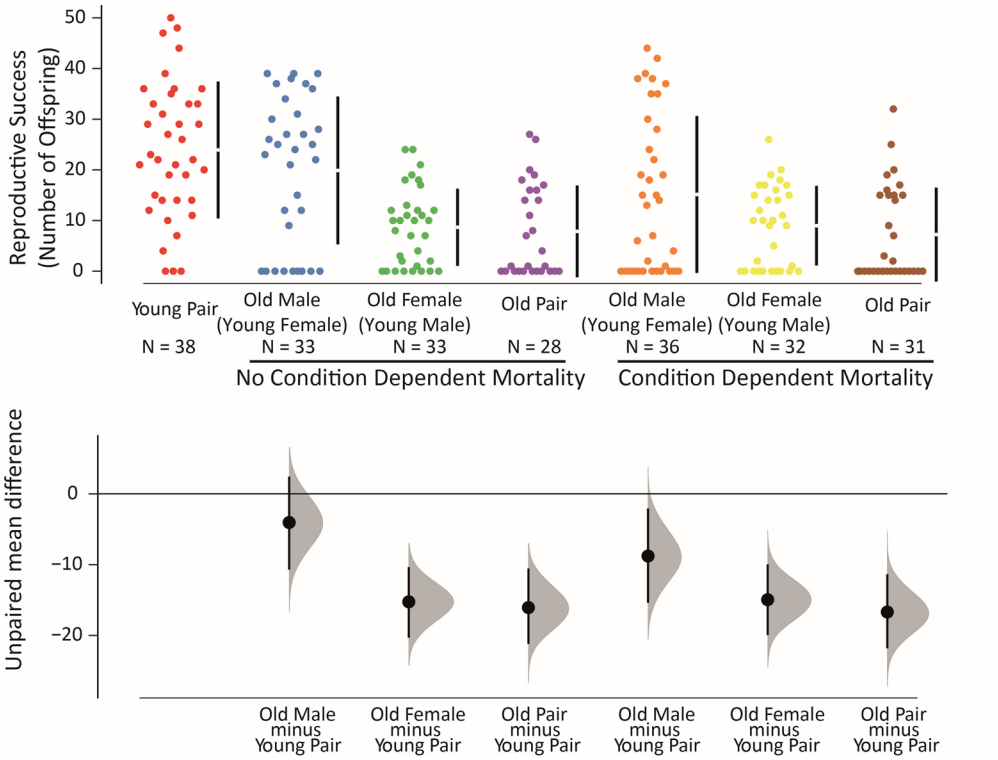

Figure 5.2. Effect of male/female age on reproductive success in the absence and presence of condition dependent mortality. Mean ± standard deviation for each treatment followed by an unpaired mean difference that uses bootstrap resampling to compute nonparametric assumption-free 95% confidence intervals which compares each treatment with the control (young pair).

Table 5.1. Age and mortality effects on reproductive success. Effect of male age, female age and couple age on reproductive success compared to the young control pair.

Response Variable	Treatment	Condition Dependent Mortality	Z test statistics	P
Reproductive Success (Number of Offspring)	Male Age (Old male-Young female)	Absent	-1.2109	0.271
		Present	-2.8212	0.010
	Female Age (Old female-Young male)	Absent	-4.4591	< 0.001
		Present	-4.2190	< 0.001
	Pair Age (Old male-Old female)	Absent	-4.6029	< 0.001
		Present	-4.9757	< 0.001

Age effects on fecundity

The effect of pair age on fecundity was significant in both the absence and presence of condition dependent mortality (Absence: Kruskal Wallis test, χ^2 = 49.862, df = 3, p < 0.001, Presence: Kruskal Wallis test, χ^2 = 60.459, df = 3, p < 0.001). Our results showed that fecundity decreases due to female age similarly in both the absence and presence of condition dependent mortality, while we did not detect an effect of male age on female fecundity (Figure 5.3 & Table 5.2).

Age effects on egg-to-adult viability

The effect of pair age was significant in both the absence and presence of condition dependent mortality (Absence: Kruskal Wallis test, χ^2 = 10.490, df = 3, p = 0.015, Presence: Kruskal Wallis test, χ^2 = 9.188, df = 3, p = 0.027). Post-hoc contrasts showed that male age tended to cause a decline in egg-to-adult viability only in the presence of condition dependent mortality. In contrast, the

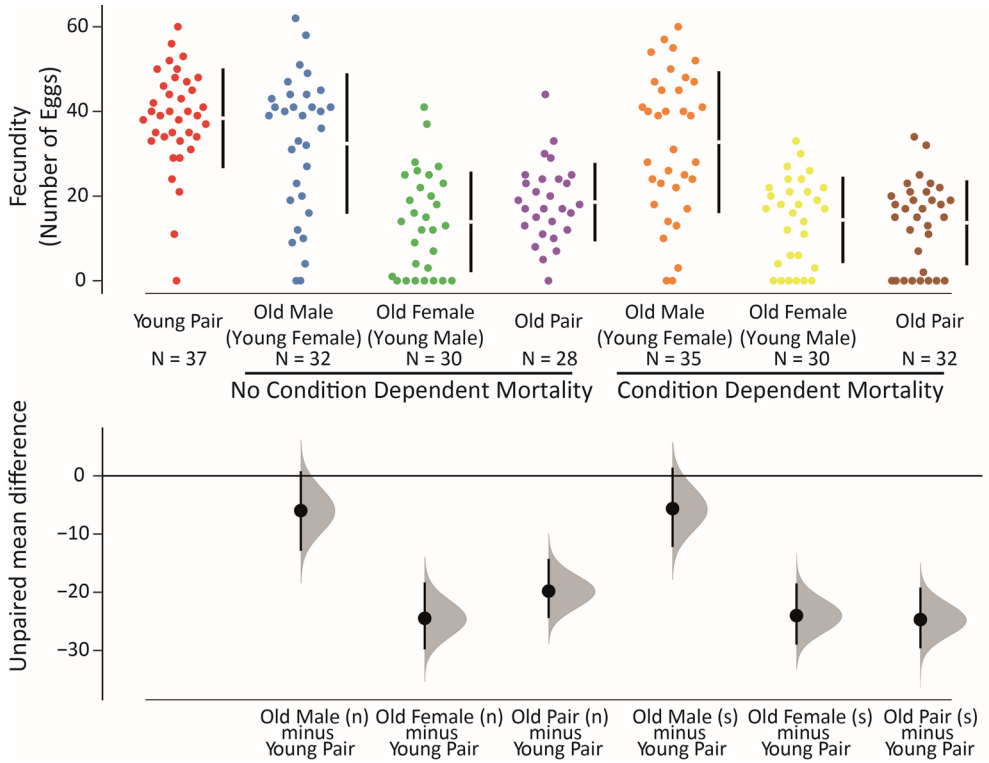

Figure 5.3. Effect of male/female age on fecundity in the absence and presence of condition dependent mortality. Mean ± standard deviation for each treatment followed by an unpaired mean difference that uses bootstrap resampling to compute nonparametric assumption-free 95% confidence intervals which compares each treatment with the control group.

effect of female age on viability was not significant in either the absence or presence of condition dependent mortality (Figure 5.4 & Table 5.3).

Table 5.2. Age and mortality effects on fecundity. Effect of male age, female age and couple age on fecundity compared to the young control pair.

Response Variable	Treatment	Condition Dependent Mortality	Z test statistics	P
Fecundity	Male Age (Old male-Young female)	Absent	-1.5419	0.148
		Present	-1.4915	0.163
	Female Age (Old female-Young male)	Absent	-6.1470	< 0.001
		Present	-5.9380	< 0.001
	Pair Age (Old male-Old female)	Absent	-5.0198	< 0.001
		Present	-6.3121	< 0.001

Table 5.3. Age and mortality effects on egg-to-adult viability. Effect of male age, female age and couple age on egg-to-adult viability compared to the young control pair.

Response Variable	Experiment	Condition Dependent Mortality	Z test statistics	P
Egg-to-Adult Viability	Male Age (Old male-Young female)	Absent	-0.9287	0.353
		Present	-2.3476	0.056
	Female Age (Old female-Young male)	Absent	1.4996	0.201
		Present	0.6802	0.496
	Pair Age (Old male-Old female)	Absent	-1.8813	0.120
		Present	-0.8152	0.498

Age effects on mating success

There was no significant interaction between male and female age for mating success in either the absence or presence of condition dependent mortality (Absence: $\chi^2 = 0.073$, df = 1, p = 0.787, Presence: $\chi^2 = 0.023$, df = 1, p = 0.880). In the absence of condition dependent mortality, male age was non-significant ($\chi^2 = 1.669$, df = 1, p = 0.196), but female age was ($\chi^2 = 6.459$, df = 1, p = 0.011). Similarly, in the presence of condition dependent mortality, there was no significant male age effect ($\chi^2 = 0.042$, df = 1, p = 0.838) but there was a significant female age effect ($\chi^2 = 5.864$, df = 1, p = 0.015). In general, mating success tended to decrease with female age in both the absence and presence of condition dependent mortality (Figure 5.5).

Age effects on mating latency

The effect of pair age was significant in the absence of condition dependent mortality (Kruskal Wallis test, $\chi^2 = 8.092$, df = 3, p = 0.044), but not the presence of condition dependent mortality (Kruskal Wallis test, $\chi^2 = 5.903$, df = 3, p = 0.116). Post-hoc contrasts showed that ageing tended to increase mating latency in the absence of condition dependent mortality but this trend was only evident when the mating pair was old (Table 5.4 and Figure 5.6).

Age effects on mating duration

There was no significant interaction effect between male and female age in the absence or in the presence of condition dependent mortality (Absence: $F_{1,129}$= 1.013, p = 0.316, Presence: $F_{1,133}$= 0.015, p = 0.902). In the absence of condition dependent mortality, male age significantly increased mating duration ($F_{1,130}$= 4.381, p = 0.038) while female age had no effect ($F_{1,130}$= 0.002, p = 0.965, Figure 5.6). Similarly, in the presence of condition dependent mortality, there was a significant increase in mating duration with male age ($F_{1,134}$= 15.809, p < 0.001) but not female age ($F_{1,134}$= 0.010, p = 0.921, Figure 5.7).

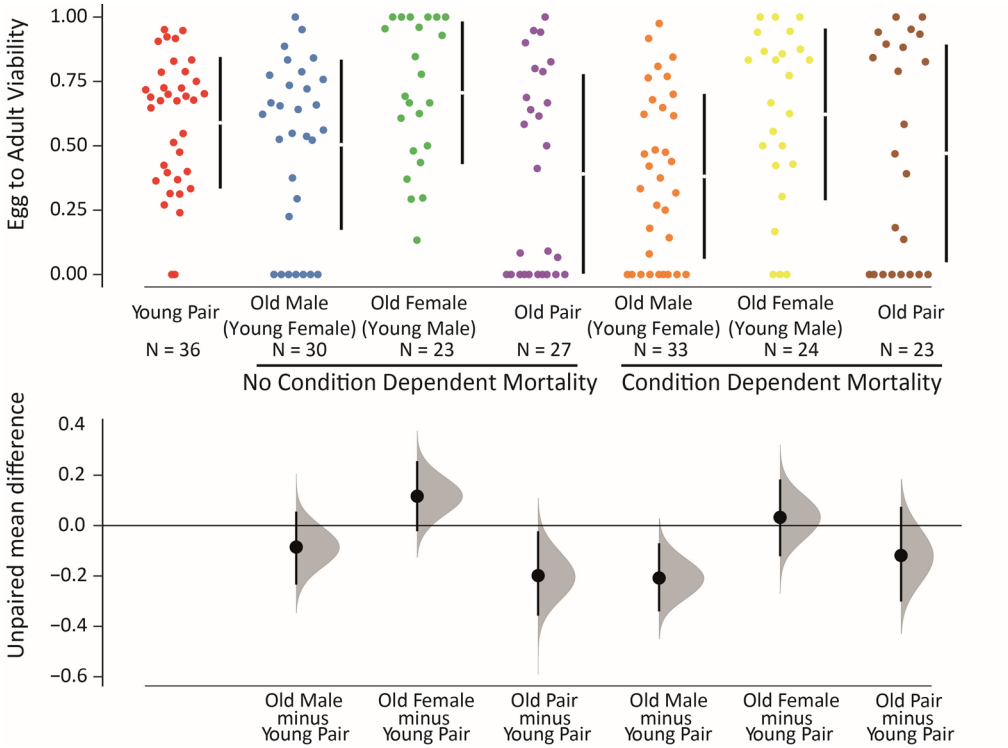

Figure 5.4. Effect of male/female age on egg-to-adult viability in the absence and presence of condition dependent mortality. Mean ± standard deviation for each treatment followed by an unpaired mean difference that uses bootstrap resampling to compute nonparametric assumption-free 95% confidence intervals which compares each treatment with the control group.

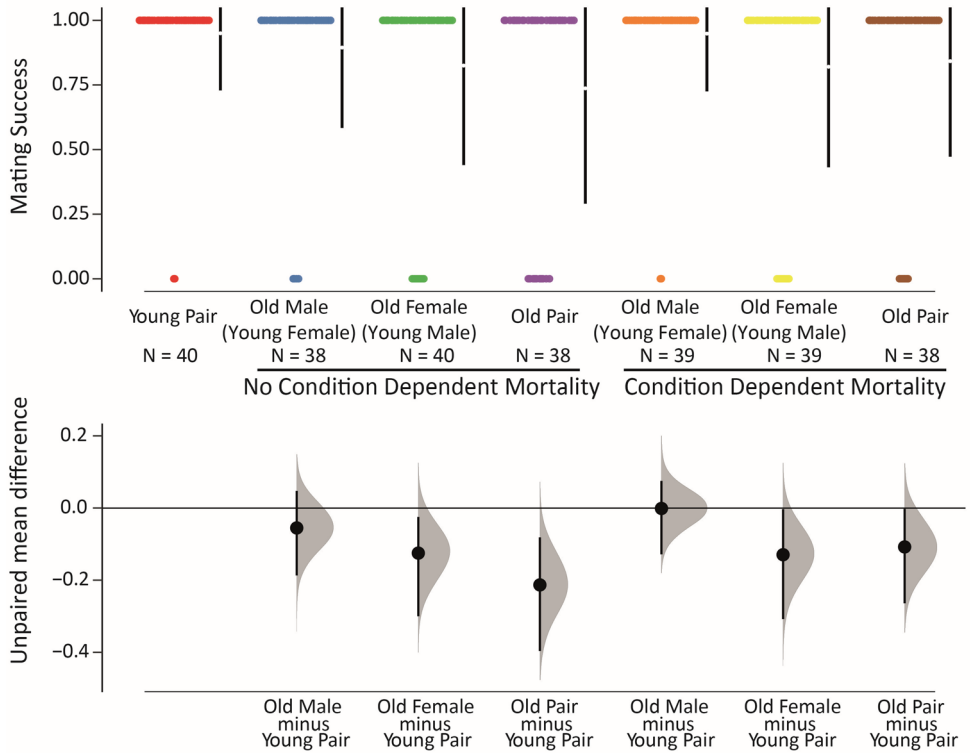

Figure 5.5. Effect of male/female age on mating success in the absence and presence of condition dependent mortality. Mean ± standard deviation for each treatment followed by an unpaired mean difference that uses bootstrap resampling to compute nonparametric assumption-free 95% confidence intervals which compares each treatment with the control group.

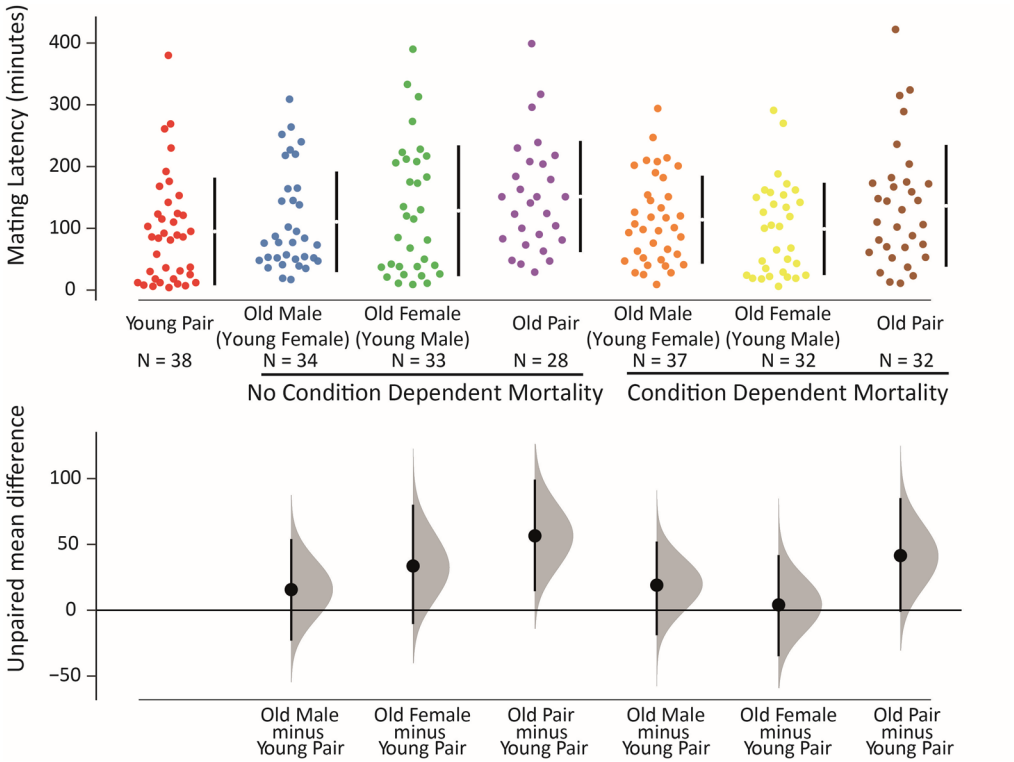

Figure 5.6. Effect of male/female age on mating latency in the absence and presence of condition dependent mortality. Mean ± standard deviation for each treatment followed by an unpaired mean difference that uses bootstrap resampling to compute nonparametric assumption-free 95% confidence intervals which compares each treatment with the control group.

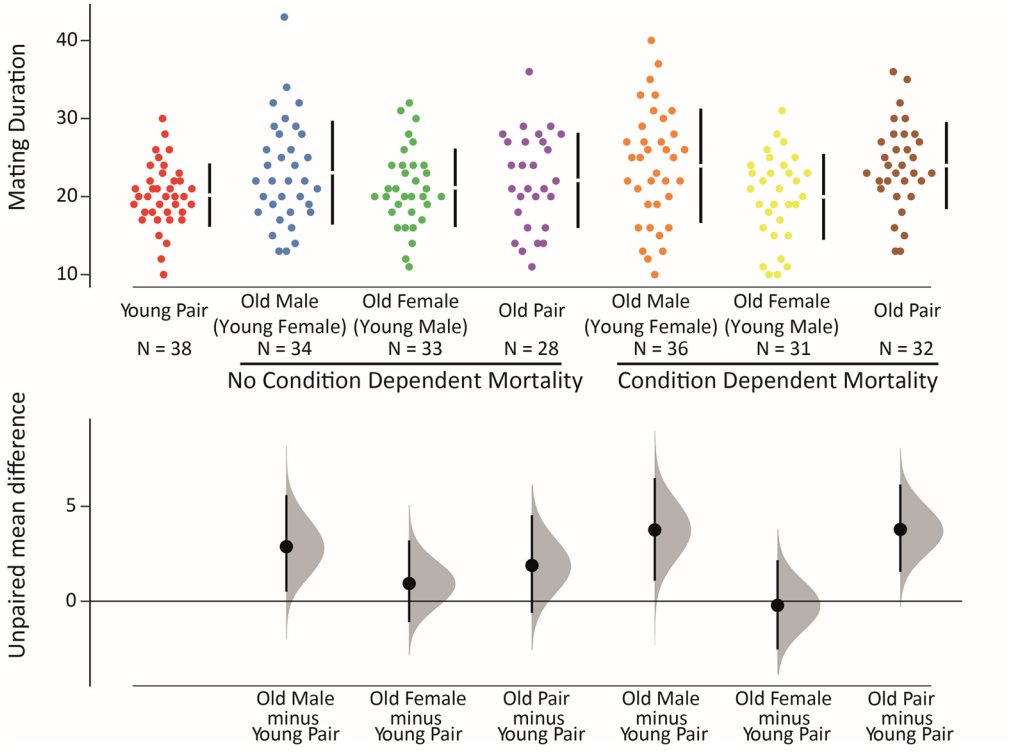

Figure 5.7. Effect of male/female age on mating latency in the absence and presence of condition dependent mortality. Mean ± standard deviation for each treatment followed by an unpaired mean difference that uses bootstrap resampling to compute nonparametric assumption-free 95% confidence intervals which compares each treatment with the control group.

Table 5.4. Age effects on mating latency in the absence of condition dependent mortality. Effect of male age, female age and couple age on mating latency compared to the young control pair in the absence of condition dependent mortality.

Response Variable	Experiment	Z test statistics	P
Mating Latency	Male Age (Old male-Young female)	1.1024	0.324
	Female Age (Old female-Young male)	1.3602	0.260
	Male + Female Age (Old pair)	2.8324	0.028

5.4. Discussion

In this study, we explored how condition dependent extrinsic mortality can modulate reproductive senescence in male and female *D. melanogaster*. For this purpose, we compared the reproductive success and mating behaviour of males and females from cohorts exposed (or not) to condition dependent mortality via simulated predation. We found that, while female reproductive senescence was unaffected by condition dependent mortality, the age-related decline in male reproductive success was higher when condition dependent mortality was present. Interestingly, accelerated reproductive ageing in surviving males seems driven mainly by ageing effects on post-copulatory processes, given that mating success was not differentially affected by age in the presence of condition dependent mortality, but egg-to-adult viability was. We discuss our findings in the context of sex differences in survival vs. reproduction life history trade-offs and their interaction with condition dependent mortality.

Selective disappearance modulates age effects on reproductive senescence

We found that female ageing caused a sharp decline in female reproductive success and that this effect was not modulated by condition dependent mortality.

As expected, female decline in reproductive success with age was mainly due to a decrease in fecundity. The effects of age on female reproductive success have been studied across taxa (i.e., in mammals, Packer *et al.* 1998; birds, Holmes *et al.* 2003; reptiles, Patnaik 1994; amphibians, Kara 1994 and fish Reznick *et al.* 2002). In *D. melanogaster*, we have long known that female fecundity declines with age (David *et al.* 1975). Although previous studies have also reported a decrease in female egg-to-adult viability with age (Fricke *et al.* 2013; Bloch Qazi *et al.* 2017), we did not find evidence of such effects in this study. Our failure to find similar results could be due to the use of different populations kept under different rearing conditions (e.g. diet).

In contrast to females, we only observed an age-related decline in male reproductive success in the presence of condition dependent mortality. Interestingly, this effect was mainly driven by a steeper decline in the egg-to-adult viability of surviving males' offspring, but not their mating success, suggesting a potential role for pre/post-meiotic senescence of male gametes. Pre-meiotic sperm senescence is the ageing of the male diploid genome, which could happen in both somatic (e.g. nurse cell degeneration) and germ cells (e.g. DNA damage of the germline) of males (Pizzari *et al.* 2008). This process could cause old males to produce sperm with lower fertilising ability, and/or offspring with poor genetic quality. Post-meiotic sperm senescence refers to the ageing of sperm after meiosis, potentially leading to sperm transport deficiencies, lower fertilizing efficiency and/or a decline in offspring quality (Pizzari *et al.* 2008). Moreover, pre-meiotic senescence might enhance post-meiotic senescence by causing old males to produce sperm that is more vulnerable to individual ageing, eventually causing old males to have low viability offspring. In line with these predictions, male age has been previously shown to affect offspring viability in *D. melanogaster* (Price and Hansen 1998) and other species (Goriely and Wilkie 2012; Fay *et al.* 2016).

Our finding that male reproductive senescence is on average higher when subject to condition dependent mortality can be explained by sex-specific selection pressures on life histories. Sexual selection is generally stronger in males than females (Bateman 1948; Trivers 1972) and, in many species, strong intrasexual competition selects for male adaptations, favouring higher reproductive success at the expense of somatic maintenance (Clutton-Brock and Isvaran 2007). In line with this idea, males generally exhibit "live fast die young strategies" compared to females (Promislow 1992; Maklakov and Lummaa 2013). In addition, precisely due to stronger sexual selection, males are generally more prone to extrinsic mortality than females (Christe *et al.* 2006; Costantini *et al.* 2007). If the source of extrinsic mortality is condition dependent, it should favour males that allocate more resources to survival (e.g. better anti-predatory escape abilities). It follows that, if males are both under stronger selection for early reproduction and condition dependent extrinsic mortality, trade-offs against reproductive maintenance will be steeper in males than in females (Chen *et al.* 2016; Maklakov and Immler 2016). The idea that survival-reproductive trade-offs might be stronger in males fits nicely with both our results, looking at intra-generational effects of selective disappearance in cohorts, as well as those from recent experimental evolution studies, looking at long-term evolutionary responses. In the nematode *C. remanei*, condition dependent extrinsic mortality leads to the evolution of longer male/female lifespan and higher female fecundity, but also resulted in a sharper decline in male reproductive success, suggesting a trade-off between ageing and reproduction in males, but not females (Chen and Maklakov 2012, 2014; Chen *et al.* 2016). We believe these findings underline the importance of understanding the interplay between sex-specific selection pressures with respect to condition dependent mortality and sexual selection, and the resulting evolution of sex-specific life histories.

Selective disappearance and the potential for sexual conflict with age

In addition to its role in shaping sex-specific life histories, condition dependent mortality could also play a role in modulating age-related sexual conflict. Male ageing has been suggested to be a potential source of sexual conflict (Dean *et al.* 2007), which has been corroborated in at least two species (Dean *et al.* 2007, 2010; Carazo *et al.* 2011). Our results add to this scarce literature by showing that, in *D. melanogaster*, male age effects on female reproductive success are exacerbated in the presence of condition dependent mortality. Furthermore, we did not find evidence that, under condition dependent mortality, male age affected mating behaviour at all, which could have diminished the net costs to females if accelerated ageing of males under condition dependent mortality made them less successful at mating with females. Average male mating success was not only not significantly lower in the presence of selective disappearance but, if anything, our results show a trend in the opposite direction. In line with this finding, we found some evidence that the mating success of old pairs (i.e., combined effects of male and female age) relative to control pairs was lower in the absence of condition dependent mortality (Figure 5.4). We found a similar (though clearer) trend for mating latency, whereby the mating latency of old pairs was significantly higher than young pairs only in the absence (but not presence) of condition dependent predation. In conjunction, these results suggest that, as might be expected, males that are able to maintain a high escape speed over time (i.e., survive simulated condition dependent predation) are if anything better at mating than average males in the population. This might be expected if males that are able to maintain a high escape speed over time maintain a generally high physiological condition. Interestingly, this implies that old males with good survival ability (i.e., old males under condition dependent predation) will be at least as successful at mating and quicker to mate with females than the average old male (i.e., old males in the absence of condition dependent predation). Overall, we show that, for females, the net costs of mating with old males is thus predicted to be higher under

condition dependent selection, which is likely to be common in nature. Hence, our results do not only reinforce the idea that ageing might act as a source of sexual conflict, but actually suggest that, in nature, this phenomenon may be more important than previously surmised. The scarcity of studies looking at aging and sexual conflict is surprising, and we contend this may be a fruitful avenue for future research.

As a corollary, we found that old males in general tended to have higher mating duration across treatments. Our findings are in line with previous reports in *D. melanogaster*, where mating duration was found to increase with male age (Bretman *et al.* 2013). Longer matings are associated with the transfer of at least two key seminal fluid proteins (sfps): sex peptide and ovulin (Wigby *et al.* 2009), and mating duration can be used as a proxy for ejaculate investment (Friberg 2006; Bretman *et al.* 2009). Sfps in the ejaculate are associated with several female post-mating responses. For example, sex peptide decreases female receptivity and stimulates fecundity while ovulin stimulates the release of oocytes from the ovary of females (Chapman *et al.* 2003; Liu and Kubli 2003). Besides, sfps are responsible for several mating costs for females such as lower lifetime reproductive success and higher mortality (Chapman *et al.* 1995; Wigby and Chapman 2005). Thus, on the one hand longer matings in old males may reflect a higher investment in their ejaculate due to terminal investment (Clutton-Brock 1984). On the other hand, however, longer mating durations may be due to age effects on male ejaculate transfer ability, so that old males may be worse at transferring their ejaculates.

Last but not least, it is important to note that our study population has been maintained in cages (both sexes together) with overlapping generations for decades and we know that males/females live around 40-50 days when they are maintained under mixed sex groups (Zajitschek *et al.* 2013). This means ageing treatments in our experiment (young flies: 4 days old, old flies: 38 days old)

resemble the conditions under which they have evolved for thousands of generations. Yet, future studies replicating this experiment with wild flies and at different ageing treatments would be very informative.

Chapter 6. Male life history traits and gut microbiota in *Drosophila melanogaster*

6.1. Introduction

Exploring the mechanisms underlying life history traits in males and females may contribute to our understanding of sex-specific life history evolution. Sex-specific selection may favour different life history traits in males and females, but both the degree to which this is so and how the sexes respond to these pressures will depend on underlying mechanisms. If the mechanisms mediating life history trade-offs differ in males and females, this can influence both sex-specific fitness peaks and responses to selection (e.g. constraints). For example, there is good evidence of trade-offs between male reproductive abilities (e.g. testosterone levels, sperm viability) and immunity in a broad range of species, from invertebrates to humans (Slater and Schreck 1993; Simmons and Roberts 2005; Radhakrishnan and Fedorka 2012), whereas similar trade-offs are not so clear in females (Adamo *et al.* 2001; Mcnamara *et al.* 2013). Therefore, characterizing the mechanisms that mediate life history traits and, in particular, the degree to which they have sex-specific effects, may contribute to our understanding of life history evolution.

An arising line of research in the study of the mechanisms of ageing, and life history mechanisms at large, is the role of gut microbiota. The effect of gut microbiota on female/male lifespan and female reproduction has been well studied in model organisms such as *D. melanogaster* (Brummel *et al.* 2004; Ren *et al.* 2007; Clark *et al.* 2015; Gould *et al.* 2018) and *C. elegans* (Houthoofd *et al.* 2002; Cabreiro and Gems 2013), as well as in humans (Tiihonen *et al.* 2010; Insenser *et al.* 2018). Despite the large number of studies indicating the contribution of gut microbiota in female ageing and fitness, the link between gut microbiota and male reproduction has not received much attention and is not well

understood. The relatively few studies that have explored how gut microbiota can shape male reproduction do hint at the importance of gut microbiota in both male mating behaviour and reproductive success (Ami *et al.* 2010; Morimoto *et al.* 2017). For example, Morimoto (2017) manipulated the gut microbiota of *D. melanogaster* by infecting them with two different species of bacteria: *Acetobacter pomorum* or *Lactobacillus plantarum*. This two species were chosen because they are known to: (1) be among five most abundant species in wild fruit flies (Wong *et al.* 2011) and (2) affect the physiology and behaviour of *D. melanogaster* (Erkosar *et al.* 2013). They found that males infected with *L. plantarum* had longer mating duration and caused the females to produce more offspring in the short-term. Moreover, when females mated with males infected with *A. pomorum*, they were less likely to produce viable offspring. Likewise, Ami *et al.* (2010) studied how gut microbiota can affect the mating behaviour of the Mediterranean fruit fly (*C. capitata*). They found that sterilized males had damaged gut bacterial community structure due to the radiation used to sterilize them, and that regenerating their original microbiota community (by feeding them with bacteria enriched diet) enhanced the mating performance of these males compared to controls (Ami *et al.* 2010).

Our aim in this study was to explore the potential role of gut microbiota in male life history traits by examining the co-variation between life history traits and gut microbiota across life. In order to do so, we first characterized the life history traits of male fruit flies from 29 different DGRP inbred isolines: i.e., lifespan, early/late life reproduction and early/late life physiological performance/condition (i.e., anti-predatory escape ability). We then characterized the early and late life gut microbiota of these isolines and investigated how gut microbiota composition changes with age. Finally, we explored the potential link between male life history traits and gut microbiota.

6.2. Materials & Methods

Experimental population

As focal flies, we used flies from the *Drosophila melanogaster* genetic reference panel (DGRP) (see details in Chapter 3). Hence, individuals within each isoline can be considered as clones. Using DGRP isolines (many individuals with the same genotype) instead of wild-type flies allowed us to characterize the life history traits of different genotypes in standard conditions while characterizing the early and late life gut microbiota associated with these same genotypes.

Life history assays

We set up replicate vials containing 10 males in same-sex groups for each of the 29 different DGRP isolines (Table 6.1). We transferred these flies to new vials with fresh food once a week throughout their lifespan (or until sacrificed, see below), and checked mortality 5-6 days a week by recording the number of dead individuals in each experimental vial. Density within vials was kept constant between 8-11 individuals until all flies died (Figure 6.1). To estimate the reproductive success of focal males, we measured the relative paternity of all experimental males competing against standard rivals at two different time points: early (4 days old) and late (25 days old) in life. Namely, we introduced 10 focal males with 10 *spa* males and 10 *spa* females into new vials and let them interact and lay eggs for 24 hours. At the end of this period, we recovered the focal males belonging to the isolines and discarded the individuals with the *spa* mutation. Following the first reproductive assay, on day 5, we sacrificed 15-20 males per isoline for gut dissection and kept remaining flies for life history characterization. We incubated the eggs from the first reproductive assay and left them to develop into adults for 16 days, froze them and then counted the proportion of wt/*spa* offspring as a measure of their reproductive success. We repeated this procedure on day 25, and again sacrificed 15-20 males per isoline for gut microbiota analyses. We calculated reproductive ageing by

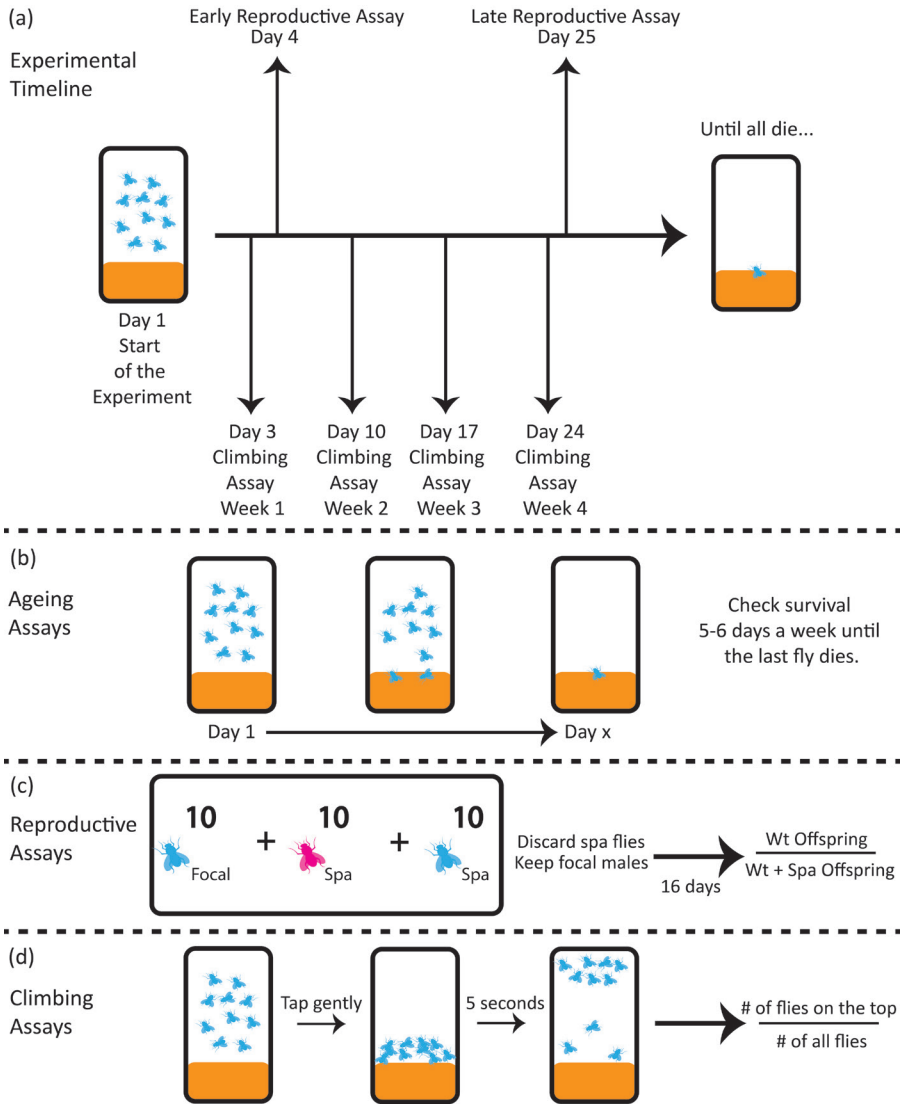

Figure 6.1. Schematic illustration of the experimental design. (a) We monitored replicate vials that contain 10 males in same-sex groups for each of the 29 different DGRP isolines. We measured climbing speed once a week for the first 4 weeks and reproductive success early and late in life (day 4 and day 25). (b) For ageing assays, we checked survival 5-6 days a week until all flies died. (c) For reproductive assays, we put 10 focal flies with 10 *spa* females and 10 *spa* males, counted the number of wt/spa offspring after 16 days and calculated the relative number of wt offspring (d). For climbing assays, we tapped each vial and measured the proportion of flies that were able to climb to the top in 5 seconds.

Table 6.1. Sample sizes used in this experiment. Isoline codes represent the codes in Bloomington Drosophila Stock Center, while isoline ID represent the codes for our experiment. Number of replicates per isoline include the number of total flies in brackets, while number of flies sacrificed early and late in life include the number of biological replicates (also in brackets).

Isoline Code (Bloomington)	Isoline ID	Number of Replicates in Life History Assays	Number of Sacrificed flies	
			Early	Late
38	2	7 (70)	NA	NA
88	4	5 (50)	NA	NA
101	6	10 (100)	15 (3)	20 (4)
109	8	6 (60)	NA	NA
208	10	10 (100)	15 (3)	20 (4)
229	11	10 (100)	15 (3)	20 (4)
239	12	10 (100)	15 (3)	20 (4)
313	14	10 (100)	15 (3)	20 (4)
324	15	10 (100)	15 (3)	15 (3)
357	17	10 (100)	20 (4)	20 (4)
359	19	10 (100)	20 (4)	20 (4)
365	20	8 (80)	15 (3)	15 (3)
375	22	10 (100)	20 (4)	20 (4)
379	23	10 (100)	20 (4)	15 (3)
390	24	9 (90)	15 (3)	15 (3)
391	25	10 (100)	20 (4)	20 (4)
399	26	10 (100)	20 (4)	20 (4)
427	27	9 (90)	15 (3)	15 (3)
437	28	10 (100)	20 (4)	20 (4)
443	29	10 (100)	20 (4)	20 (4)
492	30	10 (100)	20 (4)	20 (4)
517	31	10 (100)	20 (4)	20 (4)
703	32	7 (70)	NA	NA
732	33	10 (100)	20 (4)	20 (4)
808	35	10 (100)	20 (4)	15 (3)
812	36	10 (100)	20 (4)	20 (4)
857	37	7 (70)	NA	NA
900	38	10 (100)	20 (4)	20 (4)
911	39	10 (100)	20 (4)	20 (4)

subtracting average late life reproductive success from average early life reproductive success per isoline.

We also estimated the climbing speed of each isoline once a week for 4 weeks (i.e., on days 3, 10, 17 and 24) by measuring the climbing speed of each group of males in their experimental vials. We repeated this procedure three times per vial and took the average. Then, we calculated the average across all replicate groups within each isoline as a measure of the climbing speed of each isoline at each time point, and estimated functional senescence as the slope of the age-related decline in climbing speed in four weeks.

Gut dissection, bacterial DNA isolation and sequencing

For gut dissections, 15-20 flies per isoline were sacrificed right after the two reproduction time points early and late in life (Table 6.1). The flies were taken from the two vials per isoline, the remaining unsacrificed flies in the vials were divided to other vials of the isoline to control for density. Dissection of each fly was done separately inside PBS droplets under the microscope by using sterilized forceps. Isolated guts were collected in groups of five in order to have 3-4 biological replicates per isoline and immediately flash-frozen in liquid nitrogen prior to DNA extraction. DNA extraction from gut tissue was performed with the JetFlex ™ Genomic DNA Purification Kit. The DNA was quantified with Nanodrop-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and then sent for sequencing. DNA obtained from gut was used as a template for amplification of the bacterial 16S rRNA genes and sequenced using the Illumina MySeq technology at the FISABIO Foundation.

Processing and analyses of the 16S rRNA reads

Sequence data came in two batches, from independent sequencing runs. Almost 80% of the original read pairs were successfully merged in the pre-processing step provided by the sequencing center. We trimmed the last 3 bases of all reads

to remove a large number of Ns, and filtered out the remaining sequences with any N. Then we used the dada2 R package (Callahan *et al.* 2016) to *denoise* the dataset. That is, to infer and correct sequencing errors and to remove potentially chimeric reads. Using BLAST (Altschul *et al.* 1990), we noticed that sequences shorter than 433 or larger than 463 bases were mostly artefacts produced by unspecific hybridization of primers to the nuclear genome of the host fly, and we filtered them out. The resulting dataset consisted of ~12 million reads corresponding to 2683 unique sequences (amplicon sequence variants, or ASV) and distributed in 177 samples, including 3 or 4 biological replicates of each isoline and age combination (Table 6.1).

We used an implementation of the RDP Naive Bayesian Classifier algorithm (Wang *et al.* 2007) available in the dada2 package and the Silva taxonomic databases (Callahan 2018) to attribute taxonomy information to the ASVs. Then, we further removed 24 ASVs either missing kingdom information or spuriously assigned to the Eukaryota kingdom. Only 16 rDNA ASVs were identified at the species level, while 97% of them got a genus assigned.

The number of times an ASV got sequenced in a sample is assumed to be a proportional indication of the ASV's abundance in that microbiome. Relative abundances were computed before further filtering. Because most ASVs had a very low abundance, and were suspected to be noise, we used only ASVs present in at least 10 samples either early or late in live, and with average relative abundances higher than 10^{-5}, in at least one of the two age classes. This is the subset, comprising 1236 ASVs, used in all subsequent analyses, unless otherwise stated.

Biodiversity analysis

We calculated Shannon and Simpson diversity indices with the vegan R package (Oksanen *et al.* 2018) for every sample. We also computed isoline and age specific indices by adding up absolute abundances of replicates of the same

isoline and age before computing the indices. Unless otherwise stated, diversity indices refer to ASV diversity, computed from ASV abundance data. We also computed genus diversity, by grouping abundances by genus.

Principle component analysis for life history traits

In order to identify potential life history trade-offs and reduce life history variables across isolines, we ran a Principal Component Analysis (PCA) on the following life history variables from our isolines: (1) Early climbing speed (how fast males climbed vertically when young, a proxy for anti-predatory escape ability), (2) Functional ageing (the slope of the decline in climbing speed with age), (3) Early reproductive success (relative reproductive success of young males competing against *spa* males for *spa* females), (4) Reproductive senescence (the difference between early and late life reproductive success), (5) Average lifespan, (6) Acceleration of mortality rate (the beta component in a Gompertz fit on the survival curve) of each isoline. These six life history measures are important fitness components for flies in the wild, related to functional performance (1 and 2), the competitive potential of males during sexual selection (3 and 4), and actuarial ageing (5 and 6).

Age effects on gut microbiota composition

To study whether gut microbiota composition changes with age we fitted Negative Binomial generalized linear models of individual ASV abundances, using the DESeq2 R package (Love *et al.* 2014). Unfortunately, this package does not fit mixed models. The three factors included in the model, namely the sequencing run, the isoline and the age, were assumed to have fixed effects. The merit of this approach is that abundance data is modelled directly, without need for normalization, and taking into account the biological variation of abundances among replicates (Mcmurdie and Holmes 2014). Then we used a Wald test to identify ASVs the abundances of which were significantly affected by age.

In order to use microbiome composition data to explain variation in life history traits, it was necessary to reduce the dimensionality of the data set. We opted for the multidimensional scaling (MDS) implemented in the phyloseq R package (Mcmurdie and Holmes 2013), and we chose a binary (presence-absence) distance measure among samples. MDS uses all the variation in the data set to represent samples in a 2-dimensional space. Using the binary distance, the ordination result separated most early samples from most late samples along one direction, and it also distinguished quite well between first and second sequencing runs along the orthogonal direction. Distance measures taking abundance into account did not produce such a neat pattern. Because the orientation of an MDS result is arbitrary, we rotated the axes (using recluster R package, Dapporto *et al.* 2013) to make coordinates correspond to the two relevant directions, renamed as "rotated axis 1" (correlated with age) and "rotated axis 2" (correlated with the sequencing run; Figure 6.2).

As an additional attempt to extract potentially meaningful information from the multidimensional abundance matrix, we grouped ASVs by genus and calculated the ratio of the total abundance of *Lactobacillus* ASVs to the total abundance of *Acetobacter* ASVs. The choice to focus on these two genera was motivated by their overall large abundance (see results), and by a study showing their potential functional roles on male reproduction (Morimoto *et al.* 2017)

Linking life history traits to gut microbiota composition

We analysed whether life history PCs 1-3 were associated with changes in the following gut microbiota variables: (1) Diversity (Simpson's index), (2) Rotated axis 1 and 2 early in life ($RA1_{early}$ & $RA2_{early}$), (3) Rotated axis 1 and 2 late in life ($RA1_{late}$ & $RA2_{late}$), (4) the change in diversity with age (using Simpson's index) and (5) The drop in the ratio of *Lactobacillus vs. Acetobacter* abundance with age. We used exhaustive screening of all candidate models in combination with model averaging, owing to a lack of a priori hypotheses about which combination

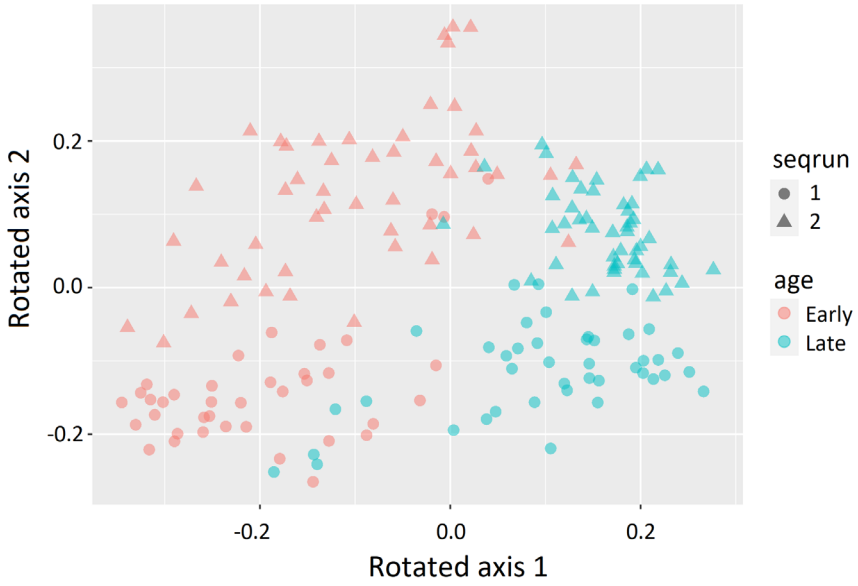

Figure 6.2. Multidimensional scaling on binary distances. The axes of multidimensional scaling (MDS) were rotated to make coordinates correspond to the two relevant directions: "rotated axis 1" and "rotated axis 2".

of variables and their interactions may represent biologically relevant models for our data (Calcagno and De Mazancourt 2010). Models were evaluated using the second-order information criterion (AICc), which is adequate when, as in our case, the ratio of parameters to sample size is less than 40 (Burnham and Anderson 2002). Model averaging was conducted over a 95% confidence set of candidate models, calculated as the set of AICc ranked models with an accumulated model weight 0.95 (i.e., a 95% probability that the best model is represented within the set; Calcagno and De Mazancourt 2010; Symonds and Moussalli 2011). Importance weights are a measure of the probability that the explanatory variable is a component of the best model (Symonds and Moussalli 2011). This model-averaging procedure incorporates uncertainty about which model is most appropriate for determining coefficient estimates and variances of relevant covariates. We allowed a maximum model complexity of 5 (5 terms in

model including intercept), including second order interactions. We identified important terms, for which we calculated model-average estimates across all models within the 95% confidence set. Finally, we ran a complementary analysis by fitting GLMs on models incorporating all the terms identified as important in the exhaustive modelling approach.

In order to assess which amplicons (ASV) or genera are associated with different life history variables (i.e., their principle components), we used Lefse (Segata *et al.* 2011). Lefse is a pipeline that evaluates the association of quantitative descriptors (e.g. ASV abundances) with a classification of samples, using linear discriminant analysis. Its goal is to identify biomarkers, that is, highly informative ASVs that could be used to predict the class a sample belongs to. In order to classify samples according to the life history traits values of the isoline they belong to, we used the signs of isolines' loadings on the three principal components. Two levels (high and low) were used for each principle component. In all cases, high indicates the higher fitness side of the range (i.e., high early climbing speed, low functional ageing, high early reproductive fitness, low reproductive senescence, high average lifespan and high acceleration of mortality rate). We used default parameters for the Lefse run, namely: alpha value for the factorial Kruskal-Wallis test among classes: 0.05; alpha value for the pairwise Wilcoxon test between subclasses: 0.05; and threshold on the logarithmic LDA score for discriminative features: 2.0.

6.3. Results

Life history traits of DGRP males

We found substantial variation across the 29 different isolines with respect to the 6 life history variables (Table 6.2). The PCA reduced these 6 life history variables into 3 principal components that jointly explained ~80% of the variability (Table 6.3). After reducing the 6 different variables into 3 principal components, we

looked at the rotated weights of each variable on each component and focused on those with > 0.5 loadings to interpret resulting PCs (Table 6.4).

PC1 was mainly explained by early climbing speed and functional ageing, predicting that the higher the early climbing speed, the more negative is the slope of functional ageing. We interpreted this as a component reflecting "anti-predator performance", although it may also reflect an interesting trade-off between early life climbing speed and functional senescence. In order to explore the latter possibility, we explored the relationship between early climbing speed (climbing speed of week 1) and climbing speed difference between week 1 and week 2 ($F_{1,27}$ = 37.947, $p < 0.001$, $r_m^2 = 0.5689$), week 1 and week 3 ($F_{1,27} = 63.483$, $p < 0.001$, $r_m^2 = 0.6906$) and, week 1 and week 4 ($F_{1,27} = 211.270$, $p < 0.001$, $r_m^2 = 0.8825$); see Figure S.13-16 and Discussion. PC2 was only explained by early reproductive success, so we interpreted this PC as "early reproduction". Finally, PC3 was explained by average lifespan and reproductive senescence, showing that isolines with higher lifespan experience lower reproductive senescence. We thus interpreted this PC as a compound measure of "ageing" (i.e., actuarial and reproductive ageing).

Age-dependent change in gut microbiota diversity & abundance

The differential abundance analysis performed with DESeq2 revealed 219 ASVs with significant abundance change between early and late ages, with a false discovery rate of 0.001. ASVs showed a significant increase in abundance with age in only two genera: *Acetobacter* and *Lactobacillus* (Figure 6.3). While *Acetobacter* overwhelmingly dominates in all isolines, *Lactobacillus* reaches a modest abundance at a late age in many isolines, while it was almost absent in early age. Indeed, the *Lactobacillus*/*Acetobacter* ratio significantly increased with age ($F_{1,23} = 8.9603$, $p = 0.0065$). The ASVs with a significant decrease in abundance with age belong to two other genera: *Ralstonia* and *Pseudomonas* (Figure 6.3).

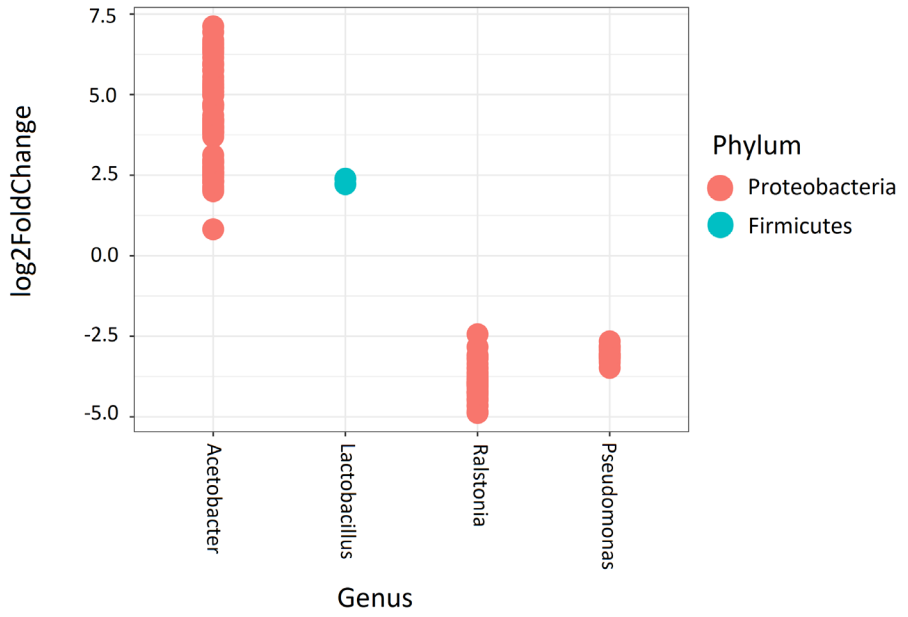

Figure 6.3. Age-related fold change and taxonomic distribution of amplicons. Age-related abundance change and taxonomic distribution of the amplicons with most differentiated abundances between early and late life samples.

The reduction is mainly due to the disappearance of species from the genus *Ralstonia* and, to a lesser extent, of *Pseudomonas.* Figure 6.4 shows the 7 most abundant classes found in the microbiomes, belonging to six different phyla, Actinobacteria (Actinobacteria); Bacilli (Firmicutes); Alphaproteobacteria and Gammaproteobacteria (Proteobacteria); Bacteroidia (Bacteroidetes); Oxyphotobacteria (Cianobacteria) and Verrucomicrobiae (Verrucomicrobia). Among them, clearly members of the Proteobacteria phylum are the most abundant. The reduction of Gammaproteobacteria with age suggests early microbiomes are more diverse. However, taking into account ASV diversity within genera, alpha diversity indices do not actually drop with age. Only when using taxonomic (genus) diversity we do notice a sharp decline in Shannon or Simpson diversity indices with age (Figure 6.5).

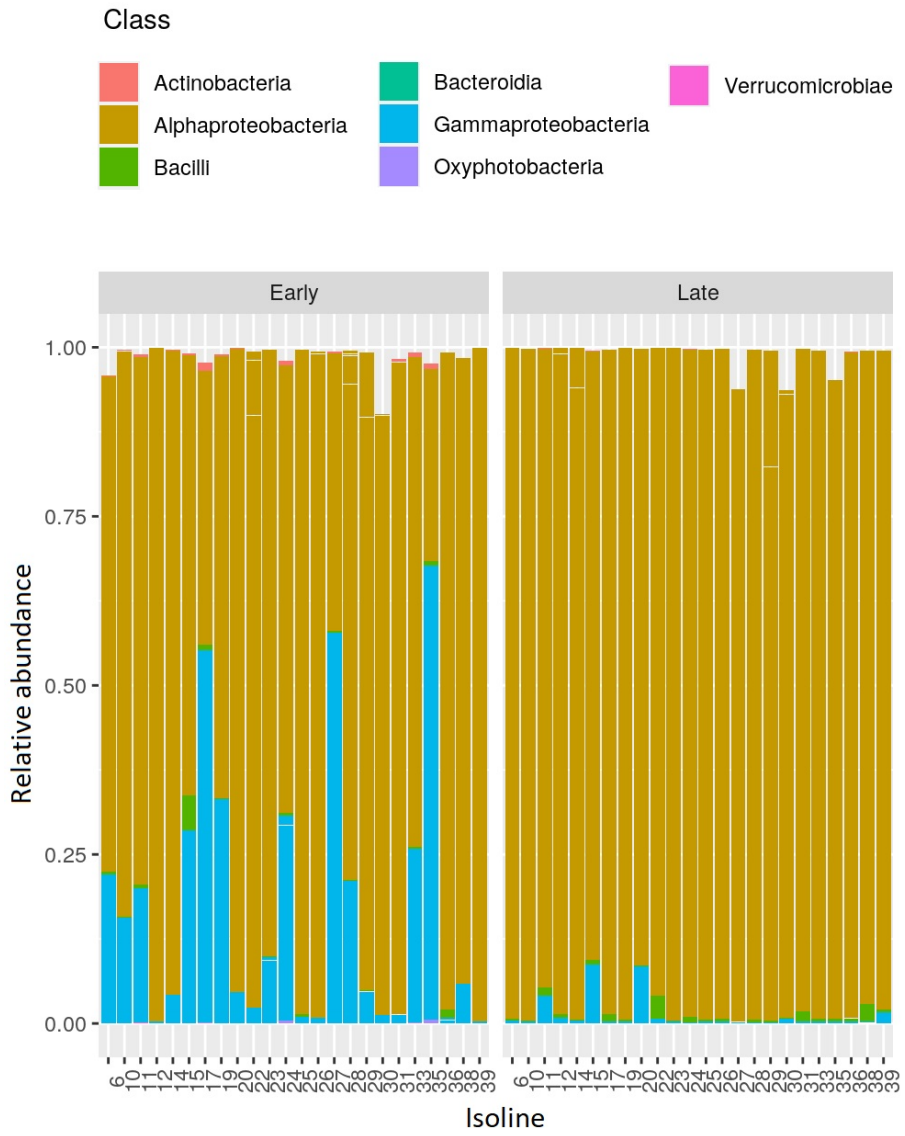

Figure 6.4. Age-related changes in the relative abundances of different bacterial classes. Early and late life relative abundances of the 7 most abundant bacterial classes across 24 different DGRP isolines.

Table 6.2. Descriptive statistics of the six life history variables. Mean, standard deviation and range of the six life history variables that were analysed in this project.

	Mean	SD	Range
Average Lifespan	37.31	7.99	25.08 - 58.61
Early Climbing Speed	0.51	0.19	0.18 - 0.82
Functional ageing	-0.41	0.16	-0.70 - -0.12
Early reproductive fitness	0.46	0.16	0.12 - 0.78
Reproductive senescence	0.15	0.14	-0.15 - 0.40
Acceleration of mortality rate	0.10	0.06	0.03 - 0.28

Table 6.3. Principle components describing male life history variation. Standard deviation, proportion of variance and cumulative proportion of the first four principle components describing male life history variation across isolines.

	PC1	PC2	PC3	PC4
Standard deviation	1.4319	1.2406	1.0958	0.8358
Proportion of Variance	0.3417	0.2565	0.2001	0.1164
Cumulative Proportion	0.3417	0.5982	0.7984	0.9148

Table 6.4. Rotated weights of male life history variables. Rotated weights of 6 male life history variables on 3 different principle components.

	PC1	PC2	PC3
Average Lifespan	0.130	-0.471	-0.597
Early Climbing Speed	0.673	0.096	0.091
Functional ageing	-0.652	-0.246	-0.009
Early reproductive fitness	-0.007	-0.647	-0.041
Reproductive senescence	0.092	-0.270	0.770
Acceleration of mortality rate	0.310	-0.464	0.200

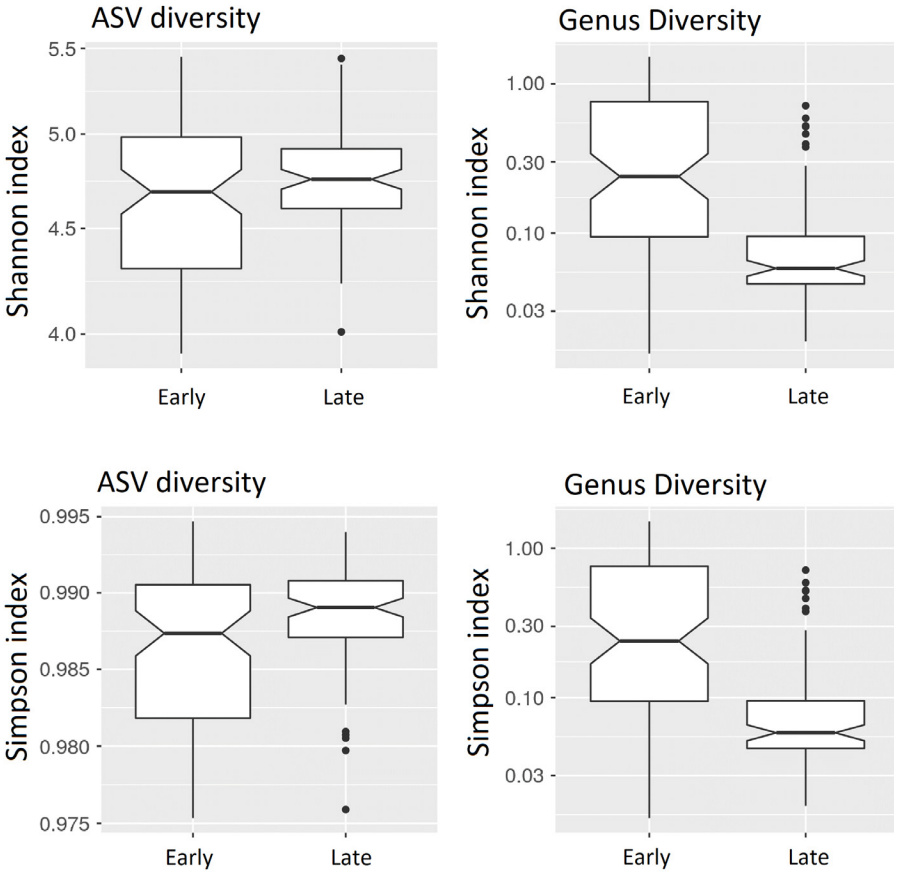

Figure 6.5. Age related change in bacterial diversity. Age related change in (a) Shannon index with respect to ASV diversity, (b) Shannon index with respect to genus diversity, (c) Simpson index with respect to ASV diversity, (d) Simpson index with respect to genus diversity.

Interactions between gut microbiota and male life history traits

Exhaustive model selection for PC1 yielded a 95% confidence set of 81 models (best model: PC1~ 1+ L/A_{drop} , AICc=80.58, wi =0.07), but no important variables. Model selection of PC2 yielded a 95% confidence set of 83 models (best model: PC2~ 1+ $RA1_{late}$+ $RA2_{early}$ + $RA2_{late}$, AICc= 54.15, wi = 0.072), with $RA1_{late}$ appearing as the only important predictor (estimate = -99.73 ± 409;

importance = 0.85). A LM on the best model showed this model was significant overall and explained a substantial proportion of variability in PC2 ($F_{3, 18} = 4.431$, $p = 0.017$, $R^2_{adj} = 0.33$). Backwards model selection confirmed $RA1_{late}$ as a significant factor in this model ($F_{1, 18} = 6.740$, $p = 0.018$), along with $RA2_{late}$ ($F_{1, 18} = 6.174$, $p = 0.023$), but not $RA2_{early}$ ($F_{1, 18} = 0.377$, $p = 0.547$). Finally, model selection of PC3 yielded a 95% confidence set of 85 models (best model: PC3~ 1+ Simpson $_{early}$ + $RA1_{early}$+ $RA2_{late}$, AICc= 61.87, wi = 0.07), with $RA1_{early}$ appearing as the only important predictor (estimate = -144 ± 613 importance = 0.93). A LM on the best model showed this model was significant overall and explained a substantial proportion of variability in PC3 ($F_{3, 18} = 4.679$, $p = 0.0138$, $R^2_{adj} = 0.35$). $RA1_{early}$ appeared as a marginally non-significant factor in this model ($F_{1, 18} = 3.687$, $p = 0.071$), while $RA2_{late}$ appear as a significant factor in this model ($F_{1, 18} = 8.076$, $p = 0.011$).

Finally, in order to understand which genera (or amplicons) might be associated with low versus high condition traits, we ran a Lefse analysis to compare isolines with positive vs. negative values of PC2 (early reproduction) and PC3 (ageing); i.e., PC2, 13 isolines with positive values and 11 with negative and PC3, 10 isolines with positive values and 14 with negative values. Lefse identified no genus significantly associated with neither high nor low levels of neither PC2 nor PC3. However, it did find significant associations when looking at ASVs. Namely, 12 ASVs were associated with low-fitness values of PC3 (shorter lifespan, faster reproductive senescence), and one ASV was associated with high-fitness values of PC3 (longer lifespan, slower reproductive senescence) (Figure 6.6). All these 13 ASVs belong to the *Acetobacter* genus.

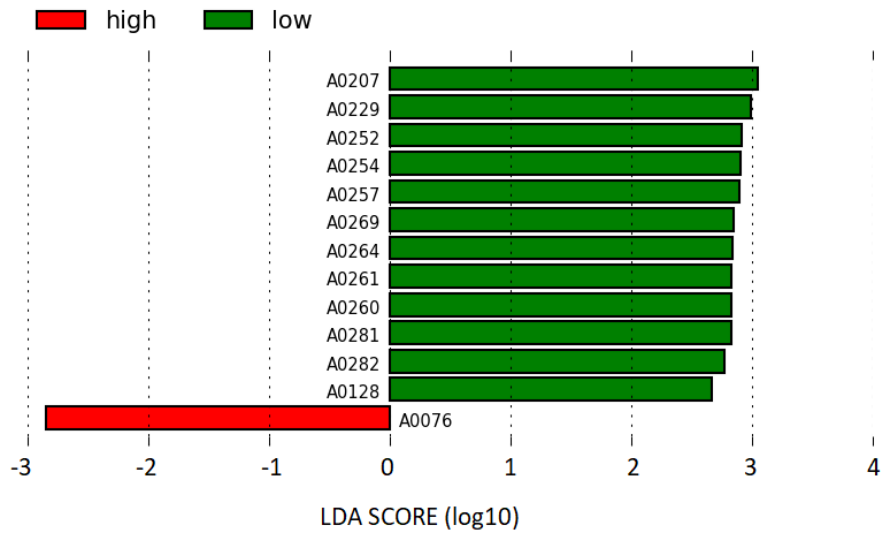

Figure 6.6. ASVs associated with high and low values of PC3 (i.e. ageing component). 12 ASVs were associated with low-fitness values of the ageing component (shorter lifespan, faster reproductive senescence; in green). One ASV was associated with high-fitness values of the ageing component (longer lifespan, slower reproductive senescence; in red).

6.4. Discussion

In this chapter, we explored male life history traits and their potential interaction with gut microbiota. First, we did not detect a clear trade-off between reproductive success and survival-associated traits (e.g. lifespan, anti-predatory escape ability), contrary to what we expected. In fact, when present, correlations between life history traits tended to be positive, so that most variability in life history traits seemed to reflect differences in quality across isolines. Second, as previously shown in the literature, we found age-related changes in male gut microbiota diversity and abundance. Finally, we found preliminary evidence of a link between male gut microbiota abundance and early reproductive success.

Life history variation and potential trade-offs across DGRP lines

We documented substantial variation in life history traits across isolines. The average lifespan across isolines was 37.31 days, with a more than two-fold difference between the longest and shortest-lived isolines (longest lived: 59 days, shortest lived 25 days). Similarly, the difference between the isoline with the highest early reproductive fitness and the one with lowest early fitness was more than six-fold (highest early fitness: 0.78, lowest early fitness: 0.12). We also found that our isolines have high variation with respect to early climbing speed (mean ± standard deviation: 0.51 ± 0.19), functional ageing (mean ± standard deviation: -0.41 ± 0.16), acceleration of mortality (mean ± standard deviation: 0.10 ± 0.06) and reproductive senescence (mean ± standard deviation: 0.15 ± 0.14; Table 6.2).

We detected a relationship between early climbing speed and our measure of functional ageing, so that isolines with high early climbing speed experienced a steeper decline in the climbing speed with age. While this may reflect an interesting trade-off, it is also possible that this relationship is due to a floor effect driven by how climbing speed decreases with age. That is, because climbing speed is bound by 0 in its lower range, it may be that isolines with a high early climbing speed necessarily have a steeper functional decline due to the fact that all old flies end up having a climbing speed close to 0 by the end of their life. Plotting how climbing speed decreases with age (Figure S.16) does seem to show a floor effect. However, we found a positive relationship between early climbing speed and the decrease in climbing speed between the first and second weeks (Figure S13), when climbing speed is unlikely to be bound by cero. Furthermore, this relationship appeared non-linear, so that the highest early climbing speeds actually showed no relationship with the decrease in early climbing speed (Figure S16). Even in the absence of a floor effect, the relationship between early climbing speed and our measure of functional ageing could simply be a spurious result of both variables being dependent (because early climbing speed is used to

calculate functional ageing). All in all, with these results we cannot firmly conclude the absence of a trade-off between early climbing speed and functional senescence in climbing speed, which suggest should be examined in detail by future studies.

We did not detect evidence of a trade-off between reproduction or survival related traits (e.g. lifespan or anti-predatory). In fact, when present, the correlation between male life history traits was positive. This might be explained by the fact that we used inbred isolines that are expected to have different phenotypes compared to the wild-type individuals due to the absence of heterozygous effects. DGRP isolines have been the target of a broad range of studies because: (1) the same isoline can be characterized with respect to several traits to look for correlations and (2) genome-wide association can be done in search of candidate genes that are associated with traits such as longevity, fecundity or stress response (Ayroles *et al.* 2009; Durham *et al.* 2014; Ivanov *et al.* 2015). However, using these isolines also has the disadvantage of looking at phenotypes that may differ from those observed in nature (i.e., all recessive alleles are expressed due to homozygosity). We suggest that a potential way to complement this study would be to replicate it using heterozygotes from DGRP line crosses and/or wild male isolines.

Age effects on male gut microbiota in Drosophila melanogaster

We found that the relative abundance of both *Acetobacter* and *Lactobacillus* increases with age. Moreover, the *Lactobacillus*/*Acetobacter* ratio was found to be higher in old males. How gut microbiota composition and diversity differs between old and young flies has been studied in outbred flies (Wong *et al.* 2011). Wong *et al.* (2011) found that the relative abundance of *Acetobacter* increased with age, which is consistent with our findings. However, they found a decrease in the abundance of *Lactobacillus* and *Lactobacillus*/*Acetobacter* ratio, in contrast to our results. One reason behind the different findings may be that Wong

et al. (2011) used outbred populations while we used highly inbred isolines. Considering that host genome can control gut microbiota composition in *D. melanogaster* (Chaston *et al.* 2016), the level of inbreeding can also affect age-related changes in relative microbial abundance. Another explanation could have to do with different diets, which could have led to different microbial compositions in these studies; particularly, concerning that diet can significantly alter the gut microbiota composition (Erkosar and Leulier 2014). In addition to the relative abundance of different taxa, we also found age-related changes in bacterial diversity. When using genus diversity, bacterial diversity was lower in old males compared to young ones (Figure 6.5). This is consistent with previous results in outbred flies, where old males had lower species diversity than young males (Wong *et al.* 2011).

Male gut microbiota and life history traits in Drosophila melanogaster

Finally, our results suggest a link between gut microbiota abundance and two principle components: PC2 "early reproductive success" and PC3 "ageing" (i.e., lifespan and reproductive senescence). Although there was no genus significantly associated with early reproductive success and ageing, 12 ASVs were associated with shorter lifespan and faster reproductive senescence. Interestingly, all 12 ASVs belonged to the *Acetobacter* genus.

Previous studies investigating the interaction between gut microbiota and life history traits have generally used germ-free flies inoculated with single or multiple bacterial species. This allowed them to determine which species/genera had an effect on certain life history traits such as lifespan, fecundity or fertility. For example, Tefit *et al.* (2017) showed that, when compared to axenic flies, mono-association with *Lactobacillus plantarum* resulted in longer lifespan in males fed with a low yeast diet; however, it did not affect female lifespan kept on a high or low yeast diet. In contrast, Fast *et al.* (2018) later found that mono-association of female flies with *L. plantarum* shortened female lifespan compared

to axenic females (Fast *et al.* 2018). These contradictory results are explained by the differential effects of sex, genomic background and diet, which can modulate the effect of gut microbiota on fitness traits (Douglas 2018). In another study, Pais *et al.* (2018) demonstrated that mono-association of female and male flies with *Acetobacter thailandicus* leads to faster development and higher fertility of emerging adults when compared to axenic flies and other bacterial species (Pais *et al.* 2018). Still, mono-association studies are not ideal for explaining how microbiota can shape host fitness because the fly gut includes more than one species of bacteria (Wong *et al.* 2011). Gould *et al.* (2018) associated female and male flies with five species of bacteria (*L. plantarum, L. brevis, A. pasteurianus, A. tropicalis,* and *A. orientalis*) that commonly exist in the wild fly gut. All combinations of bacterial association were conducted, from mono-association with each species to poly-association with all five species. They found that bacterial combinations that caused high fecundity led to lower lifespan, and combinations that caused low fecundity resulted in a longer lifespan. These results suggest that gut microbiota composition can induce a life history trade-off between reproduction and lifespan in females. As they did not observe a consistent differential effect on lifespan between males and females, they concluded that bacterial combinations have similar effects on male and female lifespan (Gould *et al.* 2018). In short, the role of gut microbiota on female/male ageing and female fecundity has been relatively well-studied using mono/poly associations. Yet, to our knowledge there is only one prior study that has investigated how bacterial association can modulate male reproduction in *D. melanogaster* (Morimoto *et al.* 2017). Morimoto *et al.* (2017) showed that, compared to males mono-associated with *A. pomorum*, males that were mono-associated with *L. plantarum* had longer mating duration and their partners produced more offspring.

In addition to mono/poly-association of germ-free flies with different bacterial species, host life history traits have also been linked to gut microbiota using

associative studies. A crucial study in this context compared how the relative abundance of acetic acid bacteria (including *Acetobacter* species) and lactic acid bacteria (including *Lactobacillus* species) changes in fruit flies from different latitudinal population that exhibit different life history strategies (Walters *et al.* 2020). Walters *et al.* (2019) found that flies from low-latitude populations (with short lifespan and high early reproduction) had more acetic acid bacteria than flies from high-latitude populations (with long lifespan and low early reproduction). Moreover, this pattern was present in the opposite direction in the case of lactic acid bacteria. Yet, this study mainly focused on female lifespan and reproduction while the link between the existing gut microbiota and male reproductive success was overlooked. The high content of acetic acid bacteria in shorter lived populations (Walters *et al.* 2020), high relative abundance of *Acetobacter* in old males (Wong *et al.* 2011) and our findings where certain ASVs from *Acetobacter* genus were associated with lifespan and reproductive ageing (see Figure 6.6), all underscore the potential importance of this genus for male ageing. In addition to ageing, the findings of Morimoto *et al.* (2017) about the effects of two bacterial species on mating duration and number of offspring suggest that gut microbiota can also affect male reproductive success. In fact, we also found an association between late gut microbiota composition and male reproductive success. However, we did not find a specific genus or ASV that was connected with high or low reproductive success in males.

Despite the inconclusive results of mono-association experiments, studies that involve the interactions of more than one bacterial species (e.g. poly-association studies) have shown that microbial composition has similar effects on the lifespan of females and males (Gould *et al.* 2018, Walter *et al.* 2020). However, this is not the case for reproductive success. For example, the *Acetobacter* genus that is generally associated with high female fecundity has been found to have negative effects on male reproductive success (Morimoto *et al.* 2017). Moreover, in our study we also found evidence suggesting that the *Acetobacter* genus is associated

with short lifespan and fast reproductive ageing in males. Hence, host-microbiome interactions underlying lifespan seem to be similar in females and males, but not so those affecting reproductive success.

Altogether, existing evidence shows that (1) gut microbiota composition can affect life history traits in both sexes and (2) both the environment and host genome can contribute to shape microbiota composition. Therefore, the link between microbiota composition and life history evolution may be complex and causally bi-directional (Macke *et al.* 2017). Our results add to this emerging literature by showing that there is a link between gut microbiota composition and male life history traits. More detailed analyses are necessary in order to understand the exact microbial interactions that shape the link between male/female life history evolution and gut microbiota. For example, metagenomics and metatranscriptomic studies can provide more information about the putative functions and gene expression profiles of bacterial communities. However, the current evidence tentatively suggests that gut microbiota may constraint male and female life histories in a different way, an exciting path for future research that we suggest may yield some insight into the mechanisms and trade-offs underlying sex-specific ageing.

Chapter 7. The “unguarded-X” and the genetic architecture of sex-specific lifespan in *Drosophila melanogaster*

7.1. Introduction

The “unguarded-X” hypothesis (hereafter UXh) posits that sex-specific ageing can be caused by the increased expression of deleterious recessive mutations in the heterogametic sex, due to the asymmetric inheritance of the sex chromosomes (Trivers 1985). While recessive mutations in the X (or Z) chromosome will be expressed unconditionally in the heterogametic sex, the same will not happen in the homogametic sex due to the second copy of the X (or Z) chromosome, which will "guard" against their expression. Hence, the “unguarded-X” effect is predicted to influence mutations accumulating both in the germline (inter-generational effect) and somatic line (intra-generational effect), and generally predicts slower ageing and longer lifespan in the homogametic sex.

In accordance with theory, it is frequently noted that female birds (heterogametic sex: ZW) tend to have shorter lifespans than males, whereas female mammals (homogametic sex: XX) tend to have longer lifespans than males; although such differences might equally be due to overall physiological or sex role differences between mammals and birds (Maklakov and Lummaa 2013). Beyond these two taxa, Pipoly *et al.* (2015) conducted a comparative study across 344 species belonging to 117 families of tetrapods (including reptiles and amphibians) and showed that adult sex-ratios tend to be female-biased in taxa with a XY sex determination system, and vice versa in taxa with a ZW sex determination system. Interestingly, this effect was particularly strong in taxa with variation in their sex determination system (amphibians and reptiles), and remained so even after controlling for sexual size dimorphism to account for sexual selection processes.

To the extent that adult sex ratios might be taken as an indirect proxy for sex-specific ageing, these results do seem to fit predictions from the UXh (Pipoly *et al.* 2015). Unfortunately, and despite suggestive correlative evidence at a broad comparative level, experimental investigations of the UXh have lagged behind, and direct empirical support for the UXh is simply scarce and inconsistent (Maklakov and Lummaa 2013; Carazo *et al.* 2016).

A fundamental prediction of UXh is that inbreeding should have a more negative effect on the lifespan of the homogametic than the heterogametic sex, because the sex chromosomes of the latter are always effectively inbred (i.e., hemizygous), and thus won't be affected by inbreeding. To date, the only available empirical studies to specifically test the UXh have used this approach in seed beetles (Fox *et al.* 2006; Bilde *et al.* 2009) and in *D. melanogaster* (Carazo *et al.* 2016). In seed beetles, results are inconsistent with the "unguarded-X" and seem better explained by sexually divergent selection (Fox *et al.* 2006; Bilde *et al.* 2009). In *D. melanogaster*, recent results have been more suggestive (Carazo *et al.* 2016), but they were based on a single study that failed to look at the effects of inbreeding on reproductive fitness, which is crucial to ascertain whether differential inbreeding effects across the sexes are maladaptive or reflect sex-specific adaptive shifts in life history. *D. melanogaster* is, for a variety of reasons, an ideal species to test predictions of the UXh. First, because it frequently exhibits a sexually dimorphic lifespan, with homogametic females usually living longer than heterogametic males (Rose *et al.* 2004). Second, because it only has two autosomal macrochromosomes, which means the relative weight of any increase in the expression of deleterious alleles in the X chromosome is likely to be important (i.e., the X chromosome includes ~20% of the total gene content; Mallet *et al.* 2011). Finally, because it has a special dosage compensation system where the expression of the X chromosome is doubled in males (Conrad and Akhtar 2012), hence predictably exacerbating the consequences of deleterious recessive mutations in the hemizygous male X chromosome.

In addition, the "Mother's curse" hypothesis also predicts sex-specific inbreeding effects on lifespan, because sustained inbreeding will tend to create an association between male fertility traits and mitochondrial matrilines, and such positive assortative mating may give rise to direct selection against mtDNA genotypes with deleterious male-specific effects (Wade and Brandvain 2009; Hedrick 2012). Thus, both the UXh and the "mother's curse" hypotheses predict sex-specific lifespan effects of inbreeding, but these predictions occur at different time-scales. The UXh predicts sex-specific differences in inbreeding load immediately following inbreeding, followed by long-term erosion of these differences if inbreeding is sustained in time, due to strong purging selection acting on the homozygous sex (Maklakov and Lummaa 2013). In contrast, the mother's curse hypothesis predicts the opposite pattern, with sex-specific inbreeding effects arising gradually as a response to selection if inbreeding is maintained through time (Wade and Brandvain 2009; Hedrick 2012).

In this study, we first used random pairs of flies from an outbred population to construct a set of isolines de novo by 10 generations of full-sib mating. We then set up a series of crosses between randomly paired isolines to set up three different inbreeding treatments: inbred, intermediately inbred and outbred (see Bilde *et al.* 2009). Finally, across inbreeding treatments we measured the reproductive fitness of males/females as well as their lifespan in different social environments: in isolation, in same-sex groups (4 individuals of the same sex) and in a mixed-sexes group social environment (2 males and 2 females). We explicitly controlled for any mitochondrial effects both by focusing on short-term effects in a novel inbreeding process, and by controlling the maternal haplotype across inbreeding treatments (Figure 7.1).

7.2. Materials & Methods

Experimental Population and Generation of Isolines

To obtain the parental flies to start up isolines with, we collected Dahomey eggs from our population cages and virgin adults emerging from those eggs using the protocol described in Chapter 3 (General Materials and Methods). We kept them in same-sex groups of 10 for 72 hours. Then, we randomly set up pairs of males and females in a vial and kept them together for 48 hours, at which time adults were discarded and eggs incubated under standard conditions (i.e., 25°C room with 60% humidity) until offspring emerged. Full-siblings from each resulting family were then mated with each other for 10 generations. The expected inbreeding coefficient after 10 generations of full-sib crosses is ~0.886, conservatively assuming that initial parental pairs were heterozygous at all their loci (Falconer 1981). For each generation of inbreeding, we set up three replicate full-sib pairings and randomly selected one out of the crosses generating viable offspring, in order to minimize line loss. The process to generate isolines took us 7 months (from March 2016 to August 2016). We started with 80 crosses and obtained a total of 50 inbred isolines.

Construction of Different Inbreeding Levels

After obtaining 50 isolines, we randomly paired them in 25 sets, within which we designed crosses to obtain three different inbreeding treatments: (a) inbred [F~0.89], (b) intermediately inbred [F~0.44] and (c) outbred [F~0] flies (Figure 1). For each set, one of the two isolines was randomly assigned a role as the maternal haplotype isoline (i.e., isoline A in Figure 1); i.e., the isoline whose cytoplasmic DNA was used in the three inbreeding treatments. Briefly, the crosses were set up as follows: a) for the inbred treatment, we collected offspring emerging from the 11th generation of inbreeding in isoline A, b) for the outbred treatment, a female from isoline A was crossed with a male from isoline B and the resulting offspring was used, and c) for the intermediately inbred treatment, a

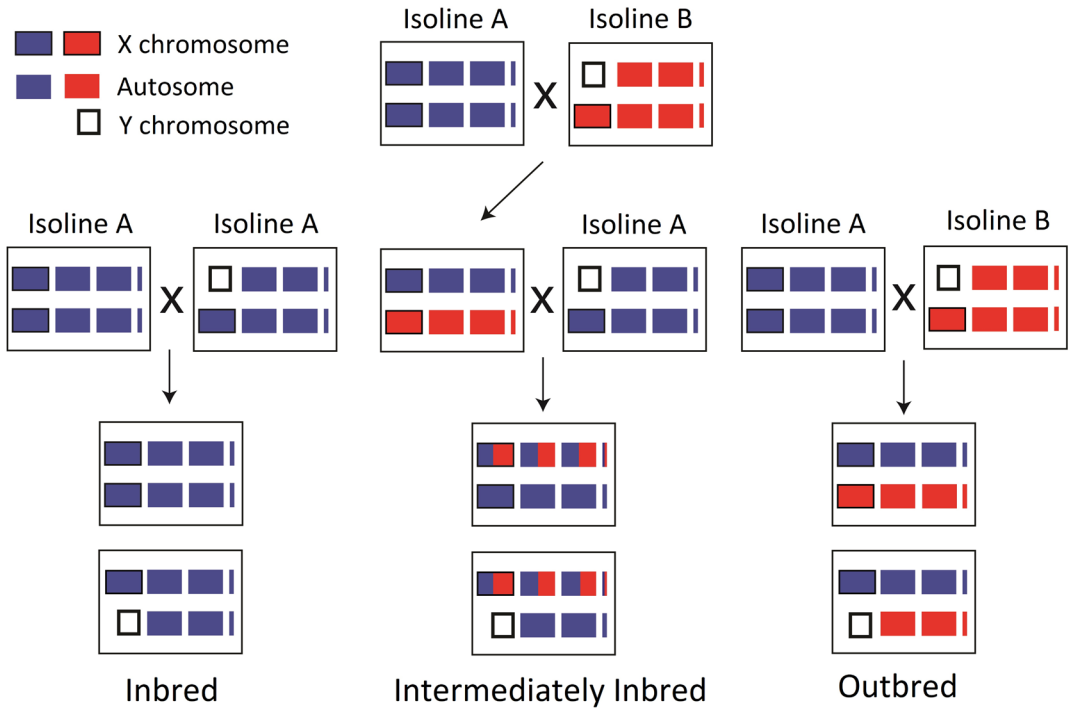

Figure 7.1. Schematic design of the crosses used to construct the three inbreeding treatments. Isoline A and Isoline B were obtained after 10 generations of inbreeding. The inbred treatment was constructed by crossing a female and male from isoline A. The outbred treatment was constructed by crossing a female from isoline A with a male from isoline B. The intermediately inbred treatment was constructed by crossing a female offspring resulting from the outbred cross with a male from isoline A.

female offspring resulting from the outbred cross above was back-crossed with a male from isoline A. For each set (n = 25) and treatment, we set up 3 pairs to hedge our bets against potential loss of the cross due to the infertility of single males/females. In all crosses, pairs were kept together for 48 hours, after which time adults were discarded and vials with eggs incubated for 16 days to allow offspring to emerge. Virgin adult males and females collected within 7 hours of eclosion from the crosses above were used for fitness and lifespan assays.

Fitness Assays

To estimate female reproductive success, we used two replicates per set and paired each focal female with an outbred wt male from our background population and left them to interact for 8 days. For wt males, eggs were collected from our population cages as described above. Pairs were transferred to a fresh vial after 4 days using light CO_2 anaesthesia, and discarded after 8 days, and for each pair both these vials were incubated at standard conditions for 16 days to allow emergence of adults, at which time vials were frozen and eventually offspring counted. Measures of early fecundity (i.e., first 5-10 days) can be taken as reasonably good indicators of a female's lifetime reproductive success (e.g. Nguyen and Moehring 2015). To estimate male reproductive success, we used two replicates per set and put a focal male with two *sparkling poliert* males and females. We left males in these groups to interact for 8 days, transferring them to a new vial on the fourth day using light CO_2 anaesthesia. At the end of this period, we discarded adults and left eggs to develop into adults for 16 days, and then froze them. We then counted the proportion of wt/spa offspring as a measure of the reproductive success of the focal male (i.e., all of the focal male's offspring should exhibit the wild-type eye phenotype). We took the average of the two replicates (per sex, per treatment), except for the intermediately inbred female treatment, where due to a contingency with an incubator we lost one of the two replicates. For that treatment, we used data from the only available replicate. Final sample size for each sex was 25.

Social Environment and Lifespan Assays

We measured lifespan of flies kept in three different social environments: in isolation, in same-sex groups, and in mixed sexes groups. For the isolation treatment, we kept isolated flies in a vial with excess food throughout their entire lifespan, flipping them to fresh vials once per week without anaesthesia. We conducted two replicates per sex and set for a total of 100 vials per inbreeding treatment. For the same-sex treatment, we kept 4 flies per sex in a vial throughout their entire lifespan and flipped them to a fresh vial once a week using light CO_2 anaesthesia to minimize censors due to escaped flies while flipping the vials. Lifespan in these vials was calculated as the mean lifespan of the four individuals. Finally, for the mixed-sexes treatment we put 2 females and 2 males from the same isoline inside a vial and transferred them to a fresh vial twice a week using light CO_2 anesthesia. Lifespan was calculated separately for males and females as the mean lifespan of the two flies of the same sex. Final sample sizes differed for each sex, inbreeding treatment and social environment due to missing data and they were as follows: inbred males (n = 24 isolation, n = 23 in same sex group and n = 25 in mixed sexes groups), intermediately inbred males (n = 23 isolation, n = 24 same sex and n = 24 in mixed sex), outbred males (n = 25 isolation, n = 24 same sex and n = 24 mixed sex), inbred females (n = 25 isolation, n = 24 same sex and n=25 mixed sex) intermediately inbred females (n = 25 isolation, n = 23 same sex group and n=24 in mixed sex), outbred females (n = 25 isolation, n = 22 same sex and n = 24 mixed sex).

Statistical Analysis

In order to determine the effect of inbreeding on reproductive success in a way that is comparable across the sexes, we first standardized data (i.e., calculated z-scores) on female early fecundity and male early reproductive success. To explore the effect of inbreeding level on reproductive success, we used a restricted maximum likelihood LMMs approach with sex, inbreeding level and their

interactions as fixed factors and set as a random intercept effect. In order to determine the effect of inbreeding on lifespan, we used raw data. To explore the effects of inbreeding level and social environment on lifespan, we used a restricted maximum likelihood LMMs approach with sex, social environment, inbreeding level and their interactions (including sex × inbreeding level × social environment treatment three-way interaction) as fixed factors and set as a random intercept effect. Significant sex interactions were explored by fitting LMMs separately for both sexes (i.e., by doing sex-specific models).

Additionally, we pooled data from the isolation and same-sex treatment in this experiment with the isolation and same-sex treatment from Carazo *et al.* (2016), which used flies from the same Dahomey background population and the same experimental procedure as described here. This resulted in a combined dataset of between 35-40 independent sets of isolines (depending on specific contrast, due to some missing values), allowing us to run a series of inter and intrasexual correlations to explore the genetic architecture underlying lifespan and lifespan × inbreeding effects. Firstly, we explored intrasexual correlations between inbred and outbred isolines in order to explore the effect of dominant alleles on the lifespan of each sex. We expected to observe a correlation due to autosomal dominant alleles because those are the ones expressed in both inbred and outbred flies. In addition, for males, we also expected an additional effect due to the recessive alleles on X chromosomes, which in this sex are always expressed irrespective of inbreeding. We used a restricted maximum likelihood LMMs approach with inbred lifespan as response variable, outbred lifespan, social environment and their interaction as fixed factors and experiment and set as random factors; separately for each sex. Secondly, we looked for intersexual correlations between males and females (of the same set) in order to explore the shared genetic architecture between the two sexes. We used a restricted maximum likelihood LMMs approach with female lifespan as response variable, male lifespan, social environment and their interaction as fixed factors and experiment

and set as random factors. We ran this analysis separately for inbred and outbred treatments, and expected a higher intersexual correlation for inbred isolines due to the additional expression of the recessive mutations in inbred isolines. Finally, to further explore for the contribution of shared recessive alleles between the sexes, we looked for an association between female and male Δlifespan (i.e., lifespan of outbred – inbred treatment). As the X chromosome does not contribute to ID in males, an intersexual correlation in Δlifespan would be caused by autosomal recessive alleles affecting lifespan in both sexes. We again used a restricted maximum likelihood LMMs approach with female Δlifespan as response variable, male Δlifespan, social environment and their interaction as fixed factors and experiment and set as random factors. All r^2 values provided for the relationships above include effects of fixed effects only (i.e., marginal r^2).

7.3. Results

Effect of Inbreeding on Fitness

We did not find a significant inbreeding × sex interaction for fitness ($\chi^2 = 0.9829$, df = 2, p = 0.612), but we did find a clear effect of inbreeding on fitness for both sexes ($\chi^2 = 16.499$, df = 2, $p < 0.001$; Figure 7.2), where outbred flies had significantly higher fitness than the inbred flies (estimate = 0.7680 ± 0.1852, z = 4.148, $p < 0.001$).

Effect of Inbreeding on Lifespan

For lifespan, we did not find a significant inbreeding × sex × social environment interaction ($\chi^2 = 1.778$, df = 4, p = 0.776). When analysing double-way interactions, we did not find a significant inbreeding × sex interaction ($\chi^2 = 4.5122$, df =2, p = 0.105), but we did find a significant inbreeding × social environment interaction ($\chi^2 = 9.7329$, df = 4, p = 0.045) and a clear social

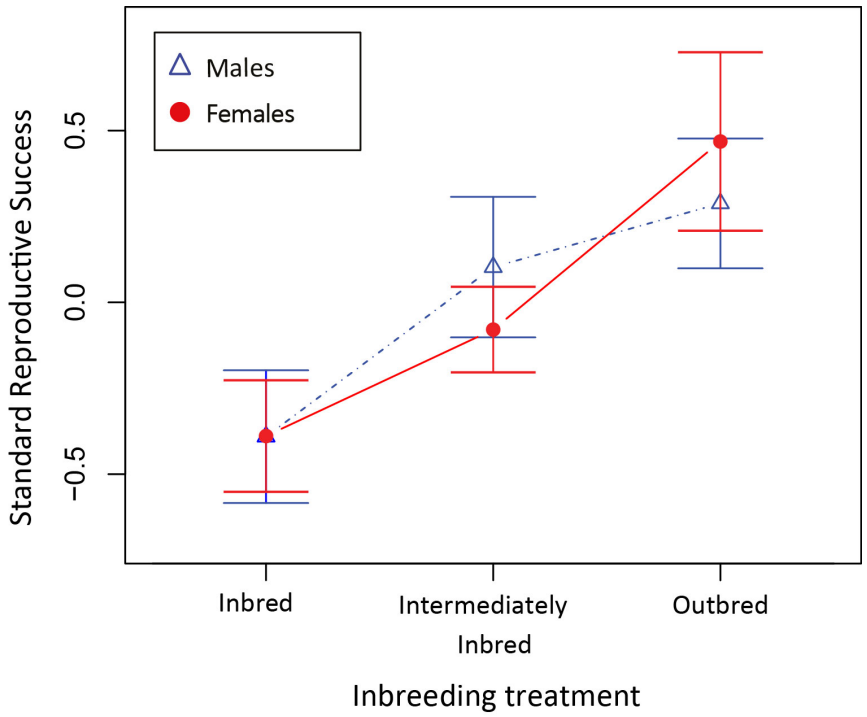

Figure 7.2. Effect of inbreeding on female and male reproductive success. Standardized female and male fitness (mean ± S.E.) across different inbreeding levels (inbred, intermediately inbred and outbred).

environment × sex ($\chi^2 = 58.217$, df = 4, $p < 0.001$) interaction. Given that these three variables are interrelated; to interpret these results we re-ran analyses for each sex separately. For males, we did not find effects of either inbreeding ($\chi^2 = 3.8377$, df = 2, $p = 0.147$), social environment ($\chi^2 = 3.6337$, df = 2, $p = 0.162$), or the social environment × inbreeding interaction ($\chi^2 = 6.2347$, df=4, $p = 0.182$) on lifespan. In contrast, in females we detected clear effects of both inbreeding ($\chi^2 = 13.136$, df = 2, $p = 0.001$) and social environment ($\chi^2 = 88.592$, df = 2, $p < 0.001$) on lifespan (Figure 7.3), whereas we did not detect an interaction between inbreeding and social environment ($\chi^2 = 5.5827$, df=4, $p = 0.233$).

We used a *post hoc* Tukey test to explore the effect of inbreeding and social environment in more detail in females. We found that outbred females lived

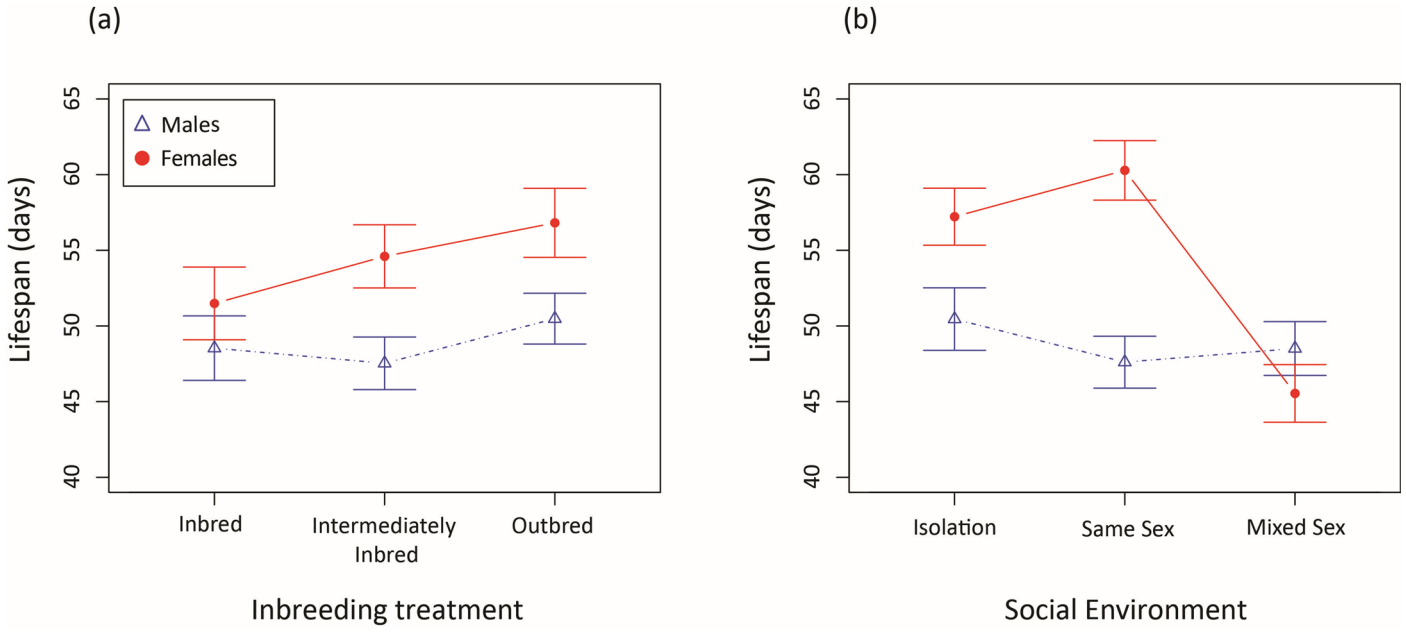

Figure 7.3. **Effect of inbreeding and social environment on female and male lifespan.** Female and male lifespan (mean ± S.E.) across different (a) inbreeding levels and (b) social environments.

significantly longer than inbred females (estimate = 5.370 ± 1.476, z = 3.639, p <0.001). However, we did not detect a significant difference between intermediately inbred females and either inbred (estimate = 2.946 ± 1.470, z = 2.004, p = 0.111) or outbred (estimate = -2.424 ± 1.487, z =-1.630, p = 0.233) females, although trends were consistent with a gradual continuous effect of inbreeding on lifespan. We also found that, as repeatedly shown in the literature (Rose *et al.* 2004), females have significantly shorter lives when they are in mixed groups when compared to both in isolation (estimate = -11.630 ± 1.459, z = -7.969, p < 0.001) and same-sex groups (estimate = -14.683 ± 1.492, z = -9.840, p < 0.001). We also found a non-significant trend for females in same sex groups to live longer than females in isolation (estimate = 3.053 ± 1.482, z = 2.059, p = 0.098).

Additional post hoc Analyses on the Sex-specific Effects of Inbreeding across Social Environments

Post hoc graphical exploration of raw data depicted on Figure 7.3, and of sex-specific lifespan data across inbreeding treatments and social environments (Figure 7.6), casted doubts about the interpretation of the main sex-specific lifespan effects detected in our general model above. The "unguarded-X" predicts inbreeding to decrease both male and female lifespan, but these effects to be stronger in females. At face value, our initial analysis seemed to confirm this prediction, but a breakdown of raw data suggested the possibility that an effect of inbreeding on male lifespan could be concealed by a male-specific increase of lifespan in mixed sexes environment (Figure 7.6). Hence, in order to explore the central prediction that inbreeding does result in a stronger reduction of female than male lifespan (while controlling for the unexpected results in the mixed sexes social environment), we re-ran our analysis separately for each sex while including or not the mixed sexes treatment. We hence fitted four separate LMMs (one for each sex with/without the mixed sexes treatment) with lifespan as a response variable, inbreeding level as a fixed factor, and isoline set and social

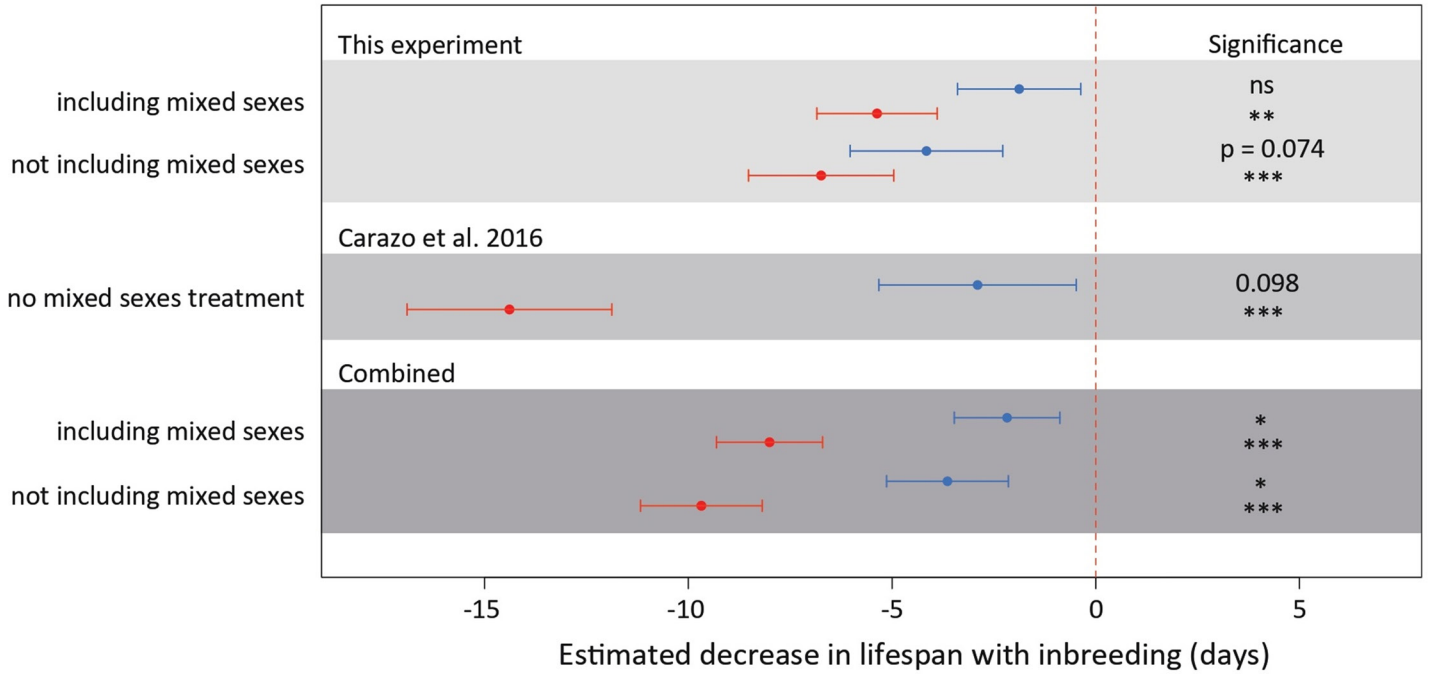

Figure 7.4. Decline in lifespan with inbreeding for females and males. Estimated decrease in lifespan (i.e., coefficients ± S.E. from adjusted LMs) with inbreeding (outbred vs inbred treatment) separately for the sexes (females in red, males in blue), including or not the mixed-sexes treatment: (A) in this experiment, (B) in Carazo *et al.* (2016) and (C) in data pooled from both experiments. In each case, we provide the significance level (or exact P-value when marginal) of the corresponding model.

environment as random factors, which allowed us to calculate a separate effect size of inbreeding on lifespan in each case (Figure 7.4).

Including the mixed sexes treatment, we failed to detect a significant effect of inbreeding on male lifespan ($\chi^2 = 3.870$, df = 2, p = 0.146), but we did detect a clear effect of inbreeding on female lifespan ($\chi^2 = 12.991$, df = 2, p = 0.002), as expected. Re-fitting this model without the mixed sexes treatment gave qualitatively similar results, with a marginally non-significant effect of inbreeding on male lifespan ($\chi^2 = 5.210$, df = 2, p = 0.074), and a clear effect on female lifespan ($\chi^2 = 14.178$, df = 2, $p < 0.001$). Because this analysis has somewhat limited power due to the modest sample size of this study (i.e., ~25 sets of isolines) we took advantage of the data reported in Carazo *et al.* 2016 (same background population of *D. melanogaster* and exact same methodology) to run more powerful models on the combined dataset across the two experiments (i.e., ~40 sets of isolines). To estimate the overall effect size of inbreeding on male and female lifespan we hence fitted, separately for each sex, a LMMs with lifespan as a response variable, inbreeding as a fixed factor, and isoline set, social environment and experiment as random factors. We repeated this analysis with and without the contribution of the mixed sexes treatment, again to guard against the potentially misleading effect of this treatment. In this case, we found clear effects of inbreeding on both sexes and in both analyses. Including the mixed sexes treatment, inbreeding significantly decreased both male ($\chi^2 = 7.611$, df = 2, p = 0.022) and female lifespan ($\chi^2 = 35.662$, df = 2, $p < 0.001$), but this effect was considerably more marked in females (estimated lifespan for inbred-outbred flies ± standard error in days; -8.007 ± 1.302) than in males (-2.177 ± 1.296 days). Not including the mixed sexes treatment actually resulted in very similar results, with inbreeding again significantly decreasing both male ($\chi^2 = 8.326$, df = 2, p = 0.016) and female lifespan ($\chi^2 = 38.660$, df = 2, $p < 0.001$), but with this effect being considerably more important in females (estimated lifespan for inbred-outbred flies ± std. error, in days; -9.680 ± 1.493) than in males (-3.643 ± 1.494 days).

Intrasexual and Intersexual Correlations for Lifespan and inbreeding depression

We found evidence of a significant (albeit weak) intrasexual relationship between the lifespan of inbred and outbred males from the same maternal isoline (χ^2=6.9568, df = 1, p = 0.008, r_m^2 = 0.122), but we did not find evidence of a similar effect in females (χ^2 =1.2469, df=1, p = 0.264; Figure 7.5a). These correlations were not modulated by an interaction with social environment in either males (χ^2= 0.0963, df = 1, p = 0.756) or females (χ^2 = 0.2145, df = 1, p =0.643). We found a significant intersexual correlation in the lifespan of males and females from the same maternal isoline for both inbred (χ^2= 15.365, df = 1, p <0.0001, r_m^2 = 0.188) and outbred (χ^2= 7.1068, df = 1, p= 0.008, r_m^2=0.130; Figure 7.5b) treatment. Again, this relationship was not modulated by an interaction with social environment in either inbred (χ^2 = 0.169, df = 1, p = 0.681) or outbred (χ^2 = 0.8013, df = 1, p = 0.371). Finally, we found a significant correlation between males and females of the same maternal isoline in the degree to which inbreeding caused a decline in their lifespan (i.e., Δlifespan, or ID for lifespan) (χ^2 = 14.952, df = 1, p < 0.001, r_m^2= 0.192; Figure 7.5c). This relationship was not modulated by an interaction with social environment (χ^2 = 1.2723, df = 1, p = 0.259).

7.4. Discussion

In this study, we found evidence supporting the idea that inbreeding decreases female lifespan to a greater degree than male lifespan across different social environments. These results were further confirmed by a more powerful analysis that included data from this study and from a recent study (Carazo *et al.* 2016) using the same population of *D. melanogaster* and experimental design, showing that inbreeding reduces both male and female lifespan across different social environments, but that effects on females are considerably larger (i.e.,

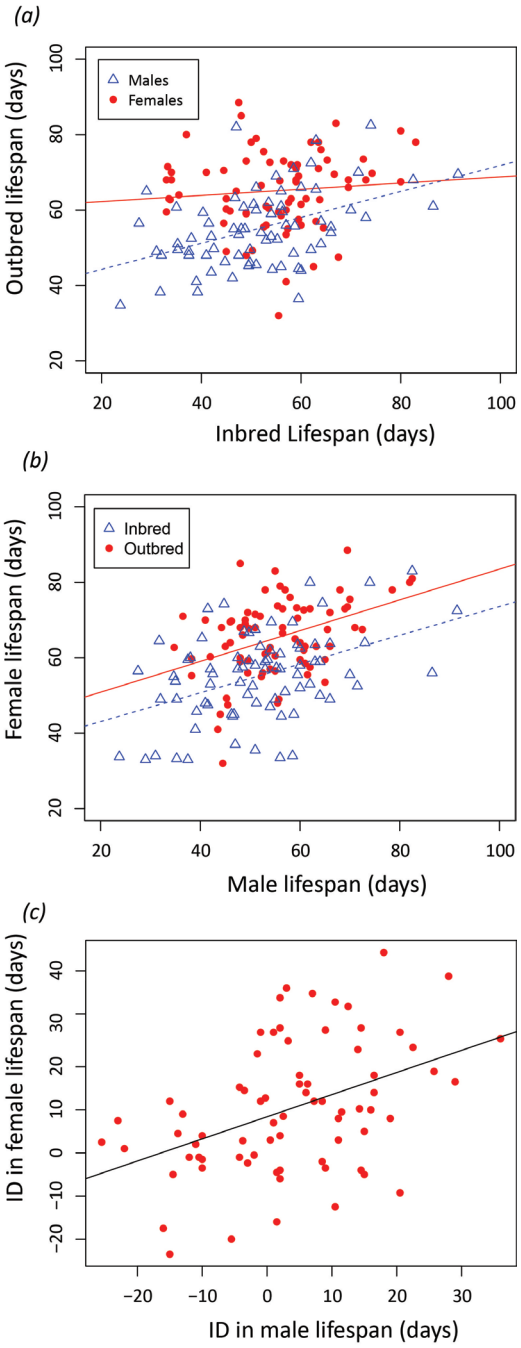

Figure 7.5. Intra/inter-sexual correlations for lifespan and inbreeding depression in lifespan. (a) Intrasexual correlation for lifespan. (b) Intersexual correlation for lifespan. (c) Intersexual correlation for inbreeding depression in lifespan.

approximately double). We further used this combined dataset to explore the underlying genetic architecture of this phenomenon using intra- and intersexual correlations on inbreeding effects on lifespan. We found evidence to suggest that, as expected, there is a common genetic architecture for the inbreeding effects on lifespan across the sexes. Finally, in our study sex-specific effects of inbreeding on female lifespan did not seem to be counterbalanced by sex-specific effects of inbreeding in the opposite direction for reproductive fitness, suggesting the former are potentially maladaptive, as predicted by the UXh.

Inbreeding, Lifespan and the "Unguarded-X" Hypothesis

We found that inbreeding resulted in a sex-specific reduction of female lifespan across social environments (Figure 7.3a), to the point of removing the female-biased sexual dimorphism that is frequently reported in this species (but see also; Chippindale *et al.* 1993). At face value, these results seem to strongly support the hypothesis that inbreeding depression for lifespan is greater in females than in males. We graphically explored raw sex-specific lifespan data across inbreeding treatments and social environments to confirm that the sex-specific inbreeding effect was consistent across the social environments (Figure 7.6), which was the case for females. In males, however, inbreeding reduced male lifespan in the same sex and isolation treatments but there was a trend for inbred males to live longer than intermediately inbred/outbred males in the mixed sex treatment (Figure 7.6c). This trend fits well with recent studies reporting relatedness effects on reproductive cooperation in male of *D. melanogaster* where males courted females less intensively and lived longer when they were reared with their brothers (Carazo *et al.* 2014; Le Page *et al.* 2017), but it may have partially driven the sex-specific effect reported above. In order to provide a more robust test of the idea that inbreeding may result in a greater reduction in female than in male lifespan per se, and not due to indirect male-specific effects in the mixed sexes

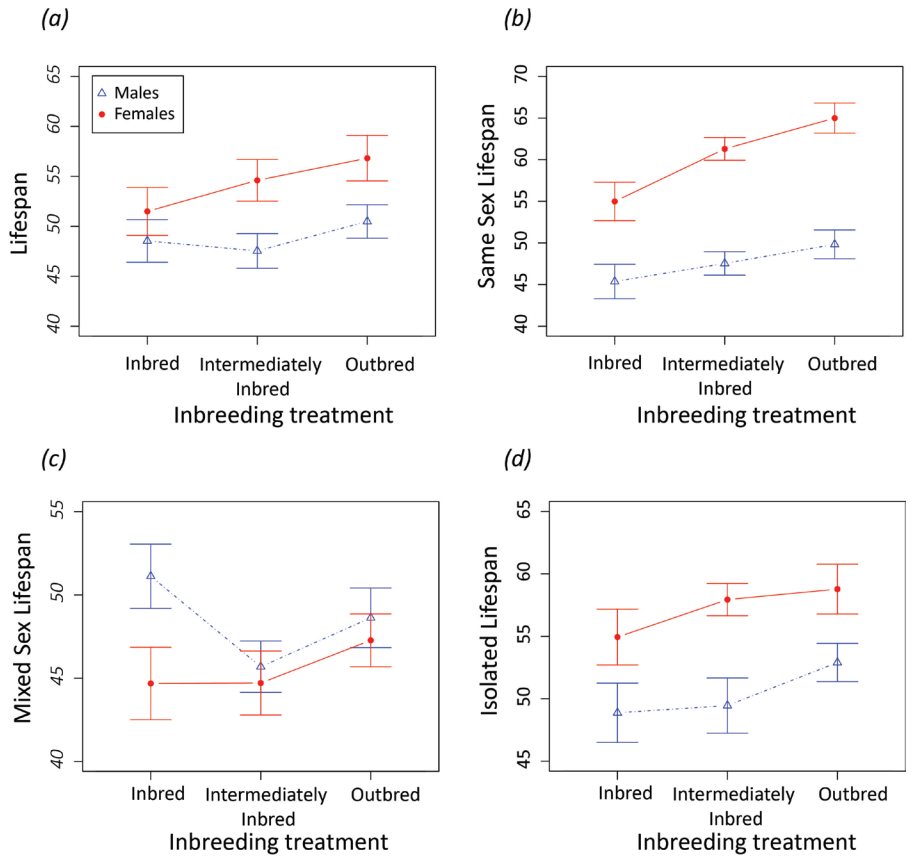

Figure 7.6. Effect of inbreeding on female/male lifespan across different social environments. Female and male lifespan (mean ± S.E.) across different inbreeding levels for (a) all social environments, (b) same sex groups, (c) mixed sexes groups and (d) isolation.

treatment, we ran a series of post hoc analyses. First, we re-analysed data from our own experiment by fitting models separately for each sex with/without taking into account the mixed sexes treatment. We detected marginal or no effects of inbreeding on male lifespan and clear effects on female lifespan irrespective of the mixed sexes treatment, showing our initial results were actually robust. Second, and in order to provide a yet more powerful test, we pooled our data with that from a previous experiment conducted following the same experimental procedure on an independently derived set of isolines from the same background population of flies (Carazo *et al.* 2016). This allowed us to estimate the overall

effects of inbreeding for each sex using a much larger overall sample size of ~40 independently derived sets of isolines (i.e., ~80 different isolines in total), while including or excluding the mixed sexes treatment. In these analyses, we detected a clear effect of inbreeding on both males and females, irrespective of the mixed sexes treatment. Importantly, however, effect sizes for females were significantly greater than for males (Figure 7.4), strongly suggesting that inbreeding does result in a stronger decrease of female than male lifespan in our population of *D. melanogaster*.

Overall, the findings above are therefore consistent with one of the main predictions from the UXh: that inbreeding will have a greater effect on the lifespan of the homogametic sex. A complementary explanation for our findings could have to do with adaptive sex-specific life history changes in response to inbreeding. In as much as inbreeding depression can affect the trade-off between current and future reproduction (Stearns 1992), it could in principle cause a sex-specific adaptive response on male and female lifespan (e.g. decline in female lifespan compensated by increase in early female fecundity). In *D. melanogaster*, it would seem unlikely that inbreeding could improve female reproductive output in a way that counterbalances the fitness loss (due to lifespan reduction), because previous studies have reported comprehensive negative effects across different fitness components (i.e., viability and fecundity; (Mallet and Chippindale 2011; Mallet *et al.* 2011). However, this does not completely discard the potential role of adaptive sex-specific responses. Provided the existence of a sex-specific relationship between reproduction and lifespan, inbred females and males could behave in a way that optimizes their fitness differently under genotoxic stress and this could mean differential effects of inbreeding on lifespan. Our results are perhaps more parsimoniously explained by the UXh, but they don't necessarily exclude a role for sexually divergent selection.

Finally, there are at least two other hypotheses that could also contribute to explain the results obtained in this study: sexually antagonistic genes and sex-specific expression patterns. Previously unexpressed sexually antagonistic recessive alleles with negative effects on females and positive effects on males will be differentially expressed after inbreeding, due to un-guarding of the X in females causing a decline in female lifespan without any effect on male lifespan. Such effects could be particularly important in the X chromosome, because classical theory predicts it will be a hotspot for the accumulation of sexually antagonistic alleles (Rice 1984). In accordance with this theory, an elegant study by Gibson *et al.* (2002) showed evidence strongly suggesting that, in at least one lab population of *D. melanogaster*, the X chromosome is enriched with sexually antagonistic alleles (Gibson *et al.* 2002). Furthermore, in the X chromosome sexually antagonistic recessive mutations with positive effects on males and negative effects on females (under positive selection in males and mostly unselected in females) are expected to be relatively more abundant than recessive mutations with negative effects on males and positive effects on females (under negative selection in males and mostly unselected in females). In contrast, other studies have suggested that sexually antagonistic polymorphisms may actually be less frequent in the sexual chromosomes than in the autosomes (Fry 2010; Barson *et al.* 2015), and a recent study reporting the first genome-wide identification and characterization of sexually antagonistic SNPs in *D. melanogaster* found that antagonistic SNPs were underrepresented in the X chromosome (Hill *et al.* 2018; but see also Innocenti and Morrow 2010). In any case, the fact remains that any recessive sexually antagonistic alleles in the X chromosome could produce sex-specific lifespan effects in the same direction as those detected in this study, as could also similar processes leading to X chromosomes enriched for deleterious recessive alleles with female-specific expression.

Inbreeding Effects on Male and Female Reproductive Success

Unlike lifespan, we did not observe a clear sex-specific effect of inbreeding on reproductive success, but an apparently similar decrease of fitness with inbreeding across the sexes. This finding begs the question of why wouldn't "unguarded-X" phenomena affect female reproductive success in a sex-specific way. There are two main reasons why we may not expect fitness to behave as lifespan in response to inbreeding. First, the UXh predicts an increase in the expression of deleterious recessive mutations in the heterogametic sex. To the extent that such mutations have a similar effect in both sexes (i.e., a similar effect in inbred individuals of the homozygous sex than in hemizygous individuals of the heterozygous sex), they could explain differences in ageing between the sexes. However, while we expect mutations affecting lifespan to produce effects in the same direction in both sexes, mutations affecting reproductive success will frequently produce different effects in males and females. This is mainly because the components and measures of female and male reproductive success differ markedly, with fecundity being cornerstone for females and intra- and intersexual competition being relatively important for males. Because distinct genes and adaptations are likely to be involved in each case, the declines in male and female fitness with inbreeding may not be directly comparable in magnitude. Second, there is considerable empirical evidence that the intensity of sexual selection in *D. melanogaster* is, as is frequently the case (Wade 1979), stronger in males than in females (Bateman 1948). This, in turn, is predicted to lead to higher inbreeding depression in male fitness, and thus any UXh differential effects of inbreeding on female fitness could be partially or completely compensated by the stronger effect of inbreeding depression on male fitness. As a matter of fact, previous studies using both inbreeding (Mallet and Chippindale 2011) and mutation accumulation experiments (Mallet *et al.* 2011; Sharp and Agrawal 2013) have consistently found that this is the case in other populations of *D. melanogaster*. Mallet and Chippindale (2011) measured inbreeding load on *D. melanogaster* viability and

adult fitness (but not lifespan) in males and females. They found that, for juvenile viability, inbreeding has similar effects on males and females, but male adult fitness experienced a sharper decrease compared to females in response to inbreeding. Moreover, Mallet *et al.* (2011) further conducted a mutation-accumulation experiment on the X chromosome and observed a greater decline in male than in female fitness, even after females were made homozygous for the X chromosome. This pattern of male-biased fitness inbreeding depression has also been shown to hold when mutation-accumulation is restricted to the second chromosome (Sharp and Agrawal 2013). In our population, we did not detect any sex-specific inbreeding effects on estimates of male and female fitness, but males suffered inbreeding in only two of their three major chromosomes (i.e., their X chromosome is hemizygous) while females suffered inbreeding across their three major chromosomes. Taking this into account, we would expect a greater effect on female than on male fitness, and our results are hence not inconsistent with previous studies showing greater inbreeding depression for reproductive fitness in males.

Social Environment Effects on Male and Female Lifespan

Our results further show that the social environment has sex-specific effects on aging. This is far from surprising, as there is ample evidence, in *Drosophila* and in other organisms, that the social environment and reproduction in particular can drastically modulate aging (Bonduriansky *et al.* 2008). However, previous studies looking at the unguarded-X hypothesis have focused on studying the lifespan of males/females kept in isolation and/or same-sex groups (Fox *et al.* 2006; Bilde *et al.* 2009; Carazo *et al.* 2016). In isolation, the influence of all social behaviours on lifespan is eliminated. In same-sex groups, individuals are able to engage in social interactions, but are not able to reproduce and females are not subject to sexual conflict/female harm, which in the case of flies is very significant (Wigby and Chapman 2005; Perry and Rowe 2015). Not surprisingly, we found that the mixed sexes group had a dramatic effect on female lifespan, which is expected

given that reproduction (Fowler and Partridge 1989; Wigby and Chapman 2005) and exposure to males (Partridge and Fowler 1990) are both well-known to decrease female lifespan in fruit flies. More surprising was our finding that, in the mixed sexes group, inbred males exhibited a trend to live longer than the other inbreeding treatments and inbreeding did not affect females as clearly as it did in other social environments. This may be due to the role of within-group relatedness in reducing male-male competition for females and/or female harm levels (Carazo *et al.* 2014, 2015; Hollis *et al.* 2015; Martin and Long 2015; Le Page *et al.* 2017; but see Chippindale *et al.* 2015), which at least in some cases leads to longer male lifespan (Carazo *et al.* 2014). Similar effects on females, via reduced female harm, may explain why inbreeding affected female lifespan relatively less in the mixed than in the other social environments. In any case, interpretation of our results in the mixed sexes group is complex and inherently speculative, especially concerning that a recent study has shown that female harm and male-male competition is exacerbated in standard fly vials with respect to more natural environments (Yun *et al.* 2017). Taking all of the above into account, we suggest results from the same sex and isolation treatments may actually provide a much clearer test of the UXh prediction than mixed sexes groups.

On the genetic architecture of lifespan

In order to explore the genetic architecture of lifespan effects, we pooled data from this and a prior study (Carazo *et al.* 2016) and looked at intrasexual and intersexual associations between the lifespan of males/females of the same maternal isoline. We found an intrasexual association between the lifespan of inbred and outbred males, but not females. We suggest this difference is probably due to the fact that, in males, our analysis would pick up the joint contribution of dominant autosomal effects and recessive effects on the X chromosome (see Figure 7.1), whereas the latter effects won't be present in females. Considering that males double the expression of X chromosomes during dosage

compensation, we may expect such recessive effects to have an important bearing on male lifespan. We also found evidence of a shared genetic architecture for lifespan across the sexes, as shown by intersexual associations between inbred and outbred males and females from the same maternal isoline. We found a more pronounced relationship between female and male lifespan in inbred compared to outbred treatments, which was expected due to the higher proportion of shared genetic material and expression of recessive alleles in inbred flies. Finally, we detected a shared male/female genetic recessive background of autosomal recessive alleles affecting lifespan. We found a clear association between *Δ*lifespan (i.e., ID for lifespan) between males and females. This association would be caused by autosomal alleles because the X chromosome of males does not cause inbreeding depression (as it is always effectively inbred) and the Y chromosome is only present in males. For *D. melanogaster*, intersexual and intrasexual correlations using inbred and outbred isolines have, to the best of our knowledge, previously been reported for fitness and viability (Mallet and Chippindale 2011), but not for lifespan. Intersexual correlational analysis for lifespan and inbreeding depression on lifespan has previously been reported in seed beetles (Fox *et al.* 2006) where, surprisingly, male and female lifespan were positively correlated in outbred but not inbred individuals. Also in contrast to our experiment, this study did not find a correlation between the inbreeding depression of lifespan on males and females. Such differences may be pointing out important differences in the genetic architecture of lifespan between these two model organisms which would be interesting to explore in the future.

Final remarks

The question of why the sexes age differently is an enduring challenge with broad evolutionary implications. There is increasing evidence that sex-specific adaptive evolution is important to understand the evolution of sex differences in ageing and lifespan (Promislow 2003; Arnqvist and Rowe 2005; Bonduriansky *et al.* 2008; Maklakov and Lummaa 2013), but the mechanisms underpinning sex-

specific lifespan remain unclear. Recent evidences in *D. melanogaster* suggest that maladaptive processes such as the asymmetric inheritance of mitochondrial DNA, expression of deleterious recessive mutations on the X chromosome (UXh) and negative effects of transposable elements on the Y chromosome ("toxic Y" hypthesis) are all necessary for a comprehensive understanding of sexual dimorphism in ageing (Camus *et al.* 2012; Carazo *et al.* 2016; Brown *et al.* 2020b). Using the same species, we contribute to this budding corpus of research by testing a fundamental prediction of the UXh: that inbreeding will have maladaptive sex-specific effects on female lifespan. Our results are indeed consistent with this prediction, suggesting the UXh may be important to understand sex-specific ageing in *D. melanogaster*. However, we identify other processes, such as sexually antagonistic recessive alleles or sex specific gene expression patterns, that could at least partly explain the results obtained here. A conclusive test of the UXh has remained elusive since its original formulation a few decades ago (Trivers 1985). Despite this fact, the "unguarded-X" is not only theoretically solid, but as we show here has the potential to contribute greatly to our understanding of sex-specific ageing across a wide diversity of taxa.

Chapter 8. Genetic sex determination and sex-specific lifespan in tetrapods - evidence of a toxic Y effect

8.1. Introduction

There are several ways in which sex chromosomes can give rise to different female and male lifespans (Pipoly *et al.* 2015; Marais *et al.* 2018; Xirocostas *et al.* 2020). As discussed in the previous chapter of this Thesis, the unguarded-X hypothesis (UXh) predicts increased mortality of the heterogametic sex due to the expression of deleterious recessive mutations that accumulate in the non-recombining parts of the X (or Z) chromosome (Trivers 1985). This idea has received indirect support so far. In Chapter 7 we showed that "unguarding" the X chromosome in females erases the sex gap in lifespan in *D. melanogaster* (Carazo *et al.* 2016; Sultanova *et al.* 2018) but see (Brengdahl *et al.* 2018). Using a comparative approach, Pipoly *et al.* (2015) showed that adult sex-ratios are typically female-biased in tetrapods with XY systems, but male-biased in taxa with ZW systems, as expected if biased adult sex ratios result from sex-specific mortality. A recent study further shows that the heterogametic sex tends to exhibit lower mean/maximum lifespan across a wide taxonomic range, but phylogenetic signal and sexual selection could contribute to explain this relationship (Xirocostas *et al.* 2020).

A recently postulated complementary hypothesis focuses on the role of the heteromorphic Y (or W) chromosome. Following recombination suppression, the non-recombining regions of Y (or W) chromosomes tend to accumulate deleterious mutations through evolutionary time, via processes such as Muller's ratchet, genetic hitchhiking or "Ruby in the rubbish" (Bachtrog 2013; Wright *et al.* 2016). Recombination suppression also leads to an accumulation of repetitive DNA in the Y and W chromosomes (Bachtrog 2013; Wright *et al.* 2016).

Interestingly, recent evidence has shown that repetitive DNA on the Y chromosome can contribute to sex-specific ageing in *D. melanogaster* (Brown *et al.* 2020b,a). Brown *et al.* (2020b) found that repetitive DNA sequences (e.g. transposable elements), that are normally repressed with heterochromatin structures, were de-repressed with age in both sexes, but more rapidly in males. This led to the mis-expression of repetitive sequences across the whole genome including the repeats-rich Y chromosome. In order to test if the mis-expression of the repetitive sequences in the Y chromosome causes faster ageing, Brown *et al.* (2020b) generated flies with different sex chromosome karyotypes: XXY females; X0 and XYY males in addition to wild-type karyotypes: XX females and XY males. They found a positive correlation between the de-repression of repeats and the number of Y chromosomes, and a negative correlation between average lifespan and the number of Y chromosomes (Brown *et al.* 2020b). Moreover, in another study conducted by the same group, Brown *et al.* (2020a) found that Y-chromosome also affects heterochromatin integrity genome-wide by decreasing the heterochromatin protection on other normally silenced repeat-rich sequences (Brown *et al.* 2020a). In short, in *D. melanogaster* there is solid evidence of substantial "toxic Y" effects via the expression of repetitive DNA elements, resulting in increased mortality of the heterogametic sex. This finding in fruit flies could be critical to understand sex-specific ageing across taxa because both Y and W chromosomes generally accumulate high amounts of repetitive sequences during their evolution (Bachtrog 2013; Wright *et al.* 2016). To our knowledge, the role of the "toxic Y" hypothesis in explaining broad patterns of sex differences in ageing has yet to be addressed.

The UXh and the toxic Y hypothesis are not mutually exclusive as both predict that sex differences in lifespan result from increased mortality of the heterogametic sex. However, for the former this is due to deleterious mutations in the X (or Z) chromosome, while for the latter this is due to the toxic effects of the Y (or W). This difference makes it possible to examine specific predictions

regarding the relationship between female/male lifespan and the relative size of the sex chromosomes (Figure 8.1). Namely, the UXh predicts that the sex gap in lifespan (i.e., female - male lifespan) will be positively associated with: a) the degree to which the X chromosome is larger than the Y chromosome (and vice versa with Z/W), because "unguarded" recessive mutations have to accumulate in the non-recombining regions of the X (or Z) chromosome, and the overall size of these regions increases with the size difference between the sex chromosomes (Figure 8.1A), and b) the relative size of the X chromosome with respect to the rest of the genome (or vice versa for the Z), because this provides a measure of how much genetic variation in lifespan we expect the X (or Z) chromosome to explain (Figure 8.1B). For example, X-linked effects are expected to be significant in *D. melanogaster*, where the X chromosome constitutes ~20% of the genome (Mallet *et al.* 2011), but relatively minor in polar bears (*Ursus maritimus*), where the X chromosome is < 5% of the genome (O'Brien *et al.* 2006). In contrast, toxic Y (or W) effects depend exclusively on the size of the non-recombining region in the Y (or W) chromosomes. Thus, toxic Y effects specifically predict lower male survival with increasing size of the Y (or W) chromosome relative to the autosomes (Figure 8.1C).

We first collected data on sex-specific survival across 138 species of birds, mammals, reptiles and amphibians, representing 6 independent origins of XY systems and 6 independent origins of ZW system, and used phylogenetic meta-analytic models to test the general prediction that females are longer lived than males in XY systems and *vice versa* in ZW systems. To tease apart whether differences in survival between the sexes are driven by unguarded-X or toxic Y effects, we then collected published karyotype data for 31 mammal and 15 bird species – the number for which we also had data on sex differences in survival. We focused on birds and mammals as we needed substantial variation in the sizes of sex chromosomes across species and this was lacking for amphibians and reptiles. We used this data to examine the following predictions. If the UXh

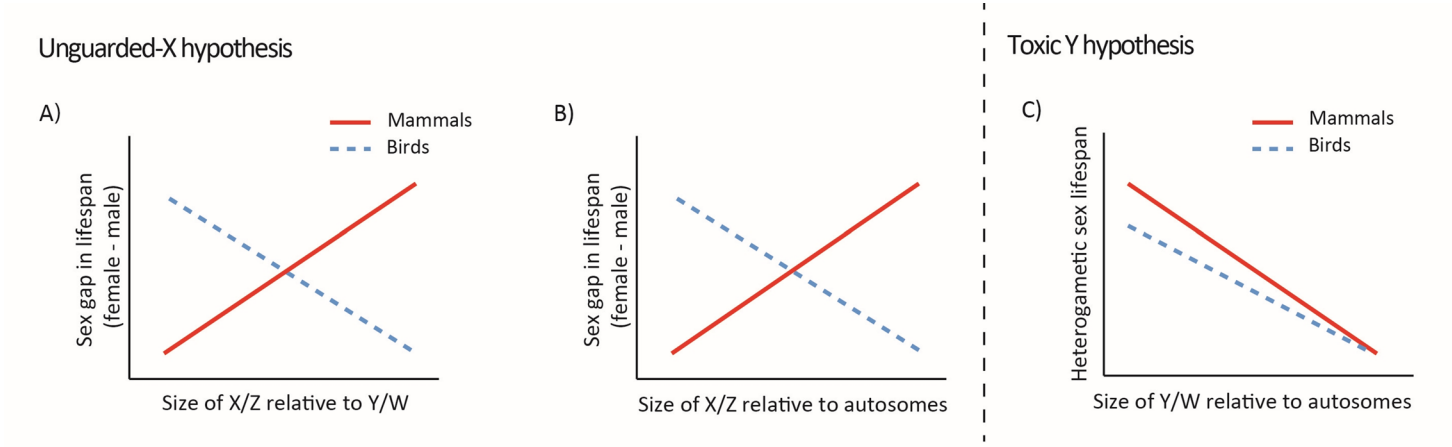

Figure 8.1. The correlations between sex-specific lifespan and relative sizes of the sex chromosomes predicted by unguarded-X and toxic Y hypotheses. The unguarded-X hypothesis predicts a positive relationship between the lifespan gap (i.e. female – male lifespan) and the size of the X relative to both the Y chromosome (A) and to the autosomes (B), and vice versa in the case of the Z chromosome. The toxic Y hypothesis, on the other hand, predicts a direct negative relationship between the size of Y and W chromosomes and the lifespan of the heterogametic sex (C). Toxic Y/W effects are expected to be stronger in mammals than in birds (e.g. because of the lower effective population size of Y compared with W).

contributes to the evolution of sex-specific lifespans, we expect: *i*) female mammals to have increasingly higher survival than males as the size of X relative to Y increases and as the size of X relative to the autosomes increases and *ii*) male birds to have increasingly higher survival than females as the size of Z relative to W increases and as the size of Z relative to the autosomes. If toxic Y (or W) effects contribute to the evolution of sex-specific lifespans, we expect: *i*) female mammals to have increasingly higher survival than males as the size of Y relative to the autosomes increases and *ii*) male birds to have increasingly higher survival than females as the size of W relative to the autosomes increases.

8.2. Materials & Methods

Data Collection

To detect unguarded-X and toxic Y effects using comparative analyses, we required data on sex-specific lifespans across multiple independent origins of XY (male heterogametic) and ZW (female heterogametic) systems, as well as variation across species in the extent of recombination suppression between the sex chromosomes. We therefore limited our search to tetrapods (amphibians, reptiles, birds and mammals) with XY or ZW systems because of the abundance of species that have been relatively well studied with respect to sex-specific lifespans (or proxies thereof, see below). We excluded fish because we did not find enough concurrent data on both sex-specific lifespan and genetic sex determination system. Data analysed in this study will be available in Dryad Digital Repository: [https://doi.org/10.5061/dryad.70rxwdbv1].

Sex differences in lifespan

We used sex-specific survival rates and mean ages as proxies for sex differences in lifespan to maximise available data across multiple independent origins of XY and ZW systems. Average lifespans are difficult to measure in wild vertebrate populations and life-tables (from which adult life expectancy can be calculated)

have been reported for relatively few species, primarily mammals and birds (Clutton-Brock and Isvaran 2007; Jones *et al.* 2014). Both survival and mean age are directly related to lifespan: life history theory predicts a longer lifespan when extrinsic mortality is low and thus survival is high while mean age will be lower in populations with a higher proportion of young individuals. Furthermore, survival and mean age are typically estimated using mark-recapture methods and so have the advantage of being reported with measurement error, which can be incorporated into statistical analyses. We excluded maximum lifespan as a proxy for average lifespan because it is strongly dependent on sampling effort, it is reported without error (by definition) and currently available data come from a mixture of wild and captive populations frequently based on very few individuals (De Magalhaes *et al.* 2005).

We used Scopus and Web of Science (WoS) to identify studies reporting sex-specific survival rates and mean ages. First, we used the following topic search term: "(male or female) AND mark-recapture" (studies published up to 31/12/2018). This returned 1647 studies from WoS and 2227 studies for Scopus. We also conducted backward and forward citation searches on the following review papers of survival rates (Karr *et al.* 1990; Yom-Tov *et al.* 1992; Siriwardena *et al.* 1998; Peach *et al.* 2001; DeSante and Kaschube 2007; McCarthy *et al.* 2008). This returned 168, 42, 118, 91, 8 and 31 studies from each review respectively. This search strategy primarily returned studies with suitable data on birds and mammals. To increase the number of amphibians and reptiles in our sample, we therefore conducted two additional searches in WoS and Scopus using the following topic search terms “mean age OR average age AND male AND female AND amphibian OR reptile” and “skeletochronology AND male AND female”. The first search terms returned 25 studies from WoS and 32 studies from Scopus and the second search terms returned 205 studies from WoS and 207 studies from Scopus. We included “skeletochronology” in our second search term because this is a widely-used technique for determining mean age in

reptiles and amphibians and is typically reported with error for females and males. We screened the title of each study in our sample (N = 4801 studies) and the abstracts of those which appeared to contain relevant data and identified 157 studies with suitable data (female and male survival / mean age reported with error) and that matched with the reptile and amphibians with known genetic sex-determination systems (see below).

Genetic sex-determination system

We collected data on the genetic sex determination system for each species in our database using the tree of sex database (Ashman *et al.* 2014). We complemented this with individual searches for species for which we had data on sex-specific lifespan (i.e., survival/mean age), but that did not appear in the tree of sex database; using the keywords "species name" AND "karyotype" OR "sex chromosome" in Google Scholar, Scopus and WoS and then examining the abstracts in search for data on the genetic determination system. All birds were assigned as ZW ($N_{species} = 69$) and all mammals as XY ($N_{species} = 45$). We assigned six amphibian and seven reptile species as ZW and ten amphibian and two reptile species as XY. We estimated the number of independent origins of XY and ZW systems in our sample of species using data from published sources (Evans *et al.* 2012). There were 2 independent XY origins and 2 independent ZW origins in our sample of reptiles and 3 independent XY origins and 3 independent ZW origins in amphibians. Therefore, in total, our sample included six XY origins and six ZW origins.

Karyotypes

We collected karyotype data for all available bird and mammal species for which we also had sex-specific survival/mean age data. We focused on birds and mammals as we needed variation in the sizes of the recombining and non-recombining chromosomes across species that share the same origin of a genetic sex determination system. This was not possible for amphibians and reptiles

where each independent origin of XY or ZW systems included seven or fewer species. We began by searching 14 chromosome atlases (Hsu and Benirschke 1967; Benirschke and Hsu 1971; O'Brien *et al.* 2006) for the karyotypes of species for which we had data on sex differences in survival. We identified karyotype images for 22 mammal and 4 bird species. For the species that were not present in these atlases, we did an additional search using the keywords "species name AND karyotype" in Web of Science and Scopus. We then examined the content of each article for karyotype images. From these searches, we found karyotype images for a further 9 mammal and 11 bird species. In total, we found suitable data for 32 mammal and 15 bird species. For each of these species, we used the karyotype image to calculate three ratios: i) the ratio of X or Z to the rest of the chromosomes (X / autosomes or Z / autosomes), ii) the ratio of Y or W to the rest of the chromosomes (Y / autosomes or W / autosomes) and iii) the ratio of X to Y and W to Z (X / Y or Z / W). We used ImageJ for calculating the relative size of the sex chromosomes from karyotype images (Schneider *et al.* 2012).

Sexual size dimorphism

We used sexual size dimorphism as a proxy to control for the intensity of sexual selection by including this variable as a co-variate in our statistical models (see below). We aimed to collect data on female and male head to body length for mammals, amphibians and reptiles. However, sex-specific body lengths were not available for most of the birds and some of the mammals in our sample, in which cases we extracted data on female and male body mass instead. For mammals, we compiled data from two online resources: http:// www.arkive.org and http://eol.org. For the species that were not present in these databases, we did an additional search using the keywords "*species name AND body size AND male AND female*" in WoS and Scopus, and then examining the whole content of the articles in search of male/female body length data. For amphibians and reptiles, the studies from which we extracted sex-specific mean age data from also

provided data on sex-specific body size. For birds, sexual size dimorphism was taken from the Handbook of the Birds of the World (del Hoyo *et al.* 2018). Finally, we calculated sexual size dimorphism as the natural logarithm of the ratio of female to male body size: *ln (female value / male value)*.

Phylogenetic trees

We used the rotl R package, an interface to the Open Tree of Life (McTavish *et al.* 2015), to estimate a phylogenetic tree of the relationships among species in our sample. Branch lengths were estimated using (Grafen 1989) method in the APE package in R, with each node height raised to the power of 0.5. This tree was used in the analysis of sex differences in lifespan across tetrapods (see below). For the analysis involving birds only, we downloaded a sample of 1300 phylogenies (out of 10 000) from the birdtree.org (Jetz *et al.* 2012). Similarly, for the analysis involving mammals only, we downloaded a sample of 1300 phylogenies (out of 10 000) from the supporting information of Kuhn *et al.* (2011). We calculated a phylogenetic covariance matrix (evolutionary distances between species) from each of these trees, which were then used to account for dependencies due to shared evolutionary history in our statistical models.

Effect size calculation

We compared female and male lifespans using an effect size which allows us to take a standardised measure of the magnitude of the statistical difference between the sexes that is comparable across studies (Koricheva *et al.* 2013). We used the natural logarithm of the response ratio:

$$lnR = \ln(\acute{X}_1/\acute{X}_2)$$

where $\acute{X}_1$ is either female mean age or female annual survival and $\acute{X}_2$ is either male mean age or male annual survival depending on which measure was available for a given species. Positive values indicate that females live longer than

males and negative values that males live longer than females. Each effect size was weighted by the inverse of its sampling variance in our statistical models to account for differences in sampling effort between studies:

$V_{lnR} = (SE_1\text{^}2 / \acute{X}_1\text{^}2) + (SE_2\text{^}2 / \acute{X}_2\text{^}2)$

where SE_1 is the standard error of the female value and SE_2 is the standard error of the male value. In total, we obtained 255 effect sizes from 157 studies on 138 species across 6 independent origins of XY systems and 6 independent origins of ZW systems. There was significant between-study variance ($\tau^2 = 0.01$, $I^2 = 97.1\%$, Cochran's $Q = 8073.7$, $p < 0.001$, $N_{effect\ sizes} = 255$). We detected no evidence of publication bias using Egger's regression method (intercept = 0.00, p = 0.96). However, a trim and fill analysis suggested that 54 effect sizes were missing from our sample. There was no difference in mean *lnR* between studies reporting annual survival and those reporting mean age in XY or ZW systems (XY difference = -0.09, se = 0.06, p = 0.13, $N_{effect\ sizes} = 120$; ZW difference = -0.07, se = 0.05, p = 0.15, $N_{effect\ sizes} = 135$) suggesting that the proxy used to estimate sex differences in lifespan does not bias our effect sizes. We calculated heterogeneity statistics for each of the meta-analytic models, including the percentage of variation in *lnR* attributable to phylogenetic history and repeated observations made on the same species (Nakagawa and Santos 2012).

Statistical models

We used the metafor (Viechtbauer 2010) and MCMCglmm (Hadfield 2010) R packages for model fitting when analysing *lnR*. Metafor uses restricted maximum likelihood for parameter estimation while MCMCglmm uses the Markov chain Monte Carlo (MCMC) method in a Bayesian framework. For analyses of non-Gaussian response variables (i.e., sex-specific annual survival rates, described below) we used MCMCglmm only which has greater flexibility when fitting models with non-normal distributions. We therefore report parameter estimates

from MCMCglmm in the results section for consistency between analyses but show those from metafor. Parameters are reported as the posterior mode (ß) and 95 % credible interval (CI) of the posterior distribution of the Markov chain and significance is assessed by whether the CI includes zero. For the MCMCglmm models, we used uniform priors for fixed effects and inverse-Wishart priors (v = 1 and nu = 0.002) for random effects and ran each model for 1,300,000 iterations with a burn in period of 300,000 and saving every 1000th iteration of the chain.

Sex differences in lifespan and genetic sex determination system across tetrapods

To test whether females are longer-lived than males in XY systems and males are longer-lived than females in ZW systems we modelled *lnR* (treated as Gaussian) as a function of genetic sex determination system (2 level fixed effect: ZW or XY) with sexual size dimorphism included as a covariate (z transformed: mean = 0 and sd = 1) and the phylogenetic covariance matrix from the tetrapod phylogeny (see above) as a random effect. We also included a species-specific random effect to account for repeated measures made on the same species. Each effect size was weighted by the inverse of its sampling variance, V_{lnR}.

Sex differences in lifespan and the difference in size between the sex chromosomes

We modelled the relationship between sex differences in lifespan and the difference in size between the sex chromosomes (X vs. Y and Z vs. W) to test for an unguarded-X effect. If recessive mutations accumulate in the non-recombining regions of X or Z chromosomes then the larger the size difference between the X and the Y (or between Z and W), the larger the non-recombining region, resulting in more recessive mutations and greater sex differences in lifespan. First, we modelled *lnR* (treated as Gaussian and with each effects size weighted by the inverse of its sampling variance) as a function of the X/Y ratio (log and z

transformed) in mammals. We included sexual size dimorphism as a covariate (z transformed) and a species identifier and a phylogenetic covariance matrix as random effects. We replaced the phylogenetic covariance matrix used in each model every 1000 iterations of the Markov chain with the next one in the sequence calculated from the 1300 mammal phylogenies downloaded from from the supporting information of Kuhn *et al.* (2011). The values of the variance components and latent variables estimated using the previous phylogenetic covariance matrix were used as starting values for the next one in the sequence. This allowed us to incorporate uncertainty in the mammal phylogeny into our analyses. Note that this was not possible for the metafor analysis and parameters were calculated based on one randomly sampled phylogenetic covariance matrix. Next, we modelled *lnR* (treated as Gaussian and with each effect size weighted by the inverse of its sampling variance) as a function of the Z/W ratio (log and z transformed) in birds. Sexual size dimorphism was included as a covariate (z transformed) and a phylogenetic covariance matrix and a species identifier were included as random effects. The phylogenetic covariance matrix used in each model was updated as described for the mammal analyses using the phylogenetic covariance matrices calculated from the 1300 bird phylogenies.

Sex differences in lifespan and the relative sizes of the sex chromosomes

The UX hypothesis predicts that sex differences in lifespan should correlate with the size of the X (or Z) chromosome relative to the rest of the genome, as this ratio measures the potential impact of recessive mutations on survival. Similarly, the toxic Y hypothesis predicts that sex differences in lifespan should correlate with the size of the Y (or W) chromosome relative to the rest of the genome. First, we tested for UX effects by constructing two models. In mammals, we modelled *lnR* (treated as Gaussian and with each effect size weighted by the inverse of its sampling variance) as a function of the X/autosomes ratio (z transformed) and sexual size dimorphism (z transformed) with a phylogenetic covariance (updated as described above) and a species identifier included as random effects. We

repeated this model for birds, substituting the X/autosomes ratio for the Z/autosomes ratio. We then tested for toxic Y effects in mammals and birds by repeating the above models but replacing the X/autosomes ratio with the Y/autosomes ratio (z transformed) in the mammal analysis and the Z/autosomes ratio for the W/autosomes ratio (z transformed) in the bird analysis. All other fixed and random effects were the same. Finally, to tease apart toxic Y and unguarded-X effects we modelled *lnR* as a function of both the X/autosomes and the Y/autosomes ratios (both z transformed) in mammals and both the Z/autosomes and the W/autosomes ratios (both z transformed) in birds. We included sexual size dimorphism as a covariate (z transformed) and a phylogenetic covariance matrix (updated as described above) and a species identifier as random effects in each model. Each effect size was weighted by the inverse of its sampling variance. The X/autosome and Y/autosome ratios were weakly correlated in mammals (r = 0.23, lwr = -0.23, upr = 0.61, p = 0.32) and Z/autosome and W/autosome ratios were weakly correlated in birds (r = -0.31, lwr = -0.72, upr = 0.26, p = 0.29).

Sex-specific survival and the sex chromosomes

The unguarded-X and toxic Y hypotheses predict reduced survival of the heterogametic sex specifically. We tested this in mammals by comparing male annual survival rates (males are XY) across species in relation to *i*) the X/Y ratio (log and z transformed), *ii*) the X/autosomes ratio (z transformed) and *iii*) the Y/autosomes ratio (z transformed). Male annual survival was modelled using a binomial distribution (number alive vs. number dead) with a logit link function. In each of these three models we included male body mass (z and log transformed) and sexual size dimorphism (z transformed) as covariates to control for variation in male annual survival rates across species explained by differences in the strength of sexual selection and size differences. A phylogenetic covariance matrix (updated as described above) and a species level identifier were included as random effects in each model. Females are the heterogametic sex in birds,

therefore we modelled female annual survival rates (using a binomial distribution with a logit link function) as a function of *i*) the Z/W ratio (log and z transformed), *ii*) the Z/autosomes ratio (z transformed) and *iii*) the W/autosomes ratio (z transformed). In each of these three models, we included female size as a covariate, to account for variation in female survival rates across species explained by differences in size, and a phylogenetic covariance matrix (updated as above) and a species level identifier were included as random effects.

8.3. Results

Sex differences in lifespan and genetic sex determination system across tetrapods

Females lived longer than males on average in XY systems while males lived longer than females on average in ZW systems (parameter estimate [β] = -0.13, 95% Credible Interval [CI] = -0.20 to -0.06; $N_{effect\ sizes}$ = 255; $N_{species}$ = 138; Figure 8.2). The effect of the genetic sex determination system on sex differences in lifespan was independent of phylogeny, which explained 33% of the variance in sex-specific lifespans across species, and sexual size dimorphism (β = 0.09, CI = -0.05 to 0.18; $N_{effect\ sizes}$ = 255; $N_{species}$ = 138), suggesting that differences in lifespan between the sexes are not due to overall differences in sexual selection. Considering each origin separately, in 6/6 XY systems females lived longer than males on average (in only one of these was the difference significant) and in 4/6 ZW systems males lived longer than females on average (in none of these was the difference significant).

Sex differences in lifespan and the difference in size between the sex chromosomes

The relative size of the X vs. Y chromosomes across mammals was not associated with sex-differences in lifespan (β = -0.01, CI = -0.06 to 0.03; $N_{effect\ sizes}$ = 55;

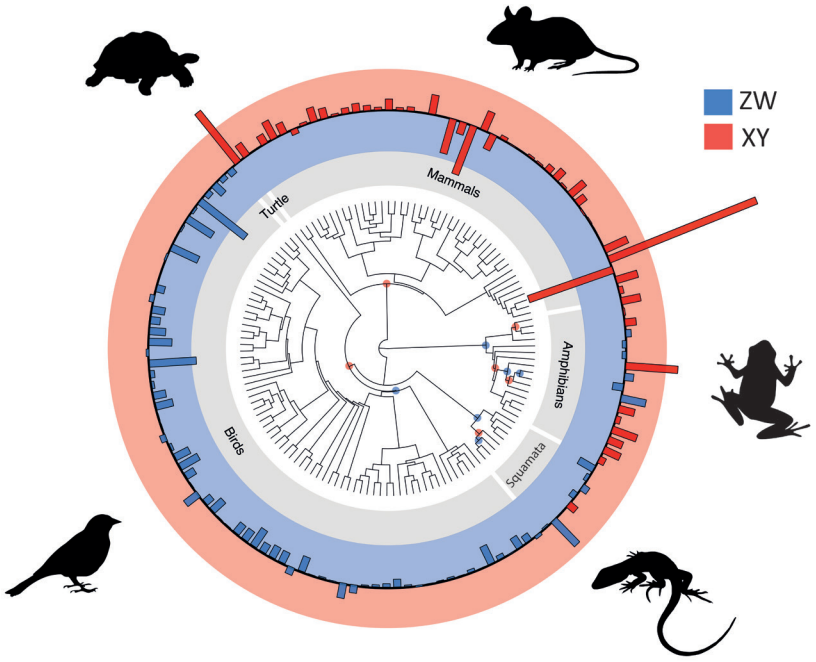

Figure 8.2. Sex differences in lifespan across 138 species of birds, mammals, reptiles and amphibians. A statistical effect size of the ratio of female and male lifespan (lnR) is plotted for each species. Bars above the black line (the red ring) indicate that females live longer than males, while bars below the black line (the blue ring) indicate that males live longer than females. ZW systems are coloured in blue and XY systems are coloured in red. The blue points in the phylogeny show the 6 independent origins of ZW systems and the red circles show the 6 independent of XY systems in our sample of species.

$N_{species}$ = 21; Figure 8.3a). In fact, the largest sex-differences in survival in mammals occur in species where X and Y appear to be similar in size (Figure 8.3a), as expected if large Y chromosomes have a negative effect on male survival rather than large X chromosomes. Sexual dimorphism in body size was also unrelated to lifespan differences between the sexes (β = -0.02, CI = -0.09 to 0.04; $N_{effect\ sizes}$ = 55; $N_{species}$ = 21). Similarly, there was no relationship between sex-differences in lifespan and size differences between the Z and W chromosomes

(β = 0.02, CI = -0.07 to 0.10; $N_{effect\ sizes}$ = 28; $N_{species}$ = 14; Figure 8.3b) or sexual dimorphism in body size (β = 0.02, CI = -0.04 to 0.10; $N_{effect\ sizes}$ = 28; $N_{species}$ = 14) across birds.

Sex differences in lifespan and the relative sizes of the sex chromosomes

Across mammals, both the size of the X chromosome relative to the autosomes and the size of the Y chromosome relative to the autosomes were positively associated with sex differences in lifespan (X/autosomes: β = 0.05, CI = 0.01 to 0.09; $N_{effect\ sizes}$ = 65; $N_{species}$ = 32; Figure 8.4a; Y/autosomes: β = 0.05, CI = 0.00 to 0.11; $N_{effect\ sizes}$ = 50; $N_{species}$ = 20; Figure 8.4b). The larger the X and Y chromosomes relative to the autosomes, the larger the sex difference in lifespan, with females being increasingly long-lived compared with males. This, however, would be expected if the relative sizes of the X and Y chromosomes are themselves correlated. When modelling the effects of the relative sizes of the X and Y chromosomes together, the relationship between relative X chromosome size and sex differences in lifespan disappeared (β = 0.01, CI = -0.05 to 0.06; $N_{effect\ sizes}$ = 50; $N_{species}$ = 20), while the relationship between relative Y chromosome size and sex differences in lifespan remained positive, although it was not statistically significant (β = 0.04, CI = -0.01 to 0.11; $N_{effect\ sizes}$ = 50; $N_{species}$ = 20). Across birds, neither the size of the Z or W chromosomes relative to the autosomes were associated with sex differences in lifespan (Z/autosomes: β = 0.01, CI = -0.07 to 0.07; $N_{effect\ sizes}$ = 29; $N_{species}$ = 15; Figure 8.5a; W/autosomes: β = 0.00, CI = -0.09 to 0.07; $N_{effect\ sizes}$ = 28; $N_{species}$ = 14; Figure 8.5b).

Sex-specific lifespan and the relative sizes of the sex chromosomes

Male mammals with high rates of annual survival tend to have small Y chromosomes relative to the autosomes whereas males with low annual survival had relatively large Y chromosomes (β = -0.72, CI = -1.19 to -0.26; $N_{observations}$ = 50; $N_{species}$ = 20; Figure 8.6a). This was independent of the strength of sexual size dimorphism (β = -0.23, CI = -0.87 to 0.25; $N_{observations}$ = 50; $N_{species}$ = 20), a proxy

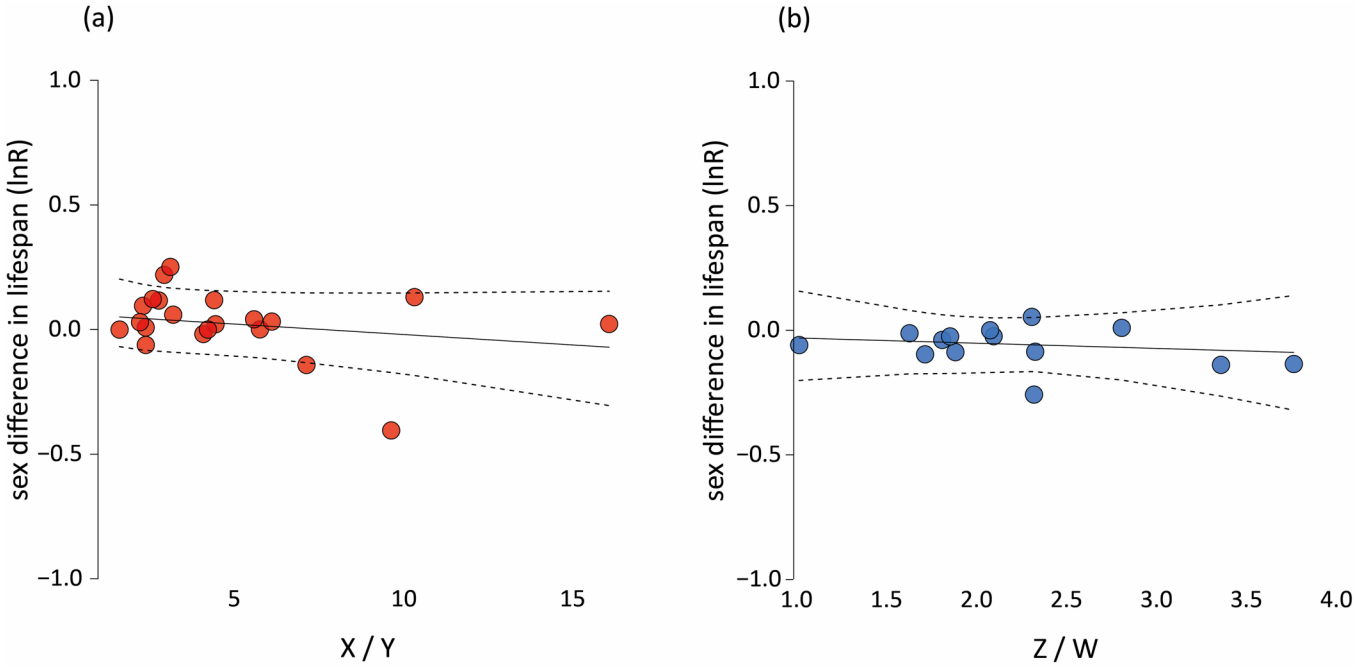

Figure 8.3. Sex differences in lifespan and the difference in size between the sex chromosomes. Sex differences in lifespan and the size of X relative Y chromosome in mammals (a) and the size of Z relative to W chromosome in birds (b).

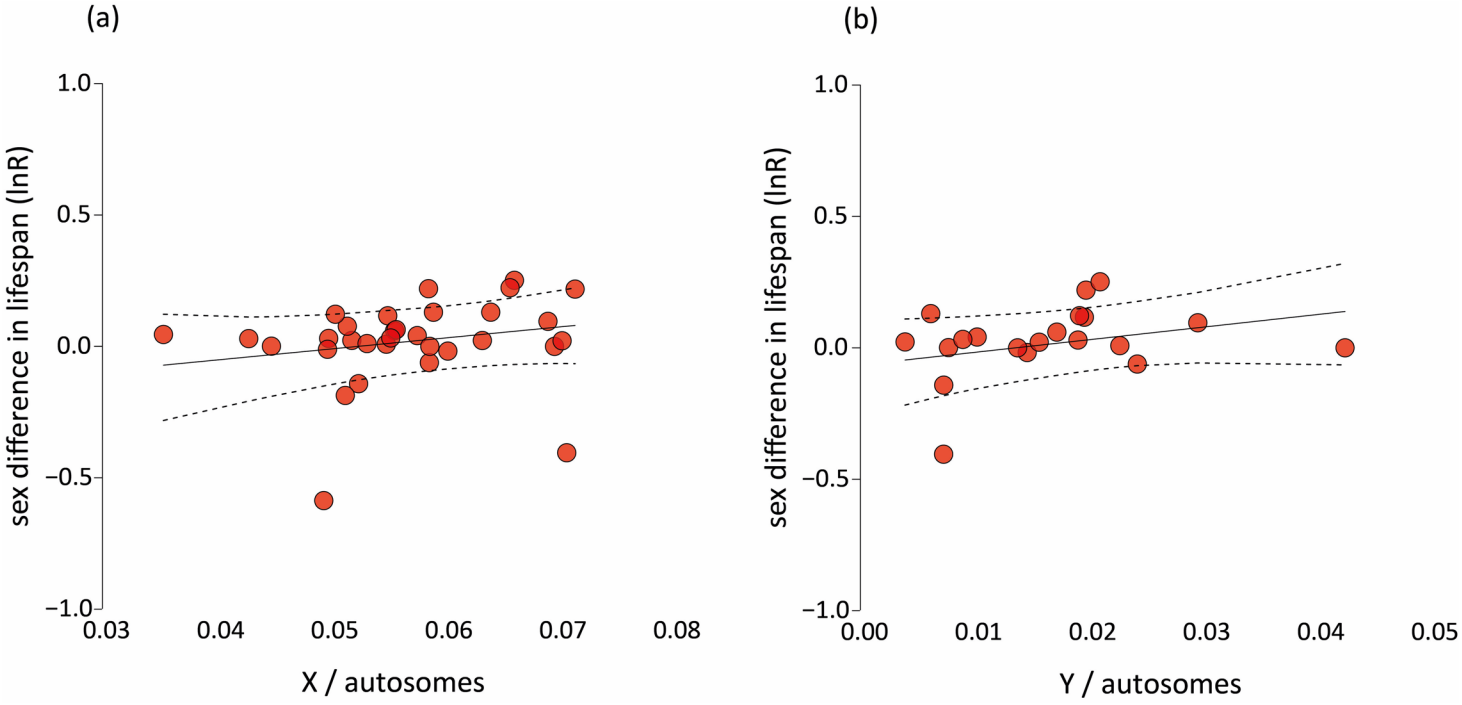

Figure 8.4. Sex differences in lifespan and the relative sizes of the sex chromosomes in mammals. Sex differences in lifespan and the size of X relative to the autosomes (a) and Y relative to the autosomes (b) in mammals.

for the strength of sexual selection, and differences in body size between species (β = 0.35, CI = -0.01 to 0.76; $N_{observations}$ = 50; $N_{species}$ = 20). Moreover, male annual survival rates in mammals were not associated with the size of X chromosomes relative to autosomes (ß = -0.10, CI = -0.50 to 0.31; $N_{observations}$ = 65; $N_{species}$ = 32; Figure 8.7a) or with size differences between the X and Y chromosomes (ß = 0.17, CI = -0.33 to 0.72; $N_{observations}$ = 55; $N_{species}$ = 21; Figure 8.7b). In contrast, rates of female annual survival across bird species were not associated with the sizes of W chromosomes relative to autosomes (W/autosomes: ß = 0.13, CI = -0.51 to 0.67; $N_{observations}$ = 28; $N_{species}$ = 14; Figure 8.6b). Similarly, female annual survival in birds was not associated with the size of Z chromosomes relative to the autosomes (Z/autosomes: ß = -0.05, CI = -0.39 to 0.37; $N_{observations}$ = 29; $N_{species}$ = 15; Figure 8.8a) or with differences in size between the Z and W chromosomes (ß = -0.15, CI = -0.63 to 0.44; $N_{observations}$ = 28; $N_{species}$ = 14; Figure 8.8b).

8.4. Discussion

In this chapter, we show that there is a clear link between genetic sex determination systems and the sex gap in lifespan. Across 138 species of vertebrates reflecting 6 independent origins of XY and ZW systems, females survived longer than males on average in XY systems, while males survived longer than females on average in ZW systems. Previously, Pipoly *et al.* (2015) identified female-biased adult sex ratios in taxa with XY systems and male-biased adult sex ratios in taxa with ZW systems. Along the same line, Xirocostas *et al.* (2020) recently found that mean/maximum lifespan is shorter in the heterogametic sex across the tree of life, but they failed to control for the effects of phylogenetic signal and the strength of sexual selection. Our results build on these studies by showing that, in vertebrates, the relationship between sex differences in survival and sex determination system remains after accounting for both phylogenetic signal (which in our sample explained ~33% of the variance in the sex gap in lifespan) and sexual size dimorphism (a proxy for sexual selection).

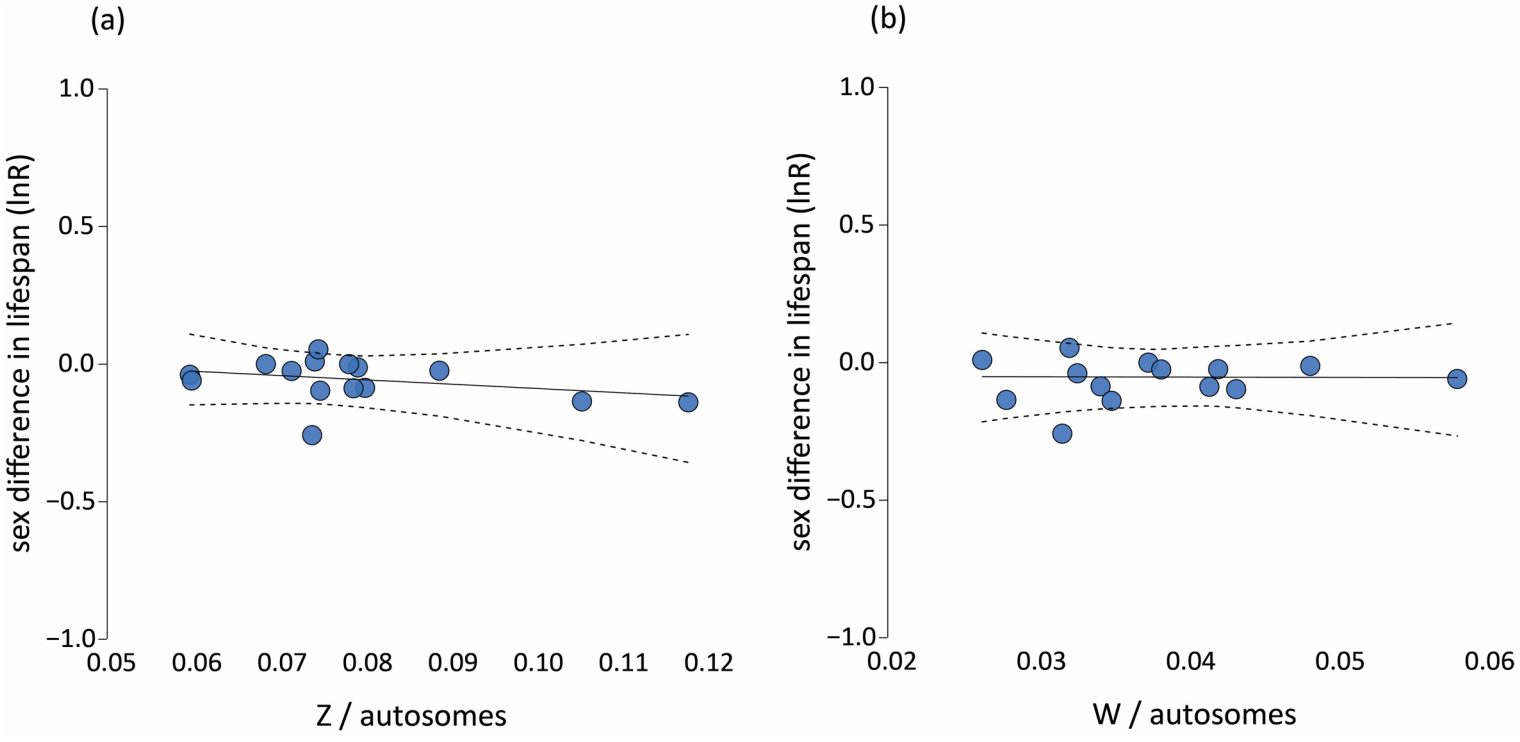

Figure 8.5. Sex differences in lifespan and the relative sizes of the sex chromosomes in birds. Sex differences in lifespan and the size of Z relative to the autosomes (a) and W relative to the autosomes (b) in birds.

Furthermore, we then looked at the relationship between the relative importance of the sex chromosomes and sex-specific lifespan in mammals and birds. Our results suggest that this relationship is better explained by "toxic Y" rather than UXh effects, the prevailing hypothesis to date.

The most commonly cited hypothesis to explain broad patterns of sex-specific lifespan across taxa is the UXh hypothesis put forward by Trivers (Trivers 1985). Recent work reporting a link between sex determination systems and different proxies for sex-specific lifespan have been interpreted as supporting this hypothesis (Pipoly *et al.* 2015; Xirocostas *et al.* 2020). Here, we specifically tested for the UXh by asking whether there is a correlation between the sex-gap in survival and the relative size of the sex chromosomes (X/Z relative to Y/W and autosomes; Figure 8.1). We found limited evidence in its support (Figures 8.3 & 8.4). Despite its long history and intuitive appeal, our results suggest that the UXh may in fact not be a fundamental driver of sex-specific mortality. Since it was first formulated, more than three decades ago, support for the UXh has been scant and indirect (Maklakov and Lummaa 2013; Pipoly *et al.* 2015; Sultanova *et al.* 2018; Xirocostas *et al.* 2020) and, while this may be due to the inherent difficulties in testing this hypothesis, there are also frequently overlooked reasons to doubt it plays a major role in explaining sex-specific lifespans. For example, the fact that there is likely to be strong selection against big effect X-linked recessive mutations in nature (Vicoso and Charlesworth 2006), meaning that any mutations that do accumulate on the recombining chromosome should have relatively minor effects. Of course, it is possible that the lack of evidence in favour of the UXh hypothesis is simply due to a lack of statistical power in our study, given the relatively scarce karyotypic data generally available for vertebrates. This limitation is bound to be particularly important given the complex relationship between the size of the two sex chromosomes. For example, due to the accumulation of repetitive and non-functional regions in the Y/W chromosomes, the size of the non-recombining region between the two sex

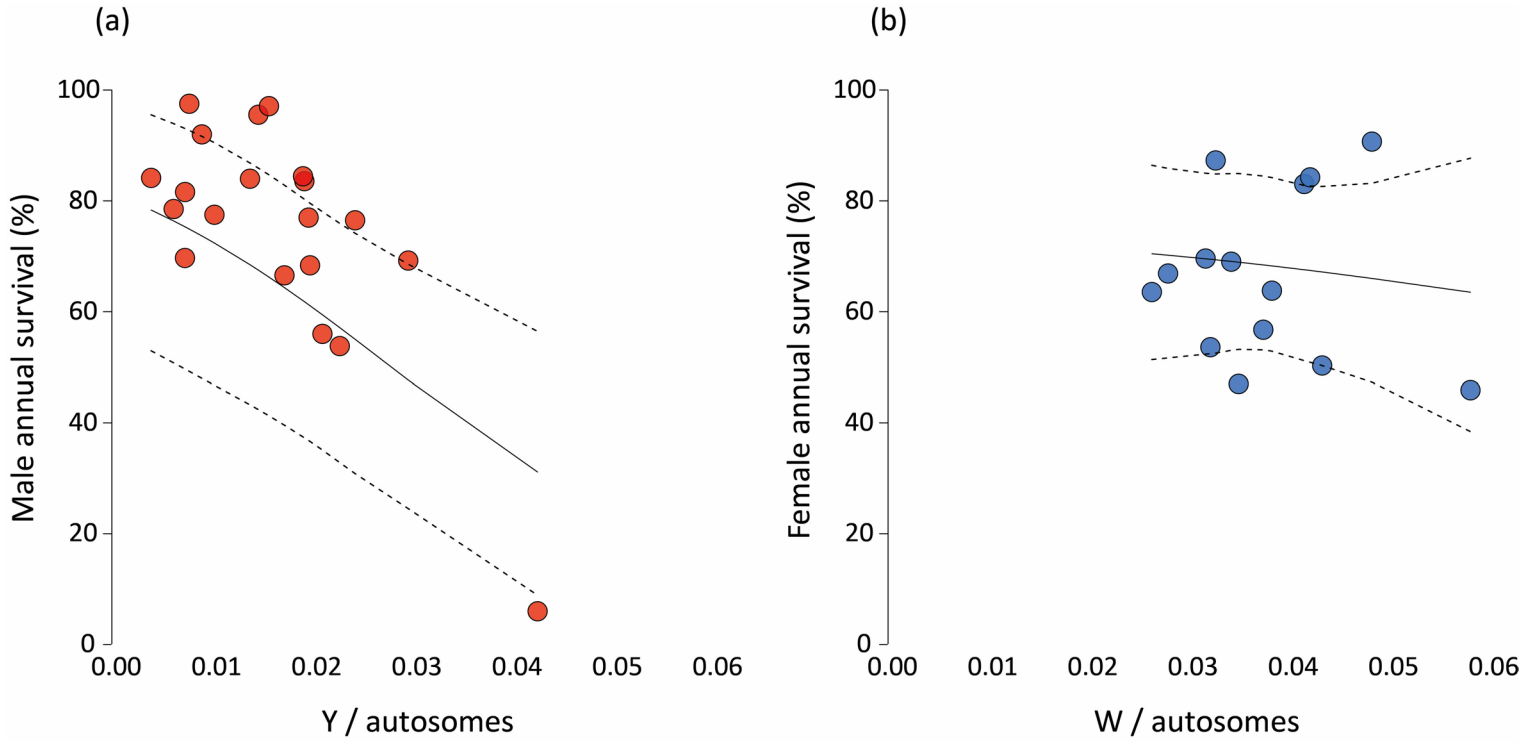

Figure 8.6. Sex-specific annual survival in relation to the relative size of the heteromorphic sex chromosome. Male annual survival and the relative size of the Y chromosome in mammals (a). Female annual survival and the relative size of the W chromosome in birds (b).

chromosomes can vary considerably even across species with the same absolute X/Z vs. Y/W size differences (Bachtrog 2013; Wright *et al.* 2016; Brown *et al.* 2020b). We thus suggest that future analysis with more exhaustive datasets should aim to replicate our analysis. We note, however, that this same lack of power should affect our ability to detect "toxic Y" effects, a recent and until now empirically untested alternative that fits with the findings reported here.

According to theory, toxic Y effects result in reduced survival of the heterogametic sex due to the accumulation of both deleterious mutations and repetitive DNA elements in the Y and W sex chromosomes (Bachtrog 2013; Wright *et al.* 2016; Brown *et al.* 2020b). Two main predictions arise. First, that genetic sex determination systems predict the sex-gap in lifespan so that the heterogametic sex tends to live longer than the homogametic sex across a wide range of taxa, as reported here and in a recent study (Xirocostas *et al.* 2020). Second, that the size of the Y or W sex chromosome inversely predicts the lifespan of the heterogametic sex in XY and ZW systems respectively. Our results show that there is indeed a negative correlation between the relative size of the Y chromosome and male lifespan in mammals, although we did not find this effect in birds. However, a stronger toxic Y effect is expected in XY than in ZW systems. This is due to the Y chromosome having a smaller effective population size than the W chromosome (higher variance in male than female reproductive success), and to Y chromosomes accumulating more mutations than W chromosomes because the male germ line undergoes more cell divisions than the female germ line (Drost and Lee 1995). This, in turn, makes degeneration of the Y chromosome (and hence accumulation of repetitive DNA) more likely (Bachtrog 2013). For example, the mammalian Y chromosome is known to be significantly enriched in repetitive DNA compared to the W chromosome in birds (Rutkowska *et al.* 2012; Bachtrog 2013; Zhou *et al.* 2014; Wright *et al.* 2016). Available evidence also suggests that non-recombining regions are larger in mammalian than in bird sex chromosomes, which also seem to exhibit less

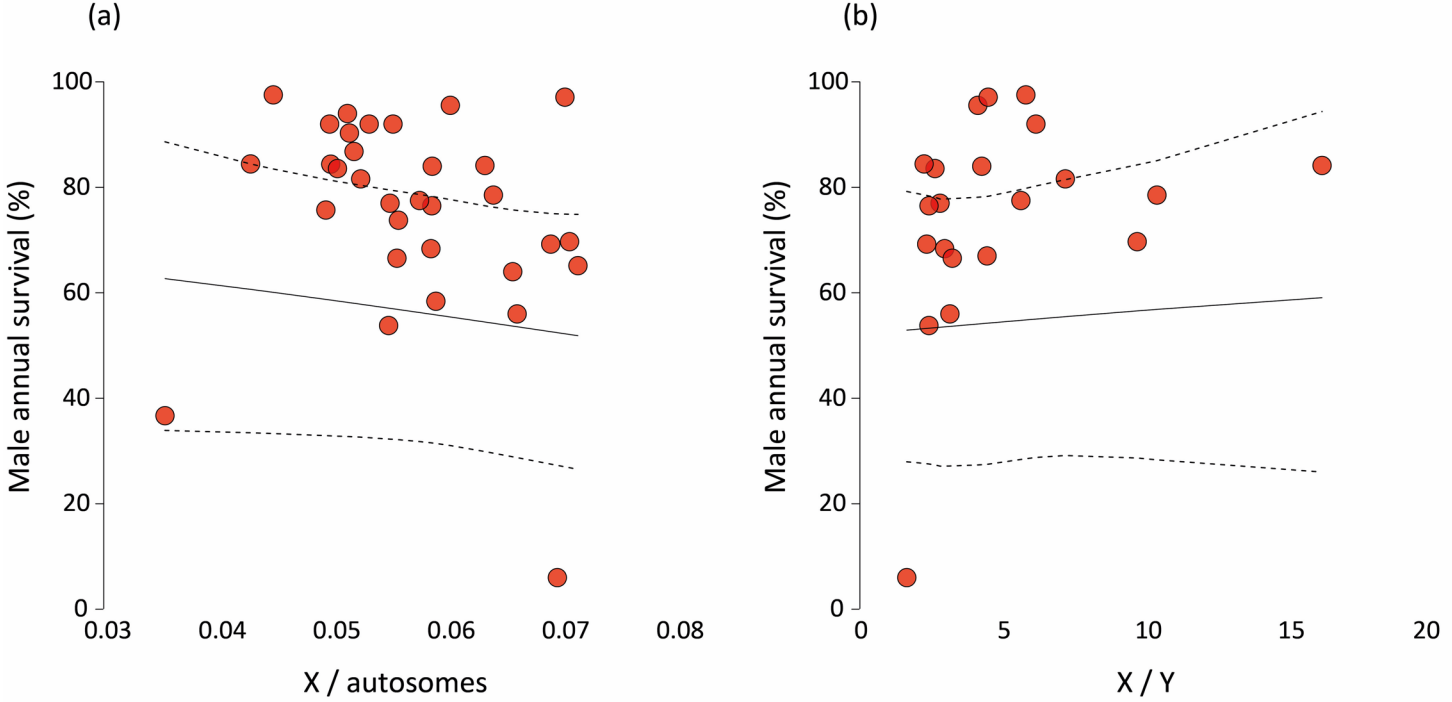

Figure 8.7. Sex-specific annual survival in relation to the relative size of the homomorphic sex chromosome and to the difference in size between the sex chromosomes in mammals. Male annual survival and the relative size of the X chromosome (a) and the difference in size between X and Y (b) in mammals.

variability in the Z/W (vs. mammalian X/Y) size ratio (Rutkowska *et al.* 2012; Bachtrog 2013; Zhou *et al.* 2014; Wright *et al.* 2016). In agreement with this, the X to Y chromosome ratio was twice as high on average than the Z to W chromosome ratio in our dataset (X to Y estimate = 4.96 ± 0.74 (sem), $N_{species}$ = 21; Z to W estimate = 2.23 ± 0.18, $N_{species}$ = 14, Figure 8.9). This is unlikely to be a sampling artefact as evidence from the karyotypes of 200 bird species shows that the Z to W chromosome ratio does not extend beyond the limit we detected (Rutkowska *et al.* 2012).

An exciting possibility is that cyto-nuclear interactions may contribute to explain marked "toxic Y" effects in mammals, but not toxic W effects in birds. Given that Y chromosomes are not inherited along with mitochondrial DNA (and other cytoplasmic products), there is less scope for cyto-nuclear co-evolution in males vs. females with X/Y genetic determination systems (but see Keaney *et al.* 2020). This is not the case in males of species with ZW sex-determination systems because in these species females are the heterogametic sex, and hence copies of both sex chromosomes are inherited maternally along with the cytoplasm. Indeed, recent evidence shows that cyto-nuclear interactions have sex-specific lifespan effects in *D. melanogaster* (Vaught *et al.* 2020), which opens yet another exciting line of research for future studies. Although it is tempting to interpret our negative finding of a toxic W effect in line with the predictions above, this finding must be taken with caution for two methodological reasons: our sample size for birds was approximately half that of mammals, and autosome size (on which many of our relative measures are based) is more difficult to estimate in birds than in mammals due to bird karyotypes often including a large number of micro-chromosomes that increased measurement error in our estimations of bird chromosome sizes.

Other complementary hypotheses that could indirectly explain a role of sex chromosomes in determining the sex gap in lifespan have to do with sexual

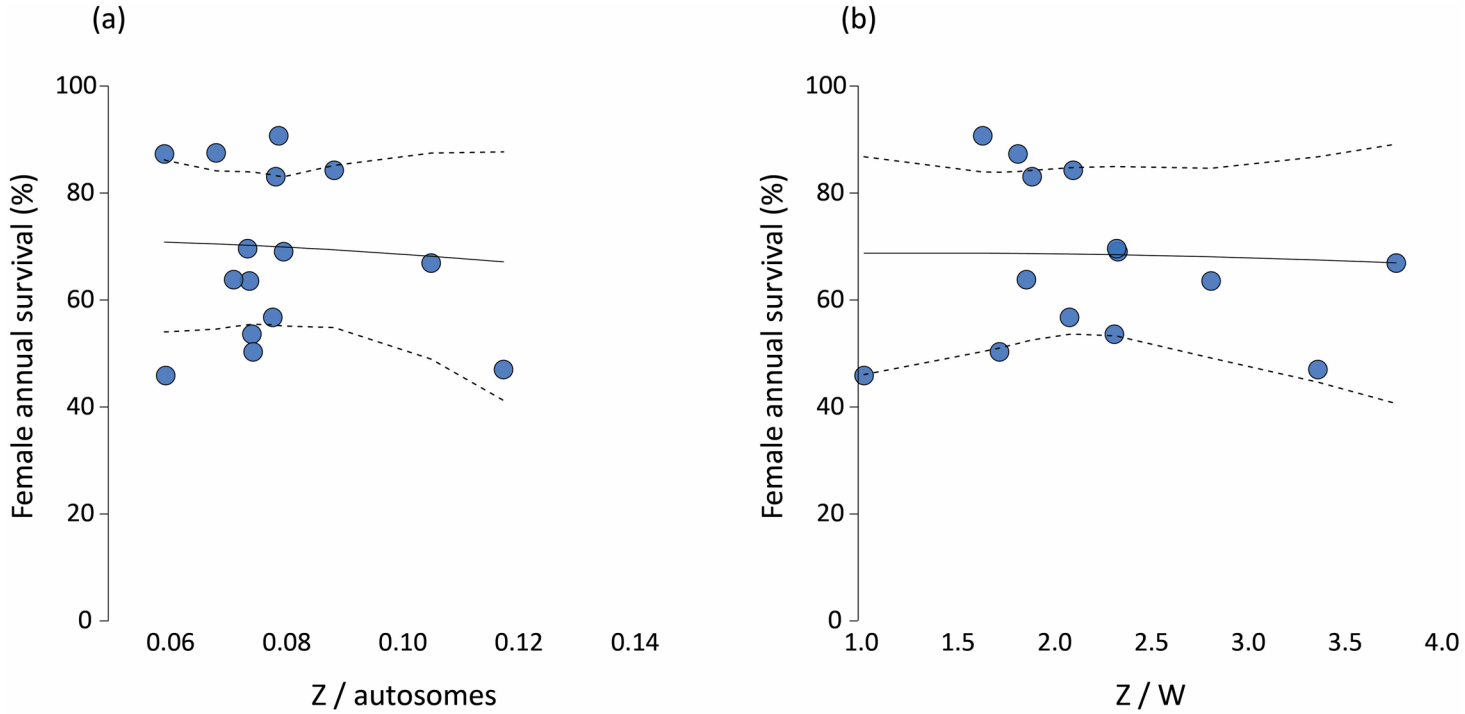

Figure 8.8. Sex-specific annual survival in relation to the relative size of the homomorphic sex chromosome and to the difference in size between the sex chromosomes in birds. Female annual survival and the relative size of the Z chromosome (a) and the difference in size between Z and W (b) in birds.

selection and imperfect dosage compensation. First, sexual selection frequently favours the accumulation of mutations that increase male reproductive success at the expense of male survival (Bonduriansky *et al.* 2008), but in both our study and in Pipoly *et al.* (2015), correlations between sex determination systems and survival/adult sex ratios were independent of sexual size dimorphism, a commonly used proxy for sexual selection intensity. Second, imperfect dosage compensation is deleterious for the heterogametic sex, explaining why this sex is short-lived relative to the homogametic sex (Mank 2013). However, imperfect dosage compensation predicts that the sex gap in lifespan should be related to the

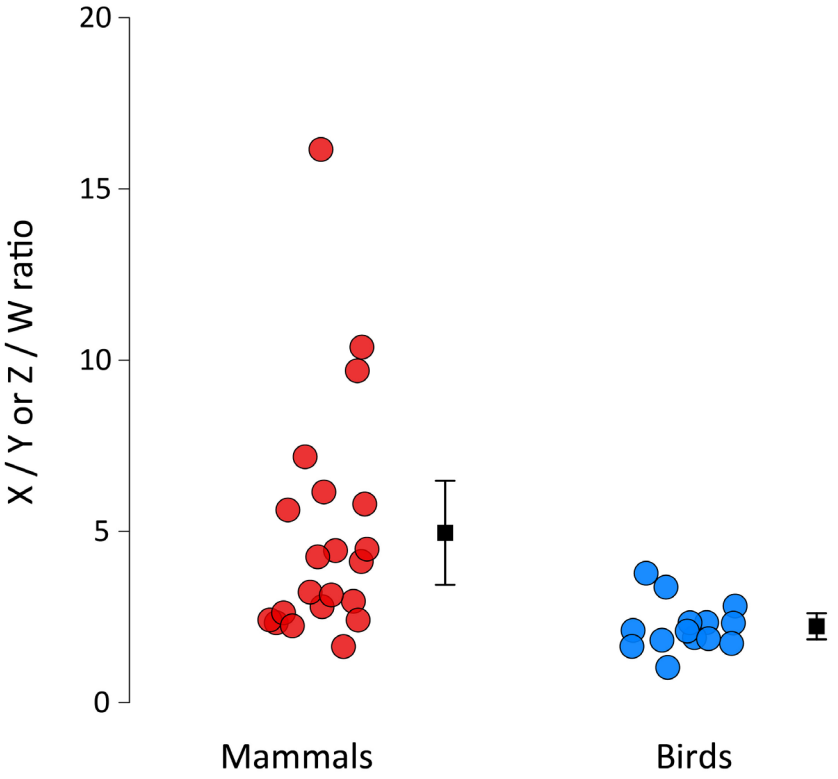

Figure 8.9. The relative size of X (or Z) chromosome with respect to Y (or W) chromosome. The relative sizes were calculated as the size of X chromosome divided by the size of Y chromosome in mammals and the size of Z chromosome divided by the size of W chromosome in birds (also shown as mean ± S.E.).

size difference between the two sex chromosomes (X relative to Y and Z relative to W) because the size difference will be proportional to the imbalance in gene dose between males and females, and hence the degree of dosage compensation (Mank 2013). Our results show, however, that this was not the case (Figure 8.3).

To conclude, we report compelling evidence of a link between sex determination systems and the sex-gap in survival across vertebrates, which along with recent evidence (Xirocostas *et al.* 2020) strongly suggests that sex chromosomes play a role in understanding broad patterns of sexual differences in lifespan. Our data suggest that "toxic Y" effects, rather than unguarded-X effects explain this link. Future research should aim to explicitly test for toxic Y effects by means of direct empirical manipulations (Brown *et al.* 2020b,a) and by expanding our comparative framework to other taxa (e.g. invertebrates).

Chapter 9. General Discussion

Sex-specific ageing is ubiquitously observed across the tree of life (Maklakov and Lummaa 2013). In this thesis, I have tried to plug some of the existing gaps in our knowledge of the evolution and function of sex-specific ageing. In doing so, I have not focused on a particular hypothesis but aimed to explore adaptive and maladaptive processes (Maklakov and Lummaa 2013), both of which I believe are crucial for a complete understanding of why males and females age differently. The overarching idea has been to shed some light into areas of research I believe hold more open (and more interesting) questions. This has led me to address a broad range of questions, and to combine quite different empirical approaches, from classic behavioural and fitness assays to more complex inbreeding designs, comparative meta-analyses and the study of gut-microbiota. So, what have we learnt from this thesis?

Sex-specific ageing as a result from adaptive processes

Due to the presence of sex-specific selection pressures, males and females are expected to have different life history strategies resulting in sex-specific ageing patterns (Vinogradov 1998; Carranza and Pérez-Barbería 2007; Clutton-Brock and Isvaran 2007; Bonduriansky *et al.* 2008; Berg and Maklakov 2012; Adler and Bonduriansky 2014). This means that external factors such as social context, predation or diseases should also have different effects on actuarial/reproductive/functional ageing of females and males. Moreover, this may also lead to the evolution of different mechanisms that underlie the life history strategies of females and males. Within this framework, we set out to contribute to three research questions that we believe hold great potential to further our current understanding about how adaptive processes can shape sex-specific ageing. We first investigated how social context (Chapter 4) and condition dependent mortality (Chapter 5) can modulate age effects on fitness. We then explored the link between gut microbiota and male life history traits:

lifespan, actuarial/reproductive/functional ageing and reproductive fitness (Chapter 6).

In Chapter 4, we investigated whether social context can modulate age effects on male/female reproductive success in *D. melanogaster*. A general prediction is that pre-/postmeiotic ageing processes will lead to a decline in the pre- and post-copulatory abilities of both males and females (David *et al.* 1975; Economos *et al.* 1979; Service and Fales 1993; Holmes *et al.* 2003; Kühnert and Nieschlag 2004; Pizzari *et al.* 2008; Deng 2012; Tan *et al.* 2013; Firman *et al.* 2015). However, inasmuch as the sexes have different strategies to optimize their fitness, the decline in reproductive success late in life can be modulated by social context, such as sex ratio, in a sex-specific manner. Although social context has been found to modulate lifespan and ageing in a sex-specific way (Botev 2012; Leech *et al.* 2017), how it affects reproductive senescence has not been well-studied. As expected, we found that male and female age caused a decrease in reproductive success across male-biased and female-biased social contexts but, contrary to previous findings, we did not find social context to modulate the age-related fitness decline in either of the two sexes. The fact that we did not find a sex-specific effect of social context does not prove the absence of such an effect. This experiment was preliminary and future studies are essential to explore the very interesting possibility that social context may change the relative importance of different sexual selection mechanisms in a sex-specific way. Specifically, behavioural and fitness assays aimed to understand how age-related mate choice changes across a wider set of social contexts than we tested (e.g. manipulating sex ratio and density) are necessary. We believe this type of studies are essential for a better understanding of how sexual selection operates in complex (natural) environments.

In Chapter 5, we explored whether condition dependent mortality can modulate reproductive senescence. Recent studies show that non-random mortality of 'low-

condition' individuals can lead to an increase in average lifespan (Chen and Maklakov 2012; Maklakov *et al.* 2015). This is a relatively novel finding that can contribute significantly to our understanding of how extrinsic mortality can shape ageing, a classic conundrum. However, selective disappearance of low-condition individuals may also affect average reproductive senescence at the population level due to trade-offs between physiological functions related to survival/lifespan and the maintenance of reproductive functions (Chen *et al.* 2016). Here, we addressed the idea that condition dependent extrinsic mortality (i.e., simulated predation) may, through selective disappearance, increase the average age-related decline in male reproductive success. Although female reproductive senescence was not affected by predation, male reproductive senescence was considerably higher under predation, due mainly to an accelerated decline in offspring viability of 'surviving' males with age (vs. non-surviving males). This sex-specific effect suggests that condition dependent extrinsic mortality can exacerbate survival-reproduction trade-offs in males, which are typically under stronger condition dependent selection than females. An exciting future line of research here would be to confirm the existence of trade-offs between (1) anti-predatory escape ability and functional ageing and (2) anti-predatory escape ability and reproductive ageing in males. It would also be exciting, and potentially fruitful, to explore the mechanisms underlying these trade-offs.

In addition to underlining the importance of potential sex-specific life history trade-offs, our results also support the recent proposal that male ageing can be an important source of sexual conflict (Dean *et al.* 2007, 2010; Carazo *et al.* 2011). Inasmuch as the 'surviving' males have lower offspring viability, mating with an old male becomes particularly costly for females in the presence of condition dependent mortality. Therefore, our results suggest that male ageing can lead to sexual conflict and this effect can be exacerbated in the wild (where condition dependent extrinsic mortality is likely common).

In Chapter 6, our main aim was to explore the role that male gut microbiota composition may play in relation to male life history traits. We believe gut microbiota could play a particularly relevant role given its dependence on ecology and, particularly, given how inter-individual differences in rearing environment, foraging ability and condition are likely to affect it (Erkosar and Leulier 2014; Macke *et al.* 2017). All of these factors are likely to change across the sexes. *Drosophila melanogaster* is a good model to address this because its gut microbiome is particularly plastic and relatively simple, compared to other insects such as cockroaches (Domínguez-Santos *et al.* 2020). We did not find clear evidence of trade-offs between any of the life history traits that we investigated, but we did find age-related changes in bacterial abundance and diversity that were generally consistent with what has been found previously (Wong *et al.* 2011). We also found tantalizing evidence that gut microbiota composition and male life history traits (mainly early reproductive success and ageing) might be linked. The most interesting result is that the genus *Acetobacter* (Proteobacteria phylum) seemed to be particularly important for ageing because (1) its abundance changed with age in general and (2) amplicon sequence variants (ASVs) associated with short and long-lived isolines were all from the *Acetobacter* genus. In this study, we used DGRP isolines instead of wild-type flies because this allowed me to characterize the life history traits of different genotypes while sacrificing flies with the same genotype early and late in life for gut microbiota characterization. In the future, we think it will be tantamount to replicate this study using heterozygotes from DGRP line crosses or wild-type males from isofemale inbred lines to see whether different genetic backgrounds will lead to different findings.

Sex-specific ageing and maladaptive processes

It has long been noted that asymmetric inheritance of different genetic elements (from mitochondria to the sex chromosomes) can contribute to sex-specific ageing (Camus *et al.* 2012; Maklakov and Lummaa 2013; Carazo *et al.* 2016;

Brown *et al.* 2020b). In this thesis, we focused on the potential role of sex-chromosomes, and more specifically on the "unguarded-X" and "toxic Y" hypotheses. It is important to note that these two hypotheses are complementary, and hence both are likely to contribute to explain broad taxonomic patterns in sex-specific ageing. In Chapter 7, we did an empirical study to test a fundamental prediction of the "unguarded-X" hypothesis in *D. melanogaster.* Whereas in Chapter 8, we did a meta-analysis across vertebrates to test how sex determination system and the relative importance of sex chromosomes (X vs. Y in mammals and Z vs. W in birds) might affect sex-specific ageing. Therefore, in Chapter 8 the predictions of both the "unguarded-X" and "toxic Y" hypotheses were tested.

First, in Chapter 7, we examined whether inbreeding bridges the lifespan gap in *D. melanogaster*, as predicted by the UXh, across three different social environments (i.e., isolation, same sex groups and mixed sexes groups). We found that, across social environments, inbreeding resulted in a greater reduction of female than male lifespan, and that inbreeding effects on fitness did not seem to counterbalance sex-specific effects on lifespan, suggesting they are maladaptative. A potential caveat here is that these results might also be explained by sexually antagonistic genes and sex-specific expression patterns (discussed in details in the discussion part of Chapter 7). In addition, a recent attempt to replicate this in a different lab population of *D. melanogaster* failed to find a similar effect (Brengdahl *et al.* 2018). All in all, the jury is thus still out on the possibility that the "unguarded-X" may play an important role in explaining sex-specific lifespan in *D. melanogaster*. Studying how inbreeding affects sex-specific lifespan in other species is a crucial line of future research.

Second, in Chapter 8, we conducted a comparative meta-analysis where we collected sex-specific lifespan data and correlated it with the sex determination system across vertebrates. We found clear evidence that the heterogametic sex has a relatively shorter lifespan than the homogametic sex across 138 species of

birds, mammals, reptiles and amphibians, as expected if sex chromosomes shape sex-specific lifespans. We also did further analyses with the karyotypes of birds and mammals where we looked at the relationship between the relative size of the sex chromosomes and sex-specific survival. We found that the relative sizes of the X and Z chromosomes are not associated with sex-specific lifespans, contrary to UXh predictions. In contrast, we found that the size of the Y chromosome correlated negatively with male survival in mammals, where toxic Y effects are expected to be particularly strong. This interesting finding suggests that small Y chromosomes benefit male lifespan in mammals. Overall, these results confirm the role of sex chromosomes in explaining sex differences in lifespan (Pipoly *et al.* 2015; Xirocostas *et al.* 2020), but indicate that, at least in mammals, this is better explained by "toxic Y" rather than UXh effects. The findings from the meta-analysis across vertebrates, together with the results of the empirical study in *D. melanogaster*, suggest that the asymmetric inheritance of the sex chromosomes can be a significant contributor to sex-specific ageing. With the progress in molecular methods, hopefully we will learn more about the content of Y or W chromosomes in heterogametic sex and hence understand how their size can affect sex-specific ageing.

To conclude, for a complete understanding of sex-specific life history evolution, both adaptive and maladaptive hypotheses should be studied in depth. Adaptive processes seem fundamental to understand sex-specific ageing at both the microevolutionary (i.e., within species and/or closely related taxa sharing mating systems and similar ecological niches) and macroevolutionary (i.e., convergence evolution in distant taxa with a similar ecology of sexual selection) levels. Maladaptive asymmetric inheritance mechanisms likely overlay such adaptive processes to produce patterns of sex-specific ageing linked to the evolution of sex chromosomes and, potentially, mitochondria and mito-nuclear interactions. Within this general picture, sex-specific effects of social and environmental factors on reproductive ageing, the mechanisms underlying sex-specific life

history traits and asymmetric inheritance of sex chromosomes all are different pieces of this puzzle to which I hope I have contributed in this thesis.

Chapter 10. Conclusions

The main conclusions of this thesis are:

Chapter 4

- Sex-ratio at mating did not modulate the decline in reproductive success with age in *D. melanogaster* neither in males nor in females. This finding does not imply sex-ratios are not important, but rather suggests that different sexual selection mechanisms could be counterbalancing each other in different ways across different social contexts. Therefore, additional experiments involving behavioural observations are required to fully resolve the complex link between sex-ratio, age and reproductive success.

Chapter 5

- In *D. melanogaster*, we found that condition dependent mortality led to a steeper reproductive senescence in a male, but not a female cohort, indicative of strong survival/reproduction trade-offs in males. This male-specific effect of condition dependent mortality on reproductive senescence fits nicely with current theories. Because males are under stronger selection for both early reproduction and condition dependent extrinsic mortality, trade-offs against reproductive maintenance are expected to be steeper in males than in females. In addition, by imposing reproductive costs to females, male ageing can be a source of sexual conflict, particularly relevant under natural conditions where condition dependent mortality is likely.

Chapter 6

- We found that gut microbiota abundance and diversity changed with male age in *D. melanogaster*. Our results suggest that the genus *Acetobacter* is associated with male ageing. Moreover, the abundance of some taxa was associated with early reproduction and both actuarial and reproductive ageing, suggesting a link between gut microbiota and male life history.

Chapter 7

- As predicted by the "unguarded-X" hypothesis (UXh), inbreeding caused a greater reduction of female than male lifespan in *D. melanogaster*. Moreover, inbreeding effects on fitness did not seem to counterbalance sex-specific effects on lifespan. These results suggest that the former was maladaptative and that UXh may play an important role in the evolution of sex-specific lifespan in this species.

Chapter 8

- Across 138 species of tetrapods including mammals, birds, amphibians and reptiles, we found the heterogametic sex to have lower survival than the homogametic sex after correcting for phylogeny and the intensity of sexual selection. This strongly suggests that sex chromosomes shape sex-specific ageing across vertebrates. Moreover, we found a clear negative correlation between the Y (but not X) chromosome size and male survival in mammals, which could mean that large Y chromosomes shorten male lifespan. This indicates that, at least in mammals, sex-specific lifespan may be affected more by "toxic Y" effects than UXh effects.

Chapter 11. Resumen en castellano

11.1. Antecedentes

Uno de los problemas más complejos en la biología del envejecimiento radica en explicar por qué los machos y las hembras envejecen de manera diferente (Maklakov and Lummaa 2013). El envejecimiento diferencial en los sexos se observa ampliamente a lo largo del árbol de la vida. Por ejemplo, las hembras viven tres veces más que los machos en el antechinus marrón, un pequeño marsupial, mientras que los machos tienen el doble de probabilidades que las hembras de sobrevivir en los charlatanes árabes, un ave paseriforme (Keller and Waller 2002; Clutton-Brock and Isvaran 2007). Se han propuesto varias hipótesis para explicar este fenómeno, derivadas de dos tipos de procesos complementarios pero fundamentalmente distintos: procesos adaptativos y maladaptativos (Maklakov and Lummaa 2013).

En esta Tesis, he abordado algunas de las lagunas existentes en nuestro conocimiento sobre la evolución y la función del envejecimiento diferencial en los sexos. Al hacerlo, no me he centrado en una hipótesis particular, sino que he buscado explorar procesos adaptativos (capítulos 4, 5 y 6) y maladaptativos (capítulos 7 y 8), ambos de los cuales creo que son cruciales para una comprensión completa de por qué los machos y las hembras envejecen de manera diferente. Mi principal objetivo ha sido arrojar algo de luz sobre áreas de investigación que creo que contienen preguntas más abiertas (e interesantes). Esto me ha llevado a abordar una amplia gama de preguntas y a combinar enfoques empíricos muy diferentes, desde ensayos clásicos de comportamiento y eficacia biológica hasta diseños de endogamia más complejos, meta-análisis comparativos y el estudio de la microbiota intestinal.

11.2. Procesos Adaptativos

El envejecimiento diferencial en los sexos con frecuencia resulta de diferencias sexuales en presiones selectivas (Vinogradov 1998; Carranza and Pérez-Barbería 2007; Clutton-Brock and Isvaran 2007; Bonduriansky *et al.* 2008; Berg and Maklakov 2012; Adler and Bonduriansky 2014). Debido a la evolución de la anisogamia (espermatozoides pequeños, baratos pero numerosos de machos, frente a los grandes, caros y escasos óvulos de hembras) y los costes de gestación posteriores, las hembras suelen ser el sexo con mayor inversión parental (Shuster and Wade 2003). Como consecuencia, la selección intrasexual tiende a ser más fuerte en los machos (Bateman 1948, Trivers 1972). A su vez, esto suele favorecer la evolución de diferentes estrategias reproductivas en los sexos (Janicke *et al.* 2016) y, con frecuencia, de historias de vida divergentes. Por ejemplo, comparado con las hembras, la intensa competencia intrasexual tiende a promover estrategias de vida rápida y muerte joven ("live fast die young") en machos (Trivers 1972; Promislow 1992; Kruger and Nesse 2004; Clutton-Brock and Isvaran 2007).

En este contexto, factores externos como el contexto social, la depredación o las enfermedades pueden ser imprescindibles para entender el envejecimiento de hembras y machos. Dentro de este marco, en esta tesis me propuse contribuir a tres preguntas de investigación que creo que tienen un gran potencial para ampliar nuestra comprensión actual sobre cómo los procesos adaptativos pueden moldear el envejecimiento de forma diferencial en los sexos. En primer lugar, investigué cómo el contexto social (Capítulo 4) y la mortalidad dependiente de la condición física (Capítulo 5) pueden modular los efectos de la edad sobre la eficacia biológica. Con respecto a los mecanismos, exploré el vínculo entre la microbiota intestinal y los rasgos de la historia de vida en machos (i.e., la esperanza de vida, el envejecimiento actuarial/reproductivo/funcional y el éxito reproductivo, Capítulo 6).

11.2.1. Capítulo 4. Contexto social y envejecimiento reproductivo en Drosophila melanogaster

Introducción

Comprender los efectos de la edad de machos y hembras en el éxito reproductivo es vital para explicar la evolución de los rasgos de la historia de vida y el envejecimiento diferencial en los sexos. Una predicción general es que los procesos de envejecimiento pre/post-meiótico conducirán a una disminución en las capacidades pre y post-copulatorias de machos y hembras (David *et al.* 1975; Economos *et al.* 1979; Service and Fales 1993; Holmes *et al.* 2003; Kühnert and Nieschlag 2004; Pizzari *et al.* 2008; Deng 2012; Tan *et al.* 2013; Firman *et al.* 2015). Sin embargo, en la medida en que los sexos exhiben estrategias diferentes para optimizar su eficacia biológica, el envejecimiento puede ser modulado por el contexto social, como la proporción de sexos, de manera diferencial en los sexos. Por ejemplo, al influir en factores como la tasa de encuentro de pareja, la elección de pareja o la competencia intrasexual (Kvarnemo and Ahnesjo 1996; Kokko and Rankin 2006), el contexto social tiene el potencial de modular la senescencia reproductiva.

Métodos y Resultados

En este estudio, utilizamos *D. melanogaster* para investigar si la proporción de sexos modula los efectos de la edad en el éxito reproductivo en machos y hembras. Como esperabamos, encontramos que la edad causó una disminución en el éxito reproductivo tanto de machos como hembras, en todos los contextos sociales. Sin embargo, el contexto social no moduló la disminución de la eficacia biológica con la edad en ninguno de los dos sexos.

Discusión

Como era de esperar, descubrimos que la edad causa una disminución en el éxito reproductivo en machos y hembras, pero, en contra de lo esperado, no encontramos un efecto claro del contexto social. No obstante, esto no prueba la ausencia de tal efecto porque los diferentes mecanismos de selección sexual podrían estar equilibrándose entre sí de diferentes maneras en diferentes contextos sociales. Este experimento fue preliminar y harán falta estudios futuros para explorar la posibilidad de que el contexto social pueda cambiar la importancia relativa de los diferentes mecanismos de selección sexual en los sexos, y moldear su envejecimiento.

11.2.2. Capítulo 5. Mortalidad dependiente de la condición, senescencia reproductiva y conflicto sexual en D. melanogaster

Introducción

Estudios recientes sugieren que considerar el papel de la mortalidad extrínseca dependiente de condición es clave para comprender la evolución de las historias de vida (Reznick *et al.* 2004; Chen and Maklakov 2012; Maklakov *et al.* 2015). Por ejemplo, la mortalidad diferencial de individuos de baja condición puede conducir a un aumento en la esperanza de vida promedio en la población al seleccionar individuos en buena conducción (Chen and Maklakov 2012; Maklakov *et al.* 2015). Sin embargo, esta desaparición selectiva también puede afectar la senescencia reproductiva a nivel de la población debido a compromisos adaptativos entre funciones fisiológicas relacionadas con la supervivencia/esperanza de vida y el mantenimiento de las funciones reproductivas (Chen *et al.* 2016). En otras palabras, los individuos que son buenos para sobrevivir podrían ser los que son malos para el mantenimiento reproductivo, lo que lleva a una población que consiste en individuos con alto nivel de senescencia reproductiva.

Métodos y Resultados

En este capítulo estudiamos el efecto de la mortalidad extrínseca dependiente de la condición (i.e., depredación simulada dependiente de la velocidad de escape) sobre el envejecimiento reproductivo. Para ello, estudiamos el envejecimiento reproductivo en cohortes de machos y hembras de *D. melanogaster* expuestas (o no) a depredación simulada dependiente de la condición a lo largo de su vida. Aunque la senescencia reproductiva no se vio afectada por la depredación en hembras, en machos fue considerablemente mayor con depredación, debido principalmente a una disminución acelerada (con la edad) en la viabilidad de la descendencia de los machos "supervivientes".

Discusión

Los resultados demuestran que la mortalidad extrínseca dependiente de la condición (es decir, la depredación simulada) exagera, a nivel de la cohorte, el envejecimiento reproductivo de los machos. Aunque la senescencia reproductiva en hembras no se vio afectada por la depredación, la senescencia reproductiva en machos fue considerablemente mayor con depredación simulada sobre los individuos de baja condición. Esto se debe fundamentalmente a que los machos supervivientes, de alta condición, muestran una disminución acelerada en la viabilidad de su descendencia con la edad. Este efecto específico de sexo sugiere que la mortalidad extrínseca dependiente de la condición puede exacerbar el compromiso adaptativo entre supervivencia y reproducción en los machos, que generalmente están bajo una selección dependiente de condición más fuerte que las hembras. Además, estos resultados apoyan la reciente propuesta de que el envejecimiento reproductivo en machos puede ser una fuente importante de conflicto sexual (Dean *et al.* 2007, 2010; Carazo *et al.* 2011) porque las hembras que se aparean con viejos machos sobrevivientes pagarán los costos de la baja reproducción. Más aún, sugieren que este efecto podría exacerbarse en la

naturaleza (donde la mortalidad extrínseca dependiente de la condición es probablemente común).

11.2.3. Capítulo 6. La relación entre la microbiota intestinal y la historia de vida en machos de D. melanogaster

Introducción

Explorar los mecanismos subyacentes a los rasgos de la historia de vida en machos y hembras puede ayudarnos a entender por qué machos y hembras con frecuencia evolucionan historias de vida distintas. Una línea de investigación emergente en el estudio de los mecanismos del envejecimiento, y los mecanismos de la historia de vida en general, es el papel de la microbiota intestinal. Estudios recientes han explorado la importancia de la microbiota intestinal en la esperanza de vida, el envejecimiento y la reproducción en hembras de una variedad de taxones como *Drosophila* (Brummel *et al.* 2004; Ren *et al.* 2007; Clark *et al.* 2015; Gould *et al.* 2018), *Daphnia* (Sison-Mangus *et al.* 2015; Callens *et al.* 2016), o *Caernohabditis* (Houthoofd *et al.* 2002; Cabreiro and Gems 2013), además de en humanos (Tiihonen *et al.* 2010; Insenser *et al.* 2018). Por el contrario, sabemos muy poco sobre el vínculo entre el éxito reproductivo y la microbiota intestinal en los machos. Si dichos vínculos divergen entre los sexos, esto podría permitirnos comprender mejor el envejecimiento específico de sexo desde una perspectiva mecanicista, y a la vez alumbrar su evolución.

Métodos y Resultados

En este estudio exploramos el papel de la microbiota intestinal en los rasgos de la historia de vida en machos al examinar la co-variación entre envejecimiento funcional, reproductivo y actuarial y los cambios en la microbiota intestinal a lo largo de la vida. Para hacerlo, primero caracterizamos los rasgos de la historia de vida de machos de *D. melanogaster* de 29 diferentes isolinas endogámicas DGRP. Luego caracterizamos la microbiota intestinal temprana y tardía de estas

isolíneas e investigamos cómo cambia la composición de la microbiota intestinal con la edad. Finalmente, exploramos el posible vínculo entre los rasgos de la historia de vida y los cambios en microbiota intestinal. En primer lugar, no detectamos evidencia clara de compromisos adaptativos entre el éxito reproductivo y los rasgos asociados con la supervivencia. Las correlaciones entre rasgos de historial de vida que detectamos tendieron a ser positivos. En segundo lugar, como se ha mostrado anteriormente en la literatura, encontramos cambios relacionados con la edad en la diversidad y abundancia de algunos taxones de la microbiota intestinal de machos. Finalmente, encontramos evidencia preliminar de un vínculo entre la abundancia de la microbiota intestinal y el éxito reproductivo temprano en machos.

Discusión

No encontramos pruebas claras de la existencia de compromisos adaptativos entre ninguno de los rasgos de la historia de vida que investigamos, pero sí encontramos cambios relacionados con la edad en la abundancia y diversidad bacteriana que generalmente son consistentes con lo que se ha encontrado en estudios previos (Wong *et al.* 2011). También encontramos evidencia de que la composición de la microbiota intestinal y los rasgos de la historia de vida en machos (principalmente el éxito reproductivo temprano y el envejecimiento) podrían estar relacionados. En nuestro estudio, el género *Acetobacter* (Alphaproteobacteria) parece tener una importancia particular con respecto al envejecimiento porque (1) su abundancia cambió con la edad en general y (2) las variantes de secuencia de amplicón (ASV) asociadas con las isolíneas de corta y larga vida eran todas del género *Acetobacter*. En este estudio, usamos isolíneas DGRP en lugar de moscas de tipo salvaje porque las isolíneas DGRP nos permitieron caracterizar los rasgos de la historia de vida de diferentes genotipos al tiempo que sacrificamos moscas con el mismo genotipo pronto y tarde en la vida para la caracterización de la microbiota intestinal. En el futuro, creo que será

valioso replicar este estudio utilizando heterocigotos de cruces de línea DGRP o machos de tipo salvaje de líneas endogámicas matrilineales para ver si diferentes antecedentes genéticos conducirán a diferentes resultados.

11.3. Procesos Maladaptativos

A diferencia de las hipótesis adaptativas, las hipótesis maladaptativas explican las diferencias sexuales en el envejecimiento como consecuencia de la herencia asimétrica de diferentes componentes del genoma (Maklakov and Lummaa 2013). En primer lugar, la hipótesis de la "maldición de la madre" (en inglés “mother’s curse”) propone que el genoma mitocondrial puede desempeñar un papel fundamental en la disminución de la esperanza de vida de los machos, lo que contribuiría a explicar el envejecimiento específico del sexo en taxones donde las hembras viven más que los machos (Camus *et al.* 2012). En segundo lugar, la hipótesis del "cromosoma X desguarnecido" (en inglés “unguarded-X”, en adelante “UXh”) postula que el envejecimiento diferencial en los sexos puede deberse en parte a la mayor expresión de mutaciones recesivas perjudiciales en el sexo heterogamético (“desprotegido” frente a su expresión por solo disponer de una copia de cada cromosoma), debido a la herencia asimétrica de los cromosomas sexuales (Trivers 1985). Finalmente, la hipótesis más reciente del “cromosoma Y tóxico" (en inglés “toxic Y”) se centra en el papel del cromosoma heteromórfico Y (o W) en el envejecimiento (Marais *et al.* 2018). En esta tesis, me concentré en las dos hipótesis que exploraron el papel de los cromosomas sexuales en la evolución del envejecimiento diferencial en los sexos.

11.3.1. Capítulo 7. La hipótesis del "cromosoma X desguarnecido" y la arquitectura genética de la esperanza de vida en machos y hembras de D. melanogaster

Introducción

La hipótesis del "cromosoma X desguarnecido" (UXh) sugiere que el envejecimiento específico de sexo puede deberse en parte a la expresión de mutaciones recesivas en los cromosomas sexuales hemizigotos del sexo heterogamético, lo que podría ayudar a explicar el envejecimiento diferencial en los sexos en multitud de taxones (Trivers 1985). Los estudios que analizan la correlación entre el envejecimiento de cada sexo y el sistema de determinación del sexo han proporcionado apoyo indirecto a esta hipótesis (Pipoly *et al.* 2015; Xirocostas *et al.* 2020). Pipoly *et al.* (2015) usaron el cociente sexual entre adultos como un proxy para la supervivencia específica de sexo y descubrieron que las proporciones de sexo de los adultos suelen estar sesgadas hacia las hembras en taxones con sistema de determinación de sexo XY, y al contrario en las que tienen un sistema de determinación de sexo ZW. Más recientemente, Xirocostas *et al.* (2020) encontraron que el sexo heterogamético tiende a tener una vida media/máxima más alta en un amplio rango taxonómico, aunque en este estudio no corrigieron ni por filogenia ni por la intensidad de selección sexual. Finalmente, la evidencia experimental reciente sugiere que desproteger el cromosoma X puede reducir la brecha de la vida sexual en *D. melanogaster* (Carazo *et al.* 2016 pero véase Brengdahl *et al.* 2018).

Métodos y Resultados

Una predicción central de la hipótesis UX es que la endogamia disminuirá la esperanza de vida del sexo homogamético más que la del sexo heterogamético, porque solo en la primera la endogamia aumenta la expresión de mutaciones deletéreas recesivas del cromosoma X (o Z). En este estudio, probamos esta

predicción examinando los efectos de la endogamia en la esperanza de vida y la eficacia biológica de *D. melanogaster* en diferentes entornos sociales. Encontramos que, en distintos entornos sociales (i.e., aislamiento, grupos del mismo sexo y grupos de sexos mixtos), la endogamia resultó en una mayor reducción de la esperanza de vida en machos que en hembras, y que los efectos de la endogamia en la eficacia biológica no contrarrestaron los efectos específicos de sexo en la esperanza de vida, lo que sugiere que los primeros son maladaptativos.

Discusión

En todos los entornos sociales, la endogamia resultó en una mayor reducción de la esperanza de vida en hembras que en machos. Además, los efectos de la endogamia en el éxito reproductivo no contrarrestaron los efectos específicos de sexo en la vida útil, lo que sugiere que estos efectos son maladaptativos. Aunque nuestros resultados apoyan la UXh, también podrían explicarse por el efecto de genes sexualmente antagónicos y patrones de expresión específicos de sexo (discutidos en detalle en la parte de discusión del Capítulo 7). Además, un intento reciente de replicar nuestro trabajo en una población de laboratorio diferente de *D. melanogaster* no logró encontrar un efecto similar (Brengdahl *et al.* 2018). Replicar este estudio en otras poblaciones y especies es, por tanto, una línea crucial de investigación futura.

11.3.2. Capítulo 8. Envejecimiento diferencial en los sexos en tetrápodos: evidencia de un efecto tóxico del cromosoma Y

Introducción

Una hipótesis alternativa a la UXh es que la acumulación de mutaciones perjudiciales y elementos repetitivos en el cromosoma Y/W podría reducir la supervivencia del sexo heterogamético ("toxic Y"). Durante la evolución de los cromosomas sexuales, la supresión de recombinación conduce a la acumulación

de mutaciones deletéreas y de ADN repetitivo (secuencias satélite y elementos transponibles) en los cromosomas Y/W (Bachtrog 2013; Wright *et al.* 2016). Estudios muy recientes demuestran que, en *D. melanogaster*, algunas secuencias repetitivas de ADN en el cromosoma Y (que se encuentran formando parte de la heterocromatina y, por tanto, no activos) se vuelven activas con la edad, lo que resulta en la expresión disfuncional de elementos transponibles (Brown *et al.* 2020b). Con el fin de probar cómo la represión de los elementos transponibles en el cromosoma Y afecta la esperanza de vida en los sexos, Brown *et al.* (2020) generaron moscas con diferentes cariotipos de cromosomas sexuales: hembras XXY, machos X0 y XYY (además de cariotipos de tipo salvaje), hembras XX y machos XY. Encontraron una correlación positiva entre la represión de los elementos transponibles y el número de cromosomas Y, y una correlación negativa entre el promedio de vida y el número de cromosomas Y (Brown *et al.* 2020b). Además, en otro estudio, Brown *et al.* (Brown *et al.* 2020a) descubrieron que el cromosoma Y afecta la integridad de la heterocromatina en todo el genoma, disminuyendo la protección de la heterocromatina en otras secuencias repetitivas que están presentes en cromosomas distintos de Y. Esto puede contribuir aún más a la expresión génica específica de sexo y al dimorfismo sexual en los rasgos de la historia de vida, incluida la esperanza de vida (Brown *et al.* 2020a). Por lo tanto, en *D. melanogaster* existe evidencia sólida de un efecto "toxic Y", donde la acumulación de elementos repetitivos de ADN causa una mayor mortalidad del sexo heterogamético (Wright *et al.* 2016; Marais *et al.* 2018). Sin embargo, el papel de esta hipótesis como factor en la evolución de patrones generales en el envejecimiento en los sexos aún no se ha abordado.

Métodos y Resultados

Mediante un meta-análisis comparado de datos de longevidad y supervivencia (en el que corregimos el efecto de la inercia filogenética y la intensidad de la selección sexual), encontramos una menor supervivencia del sexo

heterogamético a lo largo de 138 especies de aves, mamíferos, reptiles y anfibios. Esto indicaría que los cromosomas sexuales desempeñan un papel importante para entender la esperanza de vida diferencial en los sexos. A continuación, analizamos los cariotipos de aves y mamíferos y descubrimos que los tamaños relativos de los cromosomas X y Z no están asociados con la esperanza de la vida diferencial en los sexos, al contrario de lo que predice la UXh. La UXh predice esta relación porque el tamaño relativo de X (o Z) proporciona una medida de cuánta variación genética en la esperanza de vida esperamos que explique el cromosoma X (o Z). No obstante, encontramos que el tamaño del cromosoma Y correlaciona negativamente con la supervivencia de los machos en mamíferos, donde se espera un efecto “toxic Y” particularmente fuerte.

Discusión

En general, nuestros resultados confirman el papel de los cromosomas sexuales en las diferencias sexuales en la esperanza de vida observadas a lo largo del árbol de la vida (Pipoly *et al.* 2015; Xirocostas *et al.* 2020), e indican que, al menos en los mamíferos, esto parece encajar mejor con un efecto "Y tóxico" que con un efecto de la UXh. Los resultados del meta-análisis en vertebrados, junto con los resultados del estudio empírico realizado en *D. melanogaster*, implican que la herencia asimétrica de los cromosomas sexuales puede contribuir de forma significativa al envejecimiento diferencial en los sexos. Con el progreso en los métodos moleculares, con suerte aprenderemos más sobre el contenido de los cromosomas Y o W en el sexo heterogamético y, por lo tanto, entenderemos con precisión cómo su tamaño puede afectar el envejecimiento en los machos.

11.4. Conclusiones finales

Los resultados de esta tesis ponen de manifiesto cómo para una comprensión completa de la evolución de la historia de vida en los sexos se deben estudiar en profundidad tanto hipótesis adaptativas como maladaptativas. Los procesos

adaptativos parecen fundamentales para comprender el envejecimiento diferencial en los sexos tanto a nivel microevolutivo (es decir, dentro de especies y/o sistemas de apareamiento de taxones estrechamente relacionados y/o nichos ecológicos similares) como macroevolutivo (es decir, evolución convergente en taxones distantes con una ecología de la selección sexual similar). Los procesos maladaptativos derivados de la herencia asimétrica de material genético probablemente se superponen a tales procesos adaptativos para producir patrones de envejecimiento diferencial en los sexos vinculados a la evolución de los cromosomas sexuales. Dentro de esta visión general, los efectos específicos de sexo de los factores sociales y ambientales sobre el envejecimiento reproductivo, los mecanismos subyacentes a los rasgos de la historia de vida específicos de sexo y la herencia asimétrica de los cromosomas sexuales son piezas diferentes para resolver este rompecabezas, a lo que espero haber contribuido en esta tesis.

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Appendix

A.1. Pilot Study of Chapter 5

A.1.2. Materials and Methods

In the pilot study, we first aged "old" females/males in isolation with excess food for 38 days, during which time we flipped them into a new vial once a week. We maintained "young" females/males in isolation for four days followed by mating assays. Then, we aged a separate batch of flies for the same amount of time but, during this time, we simulated condition dependent mortality (i.e., extrinsic mortality) by using climbing speed as a proxy for anti-predatory escape ability. We measured the climbing speed of each experimental fly once every 8 days. Briefly, we introduced each fly into a graduated glass tube, gently tapped the fly to the bottom of the vial and then measured the distance it climbed in 10 seconds, which allowed us to calculate its climbing speed in cm/s. For flies that climbed to the top in less than 10 seconds, the total length of the tube (12 cm) was divided by the time spent to reach the top. Climbing speed was calculated as an average of three successive measurements for each fly at each time point (a total of 5 climbing assays). At the end of this procedure, we selected a cut-off point climbing speed below which 60% of flies were considered as "predated" (i.e., any fly for which we had measured a climbing speed below that point at some point during the ageing treatment). Similar to the first part, we maintained "young" females/males in isolation for four days followed by mating assays. In both ageing treatments (absence and presence of condition dependent predation), young females/males were not tested for climbing speed.

Mating assays were conducted after each ageing treatments by mating pairs with different age combinations by putting a young/old male and a young/old female together into mating vials. Observers blind to treatments measured mating latency and mating duration until the first mating in each vial. Behavioural observations

were conducted in a 25°C room, started when the lights were on (i.e., 10 a.m.), and lasted for 6 hours. Pairs that did not mate within these 6 hours were considered unsuccessful. After the completion of the first mating, males were immediately discarded, while females were kept in the same mating vials where they were allowed to oviposit for 24 hours. At the end of this egg-laying period, we also discarded females and counted the number of eggs they laid during this period. Finally, we incubated vials for 16 days to allow all viable flies to emerge, froze them and then proceeded to count the number of offspring.

To understand how condition dependent mortality modulates the effect of male and female age on reproductive senescence, we run separate analyses for each mortality treatment (i.e., absence vs. presence of condition dependent predation). As the first part (absence of predation) and second part (presence of predation) were conducted at different times, they were analysed separately. To explore how mortality affects the age-related decline in male/female reproductive success (number of offspring), distributional assumptions for linear models could not be met and transformation of the data was not helpful. Therefore, we used the nonparametric Kruskal–Wallis test to analyse whether there is an effect of pair age combination (young/old male x young/old female) on reproductive success. Similarly, we used nonparametric Kruskal–Wallis test to further understand if there is an effect of pair age combination on fecundity and egg-to-adult viability (calculated as the proportion of the number of adults to the number of eggs). For mating duration, we fitted a Linear Model (LM) with male age, female age and their interaction as fixed factors. For mating success, we used GLMs with Binomial error distribution with male age, female age and their interaction as fixed factors. Finally, for mating latency, distributional assumptions for linear models could not be met and transformation of the data was not useful. As a result, we used the Kruskal–Wallis test to analyse whether pair age combination influences latency to mate in the absence and presence of condition dependent mortality. Whenever we found significant effects of age on any given

reproductive trait, we continued with Dunn's multiple comparisons post hoc test (for non-parametric analysis) to further disentangle male and female age effects under different mortality treatments. All analyses were performed in R v. 3.3.2 (R Core Team, 2016).

A.1.2. Results

Age effects on reproductive success

The effect of pair age (i.e., young male-young female, young male-old female, old male-young female, old male-old female) was significant in both the absence and presence of condition dependent mortality (Absence: Kruskal Wallis test, $\chi^2 = 35.964$, df = 3, $p < 0.001$, Presence: Kruskal Wallis test, $\chi^2 = 54.506$, df = 3, $p < 0.001$). To disentangle the effects of male and female age on reproductive success, we run post-hoc Dunn tests (Table S.1). Briefly, old females had lower reproductive success than young ones both in the presence and absence of condition dependent mortality. Conversely, old males had lower reproductive success only in the presence of condition dependent mortality (Table S.1, Figure S.1 & Figure S.2).

Age effects on fecundity

The effect of pair age on fecundity was significant in both the absence and presence of condition dependent mortality (Absence: Kruskal Wallis test, $\chi^2 = 93.263$, df = 3, $p < 0.001$, Presence: Kruskal Wallis test, $\chi^2 = 84.367$, df = 3, $p < 0.001$). To disentangle the effects of male and female age on fecundity, we run post-hoc Dunn tests (Table S.2). Our results showed that fecundity decreases due to female age similarly in both the absence and presence of condition dependent mortality, while male age caused a decrease in female fecundity only in the presence of condition dependent mortality (Figure S.3 & Figure S.4).

Age effects on egg-to-adult viability

The effect of pair age on egg-to-adult viability was not significant in the absence of condition dependent mortality (Kruskal Wallis test, $\chi^2 = 1.126$, df = 3, p = 0.771); whereas, it was significant in the presence of condition dependent mortality (Kruskal Wallis test, $\chi^2 = 16.257$, df = 3, p = 0.001). Post-hoc Dunn test results showed that male age caused a decline in egg-to-adult viability only in the presence of condition dependent mortality. In contrast, the effect of female age on viability was not significant in either the absence or presence of condition dependent mortality (Table S.3, Figure S.5 & Figure S.6).

Age effects on mating success

We did not find a significant interaction between male and female age for mating success in the absence or presence of condition dependent mortality (Absence: $\chi^2 = 0.154$, df = 1, p = 0.695, Presence: $\chi^2 = 0.165$, df = 1, p = 0.685). In the absence of condition dependent mortality, male age effect was marginally non-significant ($\chi^2 = 3.124$, df = 1, p = 0.077), whereas female age effect was significant ($\chi^2 = 7.394$, df = 1, p = 0.006). In the presence of condition dependent mortality, there was no significant effect of male age ($\chi^2 = 0.404$, df = 1, p = 0.525) or female age ($\chi^2 = 1.464$, df = 1, p = 0.226) on mating success (Figure S.7 & Figure S.8).

Age effects on mating latency

The effect of pair age on mating latency was significant in the absence of condition dependent mortality (Kruskal Wallis test, $\chi^2 = 20.279$, df = 3, p < 0.001) but not in the presence of condition dependent mortality (Kruskal Wallis test, $\chi^2 = 6.202$, df = 3, p = 0.102). Post-hoc Dunn test showed that ageing tended to increase mating latency in the absence of condition dependent mortality but this trend was only evident when the mating pair was old (Table S.4, Figure S.9 & Figure S10).

Age effects on mating duration

There was no significant interaction between male and female age neither in the absence nor in the presence of condition dependent mortality (Absence: $F_{1,150}$= 0.011, p = 0.917, Presence: $F_{1,163}$= 1.114, p = 0.293). In the absence of condition dependent mortality, the male age effect on mating duration was significant ($F_{1,151}$= 10.092, p = 0.002) whereas the female age effect was not ($F_{1,151}$= 0.048, p = 0.827). Likewise, in the presence of condition dependent mortality, the male age effect was significant ($F_{1,164}$= 18.496, p < 0.001) whereas the female age effect was not ($F_{1,164}$= 2.696, p = 0.102) (Figure S.11 & Figure S.12).

A.2. Supplementary Tables

Table S.1. Age and mortality effects on reproductive success. Effect of male age, female age and pair age on reproductive success in the absence and presence of condition dependent mortality (pilot study).

Response Variable	Treatment	Condition Dependent Mortality	Z test statistics	P
Reproductive Success (Pilot Study)	Male Age (Old male-Young female)	Absent	-1.225272	0.265
		Present	-3.630763	< 0.001
	Female Age (Old female-Young male)	Absent	-4.462351	< 0.001
		Present	-5.685946	< 0.001
	Pair Age (Old male-Old female)	Absent	-5.007763	< 0.001
		Present	-6.762927	< 0.001

Table S.2. Age and mortality effects on fecundity. Effect of male age, female age and pair age on fecundity in the absence and presence of condition dependent mortality (pilot study).

Response Variable	Treatment	Condition Dependent Mortality	Z test statistics	P
Fecundity (Pilot Study)	Male Age (Old male-Young female)	Absent	-0.916443	0.431
		Present	-2.775416	0.007
	Female Age (Old female-Young male)	Absent	-7.413340	< 0.001
		Present	-7.890024	< 0.001
	Pair Age (Old male-Old female)	Absent	-7.161654	< 0.001
		Present	-7.145784	< 0.001

Table S.3. Age and mortality effects on egg-to-adult viability. Effect of male age, female age and pair age on egg-to-adult viability in the absence and presence of condition dependent mortality (pilot study).

Response Variable	Experiment	Condition Dependent Mortality	Z test statistics	P
Egg-to-adult Viability (Pilot Study)	Male Age (Old male-Young female)	Absent	-0.6411	1.000
		Present	-3.0188	0.008
	Female Age (Old female-Young male)	Absent	-0.1135	0.910
		Present	-0.3902	0.836
	Pair Age (Old male-Old female)	Absent	-0.9472	1.000
		Present	-3.1584	0.009

Table S.4. Age effects on mating latency in the absence of condition dependent mortality. Effect of male age, female age and pair age on mating latency in the absence of condition dependent mortality (pilot study).

Response Variable	Experiment	Z test statistics	P
Mating Latency (Pilot Study)	Male Age (Old male-Young female)	3.5468	0.001
	Female Age (Old female-Young male)	1.8408	0.098
	Male + Female Age (Old pair)	2.8324	0.028

A.3. Supplementary Figures

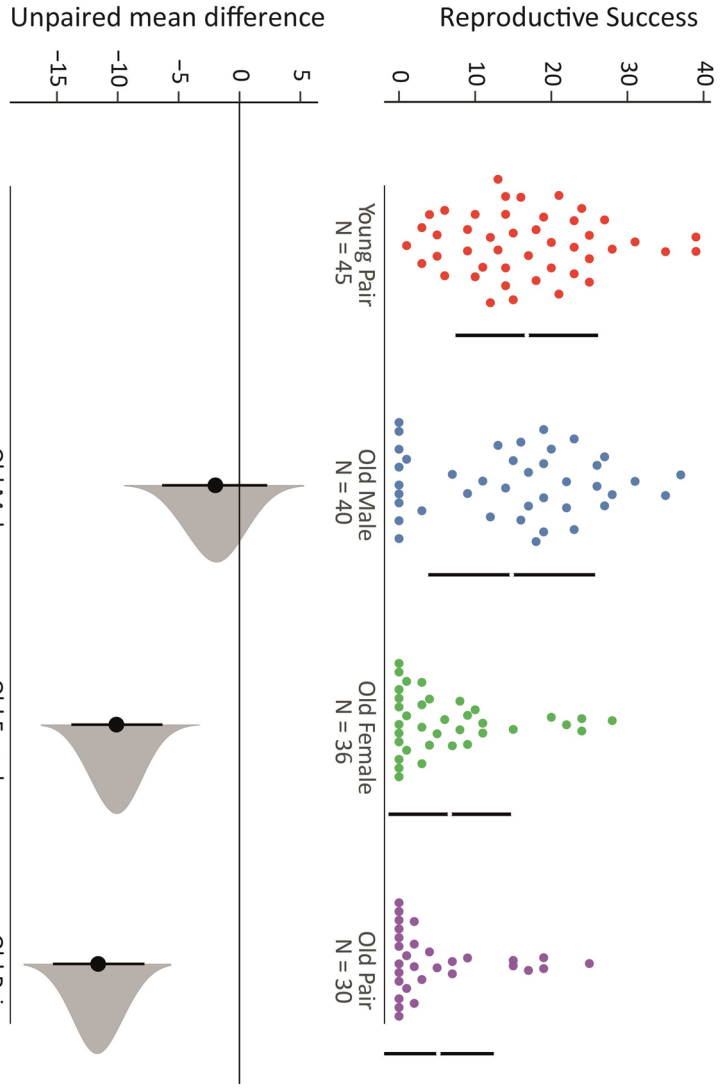

Figure S.1. Effect of male/female age on reproductive success in the absence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

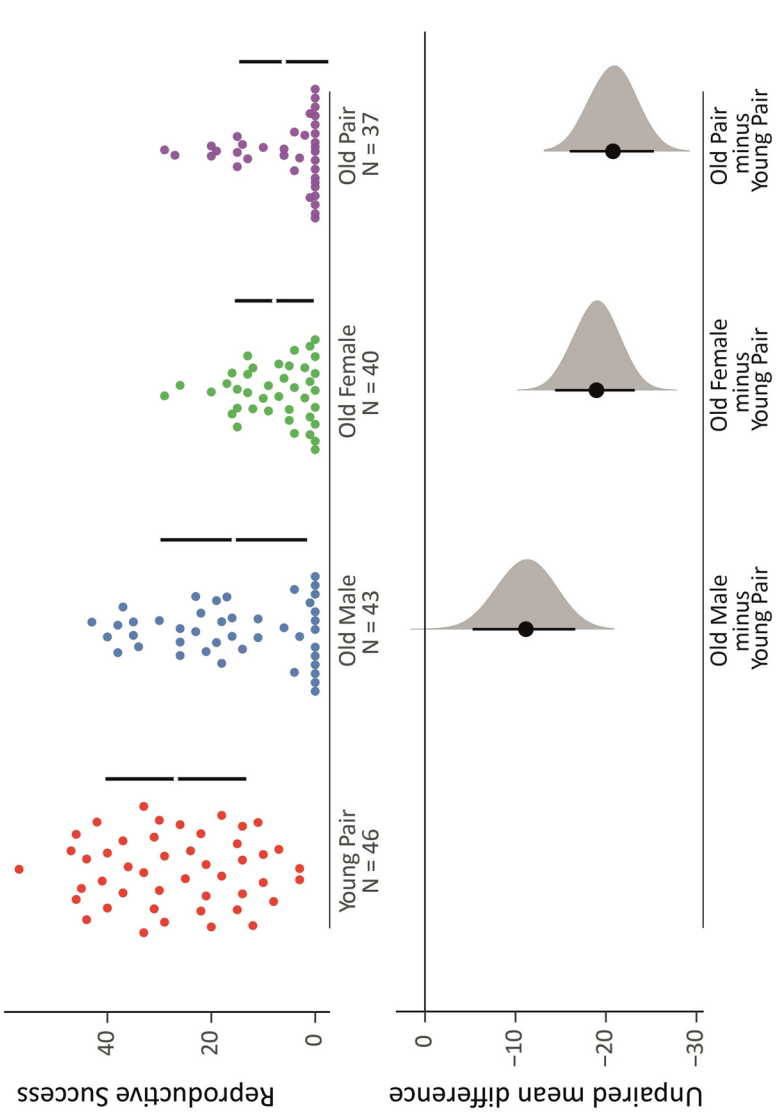

Figure S.2. Effect of male/female age on reproductive success in the presence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

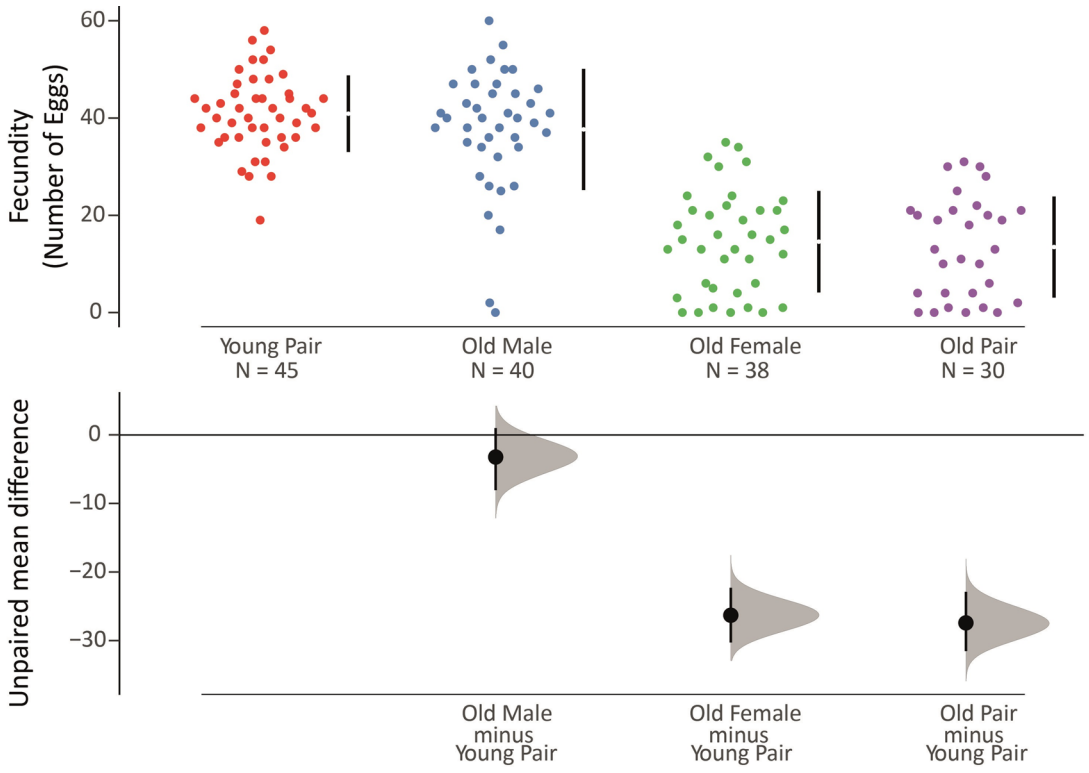

Figure S.3. Effect of male/female age on fecundity in the absence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

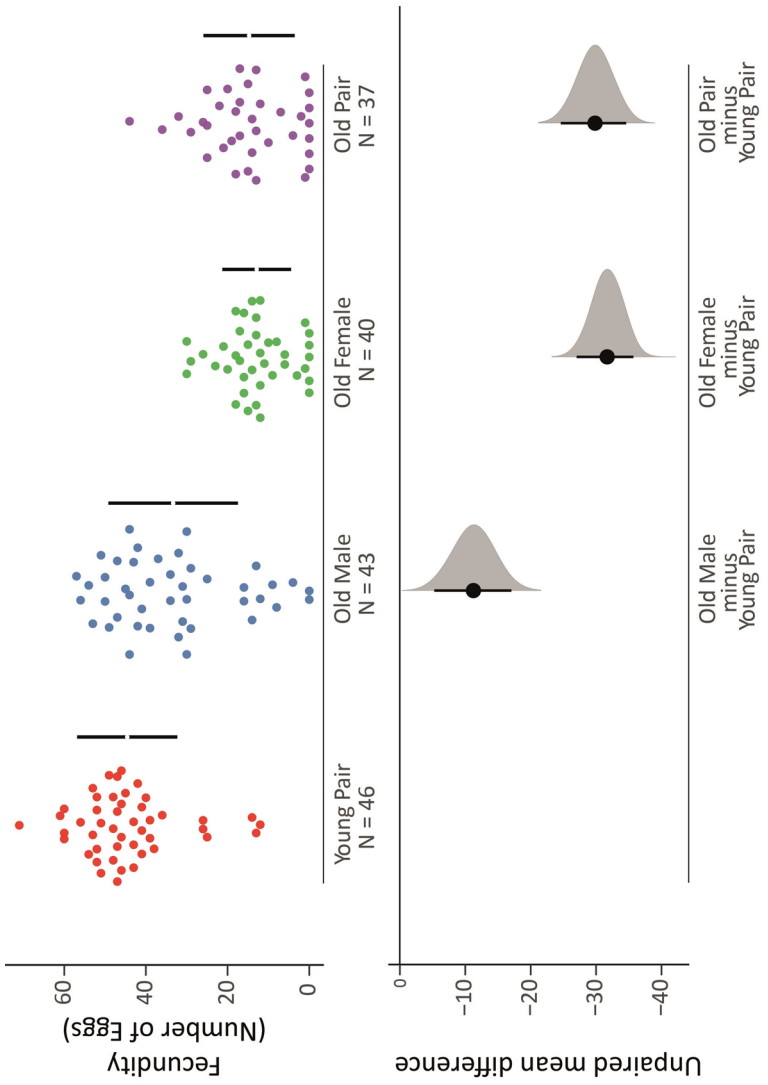

Figure S.4. Effect of male/female age on fecundity in the presence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

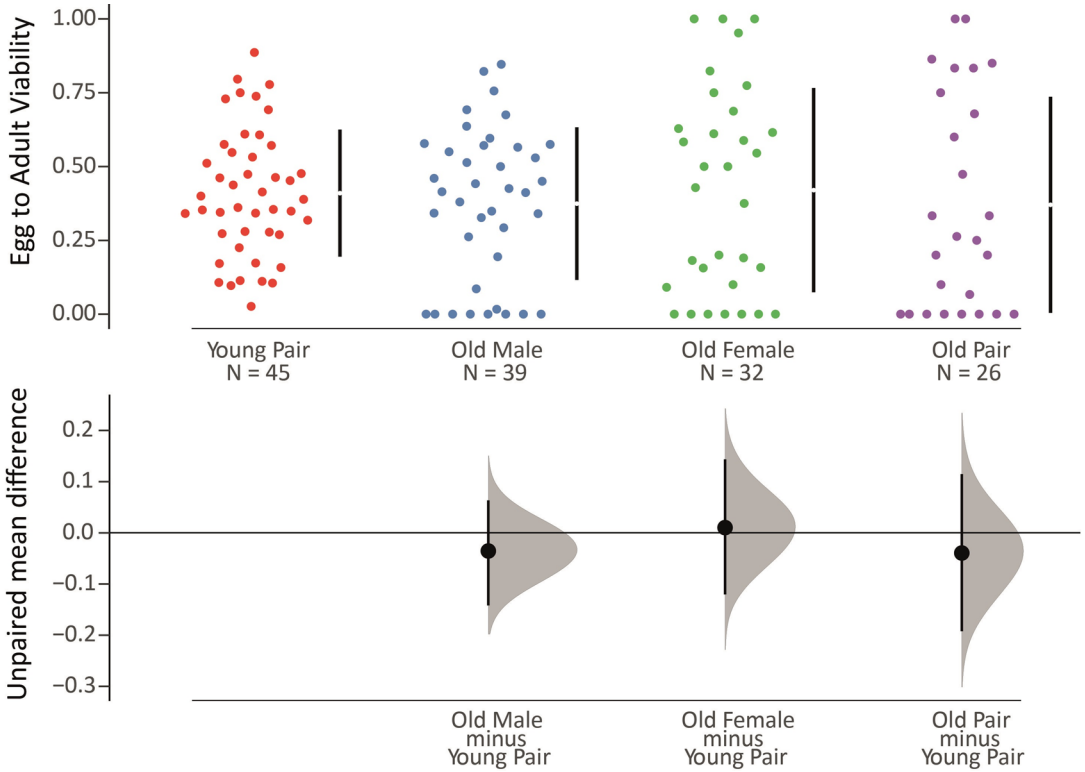

Figure S.5. Effect of male/female age on egg-to-adult viability in the absence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

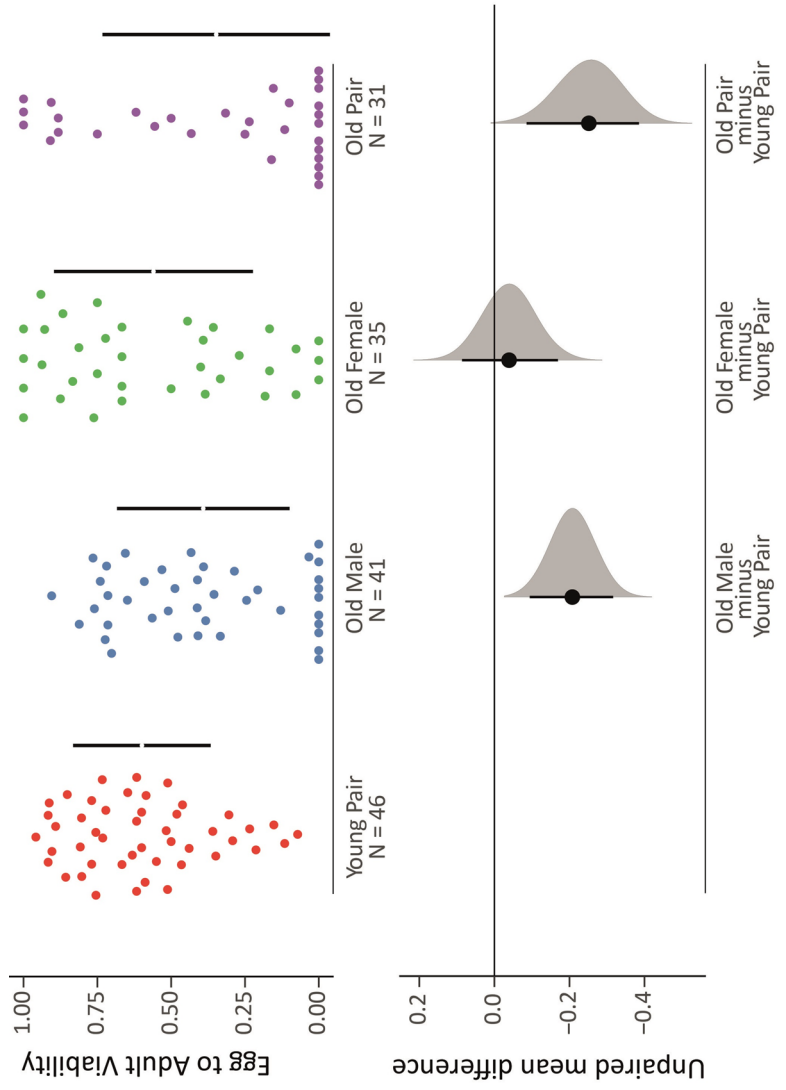

Figure S.6. Effect of male/female age on egg-to-adult viability in the presence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

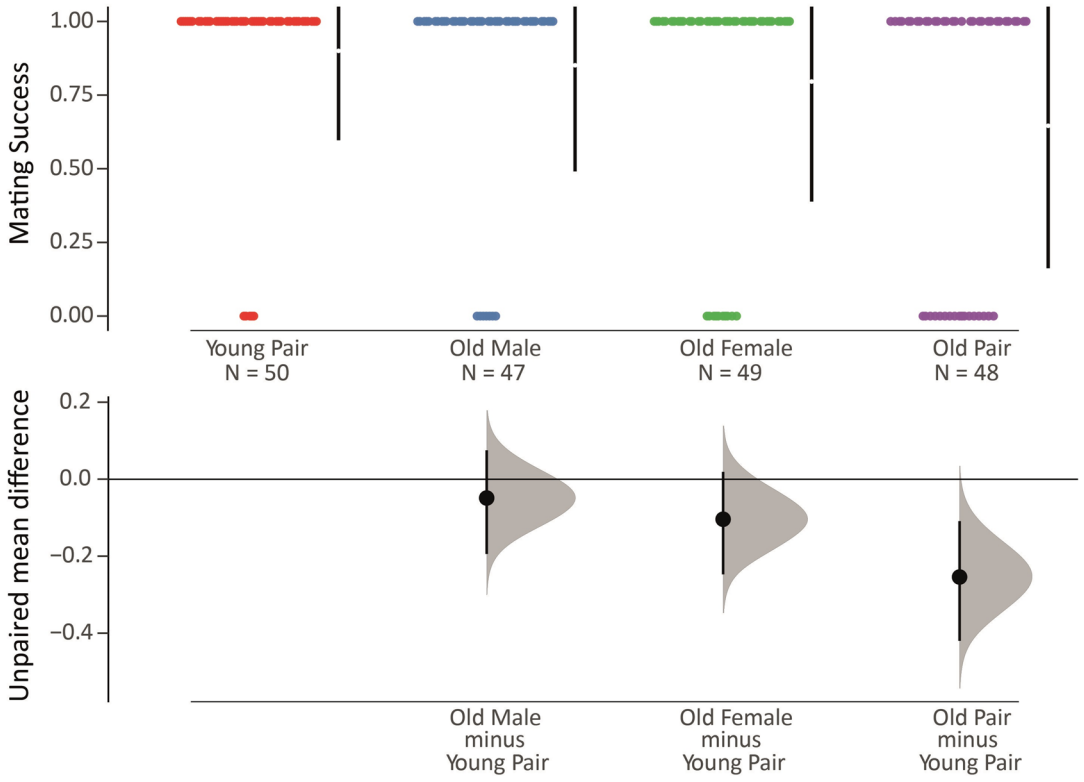

Figure S.7. Effect of male/female age on mating success in the absence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

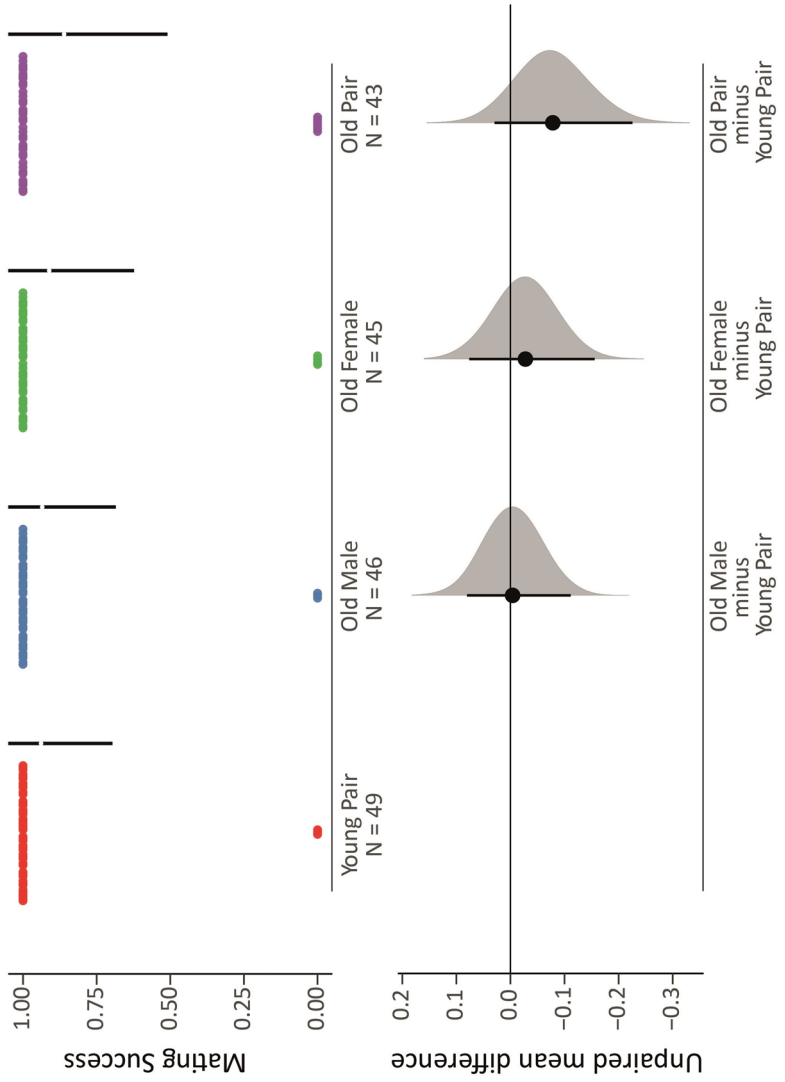

Figure S.8. Effect of male/female age on mating success in the presence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

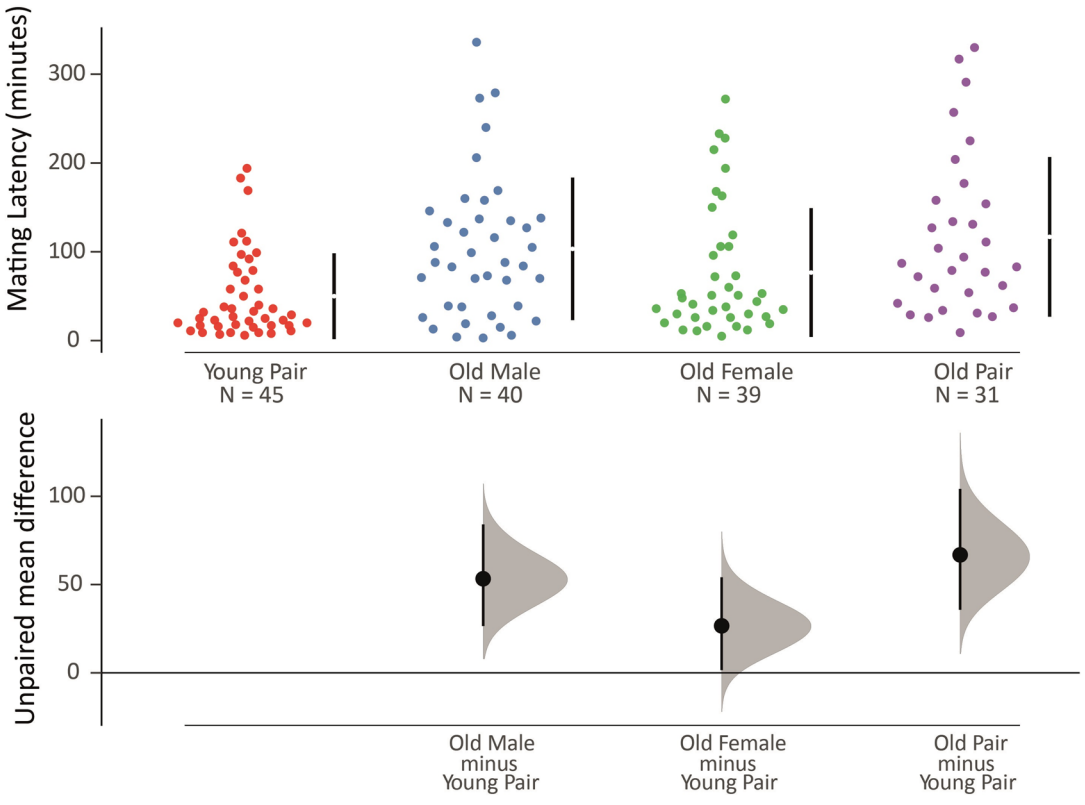

Figure S.9. Effect of male/female age on mating latency in the absence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

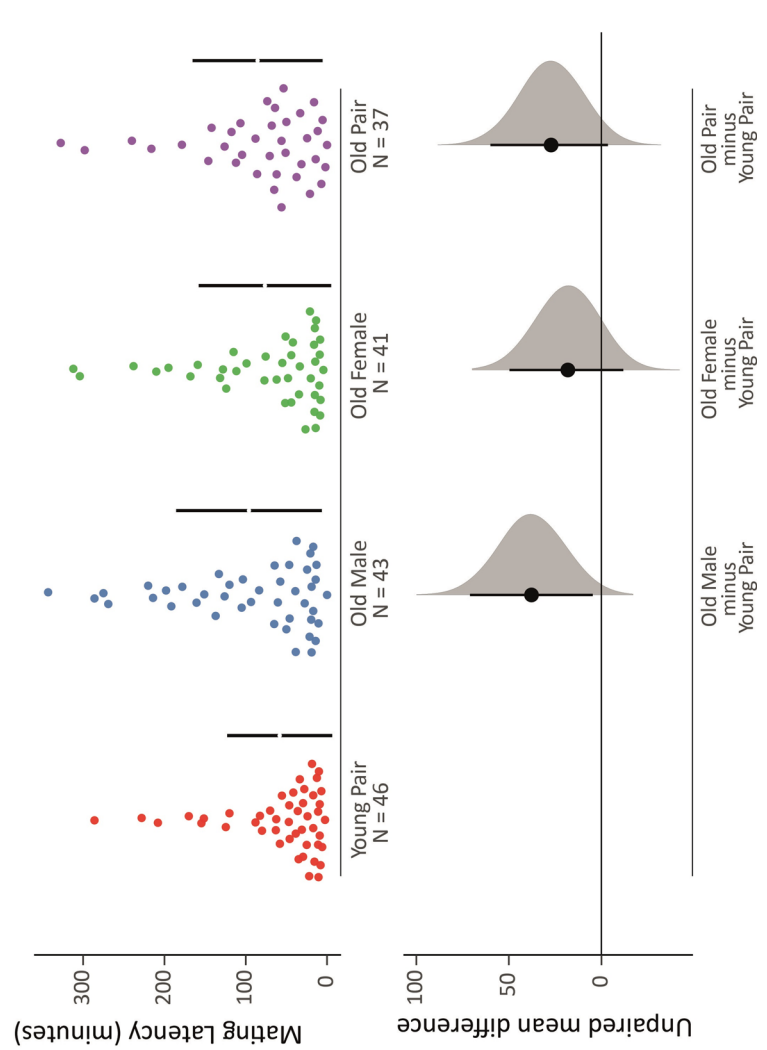

Figure S.10. Effect of male/female age on mating latency in the presence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

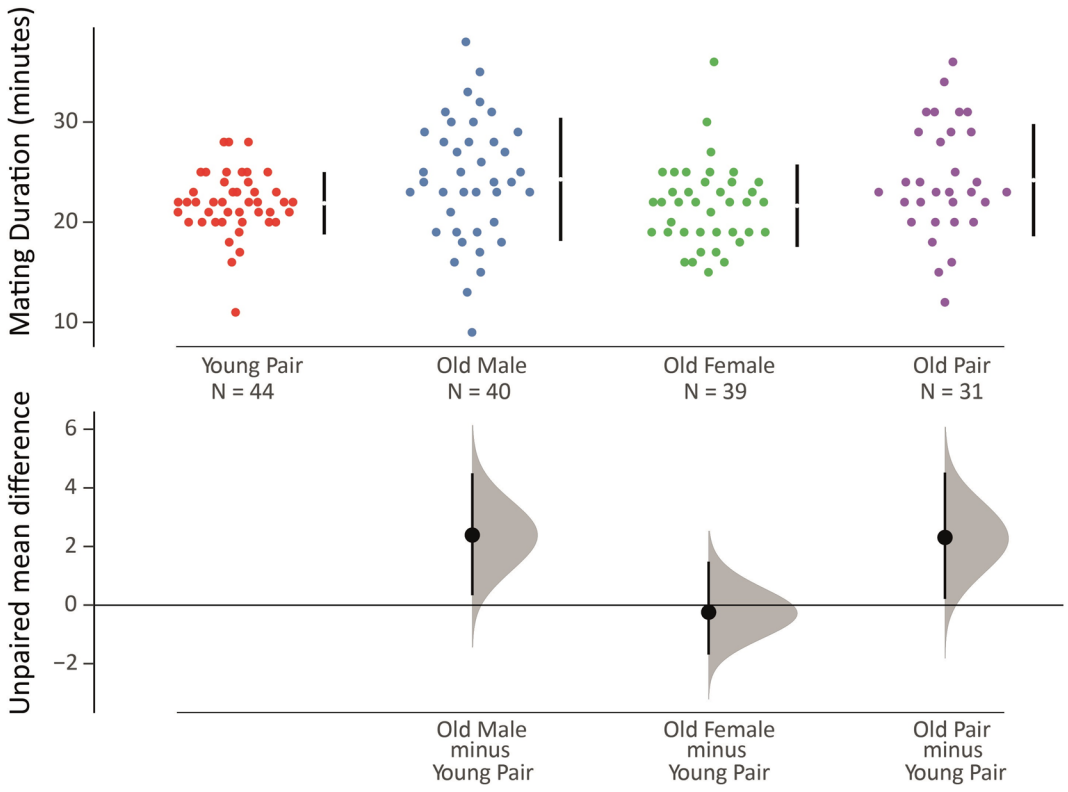

Figure S.11. Effect of male/female age on mating duration in the absence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

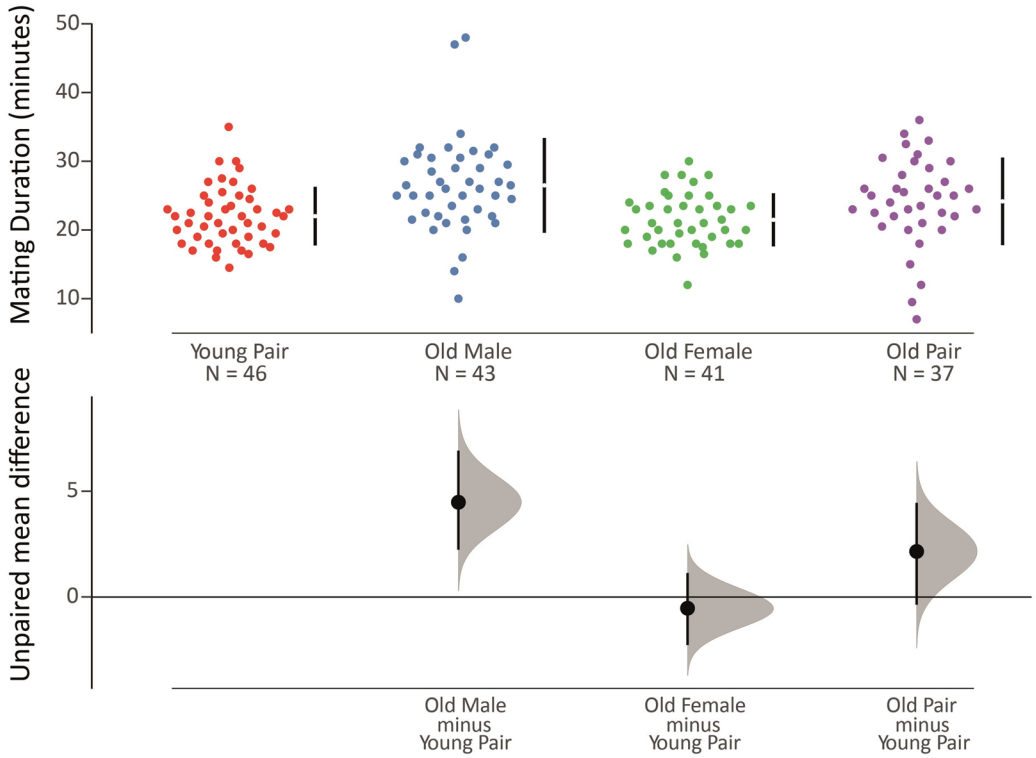

Figure S.12. Effect of male/female age on mating duration in the presence of condition dependent mortality (pilot study). Mean ± standard deviation for each treatment followed by an unpaired mean difference that computes 95% confidence intervals to compare each treatment with the control group (young pair).

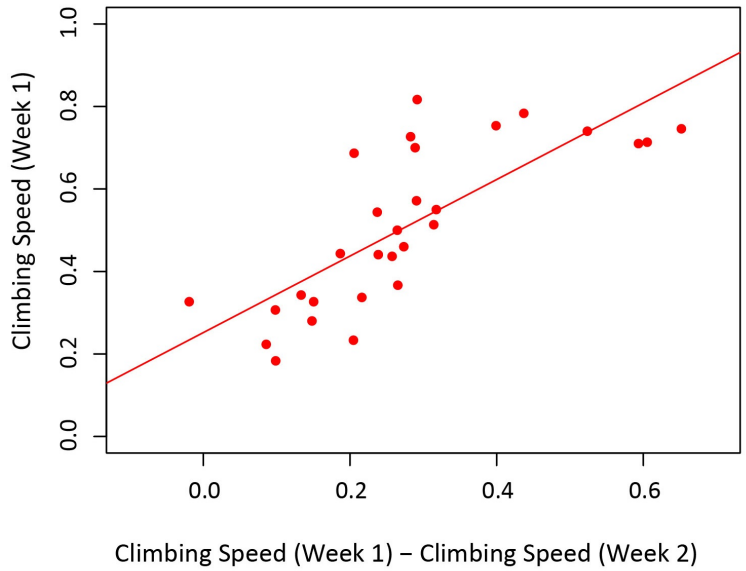

Figure S.13. Correlations between the climbing speed of the first week vs. the decline in climbing speed in two weeks. Climbing speed of the week 1 vs. the difference between week 1 and week 2 for each isoline.

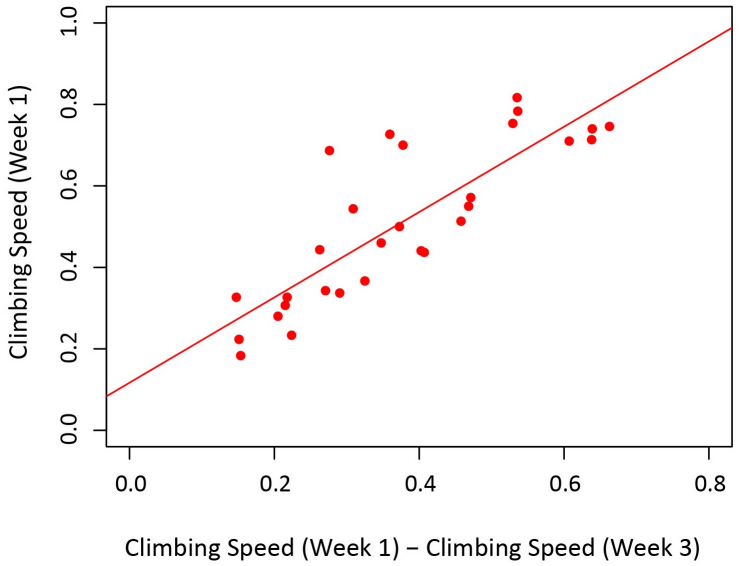

Figure S.14. Correlations between the climbing speed of the first week vs. the decline in climbing speed in three weeks. Climbing speed of the week 1 vs. the difference between week 1 and week 3 for each isoline.

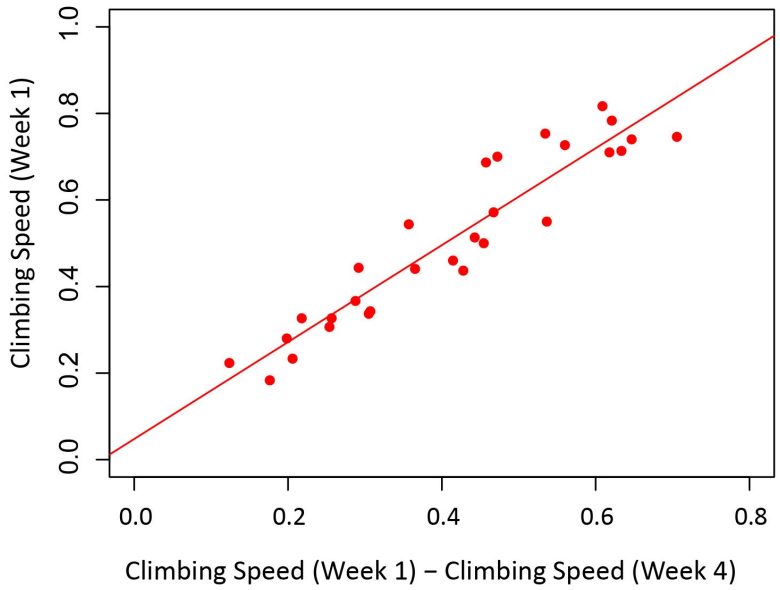

Figure S.15. Correlations between the climbing speed of the first week vs. the decline in climbing speed in four weeks. Climbing speed of week 1 vs. the difference between week 1 and week 4 for each isoline.

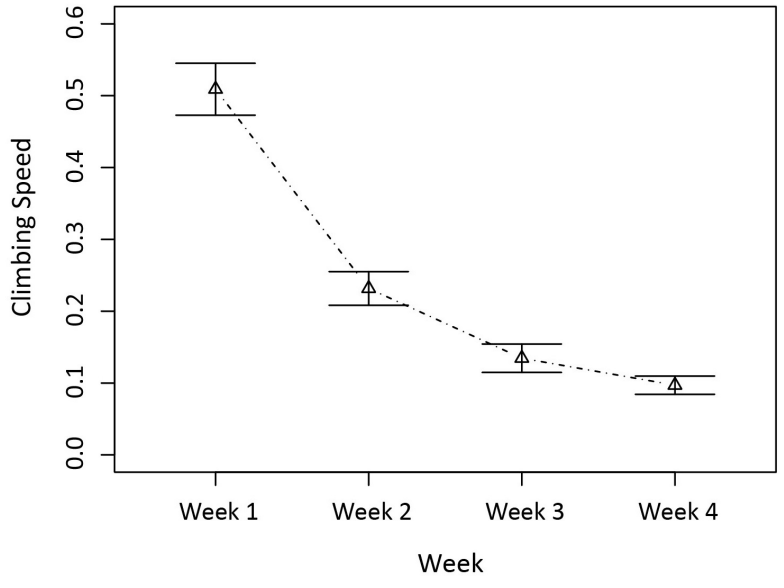

Figure S.16. Age-related decline in average climbing speed. Decline in average climbing speed of isolines per week (mean ± S.E.).