THE EVOLUTIONARY CONSEQUENCES

OF SPERM SENESCENCE IN *Drosophila melanogaster*

by

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Thesis Abstract

Sperm senescence, a decline in sperm quality caused by male ageing and by sperm ageing before or after copulation, may have fitness costs manifested as infertility or lowered genetic quality of offspring. This thesis tested the distinct evolutionary roles of sperm senescence using a laboratory-adapted population of *Drosophila melanogaster*. We developed a practical approach to avoid confounding male age with sperm age by standardizing pre-copulatory sperm age and mating history in young and old male age groups. Applying this approach, we documented sperm senescence in *D. melanogaster* and discussed its potential evolutionary importance. First, ageing males declined in fitness as evidenced by the reduction in fertilization potential of their ejaculates but not by decreased offspring fitness (the ability that a fly can survive to adulthood, successfully mate and produce viable offspring). This suggests a decline in the quality or quantity of seminal fluid or spermatozoa, with no decline in the genetic quality of sperm that actually fertilized ova. Second, post-copulatory sperm senescence has significant negative impacts on offspring fitness, indicating degraded genetic integrity of the spermatozoa stored in females. In both cases, male ageing and sperm ageing had similar fitness impact on male and female offspring, different from what has been suggested by previous work. In addition, We demonstrated that female fecundity, fertility, and length of the fertile period after a single mating were positively associated with the concentration of yeast in their food, and were negatively associated with the duration of yeast restriction in their diet, which suggested that sperm storage is affected by the nutritional status of the females. By revealing the significance of sperm senescence on male and female fertilization success and the fitness of the next generation, this thesis sheds light on a number of evolutionary and applied issues, and provokes new questions for future research on sperm senescence.
Co-Authorship

All data chapters (chapters 2-4) are written in publication format. I am solely responsible for chapter 1 (Thesis Introduction) and chapter 5 (General Discussion). All data chapters (chapters 2-4) are co-authored with my co-supervisors, Drs. Robert Montgomerie and Adam Chippindale. Dr. Robert Montgomerie commented on the data analyses; Dr. Adam Chippindale helped with the experimental designs and provided logistic resources for the experiments. They both provided intellectual support of my research and contributed to the manuscript editing.
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I would like to acknowledge my current and past labmates in the Chippindale and Montgomerie labs for teaching me fly pushing and experimental design, devoting countless hours in bench work, stimulating productive discussion, and being good accompanies. I am also grateful to the Department of Biology, especially the Behavioural Ecology and Evolution Research group for its kind social atmosphere and its supportive environment for training new scientists. Thanks as well to the Natural Sciences and Engineering Research Council of Canada, who funded this project through my co-supervisors’ research grants and my own postgraduate scholarship.

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Statement of Originality

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

Xu Han

March, 2014
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Chapter 1: Thesis Introduction

Overview

The definition and occurrence of sperm senescence

Sperm senescence may result from ageing at two levels: in the germ cells of a male before meiosis; and during and after meiosis (Pizzari et al. 2008). A number of theoretical and empirical studies have demonstrated the influence of senescence on male fertility and offspring quality in humans and other animal groups. Recently, the impact of sperm senescence on male reproductive outcomes began to receive more and more attention from biologists. Although little is known about its mechanisms and evolutionary consequences, the occurrence of sperm senescence has been widely recognized.

In this thesis, senescence is defined as a persistent increase in the probability of mortality or a decline in fertility with advancing age. Sperm senescence, specifically, refers to a decline in the fitness of a spermatozoon with the advancing age of either the male donor or the spermatozoon after production, which is expressed at two stages: fertilization and zygote. At the stage of fertilization, sperm senescence is a decline in the ability of a spermatozoon to fertilize an egg; at the stage of zygote, it is a decline in the fitness of the ensuing zygote.

The senescence of sperm cells, like all cells in sexually reproducing organisms, is a virtually inevitable biological process. Compared to external fertilizers which all have short-lived sperm (Stockley et al. 1996) and sperm mortality is often imposed by extrinsic forces, there may be a greater opportunity for sperm ageing in internal fertilizing animals. This is because females in some taxa have developed long-term sperm storage organs to enhance the survival of sperm cells between copulation and fertilization (Birkhead and Møller 1993).
The duration of sperm storage can be determined using different kinds of observations and is often recorded as the period from the last known copulation to the time when the last viable offspring is produced, given no parthenogenesis, or to the time when the last live sperm cell is observed in the female (Birkhead and Møller 1993). Some sharks and bony fish can store sperm for periods ranging from days to over a year (Pratt 1993; Darling et al. 1980; Muñoz et al. 1999). In amphibians, sperm storage has been found in species in all of the three subgroups (frogs, salamanders and caecilians). Only one species of frogs, the tailed frog, *Ascaphus truei*, is known to store sperm in the female for months or perhaps as long as a year (Sever et al. 2003). Female salamanders in all species in the suborder Salamandroidea store sperm, with a typical duration of five to six months, and a maximum duration of two and half years (Sever 2002). The caecilian *Ichthyophis cf. kohtaoensis* can store sperm for a few weeks (Kuehnel and Kupfer 2012). Sperm storage in reptiles can last from seven days in the American alligator *Alligator mississippiensis* to seven years in the Javan wart snake *Acrochordus javanicus* (Birkhead and Møller 1993). All birds examined to date store sperm. The duration ranges from eight days in the red-tailed hawk *Buteo jamaicensis* to four months in the domestic turkey *Meleagris gallopavo* (Birkhead and Møller 1993). Most mammals store sperm for five to seven days (Holt 2011), but large variations among taxa have also been observed. In marsupials, the span of sperm storage is from a half day in the opposum *Didelphis virginiana* to 16 days in the brown antechinus *Antechinus stuarti* (Birkhead and Møller 1993). The span of sperm storage of most eutherian mammals ranges from 0.6 day in the rat *Rattus norvegicus* to 30 days in the European hare *Lepus europaeus* (Birkhead and Møller 1993). Humans can maintain viable sperm in the female reproductive tract typically for a day or two, but up to about seven days (Orr and Zuk 2012). Bats are exceptional among eutherian mammals because a number of species have evolved the ability to store sperm with duration ranging from 16 days in the Japanese house bat *Pipistrellus abramus* to 6.5 months in...
the noctule bat *Nycatalus noctula* (Birkhead and Møller 1993). But an insect holds the record for duration of sperm storage: queen ants in *Formica exsecta* maintain sperm viability for up to 27 years (Pamilo 1991). In some cases, rapid sperm usage for fertilization will lead to an inability to measure the maximum possible duration of sperm survival in females. Therefore, revealing maximum sperm storage time in any organism may require direct sperm observation and experimental analysis. Nevertheless, the diversity of the duration of sperm storage in internal fertilizers provides a good opportunity to study sperm senescence. It is noteworthy that all aforementioned sperm life spans are measured after copulation and during storage in the female. One should also consider that males in many species ‘hold’ sperm before ejaculation. Although little studied or discussed, this is another stage at which sperm senescence potentially occurs.

**What causes senescence and how does it occur?**

The Evolutionary Theory of Aging explains senescence as resulting from the declining intensity of natural selection with age (Rose 1991), the genetic mechanisms of which can be explained by *mutation accumulation* (Medawar 1946; 1952) and *antagonistic pleiotropy* (Williams 1957). These two mechanisms explain senescence at the population level. Because the force of natural selection that maintains individual survival and fertility declines with increasing age, deleterious mutations that are expressed late in life are under relatively relaxed selection, and are therefore more likely to persist in a population and accumulate across generations. *Mutation accumulation* under the Evolutionary Theory of Aging is a population-level process and should not be confused with the individual-level mutation accumulation that naturally occurs in tissues with age. At the population level, mutations at advanced age cumulate over generations, whereas at the individual level, mutations cumulate within an individual’s life span. *Antagonistic pleiotropy* suggests that there is a trade-off between early survival and fecundity and later performance, resulting in
mortality. Alleles that confer fitness benefits early in life can be at a selective advantage, even though they have deleterious effects late in life. This theory is distinct from the mutation accumulation hypothesis with respect to the factors causing deleterious effects later in life: they are functional genes that have early-life benefits, rather than mutations.

“While the Evolutionary Theory of Aging predicts the existence of two broad categories of allelic variation, it says nothing specific about the mechanisms of aging. Several major ideas, such as rate of living (Sohal 1986), wear and tear (Rose 1991), and telomere shortening (Harley et al. 1992) have been proposed and investigated. In the context of sperm aging, the Free Radical Theory (Harman 1956) is probably most relevant. It is a widely accepted theory that explains the molecular, cellular and physiological manifestations of senescence. It explains senescence at the individual level and explains how senescence occurs. A free radical is any compound that contains one or more unpaired electrons. Reactive oxygen species (ROS) are free radicals that are derived from oxygen, produced mainly by mitochondria during cellular respiration. Because they are highly reactive, it is speculated that they may cause cumulative oxidative damage or "oxidative stress" resulting in senescence and the eventual death of cells. ROS may promote cumulative genetic damage in the germ-line cells or in the mature sperm cells, resulting in mutation accumulation as an individual male ages or as his sperm cells age. While the Free Radical Theory applies at the individual level, ROS-related damage in a male’s germ-line may ultimately occur because of the population-level evolutionary mechanisms of senescence: mutation accumulation, antagonistic pleiotropy, or both.

What causes sperm senescence?
Sperm senescence has an important impact on individual fitness, because genetic changes in gametes may be inherited by offspring and negatively affect offspring quality. The process of
sperm senescence can occur at both pre- and post-meiotic stages, during sperm development and after their maturation. Both theories (the Evolutionary Theory of Ageing and the Free Radical Theory) are applicable to sperm senescence to explain its occurrence and mechanisms.

Pre-meiotic sperm senescence

Pre-meiotic sperm senescence occurs in a male’s germ-line. Mutation accumulation at the individual level is likely to at least partially explain senescence at this stage, and has received a lot of empirical support (Snoke and Promislow 2003; Hughes 1995; Schmid et al. 2007). Male gametes appear to be more prone to the accumulation of deleterious mutations than female gametes are. First, more mitotic cell divisions occur for the production of sperm cells than for the production of ova, potentially leading to greater accumulation of mutations in male germ cells throughout life. This is probably due to continuous sperm production throughout a male’s adult life and a high volume of production due to sperm competition (Drost and Lee 1995; Ellegren 2007; Bartosch-Harlid et al. 2003). Mutation accumulation in the male germ-line may cause older males to have lower fertilizing efficiency or produce offspring with developmental defects and reduced viability. If older individuals are more prone to mutations, then it may be explained by greater mutation rate or lower rate of repair in senescent individuals.

In addition to senescence of sperm cells, male senescence may also result in changes in other components of ejaculate, such as a decrease in sperm production (e.g. the mite *Rhizoglyphus robini*, Radwan and Bogacz 2000) or a reduction in the number of structurally normal sperm (e.g. the black-footed ferret *Mustela nigripes*, Wolf et al. 2000; humans, Kidd et al. 2001). All these defects in ejaculates can reduce a male’s fertilization potential.

Post-meiotic sperm senescence
Post-meiotic sperm senescence may occur following meiosis. It can occur in a series of stages before fertilization. In internal fertilizers, it occurs during sperm storage inside males before copulation and inside females following copulation. Some internally fertilizing male animals also deposit their sperm externally before insemination (e.g. in the form of spermatophores, Reinhardt and Siva-Jothy 2005; and on the sperm web made by male spiders, Foelix 1996), so that sperm DNA may also experience degradation resulting from exposure to the elements.

ROS damage DNA and may be mainly responsible for post-meiotic sperm senescence. Nearly all research on oxidative stress on sperm has been conducted on mammalian species. The requirement of energy by the sperm-motility apparatus and other cellular events involved in hyperactivation, capacitation and the acrosome reaction of mammalian sperm cells demands a high level of respiratory activity (reviewed in Ramalho-Santos 2009). Both aerobic oxidative phosphorylation in mitochondria and anaerobic glycolysis contribute to this high energetic demand and produce large amounts of ROS. Low and regulated levels of ROS have been implicated in sperm hyperactivation, capacitation, acrosome reaction and oocyte interaction. However, excess of ROS creates oxidative stress and is detrimental to sperm performance (Sikka 1996). Sperm cytoplasm has limited store of antioxidant enzymes such as superoxide dismutase or glutathione peroxidase, so the genetic damage caused by excess ROS cannot be efficiently removed due to the lack of DNA repair (Siva-Jothy 2000; Olsen et al. 2005). In addition, high levels of polyunsaturated fatty acids present in sperm membranes make them particularly susceptible to the damage caused by oxidative stress, which can lead to a decline in sperm motility, sperm-oocyte fusion and the sperm’s ability to undergo acrosome reaction (Baker and Aitken 2005).

Little is known about the energy metabolism of insect sperm, but it seems that insects also use either anaerobic glycolysis or oxidative phosphorylation to produce energy, and can even
switch between these pathways (reviewed in Werner and Simmons 2008). ROS are mostly produced by mitochondria during oxidative respiration. However, despite the giant size and the fact that they are equipped with oxidative enzymes, the two mitochondrial derivatives in insect sperm are filled with paracrystalline material (Fuller 1993) and have a reduced inner membrane surface area in some species (reviewed in Werner and Simmons 2008). The relative importance of glycolysis or oxidative phosphorylation in producing energy has not been explored in most insect groups, and so the importance of ROS induced oxidative stress remains largely untested.

Despite its popularity, the Free Radical Theory mostly explain the senescence of highly motile sperm cells, which are primarily present outside of a male after an ejection. It has been largely overlooked that sperm cells of many animals have limited motility in the reproductive tract of males following development and before ejaculation (e.g. humans, Skandhan, 2004; spiders, Foelix 1996; fruit flies, author’s observation). Nevertheless, sperm senescence at this stage is also observed. For example, studies in poultry have shown that prolonged male sexual abstinence increases sperm abnormalities (El Jack and Lake 1966) and reduces fertilizing efficiency (Van Voorst and Leenstra 1995). A study of rats found that recently matured sperm from the proximal cauda epididymis have higher fertilizing capacity in vitro than those from the distal cauda, which have been stored for longer (Moore and Akhondi 1996). Similarly, in humans, the proportion of morphologically normal and motile sperm declines with periods of sexual abstinence of eleven days or longer (Levitas et al. 2005). Thermal damage, oxidative damage or both mechanisms may explain sperm senescence during male sexual abstinence, but the molecular and cellular basis of it is still not well-understood.

Reducing the rate of sperm senescence is potentially beneficial to both males and females; however, sperm storage may be a costly trait for the females in some species (e.g. in Drosophila, females that store more sperm have shorter longevity, Miller and Pitnick 2003). Thus, females in
poor condition or poor health may not be able to provide good nutrition or protection to their stored sperm, resulting in degradation in sperm quality and a reduction in sperm viability. For example, in the damsel bug, *Nabis rugosus*, females are mostly inseminated in the fall but lay eggs only in the spring (Roth and Reinhardt 2003). Food-deprived females maintain fewer sperm than well-fed females before winter hibernation, suggesting that condition may play a role in the maintenance of live sperm in this species. In the leaf-cutting ant *Atta cephalotes*, maintenance of large quantities of sperm during mating flights incurs a cost of reduced immunity during colony founding and increased risk of death for queens (Baer *et al.* 2006). In *Drosophila melanogaster*, females in stocks selected for a larger sperm storage organ were found to carry more sperm, but also suffered a reduction in adult longevity (Miller and Pitnick 2003). However, virgin females of the selected stocks had similar longevity to the control females, suggesting that storage of more sperm rather than maintenance of a larger sperm storage organ carries a longevity cost to females.

*Interaction between male age and post-meiotic sperm senescence*

Interactions between male age and post-meiotic sperm senescence can also occur whereby sperm produced by older males suffer higher rates of senescence than those produced by younger males. Consistent with this kind of interaction, old bulls have reduced activity of antioxidative enzymes in spermatozoa (Kelso *et al.* 1997). More direct support comes from the striped bass, *Morone saxatilis*, where males of intermediate age produce sperm with the highest longevity (Vuthiphandchai and Zohar 1999), and from the brown rat, *Rattus norvegicus*, where spermatozoa of older males have altered chromatin packaging and integrity and are more susceptible to oxidative challenge than spermatozoa of young males (Zubkova and Robaire 2006).
Problems in previous studies of sperm senescence

The existence of sperm senescence is well established, and its negative impacts on sperm fertilizing efficiency and embryonic viability have been demonstrated. However, the evolutionary repercussions of sperm senescence still need further exploration. Little is known about the role of sperm senescence in some fundamental aspects of sexual selection, such as sexual conflict, offspring sex ratio allocation, cryptic female choice, sperm competition, etc. In addition, problems exist in some of the earlier work on sperm senescence.

First, male age is always confounded with the age of sperm prior to ejaculation. Studies that attempt to standardize mating history routinely use the approach of sexual deprivation (e.g. using virgin males) in both young and old age classes (e.g. Avent et al. 2008; Milonas and Andow 2010). Two conflicting artifacts may arise when applying this approach: prolonged sperm storage in the males could result in reduced fertilizing efficiency, and continuous adult spermatogenesis might build up male sperm reserves and lead to a large ejaculate (Bailey and Nuhardiyati 2005).

Second, the analyses of pre-meiotic senescence are sometimes restricted to a small number of defined age classes that may not span the life of a male or even include advanced ages showing obvious senescence. For example, in studies of free-living birds, adult age has usually been assigned to two categories: young and old. Young males were always considered to be less than one year old, while the remaining males were considered old (Møller and Ninni 1998; Møller et al. 2009). However, this imposition of age class may put individuals at intermediate age in the ‘old’ category and may make the actual old males underrepresented. In the worst scenario, if the intermediate-aged males had the highest mating and fertilization success (e.g. the hide beetle Dermestes maculatus, Jones and Elgar 2004), combining intermediate- and old-age classes, or even missing the old-age class could lead to an erroneous conclusion. Therefore, it is
necessary for a study to determine at which stage of the life span a male starts to show signs of reproductive senescence before any investigations into pre-meiotic sperm senescence. Since different species have different trajectories of senescence, this evaluation needs to be species-specific.

**Study organism**

The fruit fly *Drosophila melanogaster* is an ideal system to investigate the potential contributions of sperm senescence at all stages of a sperm’s life: before meiosis in the male germ-line, after meiosis and before copulation in the male reproductive tract, and after copulation in the female sperm storage organs. First, the short generation time and short life span make the study of sperm senescence during male ageing feasible. Second, due to the absence of parental care, males almost exclusively contribute to offspring fitness through the genetic materials carried by sperm. Third, both sexes store sperm (Demerec 1965), which facilitates the study of both pre- and post-copulatory sperm senescence (Figure 1.1). Last, there is some preliminary evidence of sperm senescence in this species, as some studies have shown the influence of male ageing on the genetic quality of sperm (Price and Hansen 1998; Long and Pischedda 2005).

Here, I briefly illustrate genetic and morphological changes during spermatogenesis in *D. melanogaster*, and describe the major components in the seminal fluid that assist fertilization and sperm storage after copulation. I point out that potential factors in spermatogenesis may cause sperm senescence, and indicate that degradation of male seminal fluid may influence fertilization success of both sexes and sperm viability after copulation.
Figure 1.1. Reproductive systems in *Drosophila melanogaster*. Seminal vesicles are the sperm storage organs in males. Spermathecae and seminal receptacle are the sperm storage organs in females. Image of male from Ravi Ram and Wolfner 2007. Image of female from Bloch Qazi et al. 2003.

**Spermatogenesis in Drosophila melanogaster**

The production of sperm includes three steps: (1) proliferation of germ cells, (2) production of spermatids by meiosis, and (3) differentiation of mature sperm (Fuller 1993).

**Proliferation of germ cells**

Spermatogenesis begins when a germ-line stem cell undergoes a mitotic division, producing a stem cell and a spermatogonium (Fuller 1993). The mitotic divisions occur at the apical tip of the testis, where three types of cells can be found, a small group of somatic cells known as the hub,
germline stem cells, and somatic stem cells called cyst progenitor cells (Figure 1.2). The germ-line stem cells are arranged in a rosette around the hub, and each germ-line stem cell is enclosed in a pair of cyst progenitor cells. The hub acts as the stem cell niche promoting renewal of both germ-line and cyst progenitor cells. Meiotic division of a germ-line stem cell or a cyst progenitor cell generates two daughters, one remaining adjacent to the hub and continues to proliferate; the other daughter commits to differentiation and becomes a spermatogonium or a cyst cell. A primary spermatogonium undergoes four amplificatory mitotic divisions to produce 16 primary spermatocytes (Figure 1.3). The spermatogonium and all germ cells descended from it maintain constant contact with two daughter cyst cells throughout spermatogenesis (Figure 1.2). They comprise a cyst, the fundamental unit of the spermatogenic differentiation program. The cohort of germ-line cells within a given cyst go through cell differentiation in synchrony.

*Production of spermatids by meiosis*

The 16 primary spermatocytes go through two meiotic divisions, resulting in 64 haploid spermatids (Figure 1.3, Fuller 1993). Cytokinesis is incomplete during both mitotic and meiotic divisions. The cohort of cells (spermatogonia, spermatocytes and spermatids) in a cyst are connected by cytoplasmic bridges, which allow sharing of organelles and the entire diploid complement of gene products. The first meiotic division is achiasmate, that is, dispense with recombination. Immediately after completion of the meiotic divisions, mitochondria aggregate and fuse, producing two mitochondrial derivatives. The primary spermatocyte stage is when testis-specific transcription becomes well established and the cells grow 25 times in volume. Most of the gene products needed for the dramatic morphogenetic events that follow meiosis are transcribed at this stage. Gene expression after meiosis is extremely limited (White-Cooper *et al.* 2009).
Differentiation of mature sperm (spermiogenesis)

Spermiogenesis is the completion of spermatogenesis, producing flagellated motile sperm (Fuller 1993). During the process of flagellar elongation, most of the spermatid cytoplasm is expelled and the connections between the spermatid in a cyst are lost (Figure 1.2). The process that spermatids become fully cellularized is called individualization. The mitochondrial derivatives elongate alongside the growing flagellar axoneme. The process of crystalline inclusions begins – the paracrystalline material is deposited in the two mitochondrial derivatives and takes up most of their volume. Although oxidative enzymes are present within the mitochondrial derivatives, it is believed that they play a passive role in generating energy for sperm motility (Werner and Simmons 2008). The sperm nucleus becomes highly compacted due to a dramatic structural reorganization of sperm chromatin during sperm differentiation. Before differentiation, the structure of chromosomes in all germ-line cells is regulated by a set of histones that forms the basic platform for winding and compacting DNA. During differentiation, the most majority of histones are replaced with a set of small basic structural protein (protamines) to establish a unique sperm chromatin structure that results in DNA compaction. All DNA repair processes are inactivated at this stage of spermatogenesis; thus, DNA lesions induced during spermiogenesis remaining unrepaired (Olsen et al. 2005). Therefore, functional sperm may carry genetic defects that occur during spermiogenesis. The expulsion of cytoplasm, compaction of nucleus and elongation of flagellum drastically change the appearance of sperm, generating long hair-like spermatids extend the length of the testis. After individualization, the long sperm bundle in a cyst coils with assistance from the two cyst cells and somatic-derived terminal epithelial cells located at the base of the testis. Coiled sperm bundles are then released from the two cyst cells and passed in seminal vesicles, the organs act as a sperm reservoir before copulation. Mature sperm
are approximately as long as the male itself (~1.8mm). In seminal vesicles, they gain limited motility and so are no longer organized in bundles (reviewed in Werner and Simmons 2008). Likely, the age classes of sperm are not stratified in seminal vesicles. An ejaculate of a male probably contains sperm cells that were produced at different times, forming a mixed age cohort.

*Other characters of spermatogenesis in D. melanogaster*

Spermatogenesis is remarkably long in *D. melanogaster* and proceeds at a relatively slow speed. Spermatogenesis begins before egg hatching, the first cysts of spermatocytes appear about one day later, the first meiosis occurs at about the white prepupal stage (day 4-5 from eggs), and spermiogenesis starts in pupae (day 5-10 from eggs) (Demerec 1965; Fuller 1993). Male flies contain mature sperm at eclosion on about day 10 from eggs. Thus it takes a male almost the entire juvenile stage to generate the first ejaculate. In the adult testis, there are typically five to eleven germ-line stem cells. Spermatogenesis takes approximately 10 days, which is about a quarter of adult longevity (41.3 days (95% CI = 40.1 - 42.5 d, N = 228) in the *Ives* population, C.M. Kimber, *in prep*). At any given time, an adult testis only contains 25 cysts of differentiating germ-line cells. Spermatogenesis proceeds at a steady rate in young males, producing a gonial founder cell cycle every 32 hours (Wallenfang *et al.* 2006). However, some germ-line stem cells are lost and the division rate of the remaining stem cells decreases as an individual male ages (Wallenfang *et al.* 2006). Even without considering ageing related slow-down in spermatogenesis, a male with average longevity can only produce maximum 135,168 sperm cells in his entire life (2 testes×11 spermatogonia×64 spermatids×40 days×2.4 cell cycles). It is minuscule comparing to the 100 million sperm produced by a human male every day (White-Cooper and Bausek 2010).
Spermiogenesis is the stage when selection against abnormal spermatids occurs. Defective spermatids usually fail to individualize and are pushed aside in structures called waste bags during the coiling process (Fuller 1993). Sperm in the waste bags eventually degenerate. The testes of old males tend to have more debris in the form of degrading sperm than do those of young males.

The entire spermatogenesis process is regulated and assisted by three types of somatic cells: the hub cells, the cyst cells and the terminal epithelial cells (Fuller 1993). The hub cells regulate the proliferation of germ-line stem cells; the cyst cells influence meiosis and spermatid differentiation; and the terminal epithelial cells absorb the cyst cells and the contents of the waste bag after sperm individualization. Senescence of these somatic cells due to male ageing must have considerable impacts on spermatogenesis, therefore, resulting a decline in sperm quality.

Figure 1.2. Schematic showing the organization of hub cells, germ-line stem cells (GSC), spermatogonia, and somatic cyst cells within the apical tip of the testis. Typically, there are five to eleven spermatogonia in a testis; only three are showing here for clarity. Image from Wallenfang et al. 2006.
Figure 1.3. Major events of spermatogenesis, including proliferation of germ cells (Step 1), production of spermatids by meiosis (Step 2), and differentiation of mature sperm (Step 3). In step 1, a stem cell produces a single primary spermatogonium, which then follows four mitotic cell divisions, producing a cyst of 16 interconnected spermatocytes. In step 2, spermatocytes experience cell growth with increased gene expression, and then undergo two meiotic divisions producing 64 spermatids. In the final step, spermatids are fully cellularized. They elongate and coil, becoming motile sperm. Image drawn by author.

**Seminal fluid in Drosophila melanogaster**

Seminal fluid in a male’s ejaculate is transferred to females during copulation. It consists of a suite of proteins and other molecules. Most seminal peptides and proteins are produced in the accessory glands in *D. melanogaster* (Figure 1.1). In addition to the accessory gland proteins
(Acps), the ejaculatory duct and ejaculatory bulb also make some seminal proteins. Molecules, like lipids, are also found in the seminal fluid, which may serve as short-term energy sources for sperm during copulation (Heifetz and Rivlin 2010). Mature sperm are stored in the seminal vesicles (Figure 1.1). They are separated from seminal secretions until the onset of ejaculation. Degradation of seminal fluid may decrease fertilization success and increase the speed of post-copulatory sperm senescence. However, no data are available to support male age related degradation in the non-sperm components of an ejaculate, probably because of the difficulties in separating the effects of seminal fluid and sperm cells on sperm performance in empirical studies.

Here, I describe the major function of some well-studied seminal proteins in *D. melanogaster*, indicating their potential contribution to female sperm storage, fertilization and sperm viability.

To date, over a hundred Acps have been identified (reviewed in Pitnick *et al.* 2009). They increase the female’s food intake, enhance her egg production, increase her rate of ovulation, reduce her sexual receptivity, assist in the female’s storage of sperm, control the rate of sperm usage and also contribute to the reduced life span of the mated female (reviewed in Pitnick *et al.* 2009 and Chapman 2001). Therefore, Acps play an essential role in fertilization success in *D. melanogaster* (Ravi Ram and Wolfner 2005). They benefit both sexes by increasing female egg-laying and ensuring sperm transfer, but, at the same time, are potentially harmful to females due to the reduction of female longevity and sexual receptivity. Most of the effects of Acps are transient. Acps are only detectable within the female for several hours after mating (Ravi Ram and Wolfner 2005), and most of their effects only last for one or two days. However, some effects, like egg-laying and receptivity changes, can persist for weeks (reviewed in Pitnick *et al.* 2009).

Ovulin (also denoted Acp 26Aa) is a well-studied Acp that increases egg-laying for roughly a day after mating in virgin females by increasing the rate of release of existing eggs
from the ovaries (reviewed in Chapman 2001). The more eggs are laid after copulation, the higher the fertilization success for the females. The sex peptide (Acp 70A) also increases egg laying for one or two days after mating. Different from ovulin, it increases egg production by enhancing oogenesis and also reduces female sexual receptivity. Such effects are clearly beneficial to the male, whose sperm are resident to fertilize eggs. Again for the female there may be a longevity cost (Fricke et al. 2009). Since sex peptide can bind to sperm, it has prolonged activity that may last for the entire period of sperm storage (several weeks). Another protein, Acp 36DE, binds to sperm and is essential for sperm entry into the sperm-storage organs. Andropin and several unnamed Acps have antibacterial activity that can enhance the viability of sperm during and after ejaculation.

**Thesis goals**

The aim of this thesis is (1) to develop experimental procedures that control confounding influences of sperm age in the study of pre-meiotic sperm senescence (Chapter 2), (2) to investigate the potential contributions of sperm senescence at all stages of a sperm’s life: before meiosis in the male germ-line, after meiosis and before copulation in the male reproductive tract, and after copulation in the female sperm storage organs (Chapter 3), (3) to illustrate the cost of sperm storage to females (Chapter 4), and (4) to improve our understanding on the importance of sperm senescence on male and female fitness and its significant role in sexual selection.

I used flies from a laboratory-adapted, wild-type population, called the Ives population. This population has been extensively studied from the perspective of senescence (Rose et al. 2002; Chippindale et al. 2003). For practical purposes, I studied sperm senescence in Ives flies before and after copulation. Thus pre-copulatory sperm senescence occurs in males, which includes both pre-meiotic sperm senescence and post-meiotic sperm senescence following sperm
development and before insemination. Post-copulatory sperm senescence takes place in females, and is exclusively at the post-meiotic stage.

References


Chapter 2: Development and application of a method to standardize sperm age in studies of male fertility in *Drosophila melanogaster*

**Abstract**

In species where males store sperm for a long period, those sperm may have reduced fertilization performance due to the effects of sperm senescence. To study sperm senescence, it is important to measure sperm age independent of male age. Here, we provide a practical approach to standardizing pre-copulatory sperm age and male mating history in laboratory populations of *Drosophila melanogaster*. We first found that most four-week-old males (post-eclosion) were still fertile and alive. Therefore, we chose males from two age groups, one and four weeks old, to develop this approach. We subjected these males to serial copulations over a five-hour trial period to evacuate their stored ejaculates, and then imposed one, two or three days of sexual abstinence for development of new sperm. For these mating treatments, we used young and old males from three hemicline lines (derived by cytogenetic cloning) that had different mean lifespans. Ejaculate exhaustion required six successive copulations at both ages, on average; young males required at least three days without mating opportunities to resume approximate 65% of their initial fertility. Four-week-old males showed obvious signs of male reproductive senescence, confirming male reproductive senescence is best studied at this age group. Compared to young males, old males mated with fewer virgin females, had fewer copulations resulting in offspring, had a longer pre-copulation duration and shorter copulation time, and produced fewer offspring. Old males from the wild-type base population from which the hemiclones were derived also had lower offspring production than young males. Since pre-copulatory sperm age may be
an important factor that determines male fertility, applying our approach should advance the studies of age-related male reproductive success.
**Introduction**

Male ageing potentially exerts an important role in sexual selection. In some species, male condition may simply decline with age post-maturity, resulting in reduced attractiveness (Verburgt *et al.* 2011; Garratt *et al.* 2011), reduced intrasexual competitiveness in acquiring copulation opportunities (De Luca and Cocroft 2011) or inefficient ejaculate transfer during copulation (Jones *et al.* 2007). In others, males may have age-related advantages in size, resources, or experience in competition that are ultimately eroded by the effects of senescence (Johnson and Gemmell 2012). Potential good genes advantages for females in choosing an experienced and apparently successful older male may to be tempered or reversed by the accumulation of deleterious mutations in the male germ-line with age. Therefore, to understand the implications of male age for females that mate with them, it is critical to examine each of the levels at which partner age can affect female reproductive success.

Male senescence can occur at the level of the organism, the germ-line, and the gamete. As noted above, male age may play a varied role at the level of the organism, but below that level ageing is either neutral or harmful to sperm fitness, because positive mutations are rare. At the germ-line level, mutations accumulate with male age and are expected to steadily decrease the genetic quality of the spermatozoa that a male produces (Pizzari *et al.* 2008). At the gamete level, spermatozoa in a male’s sperm storage organs may suffer thermal and oxidative stress that damages their cellular integrity or their DNA. Such damage can reduce sperm competitiveness, female sperm storage efficiency, fertilization ability, and zygote viability (reviewed in Reinhardt and Siva-Jothy 2005), as well as can increase the mutation load in the offspring (Radwan 2003). While all males need to store sperm for some time – at least as long as it takes to assemble an ejaculate – the nature and duration of storage varies markedly across taxa, and as a function of individual copulation success. As a result of pre-copulatory sperm storage, the spermatozoa in the
storage organs (and thus ejaculates) of older males are likely to be more heterogeneous in age, and perhaps in quality, than those stored by young males.

Nearly all studies of the effects of ageing on male reproductive performance (e.g. Avent et al. 2008; Milonas and Andow 2010) have used virgin males so that male copulation history could be controlled experimentally. However, in those studies, sperm age was not standardized when examining the effects of male age. Thus, declines in the fitness of offspring sired by old fathers that have been documented in several species (fruit fly, *Drosophila melanogaster*: Long and Pishedda 2005; brown Norway rat, *Rattus norvegicus*: Serre and Robaire 1998; sandfly, *Lutzomyia longipalpis*: Jones et al., 2000) may have been due either to the senescence of stored sperm before copulation, or the senescence of the male’s spermatogenic tissue, or both, thereby conflating male age with spermatozoa age. In a few studies, males have been allowed continuous mating opportunities prior to experiments. In this case, variation in mating history, both between age treatments and among males within age treatments may influence male fertilization success (e.g. males that experienced more copulations may have lower storage of seminal fluid, thus lower fertility).

One way to control both sperm age and mating history is to deplete banked sperm through a series of copulations and to allow a controlled period of ejaculate recovery before testing. Previous studies have shown that ejaculates (sperm and seminal fluid) can be depleted by serial copulations in Soay sheep (*Ovis aries*), sandfly (*Lutzomyia longipalpis*) and fruit fly (*Drosophila pachea*) (Wedell et al. 2002 and citations therein, Sirot et al. 2009 and citations therein). After ejaculate depletion, most male animals can resume their fertility after a period of recovery, as has been demonstrated in the Mediterranean fruit fly *Ceratitis capitata* (Whittier and Kaneshiro 1991) and bedbug *Cimex lectularius* (Reinhardt et al. 2011). For each species studied, the
number of serial copulations required to deplete banked sperm and the period needed for ejaculate recovery need to be quantified.

More is known about the dynamics of sperm exhaustion and recovery in *Drosophila*. Successive copulations can reduce the amount of stored spermatozoa quite rapidly. For example, male *D. pachea* transfer fewer spermatozoa during copulations in the second day of successive matings in laboratory (Pitnick and Markow 1994), and male *D. melanogaster* typically become temporarily sterile by the fifth copulation in serial remating experiments, concomitant with reduced size of both accessory glands and testes (Hihara 1981; Linklater et al. 2007; Lefevre and Jonsson 1962; Fulker 1966). Ejaculate-depleted males show partial recovery of fertility overnight in *D. melanogaster* (Lefevre and Johnson 1962), and males are reported to transfer “virgin levels” of seminal fluid proteins after three days of sexual inactivity in this species (Sirot et al. 2009). Mating-induced temporary sterility in *D. melanogaster* suggests a useful technique for standardizing both male age and the age of the sperm transferred in experimental matings.

As far as we know, there is only one study that has attempted to differentiate between the effects of senescence of the spermatogenic tissue and the senescence of spermatozoa before copulation on male fertility. Jones and Elgar (2004) aged male hide beetles, *Dermestes maculatus*, in three age categories (11.5-day-old, 47.4-day-old, and 88.3-day-old) and applied three mating treatments to each age group: (i) kept virgin, (ii) housed with three virgin females for 24 h and isolated from females for 24 h prior to fertility testing to give the males a ‘recovery period’, and (iii) continuously housed with three females per week throughout their reproductive life until 24 h prior to the test of their fertility. The number of copulations was not quantified for the non-virgin males. While they documented senescent decline in fertility, they found no significant effect of prior mating treatments at any age, suggesting that male fertilization success
was independent of either spermatozoa age, or that neither of the mating history treatments measurably challenged the males’ sperm reserves.

We undertook the present study with an interest in the specific impact of sperm senescence on male fitness in *D. melanogaster*, we had three specific goals: (1) to find an appropriate age group to best study male reproductive senescence; (2) to develop a protocol to standardize pre-copulatory sperm age in young (three to six days after eclosion) and old males (31-34 days old after eclosion) by determining the number of successive copulations needed to empty their sperm storage organs (sperm evacuation); and (3) to measure the period of sexual abstinence needed for a male to recover his sexual potency (sperm recovery) after sperm evacuation.

In order to evaluate genetic variation among males, we selected males from three fixed-genotype lines known to exhibit different mean longevities for study. These flies were generated by a cytogenetic cloning technique (Chippindale *et al.* 2001). The flies from the same clone line carry identical haploid genotypes, including the X-chromosome and both of the two major autosomes, hereafter referred to as hemiclones. Dipteran males lack synapsis and cross-over and hence can be used to transmit these X,2,3 haplotypes (a hemiclone) to sons intact over many generations if maternally-derived chromosomes are genetically marked and replaced each generation. The use of standard genotypes is to avoid the confounding effect of selection based upon differential mortality with age when assessing reproductive senescence.

**Methods**

*Study population*

All flies used in this study were derived from the *Ives* population, a large, outbred culture of *Drosophila melanogaster* established from a wild-caught sample in Amherst, Massachusetts,
USA in 1975 (Rose and Charlesworth 1980), and adapted to constant and controlled laboratory conditions for about 900 generations prior to our experiments. The Ives population was maintained at 2000-2500 flies/generation and was cultured on a 14-d, discrete-generation schedule at 25°C under 12:12 h light:dark cycle on a banana/agar/killed-yeast medium. Mean adult male longevity was 41.3 days (95% CI = 40.1 - 42.5 d, N = 228), measured about ten generations before our experiments began (C.M. Kimber, in prep).

*Creating hemiclone males*

Males carrying an identical haplotype mate with ‘clone generator’ females with autosomal translocations to maintain the haploid genome (Chippindale *et al.* 2001). In these experiments, the two autosomes derived from the ‘clone generator’ females were a T(2;3) translocation marked with the recessive eye-color markers *scarlet* (*st*) and *pink* (*p*) and the dominant eye-color marker, *brown* (*bw*D); this genetic aberration means that there are only two, easily distinguished male offspring types, as any offspring that inherits only one of the translocated chromosomes will be aneuploid and are not viable. Therefore, hemiclone males that carry the T(2;3) translocation will produce 50% aneuploid sperm. When they mate with wild-type females, only half of the offspring are viable.

From a library of 19 hemiclones maintained by C.M. Kimber (*in prep*), we chose three lineages with different mean longevities (low (33.2 d, 95% CI = 31.8 - 34.7 d, N = 99), medium (40.0 d, 95% CI = 38.3 - 41.7 d, N = 66), high (48.4 d, 95% CI = 46.2 - 50.6 d, N = 73) spanning the longevities of the 19 lines (40.2 d, 95% CI = 39.7 - 40.7 d, N = 1511) so that we could examine if there was a genetic association between late life mortality and the reproductive characters measured here.
**Experiment One: Age-mediated decline in the percent fertile wild-type males**

We estimated the rate of decline in male fertility with age by determining whether or not viable offspring were produced after matings as males aged. To do this, we documented the percent fertile males in a sample of 1200 males from the *Ives* population every week after eclosion for six weeks (approximately the mean longevity of the population), by which time the majority of the flies were producing no viable offspring. We collected virgin males upon eclosion, maintained them in groups of ten individuals per vial, and moved them into new-food vials on the same days twice a week. We haphazardly chose 100 males on each of days 7, 14, 21, 28, 35, and 42 after eclosion, and then tested whether or not each male was capable of producing viable offspring after mating with a group of six females. After mating, females were left in their original vials for 24 hours to lay eggs. The presence of larval activity three days post-mating determined if the males were fertile. Males were kept separately from females and their survivorship was assessed four days after the matings.

**Experiment Two: Development of a protocol to standardize sperm age in studies of male fertility**

We used two male age groups for each hemiclone line: young (three to six day old) and old (31 to 34 day old). Young males were close to the age at which the *Ives* population is propagated in routine culture (about day 5 after eclosion); old males were at the age that is best to study male reproductive senescence as determined in Experiment One. At this age, there has been relatively little mortality, but some senescent decline in performance is expected based on previous observations (C. M. Kimber, *pers. comm*; Price and Hansen 1998). We collected males upon eclosion on three successive days using light CO₂ anesthesia, held them in groups of five in 25 mm × 95 mm shell vials, and randomly assigned vials to either the young- or old-male treatment.
groups. Virgin males were housed at low density per vial (five males/vial) to avoid crowding effects on pre-mating latency (Noor 1997). Both young and old males were sexually mature by the time of their first mating assays. We used 15 young and 18 old males from each hemiclone line in each assay—extra old males were included so that mortality would not adversely affect sample size. Males in the old-male treatment were transferred to fresh food vials on the same days twice a week for four weeks prior to their first mating assay at age 31-34 days.

Mating assays

Each male was used in two mating assays: sperm evacuation (SV) and sperm recovery (SR). The sperm evacuation (SV) assay was to determine how many sequential matings were needed to deplete a male’s sperm stores, and whether male age influenced the rate of sperm depletion. Three different sperm recovery assays (SR1, SR2, SR3) were conducted on each of the three days following the SV assay, to test how long a period of sexual abstinence was needed for a male to recover full fertility (i.e., to the initial level of offspring production measured by the SV assay). In all trials, mating vials contained a single virgin, three-day-old, *Ives* female that had been fed with yeast *ad libitum* from eclosion to enhance their sexual receptivity and subsequent egg-laying rate. Each male was transferred to a female vial without anesthesia. All copulations were observed for five hours to ensure no male double-mated with the same female. Immediately after a copulation, the male was gently transferred by aspiration to another vial containing a new virgin female.

To ensure that reproductive senescence had occurred in the selected old-male age group, we measured six indices of male mating success for each male from all copulations in the five-hour period in both the SV and SR assays: (i) number of copulations, (ii) number of copulations producing offspring, (iii) pre-copulation duration (time from introduction of a male into a vial
containing a female until the start of a copulation, to the nearest minute), (iv) copulation duration of each copulation (from mounting until the pair separated, to the nearest minute), (v) sterility rate at the beginning of the assays, and (vi) total number of offspring produced (total fertility).

After mating, females were housed singly while they laid eggs, and transferred into a new food vial with *ad libitum* yeast every two days until no more fertilized eggs were produced. All juveniles grew up at low-to-medium density (<100 flies per vial) to minimize crowding-related mortality. We recorded the number of progeny produced by each female and male, and assumed that this represented 50% of the viable sperm; this is because the T(2;3) translocation employed will produce 50% aneuploid sperm (see above).

We assumed that males who achieved successful copulations but had no offspring production in any copulation were already sterile at the beginning of the SV assay. Most fertile males became sterile or produced few offspring by the end of the five-hour mating trials. We then measured how long it took each male to regain his fertility (SR assay). One-third of the males that survived in each SR assay (See Table 2.1 for sample sizes) from each line were mated with a succession of virgin Ives females 24 h, 48 h and 72 h after the SV assay, constituting the sperm recovery assay, days 1, 2, 3 (SR1, SR2, SR3). The five aforementioned aspects of male fertilization success measured in the SV assay were also measured in each of the SR assays in five-hour mating observations using the same procedure.

**Experiment Three: Age-mediated fertility in wild-type Ives males**

The eye-color marker, *bw^D*, possessed by the hemiclone males has known fitness-reducing effect and so these males might have accelerated senescence and reduced fertility. Thus, we also assessed age-related fertility in wild-type males. Our previous observations indicated that when a male *D. melanogaster* was housed with a group of virgin females, he would serially mate with
the remaining virgin females. Thus, we grouped individual males with virgin females to evacuate their sperm stores without observing mating, and assumed that males would rarely double-mate the same female. From the hemiclone male assays, we learned that six copulations were usually enough to temporarily induce sterility (the sperm evacuation assay, SV), and that sperm-depleted males were able to replenish 65% of their sperm stores after three days of sexual rest (the sperm recovery assays, SRs). We collected 50 virgin wild-type males for each age treatment (three to six or 31-34 days old) from the base *Ives* population. Each male was put in a vial with six three-day-old virgin *Ives* females for six hours, to evacuate his sperm stores. Because of the stress of multiple matings, some males died after the SV matings. Four days after the initial matings, 48 young and 18 old males were still alive and fertile (having partners that produced offspring). They were allowed SR matings with another six three-day-old virgin *Ives* females and the fertility was assessed. The six females that each male mated with in the SR matings were moved into a vial with fresh food and *ad libitum* yeast every one to two days until they laid no more fertilized eggs, to determine total male fertility. Female mortality was very low during the egg-laying period (< 2%).

**Statistical analysis**

The difference in number of offspring produced between the first and the last copulations of each male in the SV assay was analysed using paired t-tests. In each mating assay, we compared young and old males on all measured reproductive characters with age and hemiclone as the factors. Mean pre-copulation and copulation durations were calculated across all copulations of each male and were log-transformed. They were analysed using two-factor ANOVAs. The number of copulations was analysed using a generalized linear model with Poisson distribution and a log link function. The number of copulations producing offspring and total number of
offspring produced were in zero-inflated Poisson distribution. For these two traits, we conducted generalized linear model only on the data of fertile males (the non-zero data) with respective distribution and a “log” link on each of them. The probability of sterility at different ages (the zero data) was compared using replicated goodness-of-fit test in R 2.14.1. We also compared reproductive characters of fertile and sterile males: for average pre-copulation and average copulation duration we used a three-factor ANOVA; and for number of copulations, we used a generalized linear model with Poisson distribution and a “log” link function. Hemiclone was included as a factor in all analyses mentioned above. Unless otherwise mentioned, all analyses were generated using JMP 9 (2010).

Results

Experiment One: Age-mediated decline in the percent fertile wild-type males

When male *Drosophila melanogaster* from the Ives population were kept virgin for different periods before being allowed to copulate, the percentage of males that were fertile and alive four days after the matings declined with age. The percent fertile was consistently high (≥ 96%) until the flies were four weeks old (89%; Figure 2.1), with a steep decline thereafter.

Experiment Two: Development of a protocol to standardize sperm age in studies of male fertility

Variation between hemiclones

Most of the components of male fertility that we examined did not significantly differ among hemiclone lines. We found genetic effects on number of copulations (Generalized linear model, \(\chi^2 = 19.3, \text{df} = 2.79, p < 0.01\)), copulation duration (Two-factor ANOVA, effect of hemiclone, \(F = 7.5, \text{df} = 2.77, p < 0.01\)), and number of offspring produced (Generalized linear model, \(\chi^2 = \).
591.0, df = 2,72, p < 0.01) in the sperm evacuation assay (Table 2.1). There were also genetic effects on number of copulations and total offspring produced in each of the sperm recovery assays (p < 0.01, Table 2.1). However, there was no certain hemiclone consistently doing better or worse than the others on each mating aspect and across all three mating aspects showing genetic effects (results not shown). The six mating indices that we examined might be independent of the factors that influence male longevity. Because we did not find consistent patterns among hemiclone lines on mating performance and offspring production, and sample sizes in each hemiclone line were small particularly in the old age group, the differences between young and old males in all measurements are present using pooled averages of the three hemiclones.

Sperm evacuation (SV) assay

All males began courtship behaviours as soon as they encountered a new female, and this typically resulted in three to five copulations during the five-hour mating observation period. Some copulations did not produce any offspring (Figure 2.2 A). The percentage of fertile copulations and the number of offspring produced by each copulation progressively declined with successive copulations in both age groups (Figure 2.2 A, B) and in all hemiclones (results not shown). In young males, fertility remained high for the first three copulations (128 -150 offspring per copulation), but dropped progressively thereafter (Figure 2.2 B). After the sixth copulation by each male, 67% (4/6) of the young males were sterile (presumably depleted of sperm), and offspring production was low in males that were still fertile—on average they produced 34.8 offspring per copulation after six copulations, compared to 153.4 offspring after the first copulation. In old males, fertility remained relatively constant for the first three copulations (average 79, 62 and 60 offspring per copulation in the first, second, and third copulation) but
declined to nearly zero after the sixth copulation. Therefore, we conclude that six copulations in sequence during a five-hour period evacuate most of the ejaculate (sperm and seminal fluid) stored by most young and old males that were virgins before the trials. Small proportion of males may be fertile after the sixth copulation, but their fertility becomes very low.

Since only half of the young males and 6% of the old males copulated at least six times during the five-hour trial, we compared first and last copulations of each male to determine sperm depletion irrespective of the number of copulations. The average fertility (number of offspring produced) of young males declined significantly from 142.4 after their first copulation to 61.6 after their last (i.e. third to ninth copulation, depending upon the male) (paired t = 4.36, p < 0.01, N = 31 males), and old males from 80.8 after their first to 45.6 after their last (second to ninth copulation, depending upon the male) (paired t = 2.20, p = 0.04, N = 17 males). This indicated that copulating with individual virgin females successively for five hours could largely exhaust a male’s sperm storage independent of the number of copulation he achieved.

Sperm recovery (SR1, SR2, and SR3) assays

During the three days without copulation after the SV assay, young males returned on average to 57% (day 1, N = 9), 42% (day 2, N = 8) and 65% (day 3, N = 9) of their initial fertility (Table 2.1, Figure 2.3). Old males also increased their offspring production during that period, but mortality (14/48 flies), sterility and the loss of flies during transfers resulted in small sample sizes for these SR assays. The recovery of fertility after periods of sexual abstinence was observed in all hemiclones, except all old males in the medium-longevity line died before SR3 (results not shown). During SR assays 8% (2/24) of old males did not copulate, and 46% (11/24) produced no offspring after copulations. In contrast all young males survived until the SR assays and copulated at least once.
Age-related male fertilization success

We found dramatic effects of age on fertilization success in all males, including those with the same genetic background (hemiclones). The use of standard genotypes helps to ensure that the observed effects are not the result of selection based upon differential mortality with age. On the six indices of male mating success that we measured in SV and SR assays, four-week-old males mated over 50% fewer times, achieved 70% fewer fertile copulations that produced offspring, took twice as long to initiate copulations, copulated for 17% less time, exhibited more than 9 times higher rates of sterility, and produced about 80% fewer offspring compared to one-week-old males. The detailed results of data analyses are listed in Table 2.1. One might speculate that the aforementioned differences in mating performances between young and old males were only due to the different sterility rates in the two age groups. However, exclusive analyses on fertile males in the SV assay also showed significant differences in the number of copulations (two-factor ANOVA, effect of age: F = 4.6, df = 1, 45, p = 0.03) and copulation duration between young and old males (two-factor ANOVA, effect of age: F = 4.0, df = 1, 45, p = 0.05) (Table 2.2), providing further evidence of the effects of senescence on fertile males. Overall, these results suggest that four-week-old males showed obvious signs of reproductive senescence and further confirm that this is the best age group for the studies of assessing age-related male fertility.

Experiment Three: Age-mediated fertility in wild-type Ives males

Using wild-type Ives males to assess age-mediated fertility, we standardized pre-copulatory sperm age using sperm evacuation matings. We observed that 18.5% more offspring were produced by one-week-old males than four-week-old males (Figure 2.4; t test, df = 64, t = 2.3, p
= 0.03). On average, young males produced 1101 offspring with six virgin wild-type females (95% CI = 1021 - 1180 offspring, N = 48); old males produced 929 offspring (95% CI = 799 - 1058 offspring, N = 18), congruent with the difference observed in young and old hemiclone males in the SV and SR assays (Table 2.1).

Discussion

The approach to standard pre-copulatory sperm age

Sperm senescence may be an important component of male age-specific fertility. Using four-week-old old males, an appropriate age to study male reproductive senescence in our base Ives population, and one-week-old young males, we developed an approach to standardize pre-copulatory sperm age in male Drosophila melanogaster of both age groups by rapid exhaustion of stored ejaculate and recovery of fresh ejaculate in which sperm age falls within a known range. We found that six consecutive copulations largely evacuate sperm stores in males, and that substantial fertility recovery takes at least three days. Since sperm evacuation and sperm recovery were observed in all hemiclones that vary in average longevity, this approach can probably be applied to flies with different genetic background. These results are consistent with previous findings on the evident decline of fertility by the fifth copulation (Hihara 1981; Linklater et al. 2007; Lefevre and Jonsson 1962; Fulker 1966). With this approach we advance the study of male reproductive senescence by dissociating the age of the gonads from the age of the sperm they produce.

Although an effective procedure to deplete banked sperm is to provide a male with a succession of at least six virgin females and to make sure copulation has occurred, this procedure requires considerable effort and will limit sample size. We suggest that a practical shortcut for sperm evacuation would be housing a male with at least six virgin females in a group for more
than five hours, accomplishing a similar outcome when less control is required. These are rough guidelines for healthy wild-type *D. melanogaster* under the specific conditions employed.

During the three days of sexual abstinence, young males partially recovered their fertility, apparently in a non-linear fashion: from recovering 57% of their initial fertility after one day of sexual rest to 65% after three days of sexual rest. Old males also showed the trend of increasing fertility over time, but might need longer recovery time because of the slower stem cell division rate (Wallenfang *et al.* 2006) and potentially faster apoptosis (Campisi 2003). Whether male *D. melanogaster* would ever replenish fertility to pre-SV ‘virgin’ levels was untested in this study, but it was clear that three days of sexual abstinence allowed males to largely recover their fertility with sperm of relatively homogeneous age.

We suggest that controlling for pre-copulatory sperm age is a necessary step in the studies of male reproductive senescence. Older males had larger sperm stores than did younger males, but expelled most their sperm by the end of sperm evacuation (SV) matings similar to younger males (author’s observation), however, they remained fertile in fewer copulations and produced fewer offspring. This suggests that among many potential factors, low quality of ejaculate due to the senescence of accessory glands or spermatozoa likely were the primary determining factors that explain age-related reduction in offspring production. There may be fewer sperm stored by females when male accessory gland secretions are lower or senesced spermatozoa have inadequate behaviour (Lefevre and Jonsson 1962; Neubaum and Wolfner 1999).

Different species vary in number of successive copulations required to eliminate their sperm reservoir, investigators on other species need to assess the male fertility potential in a series of copulations to determine an effective procedure to evacuate sperm and also to acknowledge the fact that males may tailor their ejaculate in response to social environment,
future mating opportunities, and female mating status, fecundity and age (Wedell et al. 2002; Linklater et al. 2007; Lüpold et al. 2011).

**The best age to study male reproductive senescence**

In this study, number of offspring produced by male *D. melanogaster* given ample mating opportunities in a fixed mating period decreased quickly with age, in accordance with Prowse and Partridge’s (1997) finding of complete sterility of males with a history of mating at a time when upwards of 80% of their cohort was still alive. We found that four weeks of age was an appropriate age to study male reproductive senescence in our base population because the majority of the cohort was still alive and fertile. At later ages, fertile males might have experienced selection for postponed senescent characteristics. Decreased number of copulations, number of fertile copulations, copulation duration and offspring production, increased precopulation duration, along with higher sterility and mortality during and after copulations in older males reflect the pervasive effects of senescence at this age. Also, the decline in male mating performance and offspring production was detected in all hemiclones that have different longevities, especially in the long-lived genotype, indicating four weeks of age is probably ‘old’ enough for all genotypes in the study population.

An interesting fact is that many old males continued to vigorously mate, even after they had become sterile. It may be that spermatogenetic tissue ages faster compared with other somatic tissues (Campisi 2003). Or, as noted above, there may be age-related adaptation of behaviour. For example, if older males are poorer competitors in social groupings, the conditions of the no-choice trial in which a succession of young, fertile, and sexually-receptive females is present may trigger a response in which the male “gives it all he’s got” irrespective of
fertilization probability or mortality risk. We noted an extremely high incidence of death-by-copulation in old males under these conditions.

Overall, unlike some previous studies on other *Drosophila* species, we did not find ‘old-male advantage’ in fertilization success in *Drosophila* (Avent *et al.* 2007; Somashekar and Krishna 2011; Prathibha *et al.* 2011), rather, we found evidence of pervasive senescence. Reduced behavioural performance and low offspring production in old males found in this study, in combination with available evidence showing reduced offspring fitness with age from other studies (Price and Hansen 1998) do not point to a silver lining to getting old in fruit flies. Rather, it is likely that females should avoid fertilization by older males unless there are compensatory benefits from reduced mate-harm caused by male seminal fluid (e.g. male accessory gland proteins reduce female longevity in *D. melanogaster*, Ravi Ram and Wolfner 2007). Researchers must carefully choose representative age categories when addressing senescence questions. In other words, one needs to find out how old is “old” in the particular system. Some species reach reproductive optimum in mid life (Jones and Elgar 2004, Reinhardt *et al.* 2011) or maintain a high physical condition until late in life; even different populations in the same species can have distinct senescence patterns.

**Conclusion**

Growing interest has recently focused on the evolutionary consequences of reproductive senescence. In species where males store sperm for a long period of time, the sperm are subject to ageing, and therefore potential senescence. It is important to avoid confounding male age with sperm age, which on its own plays a role in the reproductive outcomes (Radwan 2003). This study provides a practical approach to standardize pre-copulatory sperm age and mating history in different male age groups. Male-age based female mate choice along with further studies of
offspring quality using our protocol of standardizing sperm age are warranted. In addition, our protocol may be applicable to many mating systems where males bank sperm with age and senescence of gametes themselves may be important to male fitness.

References


Johnson, S. L., & Gemmell, N. J. (2012). Are old males still good males and can females tell the difference? Do hidden advantages of mating with old males offset costs related to fertility, or are we missing something else? *BioEssays*, 34, 609–619.


Table 2.1. Fertilization success examined during the five-hour observation period in sperm evacuation assay (SV; i.e. hemiclone males copulated with a succession of virgin Ives females to deplete males’ sperm store) and sperm recovery assays (SR1, SR2, and SR3; i.e. the same hemiclone males copulated with a succession of virgin Ives females one, two or three days after the SV assay).

Sperm evacuation assay (SV)

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Mean (95%CL)</th>
<th>Sample size</th>
<th>Mean (95%CL)</th>
<th>Sample size</th>
<th>Data distribution</th>
<th>Age statistics</th>
<th>Age p value</th>
<th>Clone statistics</th>
<th>Clone p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterility</td>
<td>6.5%</td>
<td>3/46</td>
<td>58.8%</td>
<td>30/51</td>
<td>Binomial</td>
<td>χ²=29.5</td>
<td>&lt;0.01</td>
<td>χ²=0.5</td>
<td>0.77</td>
</tr>
<tr>
<td>Number of copulations</td>
<td>5.3 (4.6, 5.9)</td>
<td>34</td>
<td>2.5 (2.1, 3.0)</td>
<td>49</td>
<td>Poisson</td>
<td>χ²=55.3</td>
<td>&lt;0.01</td>
<td>χ²=19.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Number of fertile copulations</td>
<td>3.9 (3.2, 4.6)</td>
<td>31[4]</td>
<td>1.2 (0.6, 1.7)</td>
<td>49</td>
<td>Zero-inflated Poisson</td>
<td>χ²=4.0</td>
<td>0.05</td>
<td>χ²=2.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Pre-copulation duration (min)[1][3]</td>
<td>26.3 (21.4, 31.2)</td>
<td>34</td>
<td>51.0 (39.4, 62.6)</td>
<td>47[4]</td>
<td>Normal</td>
<td>F=9.8</td>
<td>&lt;0.01</td>
<td>F=2.8</td>
<td>0.07</td>
</tr>
<tr>
<td>Copulation duration (min)[3]</td>
<td>21.5 (20.1, 23.0)</td>
<td>34</td>
<td>17.4 (15.8, 18.9)</td>
<td>47[4]</td>
<td>Normal</td>
<td>F=15.2</td>
<td>&lt;0.01</td>
<td>F=7.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total offspring[3]</td>
<td>492 (389, 595)</td>
<td>29[4]</td>
<td>86 (46, 126)</td>
<td>47[4]</td>
<td>Zero-inflated Poisson</td>
<td>χ²=2696.1</td>
<td>&lt;0.01</td>
<td>χ²=591.0</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
## Sperm recovery assay 1 (SR1)

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (95%CL)</td>
<td>Sample size</td>
<td>Data distribution</td>
</tr>
<tr>
<td>Number of copulations</td>
<td>6.0 (5.1, 6.9)</td>
<td>9</td>
<td>Poisson</td>
</tr>
<tr>
<td></td>
<td>(5.1, 6.9)</td>
<td></td>
<td>$\chi^2=38.7$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>$p$ value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>$&lt;0.01$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\chi^2=28.1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$&lt;0.01$</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>$\chi^2=26.4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$&lt;0.01$</td>
</tr>
<tr>
<td>Number of fertile</td>
<td>3.8 (2.4, 5.2)</td>
<td>9</td>
<td>Zero-inflated Poisson</td>
</tr>
<tr>
<td>copulations</td>
<td></td>
<td></td>
<td>$\chi^2=2.6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>$0.11$</td>
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<td></td>
<td>$\chi^2=2.0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$0.37$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Pre-copulation duration</td>
<td>27.7 (18.4, 37.0)</td>
<td>9</td>
<td>Normal</td>
</tr>
<tr>
<td>(min)$^{(1)}$</td>
<td></td>
<td></td>
<td>$F=7.0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87.5 (0, 175.9)</td>
<td>$0.02$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6$^{(2)}$</td>
<td>$F=2.1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Copulation</td>
<td>20.3 (17.8, 22.7)</td>
<td>9</td>
<td>Normal</td>
</tr>
<tr>
<td>duration (min)$^{(3)}$</td>
<td></td>
<td></td>
<td>$F=5.4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.8 (12.4, 21.2)</td>
<td>$0.04$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6$^{(2)}$</td>
<td>$F=2.4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Total offspring$^{(5)}$</td>
<td>283 (152, 414)</td>
<td>8$^{(2)}$</td>
<td>Zero-inflated Poisson</td>
</tr>
<tr>
<td></td>
<td></td>
<td>105 (0, 225)</td>
<td>$\chi^2=79.0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6$^{(2)}$</td>
<td>$&lt;0.01$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\chi^2=160.3$</td>
</tr>
<tr>
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<td></td>
<td>$&lt;0.01$</td>
</tr>
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<td></td>
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<td>N/A</td>
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<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
</tbody>
</table>
## Sperm recovery assay 2 (SR2)

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (95%CL)</td>
<td>Sample size</td>
<td>Mean (95%CL)</td>
</tr>
<tr>
<td>Number of copulations</td>
<td>6.3 (5.4, 7.1)</td>
<td>8</td>
<td>3.2 (1.5, 4.8)</td>
</tr>
<tr>
<td>Number of fertile copulations</td>
<td>3.6 (2.2, 5.0)</td>
<td>8</td>
<td>0.8 (0.2, 1.5)</td>
</tr>
<tr>
<td>Pre-copulation duration (min)</td>
<td>19.7 (13.9, 25.5)</td>
<td>8</td>
<td>65.8 (16.9, 114.7)</td>
</tr>
<tr>
<td>Copulation duration (min)$^{[3]}$</td>
<td>20.0 (15.1, 24.8)</td>
<td>8</td>
<td>18.7 (16.4, 21.0)</td>
</tr>
<tr>
<td>Total offspring$^{[5]}$</td>
<td>204 (120, 288)</td>
<td>8</td>
<td>70 (6, 132)</td>
</tr>
</tbody>
</table>
## Sperm recovery assay 3 (SR3)

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (95%CL)</td>
<td>Sample size</td>
<td>Data distribution</td>
</tr>
<tr>
<td>Number of copulations</td>
<td>5.7 (5.1, 6.2)</td>
<td>9</td>
<td>Poisson</td>
</tr>
<tr>
<td>Number of fertile</td>
<td>4.0 (2.5, 5.5)</td>
<td>9</td>
<td>Zero-inflated Poisson</td>
</tr>
<tr>
<td>Pre-copulation</td>
<td>25.1 (18.7, 31.6)</td>
<td>9</td>
<td>Normal</td>
</tr>
<tr>
<td>Copulation duration</td>
<td>20.3 (18.0, 22.7)</td>
<td>9</td>
<td>Normal</td>
</tr>
<tr>
<td>Total offspring</td>
<td>320 (152, 487)</td>
<td>8[2]</td>
<td>Zero-inflated Poisson</td>
</tr>
</tbody>
</table>

Number of copulations was analyzed using generalized linear model; pre-copulatory duration and copulatory duration were analyzed using two-factor ANOVAs, all with male age and clone line as the factors. When the interactions between the two factors were not significant, they were taken out from the final model. Clone line was always included as a factor in the model irrespective its significance to represent the genetic non-independence among males. Number of fertile copulations and total number of offspring were analyzed on fertile males only (non-zero data) using generalized linear models with corresponding distributions and “log” link functions. The statistics of both factors (age and hemiclone) are present. Significant p values are in bold.

[1] Natural logarithm transformed data
[2] Some females were lost
[3] The average of all copulations of the same male
[4] Some males did not copulate
[5] All offspring produced by the same male in all copulations
**Table 2.2.** Differences in mating behaviors between fertile males and sterile males in the sperm evacuation assay (*i.e.* hemiclone males copulated with a succession of virgin *Ives* females to deplete males’ sperm store).

<table>
<thead>
<tr>
<th></th>
<th>Fertile males (95%CL)</th>
<th>Sterile males (95%CL)</th>
<th>Effect of sterility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of copulations</td>
<td>4.6 (4.1, 5.1), n=50</td>
<td>2.3 (1.6, 3.0), n=32</td>
<td>df=1, 77, $\chi^2=20.3$, $p&lt;0.01$</td>
</tr>
<tr>
<td>Pre-copulation duration</td>
<td>33.9 (28.7, 39.1), n=49</td>
<td>51.0 (33.9, 68.0), n=32</td>
<td>df=1, 76, F=0.5, $p=0.46$</td>
</tr>
<tr>
<td>Copulation duration</td>
<td>20.9 (19.4, 22.3), n=49</td>
<td>16.4 (14.9, 17.9), n=32</td>
<td>df=1, 76, F=6.0, $p=0.02$</td>
</tr>
</tbody>
</table>

All response variables were analyzed with male sterility, age and hemiclone line as the predictors. A generalized linear model was applied to number of copulations with Poisson distribution and a “log” link function. Logarithm transformed pre-copulation duration and copulation duration were analyzed using Ordinary Least Squares models. Interactions between factors were not considered in the analyses because of small sample sizes. Age and hemiclone line were always included as a factor in the model irrespective its significance. Significant p-values are in bold.

[1] The average of all copulations of the same male
Figure 2.1. The percentage of males that were alive (A) and fertile (B) by post-eclosion age falls four days after matings with a group of eight virgin *Ives* females for six hours. A hundred males were initially used for each age group. Male fertility was determined by the presence of viable offspring. The percentage of fertile males includes dead individuals.
Figure 2.2. The percentage of fertile males by number of copulations in the sperm evacuation assay (SV, i.e. hemiclone males copulated with a succession of virgin Ives females in a five-hour period to deplete males’ sperm store), excluding males that were sterile at the beginning of the assay (A); and number of offspring produced at each copulation by number of copulations in SV (B). Numbers beside the means are the sample sizes. Error bars represent 95% confidence intervals, which were not applied to sample size < 3. The increase of percentage of fertile copulation and number of offspring produced after the sixth copulation is because the few males (n ≤ 2) who copulated for more than six times in five hours were fertile in their later copulations.
Figure 2.3. Total number of offspring produced in a succession of copulations of the same males during the five-hour observation period in the sperm evacuation assay (SV, *i.e.* hemiclone males copulated with a succession of virgin *Ives* females in a five-hour period to deplete males’ sperm store) and the sperm recovery assays (SR1, SR2, and SR3; *i.e.* the same hemiclone males copulated with a succession of virgin *Ives* females in a five-hour period, one, two or three days after the SV assay). Error bars represent 95% confidence intervals.
Figure 2.4. A comparison between the total number of offspring produced by *Ives* young and old males (one-week-old and four-week-old, respectively) mating with six *Ives* virgin females four days after sperm evacuation matings, in which the same males were housed with a group of eight *Ives* virgin females to deplete male’s sperm store. Numbers beside the means are the simple sizes. Error bars represent 95% confident intervals.
Chapter 3: Influence of male age and pre- and post-copulatory sperm senescence on offspring fitness in *Drosophila melanogaster*

Abstract

Sperm senescence, a decline in sperm quality caused by male ageing and by sperm ageing before or after copulation, may have adverse effects on offspring quality. Previous work has shown that male ageing affects the fitness of sons and daughters differentially, but it is not known whether that effect is due to sperm age or male age independent of sperm age. To disentangle the effects of male and sperm ages on offspring fitness, we studied a laboratory-adapted, wild-type population of *Drosophila melanogaster*. We used both wild-type flies and cytogenetically generated hemiclonal flies derived from the same population to control for genetic differences between young and old males. We found, contrary to previous studies of this species, that male age and pre-copulatory sperm age did not influence offspring fitness (sons or daughters), whereas post-copulatory sperm age had a negative impact on offspring fitness independent of male age and offspring sex. Our results do not support the idea that sons sired by old males or old sperm suffer a greater reduction in reproductive success than daughters, but we do provide evidence that post-copulatory sperm ageing results in deleterious genetic changes.
**Introduction**

Senescence, the persistent decline in age-specific fitness components with advancing age, is prevalent in eukaryotes (Rose 1991; Pizzari et al. 2008). Although both the mortality and fertility/fecundity of ageing individuals have been widely studied (Rose 1991; Bonsall 2006), age-dependent patterns of decline in sperm fitness have received less attention in the context of the evolution of ageing (Pizzari et al. 2008). Sperm senescence occurs at two levels: the organismal level, in the germ cells of a male before meiosis; and the gametic level, during and after meiosis (Pizzari et al. 2008). Therefore, sperm senescence is related to both male age and sperm age. Because mature sperm cells are not used instantaneously, but rather may be stored for considerable periods of time by males prior to transfer to females and by females prior to fertilization, sperm-age-mediated senescence can occur before copulation (pre-copulatory) and after copulation (post-copulatory). Sperm senescence at both organismal and gametic levels can lead to a reduction in offspring quality; hence can result in significant evolutionary consequences, such as generating inter-sexual conflict, biasing the outcomes of sperm competition, leading to cryptic female choice and inducing maternal allocation of offspring sex ratio (Pizzari et al. 2008).

At the organismal level, male senescence may contribute to both impaired sperm DNA and a decline in sperm performance related to malformation of the cellular phenotype via mutation accumulation in germ-line stem cells. This degeneration is related to the continuous replication required for ongoing sperm production at a high rate, resulting in spermatozoa of diminished quality as a male ages (Kandeel et al. 2007). The reduction in apoptosis in the testes that eliminates damaged germ cells (Brinkworth 2000), and abnormality of cellular proteins that are involved in genomic maintenance (Intano et al. 2002) may also contribute to the increased manufacturing errors in spermatogenesis with male age.
In the sexual selection literature, the idea that by choosing older males females acquire “good genes” or at least viability genes has been widely discussed (Hansen and Price 1995; Kokko 1998; Brooks and Kemp 2001), but little has been done to measure the potentially countervailing costs for progeny of mutation accumulation in the germ-line.

Some studies failed to find measurable male age effects on offspring fitness. In the fly Sepsis cynipsea, for example, there was no significant effect of male age on offspring growth or survival (Martin et al. 2003). In the seed beetle, Callosobruchus maculatus, old males had similar reproductive outcomes to young males, measured as number of offspring produced, hatching success of eggs, larval development rate and egg-to-adult survival of offspring (Fricke and Maklakov 2007). In humans, paternal age is not associated with the overall prevalence of congenital malformations in offspring (Zhu et al. 2005; Stewart and Kim 2011), but is related to a small increased risk of occurrence of some specific genetic diseases such as achondroplasia (Wyrobek et al. 2006), Apert’s syndrome, Autism, and Schizophrenia (Wiener-Megnazi et al. 2012). Other studies report decreases in progeny quality with male age. For instance, progeny of older brown Norway rat males have an increase in neonatal death rate (Serre and Robaire 1998). The fruit fly Drosophila, as major model system in the study of ageing, has been shown to have male-age-related decline in progeny fitness in several studies.

Male-ageing effects on offspring fitness were initially suspected in D. melanogaster because of a progressive decrease in the proportion of sons with father’s age (Yanders 1965; Mange 1970). Then it was indirectly and directly supported by two following studies (Price and Hansen 1998; Long and Pischedda 2005). Price and Hansen (1998) measured the quality of sons and daughters of males belonging to three age classes: young (two days old), intermediately aged (13 to 14 days old) and old (32 to 33 days old). There was no decline in daughter fecundity with paternal age. The authors suggested that a nonsignificant decline in son’s fitness might be
suggestive of a real phenomenon, but because the competitor stock used was so much weaker than the experimental flies, their power to detect differences between male age treatments may have been limited. Differential impacts of male senescence on sons and daughters suggest an adaptive function for the sex ratio skew observed in the studies of Yanders (1965) and Mange (1970): females mitigated the cost of mating with old males by producing more daughters than sons.

Long and Pischedda’s (2005) research offered direct support for this explanation. First, they confirmed Mange’s (1970) finding of a shift in offspring sex ratio towards a greater proportion of females in broods sired by 13-day-old males compared to broods sired by one-day-old males (Long and Pischedda 2005). Second, they found that the shift in offspring sex ratio might be explained by a more rapid decline in the fitness of sons than daughters as fathers aged: young fathers’ sons were about 20% fitter than old fathers’ sons in competition with competitor males for mates, but daughters’ fecundities did not change with their father’s age. Long and Pischedda’s (2005) result was exciting for several reasons, knitting together a cost to mating with older males (lower quality sons) with an apparent adaptive solution by females (sex ratio adjustment in favour of daughters). The authors suggested that although both sexes inherit the same mutations, the reduction in fitness of sons with paternal age was the result of differential impact of mutation accumulation in the germ-line upon the two sexes of offspring. Such a difference is predicted if males experience stronger net selection (e.g., due to competition for mates) than females do (Agrawal 2001) and shown for both visible “marker” mutations (Pischedda and Chippindale 2005) and spontaneous mutations (Mallet et al. 2011). The authors speculated that females might adaptively use X-bearing sperm over Y-bearing sperm to reduce son production, and therefore could help to mitigate the indirect “bad genes” cost of mating with older males from germ-line mutation. All in all, the prevalence and evolutionary significance of
changes in offspring quality with advancing male age is still unknown, and direct evidence for it is only found in limited organisms.

Male ageing affects sperm senescence by determining the genetic quality of the progenitor stem cells within the testes before meiosis. After meiosis, sperm cells also experience senescence independent of male age. Mature spermatozoa stored by males prior to copulation are potentially susceptible to degradation because of the high level of reactive oxidative species within sperm cells and the dearth of cytoplasm to repair the damage of oxidative stress at the late stages of spermatogenesis (Velando et al. 2008; Kandeel et al. 2007; and Siva-Jothy 2000). A male’s capacity to repair damage from mutagenesis may even weaken as he ages, and so an interaction between male age and sperm age is expected. For example, old bulls have reduced activity of antioxidative enzymes in seminal fluid and spermatozoa (Kelso et al. 1997). In the aforementioned Drosophila studies, the question of at which level (organismal or gametic) apparent sperm senescence occurred cannot be answered because males were aged as virgins prior to experimental mating, and sperm quality of old males might be determined by both male age and pre-copulatory sperm age.

In addition to pre-copulatory aging, sperm may senesce after being transferred to females. While extended sperm storage is rare in mammals, it is common in invertebrates (Pitnick et al. 2009; Simmons 2001). There are many reasons to expect post-copulatory sperm senescence. First, oxidative stress within the female reproductive tract is likely to induce damage to DNA in both mitochondrial and nuclear genomes, and thereby cause deterioration in offspring quality (Aitken and Krausz 2001). Second, it is known that both male and female secretions can enhance sperm survival during storage by providing antioxidants that protect sperm from degradation and nutrients that are essential for healthy sperm metabolism (Heifetz and Rivlin 2010) (e.g. ants, den Boer et al. 2008; honey bees, den Boer et al. 2009b; fruit flies, Ravi Ram et al. 2005; mammals,
Sidhu et al. 1999; Boere et al. 2011). However, the effects of these secretions from both sexes may be temporary. For example, in *Drosophila*, most male accessory gland proteins are only detected for a matter of hours in the female reproductive tract after mating (Ravi Ram et al. 2005) and most of their effects only persist for days (Xue and Noll 2000). If the senescence of sperm cells before and after copulation also causes genetic damage similar to ageing of testis, then we expect that male offspring will be more vulnerable to these mutations and thus have lower fitness than female offspring because males experience stronger sexual selection (Agrawal 2001).

*Drosophila melanogaster* is an ideal system for the study of sperm senescence for several reasons. First, the short generation time and short life span make the study of male ageing feasible. Second, due to the absence of parental care, males almost exclusively contribute to offspring fitness through the DNA carried in their sperm. Third, both males and females store sperm (Demerec 1965). In males, seminal vesicles act as pre-copulatory sperm reservoirs, where the sperm stored are isolated from seminal fluid until the onset of ejaculation (Gromko 1984). The female sperm storage organs comprise a seminal receptacle and paired spermathecae. The seminal receptacle is the primary sperm storage organ, housing approximately 70% of stored sperm cells (Manier et al. 2010). The paired spermathecae are presumed to be the long-term sperm storage organs (Fowler et al. 1968). Sperm is gradually released from the two types of organs such that a female can lay fertilized eggs up to five weeks after a single insemination under laboratory conditions (see Chapter 4). Thus we can potentially obtain offspring sired by sperm that have experienced both pre- and post-copulatory sperm senescence. We can also partition male senescence and pre-copulatory sperm senescence in this species by standardizing sperm age, which can be achieved by depleting the sperm reserves of males in different age classes and allowing a controlled recovery period. Finally, there is already some evidence for
male ageing effects on the genetic quality of sperm (Price and Hansen 1998; Long and Pischedda 2005; described above).

In this study, we examined the relative contributions of male senescence, and pre- and post-copulatory sperm senescence, to the decline in offspring fitness in *D. melanogaster*. Offspring fitness is a comprehensive measurement of both adult mating performance under controlled mating competition and the viability of progeny in the next generation. Following the earlier studies (reviewed above) we asked if the fitness of sons and daughters responded differently to these three senescence factors. To do this, we applied two male-age and two sperm-age treatments in *Ives* males, and the same two male-age treatments in hemiclone males generated by a cytogenetical technique ‘hemiclone analysis’ (Chippindale et al. 2001). This technique allowed us to examine the fitness of flies carrying two identical haploid genomes that should be identical but for the effects of senescence, and to reveal genetic variance in age-related changes. Finally, because our results were qualitatively different from Long and Pischedda’s (2005), we undertook a full-scale replication of their experiment. We expected to reproduce their results, given the reported magnitude of relative difference in son’s fitness between the two father’s age categories (20%), which was extraordinary considering 13-day-old males used in the ‘old male’ category were really not very old.

**Method**

**Study populations**

All flies were derived from the *Ives* population, a large outbred population of *D. melanogaster* (Rose 1984). This population had adapted to consistent laboratory conditions for about 900 generations prior to these experiments. In the fitness experiments, an outbred competitor population with a recessive brown-eye color (*Ives*_b) was used to distinguish them from the red-
eyed wild-type *Ives* phenotype. The *Ives* population was derived from the *Ives* population by repeatedly backcrossing the recessive brown marker *bw* into *Ives*. Both populations were maintained at 1,800 – 2,500 adults/generation and were cultured on a 14-d, discrete-generation schedule at 25°C under 12h:12h light:dark cycle on a banana/agar/killed-yeast medium. In each culture cycle, egg density was maintained at approximately 100 per 25 mm × 95 mm shell vial. Egg-to-adult development time was nine to ten days and median longevity was about six weeks after eclosion (C. Kimber, unpubl. data). Adult flies were handled using light CO₂ anesthesia. In all experiments, mating and egg-laying environments closely matched those of the routine culture of the *Ives* population in our laboratory and this defined environment provides us with an advantage to reproduce demographic treatments in experiments.

**Male age and sperm age treatments in genetically variable *Ives* males**

**Male age and pre-copulatory sperm age treatments**

We conducted offspring fitness experiments on genetically variable *Ives* males in two trials varying with somewhat different ‘old’ male ages. In trial 1, young males were four-day-old and old males were four-week-old; in trial 2, young and old males were four-day-old and three-week-old, respectively (Figure 3.1). Males aged four to five-day-old were at the same approximate age as *Ives* flies are when propagated in routine culture; at four weeks of age, males showed signs of senescence, like increase in pre-copulation duration, decrease in copulation duration, increase in number of copulations given ample mating opportunities in a standard mating period, even though over 90% of the cohort were still alive and able to reproduce (see Chapter 2). Old males were collected upon eclosion 24 days (trial 1) and 16 days (trial 2) before the collection of young males, and so the two age groups could be used synchronously for the experiments. Males in both age groups were kept unmated until the experiments. Old males were
housed in groups of 10, transferred to fresh-food vials on the same days twice a week, and then separated into single vials four days before the experiments. Young males were housed in single vials after eclosion.

Male *Drosophila* store sperm in their seminal vesicles before insemination, so old males likely transferred a mixture of old and fresh sperm in their ejaculates. To disentangle the effects of male senescence and pre-copulatory sperm senescence on offspring fitness, we used carefully controlled “sperm evacuation” and “sperm recovery” protocols, to standardize sperm age in our virgin males (see Chapter 2). In the sperm evacuation trials (SV), each young and old male was housed with eight four-day-old virgin *Ives* females for five hours. We showed earlier that these five-hour mating trials were sufficient to induce temporary sterility, as the result of depleting the stored sperm in a male’s seminal vesicles. Four days after the SV procedure, when males were predicted to have recovered > 65% of their virgin-level fertility (see Chapter 2), each male was placed in a vial with another six four-day-old virgin *Ives* females for five hours. We called this the sperm recovery (SR) protocol. After each SV and SR trial, the females were isolated from the males and allowed to oviposit for 24 h in their original groups. Some males died or became sterile after the SV trials, probably because of the stress induced by repeated mating over such a short time period. In total, there were 81% three-week-old males and 49% four-week-old males still alive and fertile during the SR trials. In rare cases, males were discarded because the females that they mated with died or got lost. This was to ensure that the products of all matings were included in the following offspring fitness tests.

The SV and SR trials resulted in offspring that we classified into four groups depending upon male age and pre-copulatory sperm age (Figure 3.1). In the SV trials, offspring were sired by young or old males with unknown sperm age. We designated these young-male old-sperm (YMOS) and old-male old-sperm (OMOS) groups, respectively. The SR trials resulted in
offspring from the young-male young-sperm (YMYS) and the old-male young-sperm (OMYS) groups, sired by males of two different ages both with known and standardized sperm ages (< four-day-old) in their ejaculates. We did not conduct the YMOS treatment in trial 1 (offspring from the SV trail were discarded) because of time limitations; we added this treatment in the trial 2 in recognition the potential exists for young males to carry somewhat aged sperm because spermiogenesis begins in the pupal stages in *Drosophila* and males carry mature sperm at eclosion (see Chapter 1, Demerec 1965; Fuller 1993).

We used the differences between the YMYS and OMYS groups to quantify the effects of male senescence on offspring fitness, independent of sperm age. We used the differences between OMOS and OMYS groups to quantify the effects of pre-copulatory sperm senescence on offspring fitness, although exact age of sperm in the fathers’ ejaculates was not quantified. The contrast between YMOS and YMYS may serve as a control for the effects of the SV procedure because the heterogeneity of sperm ages may be smaller in YMOS than OMOS.

*Post-copulatory sperm age treatments*

Post-copulatory sperm age was defined as the duration of sperm storage within the female’s reproductive tract. We created one-day-old and five-day-old post-copulatory sperm age treatments for each of the male age and pre-copulatory sperm age treatments described above, to measure the influence of post-copulatory sperm age on offspring fitness. The one-day-old sperm treatments measured the fitness of the offspring produced from eggs laid by females during the twelve-hour period immediately following the mating trials. After that period of egg-laying, the females were fed yeast *ad libitum* with fresh banana medium each day for five days. Eggs laid over the following 24-hour period were reared to adulthood to measure the effects of the five-day-old sperm treatment. This egg-laying period resulted in a standard moderate density of
approximately 100 eggs in each vial, minimizing potential artifacts of larval density on adult fitness. Thus the same females produced offspring in both the one- and five-day-old sperm treatments, from the same copulations, but in the five-day-old sperm treatments both the females and the sperm were five days older than in the one-day-old sperm treatments.

A five-day-old sperm ageing treatment was applied for two reasons. First, it was an attempt to minimize the potentially confounding effect of female senescence on offspring fitness. Second, sperm that are greater than five-day old are not likely to be present in significant numbers under normal conditions; female D. melanogaster typically remate at least once within five days of an initial mating and last-male sperm precedence is typically strong (> 75% of progeny will come from the last male) (Clark et al. 2000).

**Offspring fitness in relation to male and sperm age of genetically variable males**

Our measure of adult fitness was designed to capture the outcome of competition under Ives culture conditions, while making the results of such competition tractable. Every effort was made to simulate normal rearing conditions despite the necessary interventions. For example, mating trials were conducted in vials containing ‘old food’ that had been processed by larvae, as occurs in the normal culture routine (Figure 3.2).

The fitness of adult male and female offspring was measured separately. To measure the fitness of male offspring, we put five red-eyed wild-type sons from the same father, 20 brown-eyed Ives$_b$ male competitors, and 25 Ives$_b$ females into each mating vial. To measure the fitness of female offspring, we put five red-eyed wild-type daughters from the same father, 20 Ives$_b$ females and 25 Ives$_b$ males into each mating vial. Thus there were 50 flies in each mating vial in both sets of trials. We controlled the number and the age of the target and competitor flies of each sex, and
expected that fitness tests conducted in different days (e.g. ‘one-day-old’ and ‘five-day-old’ sperm age treatments) were comparable.

The net fitness of sons and daughters of the same father was ultimately measured by the relative frequency of red-eyed progeny (the proportion of red-eyed progeny among all red-eyed and brown-eyed progeny) in each vial from a mating trial (Figure 3.2). Hence, if the fitness of wild-type flies were equal to the Ives competitors in a vial we would see 20% red-eyed offspring. This measurement encompassed every component of reproductive performance of adult offspring, including mating ability, sperm competitiveness, fertility/fecundity, and egg-to-adult survival of the second generation; but egg-to-adult survival of the experimental flies was not covered.

**Offspring fitness in relation to male age and pre-copulatory sperm age in males with defined genotypes**

**Hemiclones**

Twelve lines of hemiclones were generated from the Ives population. Males in the same hemiclone line share a haploid genome constituting an X chromosome and the two major autosomes (Chippindale *et al.* 2001). Using males with defined genotypes (hemiclones) has an advantage over using genetically variable flies as it enables us to compare offspring from outbred males at different ages that transmit haploid genomes via sperm that should be identical but for the effects of senescence. Hemiclones are produced via successive generations of mating with cytogenetically constructed ‘clone generator’ (CG) females. Generating hemiclone males relies first on the natural lack of recombination in *D. melanogaster* males—chromosomes are inherited intact from fathers due to achiasmate meiosis (no crossover between homologous chromosomes), so can be copied from father to sons and identified using simple visible markers. Second, CG
females (denoted DX-tbs) carry a random Y chromosome from the source population, and a compound X chromosome (both X chromosomes in females form a single linkage unit). These double-X females reverse the transmission of the X and Y chromosomes, so their sons inherit the father’s intact X. Third, CG females carry an aberration consisting of several homozygous-viable translocations of the two major autosomes (T(2:3) rdgc st in ri p^r bw), which in heterozygotes cause the two autosomal chromosomes to cosegregate as a single linkage unit in surviving offspring (Chippindale et al. 2001). Each hemiclone line started with a cross between a randomly chosen Ives male and several virgin CG females (Figure 3.3, Step 1). In the second generation, all male offspring carried haploid wild-type genomes constituting an X chromosome and two major autosomes. By crossing only one of these hemiclone males to CG females, we preserved a single wild-type haploid genome from each wild-type father and made many copies (Figure 3.3, Step 2). Hemiclonal lineages were maintained or expanded by crossing males carrying the selected haploid genotype to virgin DX-tbs females each generation (Figure 3.3, Step 3).

Offspring fitness test

We generated three male age and pre-copulatory sperm age treatments OMOS, OMYS, YMYS for each of the twelve hemiclone lines described above. Males were four-day-old in the young male treatment, and four-week-old in the old male treatment, and they all mated with CG females in the SV and SR trials (Figure 3.3, Step 4). We did not test for post-copulatory sperm senescence in hemiclone males, because CG females have higher mortality and might have lower sperm storage ability than wild-type females (Rice 1996). Thus, only one-day-old post-copulatory sperm age treatment was conducted, but not the five-day-old sperm treatment. In addition, we only measured the fitness of male offspring but not female offspring, because the scale of the
experiment was large and male offspring fitness is reported to be more vulnerable to paternal senescence than female offspring fitness is (Long and Pischedda 2005).

Although cultivated with special aberrations and markers, hemiclones were crossed to flies carrying wild-type autosomes to remove these aberrations and markers prior to the fitness test, and so were tested as 100% wild-type flies with normal heterozygosity. Specifically, male offspring from the SV and SR trails (hemiclone males) were mated to DX-IV females (i.e., autosomal Ives females carrying a compound-X (DX) and a Y chromosome; Figure 3.3, Step 5). This cross generated wild-type sons who possessed a defined young/old haplotype (i.e. the hemiclone genotype) and a random haplotype inherited from the Ives population. It is noteworthy that males used in this cross in all three treatments (OMOS, OMYS, YMYS) were < three days old: they were the sons of males that had undergone those ageing treatments so that the experimental flies all had young fathers of standardized age. By standardizing the age of hemiclone males in the genetic cross a generation before the fitness tests (Figure 3.3, Step 5), the differences between treatments should therefore only be attributable to paternal germ-line mutations altering the hemiclone (i.e., genetic changes resulting from senescence in the last generation). We note that this novel use of hemiclone analysis in the study of ageing facilitated testing for germ-line senescence by excluding the influence of nongenetic age-related factors, such as somatic senescence.

For each of the twelve hemiclones, we haphazardly chose five young and old males to generate each of the OMOS, OMYS and YMYS treatments (Figure 3.3, Step 4); in the cross that returned hemiclone male offspring to wild-type genetic background (i.e. replace the haploid genome with translocations with wild-type haploid genome), we applied four replicates for each male (Figure 3.3, Step 5). This design generated 20 fitness tests for each hemiclone / experimental treatment. The procedure of the fitness test was the same as it was on Ives flies.
(Figure 3.2; Figure 3.3 Step 6-7), except it was carried out in four blocks (on consecutive days) due to the large scale of the experiment, with one replicate per male run in each day.

**Replicating Long and Pischedda’s (2005) Experiment**

In order to compare our *Ives* population to the population that Long and Pischedda (2005) used [the LH\textsubscript{M} population of Chippindale *et al.* (2001)], and to test if Long and Pischedda (2005)’s finding of a decline in the fitness of sons in relation to their father’s age was indeed replicable, we repeated Long and Pischedda’s experiment using the same fly stock (LH\textsubscript{M}) that they did, closely following their experimental protocol.

In the experiments of Long and Pischedda, offspring fitness was tested in competition with LH\textsubscript{M}-\textit{b} flies, which were derived from LH\textsubscript{M} flies and homozygous for a recessive brown-eyed marker (\textit{bw}). They created two types of experimental vials, with 15 replicates each. Each replicate contained 90 old-LH\textsubscript{M} and 90 young-LH\textsubscript{M}-\textit{b} eggs or 90 young-LH\textsubscript{M} and 90 old-LH\textsubscript{M}-\textit{b} eggs. Thus each vial contained two types of offspring that differed in both population of origin and sire age. On day 11, the flies that eclosed from these eggs were transferred to half pint bottles containing standard medium supplemented with 40 mg of yeast, and male-male competition was allowed to ensue for a further two days. Male reproductive success was estimated by sampling 20 LH\textsubscript{M}-\textit{b} females from each bottle and placing them individually into test tubes for egg laying (a total of 300 females per male age treatment), and scoring eye color of the next generation. The brown-eyed marker made it possible to determine paternity, as brown-eyed progeny resulted from fertilization by LH\textsubscript{M}-\textit{b} males, and red-eyed progeny resulted from fertilization by LH\textsubscript{M} males. The relative adult fitness of sons was measured as the proportion of broods in the next generation that contained predominantly red-eyed offspring for each of the replicate vials (15 vials with old-LH\textsubscript{M} and young-LH\textsubscript{M}-\textit{b} progeny, 15 vials with young-LH\textsubscript{M} and old-LH\textsubscript{M}-\textit{b} progeny).
measurement provides a simple approximation of reproductive success. Because there were the equal number of LH\textsubscript{M−b} and LH\textsubscript{M} eggs in each replicate, it was expected that 50% of broods contained a predominance of red-eyed offspring.

**Statistical analyses**

The proportion of red-eye progeny produced from a mating vial was angular-transformed to improve the fit to normality (Sokal and Rohlf 1995). All statistical analyses were carried out with JMP 9 (SAS Institute Inc., 2010). When analyzing the fitness of wild-type offspring, we constructed a full factorial model including individual male as a random effect, which took independence of data between OMOS and OMYS into account, and the following factors as the fixed effects: male age and pre-copulatory sperm age (OMOS, OMYS, YMOS, and YMYS), age of old males (three- and or four-week-old males), post-copulatory sperm age (one-day-old and five-day-old), and offspring sex (male and female). Fifteen vials that contained < ten progeny and two vials that had more than six dead progeny were excluded from the analyses. In the full model testing the fitness of male offspring in males with defined genotypes, we treated male age and pre-copulatory sperm age (OMOS, OMYS, and YMYS), hemiclone line and replicate of each male as the fixed effects, and individual male as random effects. Replicate was nested within male, which in turn was nested within hemiclone line. We generated full models with interactions of all fixed effects using the restricted maximum likelihood (REML) method and found the best model by sequentially removing the least significant term in the model to find the minimum adequate model.
Results

Offspring fitness in relation to male and sperm age of genetically variable males

In genetically variable males from the *Ives* population, the best statistical model included post-copulatory sperm age and offspring sex as the significant predictors of offspring fitness measured as proportion of red-eyed progeny produced in each mating vial (Table 3.1). Based on this model, offspring fitness was significantly higher in the one-day-old sperm treatment than in the five-day-old sperm treatment (Table 3.1; Figure 3.4). The fitness of sons was significantly higher than the relative fitness of daughters sired by the same male (Table 3.1). As noted in the Introduction, this result is believed to reflect the stronger negative impact of the *bw* mutation upon the male and female *Ivesb* competitors, rather than differences in the wild-type flies (Pischedda and Chippindale 2005; Mallet *et al.* 2011). There was no significant difference among the male age and pre-copulatory sperm age treatments (OMOS, OMYS, YMOS, and YMYS), and no significant interaction between factors in the factorial model (Table 3.1). The old male ages (three or four-week-old) did not significantly influence offspring fitness, indicating that the two ages that we used in the ‘old male’ category did not differentially influence the outcomes of our analyses. The lack of significant difference between the YMOS and YMYS treatments indicated that there was no effect of the sperm evacuation (SV) procedure on offspring fitness: whether or not a male had experienced sperm depletion from a successive matings did not affect his offspring fitness.

Offspring fitness in relation to male age and pre-copulatory sperm age in males with defined genotypes

Using hemiclone analysis, we only tested the effects of male age and pre-copulatory sperm age on the fitness of sons. The results were congruent with the findings using genetically variable
males, with no significant effects of male or pre-copulatory sperm age on son’s fitness. There was significant variation in adult fitness among the twelve hemiclone lines ($F = 4.5$, $df = 11,706$, $p < 0.01$) (Table 3.1), suggesting that there was some inherent variation among males in sperm quality in the *Ives* population (heritability of offspring fitness was 6.6%).

**Replicating Long and Pischedda’s (2005) Experiment**

After undertaking a full replication of the experiments performed by Long and Pischedda (2005), we found no significant difference in the relative reproductive success of male offspring sired by old versus young males ($t$-test, $t = 0.86$, $df = 28$, $p = 0.40$), estimated as the proportion of individual female broods that were predominantly sired by wild-type males in each replicate of the experiment (Figure 3.5). On average, focal (red-eyed) sons of young sires outcompeted their competitors in reproductive success in 76% (95% CI = 65 - 79%) of the broods, about 50% in excess of what would be expected (their fitness equivalent to the competitor population used), and close to the proportion (65%) reported by Long and Pischedda (2005). Focal (red-eyed) sons of old sires outcompeted their competitors in a similar proportion of broods (mean = 72%, 95% CI = 69 - 83%), but this proportion was significantly higher than Long and Pischedda’s (2005) report (55%). In summary, we did not see a decline in male fitness with sire age that the original work present. Because the expected proportion of broods containing predominantly red-eyed offspring was 50%, our data show marked superiority of wild-type males over competitor males that were genotypically the same except for the presence of the *bw* eye-color marker.

**Discussion**

Despite centuries of enduring fascination with sperm, and human concern about age-related effects on offspring quality, there have been surprisingly few formal studies of sperm senescence.
By quantifying reproductive success of male and female offspring from experimentally aged males in *Drosophila melanogaster*, we investigated the potential evolutionary impacts of sperm senescence. To our knowledge, our study is the first to attempt to assess sperm genetic quality in relation to two very different stages in a spermatozoon’s lifespan, before and after copulation. Our population-level results from genetically variable *Ives* males provided good evidence for a decline in offspring fitness in relation to post-copulatory sperm age, but did not lend any support to the idea that offspring fitness is influenced by either male age or pre-copulatory sperm age. These results run contrary to previous findings in *Drosophila* (Price and Hansen 1998; Long and Pischedda 2005) where a son-specific reduction in fitness was reported with paternal age. Our genotype-level experiment using hemiclone males, while sensitive enough to measure significant heritability for male reproductive success, further confirmed that male senescence and pre-copulatory sperm senescence did not measurably affect offspring fitness. These findings were a surprise to me, particularly because the old males that we used (three- and four-week-old) were much older than the late-age males employed in Long and Pischedda (2005) which were 13-day-old. As a result, we expected to see a more pronounced decline in male offspring fitness and possibly a reduction in female offspring fitness as well.

**Offspring fitness in relation to male age**

Our study of *D. melanogaster* suggests that male ageing does not necessarily result in adverse effects for offspring fitness, even though we looked at offspring from males at fairly advanced ages. Some details of fruit fly life history and sperm production were not addressed by our work, including the influence of continuous mating and social interaction with females upon male ageing and sperm production. However, most troubling to us was the fact that we were not able to replicate the results of Long and Pischedda’s (2005) work.
We saw no evidence for the patterns reported by Long and Pischedda (2005) in the *Ives* population and therefore undertook a careful replication, using the same protocols and the same base population (LHM). Nonetheless, as our results show, our data are different from those reported by Long and Pischedda (2005) (Figure 3.5). Our data reveal a similar competitive advantage for the wild-type sons of young experimental males – about $\frac{3}{4}$ of individual female broods showed progeny predominately sired by red-eyed males, where the expectation is $\frac{1}{2}$ – but no penalty to the sons of aged males, as seen in the work of Long and Pischedda. An additional control (data not shown) in which both competitor and focal wild-type males were sired by young fathers was not different from either experimental treatment, confirming the strong competitive advantage of the wild-type over the mutant stock.

Why was there a discrepancy in the results between the study of Long and Pischedda (2005) and those present herein? An inadvertent flaw in the experiment of Long and Pischedda (2005) may exaggerate the observed difference in the fitness of sons between young and old males. Long and Pischedda (2005) used a design that was not fully crossed for all age-related factors to assess male adult fitness. Instead of competing young male offspring against the progeny of young males from the competitor stock, they used progeny of old males; when they looked at old male offspring fitness they used young male offspring as competitors and mates. In other words, the competitor flies used in the trials designed to assess offspring fitness were heterogeneous in age such that offspring sired by old LH_M males grew up and competed with the offspring of young LH_{M-b} competitor males; and young LH_M males’ offspring were housed with the offspring of old LH_{M-b} competitor males. This design was meant to test the effect of both population of origin and sire age on offspring fitness synchronously. If the offspring of young LH_{M-b} males were of higher quality than the offspring of old LH_{M-b} males, so that the offspring of old LH_M males were competing with flies of higher quality than the offspring of young LH_M.
males, then the 20% difference in relative fitness of sons reported in their trials would be an overestimate: competitor age would interact with focal male age sympathetically with their hypothesis. Note that such an effect does not refute their findings, but rather would exaggerate a sire ageing effect, however, we have no explanation for the male ageing effects seen by the authors. We conclude that the previously reported adverse effects of male age on the fitness of male offspring is likely to be overestimated and cannot be considered to be repeatable in this species.

Our experimental design had several advantages over Long and Pischedda’s design (2005). First, the ‘old’ age group that we employed (three- and four-weeks old) was much older than the ‘old’ age group (13-day-old) used by Long and Pischedda (2005), and the old males that we used showed obvious reproductive senescence at this age (see Chapter 2). Second, we repeated our own experiments by running two rounds of tests on the offspring fathered by genetically variable Ives males, and found out the results of the two tests were highly repeatable. Third, we conducted the experiments on both genetically variable Ives flies and flies generated from hemiclone lines and showed agreement in results using the two types of flies. Unlike Long and Pischedda (2005), the hemiclone analysis used for this work has a great power to generate male flies that differ in age but otherwise share a nearly identical genome, therefore, offered a good genetic control between age treatments in our experiment. In addition, this technique eliminate the potential non-genetic effects of male senescence on offspring by collecting offspring produced by males at the same age but carrying an either young or aged genome after a generation of proliferation of young and old hemiclone males. Therefore, the results of our hemiclone analysis demonstrate exclusively genetic impacts of male ageing on offspring fitness. In contrast, Long and Pischedda (2005) used aged genetically variable flies in their ‘old’ treatment. The fitness of offspring produced by these flies might be influenced by factors other
than male germ-line senescence, such as the senescence of seminal fluid and reduction in male mating stimulation on females. It is another reason that their results might exaggerate the effect of pre-copulatory sperm senescence. The strength of our experimental design and the potential overestimation of a sire ageing effect in Long and Pischedda (2005) indicate that our finding of no significant impact of male age on offspring fitness in both sexes is a more convincing result and likely reflects the real biology in *D. melanogaster*.

Studies on humans have found an increased risk of some genetic disorders in offspring of aged fathers (Brinkworth 2000; Zhu *et al.* 2005), but why is there no detectable decline in offspring fitness sired by males at an advanced age in fruit flies? First, male fruit flies may experience a lower number of germ-line stem cell divisions at an advanced age than human males do. In humans, 23 cell divisions occur during spermiogenesis every year (Bordson and Leonardo, 1991). Thus, spermatogonia of a 50-year old father have approximately 575 more DNA-replications than a 25-year old father. However, in *D. melanogaster*, each division of germ-line stem cells takes approximately 32 h (Wallenfang *et al.* 2006). At this rate, a four-week-old male has only 18 more DNA-replications than a four-day-old male. Therefore, it is expected that there will be fewer DNA replication errors accumulated in the germ-line of old male fruit flies than of old men. Second, the impacts of mutations may not be easily measured. Even though the number of spontaneous mutations in germ cells is likely to increase with male age, most mutations are neutral with respect to the offspring phenotype. For example, in humans, the known genetic disorders that pass through the male germ-line all have rare incidence (Brinkworth 2000; Zhu *et al.* 2005) requiring massive sample sizes to detect. If deleterious mutations in the male germ-line of *D. melanogaster* have similarly low incidence, then their adverse impacts on offspring may not be revealed by the sample sizes employed in our experiments.
Although we did not measure all aspects of offspring fitness, such as egg-to-adult survival, we chose adult fitness measures known to explain the majority of variance in total fitness in these populations (e.g., Chippindale et al. 2001; Mallet et al. 2011) as predicted by genic capture theory (Rowe and Houle 1996). Moreover, earlier evidence on sire age effects on offspring viability in D. melanogaster suggests that the impacts of male age on egg-to-adult survival are at best weak (Long and Pischedda 2005), and only detectable with application of stress, like larval crowding (Price and Hansen 1998). Therefore, the sire age effects on offspring viability may be minor under the culture conditions employed.

**Offspring fitness in relation to sperm age**

**Pre-copulatory sperm senescence**

We found no difference in offspring fitness between the old male old sperm (OMOS) and the old male young sperm (OMYS) treatments, indicating that male sperm storage had no detectable detrimental effects on sperm DNA. It is possible that sperm cells are densely stored in a male’s seminal vesicles and thereby exposed to low oxidative stress. However, supportive evidence for this speculation is currently absent. The pattern found in our study is consistent with findings from mammals, where male sexual abstinence seems to have no detrimental effect on either fertilization success or offspring development (reviewed by Tarín et al. 2000). In poultry, however, prolonged male sperm storage increases sperm abnormalities (El Jack and Lake 1966) and reduces fertilizing efficiency (Van Voorst and Leenstra 1995). We suggest that approaches to standardize pre-copulatory sperm age, like our sperm evacuation trials, may be valuable in future studies of male-age-mediated sperm senescence.
Post-copulatory sperm senescence

Post-copulatory sperm senescence is expected to be prevalent in the animal kingdom due to the common occurrence of female sperm storage and frequent characterization of the female reproductive tract as a “hostile environment” to sperm. There is a lot of evidence that sperm senesce and die in the female’s reproductive tract in a variety of animals (e.g., the cricket *Gryllus bimaculatus*, Ribou and Reinhardt 2012; the leaf-cutter ant *Atta colombica*, den Boer et al. 2009a; and the honeybee *Apis mellifera*, Al-Lawati et al. 2008). Accelerating sperm mortality may decrease the probability of fertilization from a male’s ejaculate, but does not necessarily mean that the genetic quality of the sperm is declining. To date, there are very few studies that have explored reduction in sperm quality with increasing post-copulatory sperm age. In humans, there is a controversy whether *in-vivo* sperm senescence in the female genital tract is associated with embryo or fetus mortality (reviewed in Tarín et al. 2000). The most comprehensive study in other animals is on a seabird, the black-legged kittiwake (*Rissa tridactyla*) in which post-copulatory sperm senescence has been shown to negatively affect fertilization potential, the rate of embryonic development, embryonic survival, and chick condition at hatching (White et al. 2008). In the fruit fly *D. melanogaster*, Radhakrishnan and Fedorka (2011) demonstrated a steady increase of sperm death inside the female’s reproductive tract, and showed that this decline was not due to female age. They speculated that most sperm death in the female sperm storage organs was a result of sperm senescence within the female, but did not assess offspring quality. We showed evidence that the genetic quality of sperm declined over time in the female’s sperm storage organs in this species, which as far as we know is the first evidence in invertebrates.

As noted in the introduction, several mechanisms have been proposed to explain post-copulatory sperm senescence, such as oxidative stress within the female reproductive tract. Other factors, such as female age and female immune response to mating have so far not been
implicated in declining sperm viability in *D. melanogaster* (Radhakrishnan and Fedorka 2011). However, it is difficult to separate an innate sperm senescence process from the effects of senescence of the female’s supporting glands and tissues, as both nature (the original sperm phenotype) and nurture (the storage process) are likely to be crucial for the viability and the genetic integrity of stored sperm.

**Sperm senescence and sperm selection**

Sperm selection offers another potential explanation for our inability to detect the effects of male senescence and pre-copulatory sperm senescence on sperm genetic quality, and may complicate the detection of post-copulatory sperm senescence. First, even if genetic quality of sperm deteriorates with male age or pre-copulatory storage time, the outcome is likely to be a more heterogeneous ejaculate. If genetic quality is related to performance of the sperm in competition, then an ejaculate that is, on average, composed of bad sperm may still produce high quality offspring, albeit in limited numbers, because post-copulatory sexual selection, via either intra-ejaculate sperm competition or cryptic female choice (Eberhard 1996), may filter out inferior sperm. There is some evidence for such processes. In *D. melanogaster*, only about 30% of ejaculated spermatozoa can be stored in the female’s reproductive tract, with most of the surplus being extruded in the “sperm mass” (Lefevre and Jonsson 1962; Manier *et al.* 2010). This process is not immediate, taking approximately an hour, and it is possible that only sperm in good condition gain entry to the female sperm storage sites. Indeed, our Chapter 2 found support for this pattern in this species: old males produced fewer offspring in a series of exhausted copulations than young males despite their larger pre-copulatory sperm reservoir. There is also supportive evidence in other animals. In the house cricket *Acheta domesticus*, more recently produced spermatozoa were relatively more abundant in the female storage organ than in the
male reproductive duct, suggesting that there is a selection mechanism in females to filter out aged sperm from male ejaculates (Reinhardt and Siva-Jothy 2005). If sperm selection exists, then our approach will have limitations to assess sperm senescence before copulation but techniques that directly measure sperm quality, such as the Comet assay and sperm viability staining, may be particularly useful (Singh et al. 2003; Holman 2009). Second, after sperm enter female sperm storage sites, similar selection mechanisms may act upon stored sperm. If there is a relationship between sperm phenotype and genetic quality, then such mechanisms could result in a pattern that earlier broods of offspring have greater fitness than later ones will, and so could complicate our detection of post-copulatory sperm senescence. However, because DNA of spermatozoa is highly condensed and transcriptionally quiescent (White-Cooper et al. 2009), selection may not act on senesced genome of viable sperm. Therefore, we believe that post-copulatory sperm senescence is the premier source of the observed decline in offspring fitness.

So far evidence for intra-ejaculate selection is lacking in D. melanogaster and experiments suggest that sperm competitive ability is a property of the diploid male’s genotype, rather than the individual sperm haplotype (Clark et al. 2000). Indeed the issue of whether or not sperm express the genotype that they carry remains unresolved. Given our state of knowledge, we suggest that the lack of a detectable impact of either male senescence or pre-copulatory sperm senescence on offspring fitness is most likely related to the maintenance of consistent sperm genetic quality in males and post-copulatory selection that ensure only good sperm entering the fertilization pool. However, once sperm are activated and transferred to females, post-copulatory sperm senescence begins and is responsible for the decline in offspring fitness after copulation in our system.
Conclusion

Little is known about sperm senescence from the perspective of genetic quality. Adding to the controversy over the effects of male senescence on offspring quality, and the potential costs and benefits of mating with older males, our research suggests that there is little or no impact of male age on sperm genetic quality in *D. melanogaster* into ages approaching the average longevity of the males. On the contrary, we found that post-copulatory sperm senescence presents a fitness cost to females through the reduction of offspring quality. Females may therefore encounter selective pressures to evolve various mechanisms to avoid using senesced sperm, or to protect sperm from degradation in storage. Males, on the other hand, may face trade-offs with respect to sperm numbers, design and senescence rate.

Hypotheses like “trading up” for better quality mates have been suggested to explain female remating prior to sperm exhaustion from the sperm storage sites. We suggest that sperm senescence plays a role in remating behaviour: females adopt a strategy of remating to recruit fresh sperm and to displace senesced sperm in storage (Reinhardt 2007; Pizzari et al. 2008). For example, in laboratory stocks of *D. melanogaster*, about 50% females remate in one to two days after their previous matings and the sperm of the last male displaces most (typically 80%) of the residual sperm (Gromko 1984). At this stage, females still have sufficient sperm in storage and egg fertilization rate is still at its maximum (see Chapter 4). Remating within a few days after the initial mating may have substantial benefits. Among other advantages of remating, such as obtaining male accessory gland proteins that enhance female feeding, and increase egg production, ovulation and egg deposition (reviewed in Ravi Ram and Wolfner 2007), females also recruit fresh sperm from remating to limit reduction in fitness due to sperm senescence in storage. We may speculate that under harsher field conditions, with variable temperatures, drought, and food limitation routinely encountered, sperm senescence may occur more rapidly.
and remating may be more strongly selected for than it is in the laboratory. We cannot rule out the occurrence of absorption of ejaculate for energy in female Drosophila, however, our study suggests that sperm senescence is a potential factor that may influence female remating. In species where remating is not possible or probable, females may be equipped to maintain live sperm for extended periods of time. An extreme example is the ant Formica exsecta, where queens may maintain viable sperm for up to 27 years after becoming isolated from males (Pamilo 1991). We expect that females in these species evolve mechanisms to ensure sperm survival and maintain sperm genetic quality, including providing space, nourishment and antioxidants (Heifetz and Rivlin 2010).

A comparative perspective on sperm and sperm-female interactions across species with long-term sperm storage, coupled with experimental investigations may be integral to understanding not only the potentially complex trade-offs associated with sperm but also a wide range of problems in sexual selection. Our research sheds new light on the potential fitness benefits of female promiscuity and calls for more studies on sperm senescence as an important contributor to post-copulatory sexual selection. Spermatozoa are a unique cell type in having their own life-history apart from the male that produced them, but like most other eukaryotic cells, they senesce and die. The evolution of sperm senescence is certain to be determined by manifold ecological and behavioural factors. We suggest that further exploration of sperm senescence is essential to a complete understanding of post-copulatory sexual selection.

References


Sidhu, K. S., Mate, K. E., Molinia, F. C., Glazier, A. M., & Rodger, J. C. (1999). Secretory proteins from the female reproductive tract of the brushtail possum (*Trichosurus vulpecula*):


Table 3.1. Best general linear model testing the effects of age of old males, male age and pre-copulatory sperm age (OMOS, OMYS, and YMYS), post-copulatory sperm age and offspring sex on the fitness of offspring of genetically variable *Ives* males, and the best general linear model testing the effects of male age, pre-copulatory sperm age, hemiclone line and replicate on the fitness of male offspring in males with defined genotypes.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>DF</th>
<th>F</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>Genetically variable <em>Ives</em> males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-copulatory sperm age</td>
<td>1,1129</td>
<td>5.88</td>
<td>0.02</td>
</tr>
<tr>
<td>Offspring sex</td>
<td>1,1129</td>
<td>33.63</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Males with defined genotypes</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hemiclone</td>
<td>11,706</td>
<td>4.5</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The response variable was the angular-transformed proportion of red-eyed progeny produced in each mating vial.
Figure 3.1. Experimental design for measuring offspring fitness in relation to male age and pre- and post-copulatory sperm age in Ives flies. Each young (three days old) and old male (three or four weeks old) were exposed to eight virgin Ives females to deplete his sperm store (sperm evacuation matings, SV). The offspring resulting from these matings created young-male old-sperm (YMOS) and old-male old-sperm (OMOS) treatments. Four days after SV, the same young and old males housed with another six virgin Ives females. Their offspring were used in the young-male young-sperm (YMYS) and old-male old-sperm (OMOS) treatments. Females from all these matings were saved for five days. Offspring produced on day 1 and day 5 generated one-day-old and five-day-old post-copulatory sperm age treatments, respectively. The numbers beside the treatments represent the sample sizes in three- and four-week-old old male age treatments, respectively.
Figure 3.2. Offspring fitness test protocol. Vials that contained *Ivesb* eggs were inserted with acetate sleeves that covered the entire inner surface. After eight days from oviposition, when all *Ivesb* pupae had attached to the sleeves, flies were removed and transferred to separate vials. The vials containing larva-processed food were used as the ‘old-food’ vials in which mating trials of the fitness test were conducted; and the flies that emerged from the transferred pupae were used as the *Ivesb* competitors (step 1). Nine days after oviposition, when most the flies had eclosed, fixed number of target red-eyed wild-type flies and brown-eyed *Ivesb* competitor flies were counted on light CO2 and then introduced in the ‘old-food’ vials, in which they could interact over a five-day period (step 2). Fourteen days after oviposition, all flies from the ‘old-food’ vials were transferred with light CO2 anaesthesia to vials containing new food where they laid eggs for 50 min resulting in approximately 100 eggs in each vial (~4 eggs per female) (step 3). Eggs were incubated for an additional twelve days at 25°C by which time all flies had eclosed. Progeny were sorted by eye color and then counted. The progeny produced by the target flies had a dominant red-eyed phenotype, which could be distinguished from the brown-eyed progeny produced by *Ivesb* parents (step 4).
Figure 3.3. Protocol for sampling and cloning a single haploid genome (steps 1–3), creating the old male old sperm (OMOS), old male young sperm (OMYS), and young male young sperm (YMYS) treatments (steps 4), returning male offspring to wild-type genetic background (step 5), and then measuring their fitness over a full generation (steps 6–7). To save space, in steps 5 and 6, males in the OMOS, OMYS and YMYS treatments are shown in brackets, denoting that each type had the same type of mates. Steps indicate generations. The offspring of the OMOS treatment were < three days older than the offspring of the OMYS and YMYS treatments when they were used in step 5. All wild-type chromosomes making up a hemiclone were derived from the base Ives population. For clarity, only genotypes that were viable and used in a subsequent cross are shown in steps 1–6. By using this protocol, twelve haploid genomes were cloned (steps 1-3). Steps 4 and 6 were independently repeated five and four times, respectively, for each genome. White bars: (T(2:3) rdgc st in ri p° bw) translocations that result in cosegregation of two major autosomes in surviving offspring; black bars: wild-type major autosomes of the target males; grey bars: random wild-type major autosomes of Ives flies; Young males were four days old; Old males were four weeks old. SV: sperm evacuation trial; SR: sperm recovery trial; bw: a recessive brown-eyed marker carried by the Ives population.
Figure 3.4. Proportion of red-eyed progeny produced from the mating vials for testing offspring fitness at two post-copulatory sperm ages of genetically variable *Ives* males. Red-eyed progeny were expected at a frequency of 20%; the data show the slight deleterious effect of the brown-eye marker on the mating success of *Ives* males. Error bars represent 95% confident intervals. Numbers beside the means represent sample sizes. Female progeny shown with blue symbols; male progeny shown with red symbols.
Figure 3.5. The proportion of broods produced by LH$_{M}$-b females that were predominantly red-eyed from each of two combinations of matings where both red-eyed and brown-eyed males of different sire age competed for fertilizations (i.e. old-LH$_{M}$ versus young LH$_{M}$-b and young-LH$_{M}$ versus old-LH$_{M}$-b). Sample size is 15 in each treatment. Error bars represent 95% confident intervals.
Chapter 4: Duration of female sperm storage depends upon nutritional condition in

*Drosophila melanogaster*

Abstract

Prolonged sperm storage in females is common in internally fertilizing animals, including *Drosophila melanogaster*, a species known for frequent matings but also with a capacity for long-term sperm storage. Limited and indirect evidence has suggested that sperm storage may impose a cost to females in this species. Thus, females in poor condition may not be able to provide nutrition or protection to their stored sperm, resulting in degradation in sperm quality. We studied the cost of sperm storage to females in relation to their nutrition by manipulating the amount of dietary yeast in a laboratory-adapted stock. Singly mated females were put on food media that had one of four successively declining yeast concentrations, applied either until no viable eggs were produced or for periods of one, two or three weeks, followed by *ad libitum* yeast. A control group of females was always provided with *ad libitum* yeast throughout the experiment and acted as the maximum fertility treatment. We examined female fertility and fecundity by daily enumeration of egg production, number of hatched eggs, and egg-hatching rate on each female, and documented duration of the female’s fertile period, defined as the duration that females laid hatched eggs since the copulations. We found that female fecundity and fertility were linearly and positively associated with concentration of yeast in the food medium, and were negatively associated with the duration of yeast restriction. Also, both female fecundity and fertility increased after females received additional yeast. Duration of the female’s fertile period was also positively associated with the amount of dietary yeast. The decline in total fertility of females that had experienced any of the three periods of yeast restriction suggests that viability of stored sperm depends on female condition. Although there is a potential for conflating the effects
of sperm maintenance and egg quality, the experiments suggest that sperm maintenance in *D. melanogaster* carries a cost when dietary yeast is in short supply.
Introduction

Prolonged sperm storage in females is common in animals, generally occurring where there is an advantage to a delay between insemination and fertilization. One such advantage might be fertility assurance when there is uncertainty in coordinating the timing of reproduction with finding a suitable mate. Many female animals store sperm as an apparent adaptive response to unsuitable environmental conditions for producing and rearing offspring. For example, females in some species store sperm over winter, presumably in response to the difficulty of finding fertile males at the optimal time of fertilization in spring (bats: León-Galvan et al. 1999; fruit flies: Izquierdo 1991; Collett and Jarman 2001). Similarly, females that reproduce continuously may reduce sperm use by suspending egg deposition in unfavorable environments, holding onto sperm for future use when conditions improve (Bloch Qazi and Hogdal 2010).

There is emerging evidence that sperm storage can impose a considerable cost to females (Roth and Reinhardt 2003; Baer et al. 2006; Ward et al. 2008; Miller and Pitnick 2003). Although the content of the seminal fluid that males transfer to females during copulation can enhance sperm viability in a short term (den Boer et al. 2008; Holman 2009), long-term sperm survival in the female reproductive tract depends upon female secretions that may (i) maintain a low oxidative-stress environment in the sperm storage organs (e.g. Mexican big-eared bats Corynorhinus mexicanus, León-Galvan et al. 1999), (ii) supply nourishing fluid (Drosophila, Heifetz and Rivlin 2010; honey bees, den Boer et al. 2009; mammals, Sidhu et al. 1999, Boere et al. 2011), or (iii) regulate other aspects of the sperm storage environment, such as defending against pathogens (e.g. Drosophila, pigs, rats, hens and humans) and maintaining osmotic homeostasis (e.g. Drosophila) (reviewed in Heifetz and Rivlin 2010). There have been few attempts to determine whether sperm use and storage depend upon female condition. It is possible, for example, that females in poor condition or under physiological stress accelerate
sperm senescence or sperm loss rates because they cannot allocate adequate resources to nourish sperm; or they may reallocate energy from egg-production to sperm maintenance, waiting for the environment to improve.

Direct evidence on the cost of sperm storage has been found in some insect species. In the damsel bug *Nabis rugosus*, females are mostly inseminated in the fall but lay eggs only in spring (Roth and Reinhardt 2003). Food-deprived females maintain fewer sperm than well-fed females before winter hibernation, suggesting that condition may play a role in the maintenance of live sperm in this species. In the leaf-cutting ant *Atta cephalotes*, maintenance of large quantities of sperm during the mating flight incurs a cost of reduced immunity during colony founding and increased risk of death for queens (Baer et al. 2006). In addition, indirect evidence also indicates costs of sperm storage to females. For example, in the yellow dung fly *Scathophaga stercoraria*, a stock that was artificially selected for four spermathecae had lower fecundity in mated females than a stock that was selected for three spermathecae (Ward et al. 2008). However, this result does not differentiate between costs of producing more storage organs and those associated with sperm maintenance.

For sperm-female interactions, *Drosophila* is an increasingly well-studied genus. Although female *Drosophila* are known to remate frequently, they also can store sperm for a long time in remarkably elaborate reproductive organs, which include a long tubular seminal receptacle and a pair of capsule-like spermathecae. Take *D. melanogaster* for example: the seminal receptacle is similar in length to female body itself and is the primary sperm storage organ, housing 70-80% of stored sperm (Manier et al. 2010). The bulb-like spermathecae are presumed to have the function of long-term sperm storage. Each of them is lined with glandular cells containing secretory organelles, the secretions of which are important to maintain sperm viability (Iida and Cavener 2003; Schnakenberg et al. 2011; Wolfner 2011). In the field, some
species of *Drosophila* can overwinter in the adult stage in temperate regions, suspending their egg laying and maintaining viable sperm until spring (Izquierdo 1991; Collett and Jarman 2001). In the laboratory, female *D. melanogaster* can store viable sperm for up to four months at 14°C (Boulétreau-Merle and Fouillet 2002) or for several weeks at 25°C (Kaufmann and Demerec 1942; Perotti 1973). However, there is only limited and indirect evidence that sperm storage has a cost for females in this species. For example, it is well known that mated females die more rapidly than virgin females do, a result that conflates a wide range of physiological effects. Suggestively, females in stocks selected for a larger sperm storage organ were found to carry more sperm, but also suffer a reduction in longevity (Miller and Pitnick 2003). However, virgin females of the selected stocks had similar longevity to control females, suggesting that storage of more sperm rather than maintenance of a larger sperm storage organ carries a longevity cost to females.

In this taxon, energy metabolism of stored sperm is likely provisioned and regulated by females. Little is known about the energetic needs of *Drosophila* sperm in storage, but biochemical analysis in the honeybee gives us insights into the energy sources that sustain insect sperm for long-term storage. Research in honeybee suggests that there probably is an extracellular glycolytic pathway operating in the spermathecal fluid and ATP produced from this extracellular glycolysis could be used to maintain the internal metabolism of sperm (Baer *et al.* 2009). Fruit fly sperm lacks internal metabolites, like glycogen, as fuel sources to sustain activity (Anderson and Personne 1970), and so they likely require exogenous energy resources to generate energy. Therefore, sperm storage may require energetic provisioning from females after the energy sources from male seminal fluid are exhausted (Heifetz and Rivlin 2010; Bloch Qazi *et al.* 2003). The spermathecae and seminal receptacle change in metabolic pathway regulation soon after mating in response to male accessory gland proteins (Acps), suggesting that both
storage organs respond to the needs of sperm (Prokupek et al. 2009; Heifetz and Rivlin 2010). Other agents in the female’s sperm storage fluids protect sperm and help regulate their living environment (Heifetz and Rivlin 2010; Bloch Qazi et al. 2003). The secretion of these agents may depend upon adequate nutritional intake. Therefore, sperm stored by food-deprived females may experience higher mortality. If there is any cost of sperm storage due to poor nutrition, it must be traded off with other kinds of functions, including fecundity and somatic maintenance.

In *Drosophila melanogaster*, manipulating dietary yeast levels may help reveal the cost to female sperm storage. Yeast is the primary protein source in the female diet and contains the essential amino acid methionine for egg production (Trivett et al. 1988; Carey 2008; Grandison et al. 2009). Female reproduction is strongly affected by dietary yeast levels (Olivieri et al. 1970; Chippindale et al. 1993; Good and Tatar 2001). After moderate periods of dietary yeast restriction (DR) and low egg-laying rates, females can typically restart egg laying and produce more progeny than control females later in life upon returning to a full diet (Chippindale et al. 1993; Good and Tatar 2001; Trevitt et al. 1988; Bloch Qazi and Hogdal 2010). However, they also have curtailed lifetime progeny production compared to females held at constant high nutrition and high fecundity (Olivieri et al. 1970; Trevitt et al. 1988; Bloch Qazi and Hogdal 2010). The capacity to rebound in fecundity may facilitate the examination of the energetic costs of sperm storage by defining severity and duration of DR and assessing the subsequent progeny production. Curtailed lifetime progeny production suggests that female lose some stored sperm during DR, which may be because females under DR are incapable of providing adequate energy to stored sperm and thus sperm die or are lost (Bloch Qazi and Wolfner 2006; Lefevre and Jonsson 1962; Snook and Hosken 2004; Stewart et al. 2007).

In this study, we set out to extend the understanding of the costs of sperm storage using the model organism *D. melanogaster*. First, we investigated the influence of dietary yeast
restriction on female sperm storage by measuring female fecundity, fertility, and reproductive longevity after a single copulation under varied diet regimes. We then examined how the severity and duration of dietary restriction influenced subsequent reproductive success when females were switched to an ad libitum yeast treatment.

Methods

Fly husbandry and crosses

The experimental flies were taken from the Ives stock, a wild type, outbred stock of *D. melanogaster* founded in 1975 from flies collected at South Amherst, Massachusetts, USA (Rose 1984). This stock had been maintained at 2000-2500 adults/generation with discrete two-week generations at 25°C on banana/agar/killed-yeast medium for about 900 generations prior to the experiments. All experimental trials were conducted under conditions close to the standard culture protocols unless otherwise indicated.

Virgin males and females were collected upon eclosion and maintained in vials in groups of 20. Three days later, females were placed individually with a single male in a food vial, and the pairs were monitored to ensure a single copulation per pair. We then separated the females from the males by aspiration and allocated them haphazardly into different experimental treatments, placing them individually into plastic vials containing food with predetermined yeast concentration. The copulation day was recorded as day 0 in the following two experiments, and females were transferred into new food vials with the same yeast concentrations every 24 h.

Yeast levels

The yeast used in this experiment was Fleischmann’s Baker’s Active Dry Yeast (*Saccharomyces cerevisiae*) ordered at Findlay Foods (Kingston) Ltd. We used four successively declining yeast
concentrations in the banana/agar/killed-yeast food medium, Full (30 g yeast/L), Half (15 g yeast/L), Low (5 g yeast/L), and None (0 g yeast/L). Full Yeast was the level on which the flies were normally maintained, and was expected to induce low and steady fecundity, with lower yeast concentrations known to result in reduced egg-lay from a pilot experiment. In addition to the different background yeast concentrations, we generated an ad libitum control treatment in which a slurry of live yeast was put on the surface of the Full Yeast medium. We did this to induce the maximal egg deposition and sperm use (Sy 2008).

**Daily egg counting**

In our two experiments, we quantified three aspects of egg production by each female over each 24 h interval. Fecundity was measured by counting the number of eggs deposited on and around the medium surface. Fertility was estimated as the number of hatched eggs, determined by counting the empty chorions of hatched eggs after 30 h (sufficient time for all viable eggs to have hatched). Egg-hatching rate was calculated as the number of hatched eggs divided by the total fecundity.

**Experiment One: Effects of yeast restriction on female fecundity, fertility and duration of fertile period**

This experiment was designed to quantify the influence of different levels of dietary yeast on female fecundity, fertility and duration of fertile period. Females (48) were assigned to one of five yeast levels. After copulation they were placed individually into a food vial and transferred into a new food vial every 24 hours. Oviposition and egg hatch was recorded every day 7 days after the production of hatching eggs ceased. The last day that the female laid hatching eggs was used to determine the duration of a female’s fertile period. We expected that females in the ad
*libitum* yeast treatment that started with high egg-lay rates early in life would produce the most offspring, and so denoted them as the maximum fertility treatment.

Forty-eight singly-mated females were haphazardly assigned to a yeast level treatment. Females can continue to lay unfertilized eggs after they run out of sperm; once a female stopped laying hatched eggs for seven consecutive days, we assumed that she was no longer fertile, and the last day that the female laid hatched eggs was used to determine the duration of a female’s fertile period. We terminated egg counting in a treatment after all females had stopped laying hatched eggs for seven days.

**Experiment Two: Effects of yeast supplementation on the recovery of female fecundity and fertility**

This experiment was designed to test the effects of different durations of yeast restriction on a female’s subsequent fecundity and fertility. To do this we put females on twelve different yeast-restriction diet regimens, for one week, two weeks or three weeks, on each of the four base food levels described above. During the yeast restriction periods, we kept 150 singly-mated females on each yeast concentration with five flies per vial. Each vial of flies was transferred to new food every 24 h. Experiments 1 and 2 were run simultaneously so that females would experience similar environmental conditions (other than food and group size).

Thirty-six of the initial 150 females collected for each yeast treatment were assigned to one of the three yeast-restriction periods (one, two or three weeks). At the end of each period, we aspirated each female into a single vial having *ad libitum* food, and recorded total egg deposition and the number of hatched eggs every 24 h thereafter. Females who had been kept on *ad libitum* food were considered to be infertile after two days without producing hatched eggs. The
maximum fertility treatment in Experiment One served as the ‘0 week’ control in this experiment, because the females received *ad libitum* yeast right after mating.

**Data treatment and analyses**

In each experiment, any missing data of a few vials due to mishandling or miscounting were each filled by an average of the values of the same fly a day before and a day after the day with missing value. On rare occasions, females were lost during transfers between vials. Data from lost females was used for calculating daily average fecundity, fertility (measured as number of hatched eggs), and egg-hatching rate before the date that they were lost, but was not used in the calculation of mean total fecundity and fertility. Female mortality was low on any yeast concentration during yeast restriction (<10%) and after any period of yeast restriction (<6%).

In Experiment One, we compared across the five yeast treatments (Full, Half, Low, None, and *ad libitum*): (1) total and maximum daily egg deposition until day 32, by which time egg counting had terminated in the *ad libitum*, Low and No Yeast treatments, (2) total and maximum daily hatched egg deposition, (3) daily egg-hatching rate, and (4) duration of the female’s fertile period. We performed the same analyses of the four yeast restriction treatments (Full, Half, Low and None) for Experiment Two for the *ad libitum* yeast supplemented stage of the experiment. We used the total number of hatched eggs produced after yeast supplementation as an index of the number of functional sperm still available in the female’s sperm storage organs at the end of yeast restriction.

Estimating the number of sperm remaining at the end of, or lost during, the experimental diet manipulation required an estimate of the number of sperm transferred by males initially. This estimate was supplied by the maximum fertility treatment, in which *ad libitum* yeast was applied at the beginning of the assay. Initially we make the simplifying assumption that no sperm died or
were lost from storage organs during females’ fertile life in that treatment. At the end of each dietary manipulation, ad libitum yeast was supplied to stimulate rapid oviposition and, with the same assumptions, estimate the number of sperm remaining. The cumulative total fertility at the end of the dietary treatment prior to supplementation plus the total fertility after yeast supplementation provides an approximation of the total number of sperm used for fertilization. The difference between total fertility of the maximum fertility treatment and total used sperm was an estimate of the number of sperm lost during yeast restriction. Because on average more than one sperm is used per fertilization event (Bloch Qazi et al. 2003), any such estimate needs to be adjusted by the 79% efficiency of fertilization (estimated from an average of 349 hatched eggs produced from an average of 441 sperm stored immediately after a copulation in the Ives population; unpubl. data).

Results

Experiment One: Effects of yeast restriction on female fecundity, fertility and duration of fertile period

Female fecundity and fertility

The number of eggs and the number of eggs that hatched were counted for the first 32 days. Both measures increased significantly with yeast concentration and were proportionate to the level of yeast supplied (Figure 4.1 A, B & C) (Total eggs: One-way ANOVA, df = 3,156, F = 144.9, p < 0.01; Tukey-Kramer test, all yeast levels are significantly different from each other (p < 0.05). Hatched eggs: One-way ANOVA, df = 3,157, F = 143.1, p < 0.01; Tukey-Kramer test, all yeast levels are significantly different from each other (p < 0.05), except between Low Yeast and No Yeast.). Females in the ad libitum treatment had the highest total (average 1234 eggs, range 484 to 1717 eggs) and maximum daily egg deposition (average 82 eggs on day 5), and also had the
highest total fertility (average 349 hatched eggs, range 181 to 553 hatched eggs) and highest daily fertility (average 80 hatched eggs on day 4). The total hatched eggs in the ad libitum treatment represented the maximum fertility of a female after a single copulation. Therefore, females under yeast restriction only had 7-34% of the maximum fertility (Full: 118 eggs, Half: 58 eggs, Low: 29 eggs, and None: 23 eggs). In the Full Yeast treatment, daily egg deposition peaked on day 4 at 10 eggs/d, and stayed at a consistent rate after day 7 (~5 eggs/d). At all of the lower yeast concentrations, egg deposition showed rapid declines during the first five days and then stayed stable thereafter (Half: ~3 eggs/d, Low: ~1 eggs/d, and None: ~0.2 eggs/d). Daily deposition of hatched eggs showed a similar pattern. Also, during the first five days, egg-hatching rates were high (88-100%) in all five yeast treatments (Figure 4.1 D). Thereafter, it declined roughly proportionately to the level of yeast restriction. Fecundity and fertility of all females in the No Yeast treatment became zero after day 12 (Figure 4.1 A & B).

*Duration of the fertile period of females*

The average period of female fertility after a single copulation was greater at higher yeast levels (Table 4.1) (One-way ANOVA, df = 3,157, F = 79.3, p < 0.01; Tukey-Kramer test, all yeast levels are significantly different from each other (p < 0.05)). Females in the Full Yeast treatment had the highest average duration of the fertile period (25 days). The highest duration of the fertile period of individual females recorded was 39 days, in a female in the Half Yeast treatment. The average duration of the female’s fertile period was only nine days in the ad libitum treatment, probably because of fast sperm exhaustion, and was six days in the No Yeast treatment, determined by females’ ability to produce eggs.
Experiment Two: Effects of yeast supplementation on the recovery of female fecundity and fertility

Female fecundity and fertility

Switching to a diet of surplus yeast after a period of yeast restriction allowed females to quickly resume fecundity at the levels similar to that of age-matched ad libitum control females (Figure 4.2 A-C). Females from all yeast concentrations achieved the maximum fecundity around day 3 after ad libitum yeast supplementation. In general, the more yeast available to a female during dietary restriction, the higher her daily fecundity was after ad libitum yeast supplementation (Table 4.2). This increase in egg production boosted female fertility and improved egg-hatching rate. The daily pattern of hatched egg deposition was similar to that of daily egg deposition within and between treatments until females run out of sperm (Figure 4.2 D-F). Many females that had stopped laying viable eggs regained their fertility after receiving ad libitum yeast, but some females lost their fertility permanently during yeast restriction (Table 4.3 A). Among fertile females, there was a significant increase in the total number of hatched eggs produced after yeast supplementation at almost all yeast concentrations and in almost all durations of yeast restriction (Table 4.3 B, Figure 4.2 D-F, Figure 4.3). The maximum daily egg-hatching rates in yeast-supplemented treatments were generally higher than those of females in the age-matched yeast restriction treatments (Figure 4.4 A-D). In Week 1, the maximum egg-hatching rate was close to 100% in all yeast treatments, but in Weeks 2 and 3, the maximum egg-hatching rate declined with degree of yeast restriction as well as with duration of yeast restriction.

In the No Yeast treatment, where egg deposition declined to a low rate after day six and completely stopped after day 12, most females were only able to resume fecundity and fertility if they received yeast within the first two weeks of yeast restriction (Figure 4.2 A, B, D & E). A longer period with no yeast intake led to permanent sterility of most females, and all females
looked extremely emaciated. In the Three Week No Yeast treatment, only 22% of females produced eggs and 7% of females still had the capacity to produce hatched eggs after receiving surplus yeast, and both fecundity and fertility were very low in those females (Figure 4.2 C & F).

The number of fertile females and the total number of hatched eggs produced by the fertile females after yeast supplementation was positively correlated with base yeast concentration and negatively correlated with period of yeast deprivation (Fertile females by yeast levels: contingency table analysis, $\chi^2 = 35.2$, $p < 0.01$; Fertile females by period of yeast deprivation: contingency table analysis, $\chi^2 = 101.4$, $p < 0.01$) (Hatched eggs produced by fertile females: Two-way ANOVA, df = 11,523, $F = 19.5$, $p < 0.01$; effect of yeast levels: df = 2,523, $F = 9.9$, $p < 0.01$; effect of period of yeast deprivation: df = 3,523, $F = 52.7$, $p < 0.01$; interaction term: df = 6,523, $F = 4.0$, $p < 0.01$) (Table 4.4, Figure 4.3). The only exception occurred during Week 1 of the No Yeast treatment, where females laid more hatched eggs than they did following the Low Yeast treatment. This matched the pattern in daily fecundity (Figure 4.2 A & D, Figure 4.3 A).

Sperm loss

Matching with the pattern of female fertility, our estimated average sperm loss was negatively related to the level of base yeast concentration and positively related to the duration of yeast restriction (Figure 4.5). The negative association between sperm loss and yeast concentration is almost linear, except that more sperm seemed to be lost in the Low Yeast treatment than in No Yeast treatment during the first week of yeast restriction. On average, about 20 more sperm were lost with a decrease in one gram of yeast per liter of food. The speed of sperm loss slowed down with time, being the fastest in the first week and the slowest after three weeks of yeast restriction.
Duration of the female’s fertile period

Most females were only able to produce hatched eggs for short periods of time after receiving additional yeast: ~6 days in Week 1, ~4 days in Week 2, and ~2 days in Week 3 (Table 4.5). Among fertile females, their duration of fertile period after *ad libitum* yeast supplementation was independent of the base yeast concentration during the period of yeast restriction (df = 3,300, F = 0.3, p = 0.84), but was negatively correlated with the duration of yeast restriction (df = 2,300, F = 34.4, p < 0.01) (Two-way ANOVA, df = 5,306, F = 14.3, p < 0.01).

Discussion

We found that all three female reproductive traits that we measured (fecundity, fertility, and egg-hatching rate) were positively associated with yeast concentration in the food medium and negatively associated with the duration of the yeast restriction treatment. Duration of the female’s fertile period – the number of days a female could produce fertile eggs – was linearly and positively associated with yeast concentration when lifetime yeast restriction was applied. However, after *ad libitum* yeast supplementation, duration of the female’s fertile period was independent of base yeast concentration during yeast restriction, but was negatively associated with the duration of yeast restriction.

The more yeast that females received during yeast restriction, the more eggs they produced, the more eggs were fertilized, and the fewer sperm that were apparently lost; the duration of yeast restriction had the inverse effects. The level of yeast received during our dietary manipulation not only dictated a female’s current fecundity and egg-hatching rate, but also influenced her future reproduction after the nutritional condition was improved via supplementation. In general, the negative impacts of yeast restriction on female fecundity and sperm use can be readily improved after receiving supplemental yeast. The more yeast received during yeast restriction, the higher
the fecundity, fertility and egg-hatching rate would be after yeast supplementation. At the extreme, most females that received no basal dietary yeast for longer than two weeks became permanently sterile.

Although in using hatched eggs to estimate sperm numbers we must be careful to acknowledge the potential for conflating sperm maintenance and egg quality-related effects, our results do suggest that sperm are sensitive to female condition in *D. melanogaster*. Females not only achieved higher fecundity and fertility when permissive conditions followed yeast restriction, but also were able to remain fertile for longer time, and appear to use sperm at a higher efficiency (*i.e.* egg-hatching rate, data not shown) when they had received more yeast in their basic diet previously. Taken together these results suggest that the restricted protein diet treatments applied were incrementally stressful and had detrimental impact on both female reproductive characters and the capacity to maintain stored sperm.

**Female fecundity and fertility**

Consistent with other studies, we found that controlling dietary yeast alters female fecundity and fertility, with a linear association between the concentration of dietary yeast and production of total eggs and hatched eggs over the range applied here. Females on the *ad libitum* yeast treatment from the beginning of the experiment appear to have exhausted sperm after ten days at a high reproductive rate, but continued to produce eggs at a rate of over 40 eggs/d/female. However, this pattern may also be stock- or environment-specific. In some other fly stocks, female egg laying rate decreases to virgin levels (typically 0 to a few eggs/d) when they use up all stored sperm (Kaufman and Demerec 1942; Neubaum and Wolfner 1999; Bouletreau 1978). It is possible that our *ad libitum* treatment is so nutritionally rich that females pay a negligible cost of egg production. Also, such environments in nature are likely to attract many flies via olfaction.
and therefore sperm may rarely be limiting, encouraging a hard-wired response to produce eggs by females. At the other extreme, long period (≥ three weeks) of no yeast intake results in emaciation of females and permanent damage of their fertility. This finding also agrees with previous studies (Olivieri et al. 1970; Trevitt et al. 1988) and indicates that yeast deprivation is a stressful starvation condition.

Once rich dietary conditions were applied, fecundity of females previously exposed to most yeast restriction treatments reached levels similar to age-matched females in the *ad libitum* control, females that had high nutrition from the start. This was surprising because dietary restriction and reduced reproductive rate have been shown to to slow the rate of senescence (Chippindale et al. 1993), at least in the higher treatments that were not expected to induce starvation stress. Other than long-term yeast deprivation (Three Week No Yeast), the impacts of temporary dietary restriction on future fecundity were slight after ample nutrition was made available. This differs from a comparable study of the Windsor stock, where fecundity of singly mated female reached zero on full diet after 30 days (Williams and Sokolowski 1993); but upon receiving full diet, yeast-deprived females regained fecundity at levels that were much higher than age-matched females who had always been fully fed (Good and Tatar 2001).

The significant increase in the total number of eggs and hatched eggs produced after yeast supplementation suggested that female fertility during yeast restriction was limited by female fecundity, rather than by sperm storage. But once females received additional yeast, it was apparent that the more yeast that females had received during yeast restriction, the higher their total subsequent fertility was. This pattern is observed despite the fact that females on the higher-yeast diet had produced more hatched eggs previously. The quick improvement of female fecundity and fertility after yeast supplementation implies that either females adaptively curtail
their egg production for themselves or for their prospective larvae under undesirable nutritional conditions, or reproduction is highly yeast-limited on even short timescales.

Decline in egg-hatching rate with time after copulation and with degree of yeast restriction may be due to either a reduction of egg quality or the low availability of functional sperm. However, indirect evidence suggested that egg quality did not change dramatically with yeast concentration in diet and with the durations of DR applied here. Female fecundity at all base yeast concentrations increased within three days after yeast supplementation at the levels similar to the maximum possible daily fecundity (the levels of age-matched \textit{ad libitum} control females) and egg-hatching was also substantially enhanced before apparent sperm exhaustion. Our results are in accord with Drummond-Barbosa and Spradling’s (2001) finding, which suggests that pre-vitellogenic-stage oocytes remain in good condition under yeast restriction and still have full potential of yolk uptake. Female ageing is not likely an explanation of the results obtained in the current study. In another study of the \textit{Ives} population, females that were fed with normal culture food (Full Yeast), the number of sperm in the female reproductive tract over time after a single copulation (J. Duszczyszyn, unpubl. data) is similar to our estimates of sperm number at the end of any period of yeast restriction. Also, Kern \textit{et al.} (2001) indicated that for females who constantly have access to same-aged males, there is no decline in egg-hatch until females were over 30-day-old, which was near the end of our experiments. However, direct evidence of changes in egg quality under yeast restriction is currently absent because methods such as providing females with fresh sperm through remating introduces numerous confounding changes to female physiology and behaviour, such as increase in egg laying, and increases female mortality.

Assuming that egg quality does not vary dramatically with the yeast manipulation that we performed, sperm availability is more likely to explain the observed changes in egg-hatching rate.
Although female experienced sperm loss during yeast restriction, it is clear that there is an adequate number of sperm to fertilize more eggs because the egg-hatching rate can be dramatically improved after application of additional yeast. We think that lower hatching rates at lower yeast concentration are likely due to a reduction of sperm function (i.e. motility and velocity) or localization in the distal region of seminal receptacle, away from the site of fertilization (authors’ observation).

**Sperm loss**

Sperm loss during yeast restriction, estimated by the difference between average sperm usage of females that had experienced yeast restriction and average sperm usage in the maximum fertility treatment, was negatively related to the base yeast concentration and positively related to duration of yeast restriction. This reflects how well the sperm were maintained or retained during the yeast restriction period. If sperm in the female sperm storage organs require continuous female provision to maintain their viability (Heifetz and Rivlin 2010; Bloch Qazi et al. 2003), then females with low yeast intake may not be able to allocate enough energy to sperm maintenance, therefore, may lose sperm due to sperm death or female resorption. This suggests that sperm storage may have a substantial energetic cost to females.

**Duration of the fertile period of females**

Duration of the fertile period of females in this study refers to the duration that a female produces hatched eggs after copulation. Under the *Ad Libitum*, Full (30g yeast/L), Half (15g yeast/L), and Low Yeast treatments (5g yeast/L), female fecundity extended beyond the duration of fertility, but likely for quite different reasons. Our data suggest that the *ad libitum* treatment results in rapid sperm exhaustion with a high efficiency of use (79%). This efficiency is considerably high.
even among insects, an animal group that is renown for efficient sperm use (Parker 1970). This high efficiency of sperm use may be a result of correlated sperm release and ovulation in D. melanogaster (Bloch Qazi et al. 2003) in adaptation to limited sperm storage due to their giant sperm size (females typically store ~1400 sperm, Manier et al. 2010). In restricted yeast conditions (Full, Half and Low) duration of the female’s fertile period appears to roughly represent the duration of sperm viability. Within this range of yeast concentration, the more yeast available to females, the longer stored sperm could survive. These observations suggest that sperm storage depends upon female condition and imposes a nutritional cost to females.

When females received no yeast in diet, their duration of the fertile period was determined by the duration that females were able to produce eggs (on average six days). Females regaining fertility after yeast supplementation confirmed the presence of live sperm in their sperm storage organs after they stopped egg laying. The longest average duration of the female’s fertile period found in this study was 25 days, in Full Yeast; and the longest duration of the fertile period of individual female was 39 days, in Half Yeast. As far as we know, this is the longest record of sperm survival in D. melanogaster, longer than Perotti’s (1973) report of motile sperm in the spermathecae for 23 days, and Kaufmann and Demerec’s (1942) report of female producing fertile eggs for two weeks.

*If sperm storage is costly, then what is the purpose of long-term sperm storage in a promiscuous species?*

*Drosophila melanogaster* is a promiscuous species. Given the opportunity, females always remate before sperm from their previous matings are exhausted (Gromko *et al.* 1984). Hence, in both natural and laboratory populations, females typically carry sperm from more than one male (Milkman and Zeitler 1974, Harshman *et al.* 1988). If females always have access to males, then
why do they carry sperm from a single mating for as long as 25 days? Here, we propose two hypotheses. First, females may adaptively store sperm for a long time because males may not always be accessible (Demerec 1965). For example, in the temperate zone, the majority of males die and most living males become sterile over winter. Female sperm storage may be important for founding a population in spring (Izquierdo 1991; Boulétreau-Merle and Fouillet 2002). Similarly, if resources are patchily distributed, foraging females may encounter good oviposition sites where mates are absent; stored sperm would allow them to exploit these resources. This creates a potentially complex tradeoff architecture, in which the cost of maintaining sperm may be weighed against migration costs and somatic maintenance in marginal environments, as well as the cost of producing eggs. Our data suggest that females may coordinate fecundity curtailment with reduced sperm use under yeast limitation, in keeping with an adaptation to a marginal habitat. Our second hypothesis is that long-term sperm storage may be a coincidental response that is triggered by sperm movement. Since females are known to remate frequently, and last male sperm precedence is strong, the average age of resident sperm may be quite low in natural females. Removing access to males, as we did experimentally, may create the unusual situation for the female of carrying aged sperm. Females may simply respond to the presence of sperm and provision on that basis. Which of these scenarios fits D. melanogaster depends greatly upon their natural biology and evolutionary history of mate accessibility, among other factors.

The population explored in the present work has approximately 900 generations of confined laboratory evolution with early reproduction, in which there has been no selection for prolonged sperm storage. This makes it quite remarkable that these females are capable of storing sperm for the periods of time we document, particularly if maintenance of sperm carries a cost. Perhaps, there has not been enough time for the trait to become vestigial. Alternatively, genes that control sperm storage may be pleiotropic: enhancement of short-term sperm motility and
survival may also facilitate long-term sperm viability. Measurements of sperm longevity and female responses to environmental factors on wild or more recently domesticated populations would be of considerable interest.

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Company, Inc.

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Table 4.1. Duration of the fertile period of females (number of days that female produced viable eggs after copulation) on five dietary yeast levels.

<table>
<thead>
<tr>
<th>Yeast level</th>
<th>Mean</th>
<th>95% CI</th>
<th>Range</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ad libitum</em></td>
<td>9.1</td>
<td>7.3-10.9</td>
<td>6-15</td>
<td>39</td>
</tr>
<tr>
<td>Full (30 g/L)</td>
<td>25.2</td>
<td>23.3-27.0</td>
<td>7-37</td>
<td>40</td>
</tr>
<tr>
<td>Half (15 g/L)</td>
<td>20.0</td>
<td>18.2-21.9</td>
<td>5-39</td>
<td>37</td>
</tr>
<tr>
<td>Low (5 g/L)</td>
<td>9.8</td>
<td>8.1-11.5</td>
<td>0-24</td>
<td>43</td>
</tr>
<tr>
<td>None (0 g/L)</td>
<td>5.9</td>
<td>4.1-7.7</td>
<td>3-12</td>
<td>41</td>
</tr>
</tbody>
</table>
Table 4.2. Comparisons between the daily egg deposition on day 3 since receiving surplus yeast after one week, two weeks and three weeks yeast restriction on four base yeast levels and the age-matched daily egg deposition in the ‘ad libitum’ treatment using Tukey-Kramer HSD test (levels not connected by the same letter are significantly different).

<table>
<thead>
<tr>
<th>Yeast level</th>
<th>Week 1 Mean</th>
<th>95% CI</th>
<th>N</th>
<th>Tukey test</th>
<th>Week 2 Mean</th>
<th>95% CI</th>
<th>N</th>
<th>Tukey test</th>
<th>Week 3 Mean</th>
<th>95% CI</th>
<th>N</th>
<th>Tukey test</th>
</tr>
</thead>
<tbody>
<tr>
<td>ad libitum</td>
<td>44.4</td>
<td>38.7-50.1</td>
<td>39</td>
<td>B</td>
<td>34.6</td>
<td>30.4-38.8</td>
<td>35</td>
<td>A</td>
<td>27.4</td>
<td>23.1-31.7</td>
<td>35</td>
<td>A</td>
</tr>
<tr>
<td>Full (30 g/L)</td>
<td>62.9</td>
<td>57.0-68.8</td>
<td>36</td>
<td>A</td>
<td>34.9</td>
<td>30.7-39.0</td>
<td>35</td>
<td>A</td>
<td>30.2</td>
<td>25.6-34.9</td>
<td>30</td>
<td>A</td>
</tr>
<tr>
<td>Half (15 g/L)</td>
<td>53.3</td>
<td>47.3-59.3</td>
<td>35</td>
<td>AB</td>
<td>29.1</td>
<td>25.0-33.2</td>
<td>36</td>
<td>AB</td>
<td>21.9</td>
<td>18.0-25.8</td>
<td>43</td>
<td>AB</td>
</tr>
<tr>
<td>Low (5 g/L)</td>
<td>47.5</td>
<td>41.6-53.4</td>
<td>36</td>
<td>B</td>
<td>21.0</td>
<td>16.9-25.2</td>
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<td>B</td>
<td>14.3</td>
<td>9.5-19.1</td>
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<td>None (0 g/L)</td>
<td>55.1</td>
<td>49.2-61.0</td>
<td>36</td>
<td>AB</td>
<td>10.4</td>
<td>5.7-15.0</td>
<td>28</td>
<td>C</td>
<td>2.3</td>
<td>0-7.3</td>
<td>26</td>
<td>C</td>
</tr>
</tbody>
</table>
Table 4.3. A. The results of Fisher’s exact tests on number of fertile females after yeast supplementation in comparison with number of fertile females on lifetime yeast restriction from the time points of introducing additional yeast and onward.

<table>
<thead>
<tr>
<th>Yeast level</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full (30 g/L)</td>
<td>N=76, p=0.49</td>
<td>N=75, p=0.61</td>
<td>N=70, p=0.33</td>
</tr>
<tr>
<td>Half (15 g/L)</td>
<td>N=72, p=1.00</td>
<td>N=72, p=0.19</td>
<td>N=79, p=0.18</td>
</tr>
<tr>
<td>Low (5 g/L)</td>
<td>N=78, p&lt;0.01</td>
<td>N=78, p&lt;0.01</td>
<td>N=72, p&lt;0.01</td>
</tr>
<tr>
<td>None (0 g/L)</td>
<td>N=75, p&lt;0.01</td>
<td>N=69, p&lt;0.01</td>
<td>N=68, p=0.15</td>
</tr>
</tbody>
</table>

B. The results of t tests on total hatched eggs produced by fertile females after yeast supplementation in comparison with total hatched eggs on lifetime yeast restriction from the time points of introducing additional yeast and onward.

<table>
<thead>
<tr>
<th>Yeast level</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full (30 g/L)</td>
<td>df=1,72, t=10.2, p&lt;0.01</td>
<td>df=1,69, t=5.4, p&lt;0.01</td>
<td>df=1,57, t=1.4, p=0.18</td>
</tr>
<tr>
<td>Half (15 g/L)</td>
<td>df=1,65, t=8.8, p&lt;0.01</td>
<td>df=1,58, t=5.4, p&lt;0.01</td>
<td>df=1,39, t=2.8, p&lt;0.01</td>
</tr>
<tr>
<td>Low (5 g/L)</td>
<td>df=1,51, t=6.4, p&lt;0.01</td>
<td>df=1,32, t=3.8, p&lt;0.01</td>
<td>df=1,12, t=1.0, p=0.34</td>
</tr>
<tr>
<td>None (0 g/L)</td>
<td>df=1,38, t=4.4, p&lt;0.01</td>
<td>N/A*</td>
<td>N/A*</td>
</tr>
</tbody>
</table>

*No females were fertile under yeast restriction.
Table 4.4. The percentages of fertile females at the time of yeast supplementation in each yeast level and duration of dietary restriction.

<table>
<thead>
<tr>
<th>Yeast level</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full (30 g/L)</td>
<td>100.0% (36)</td>
<td>97.1% (35)</td>
<td>90.0% (30)</td>
</tr>
<tr>
<td>Half (15 g/L)</td>
<td>94.3% (35)</td>
<td>91.4% (35)</td>
<td>59.5% (42)</td>
</tr>
<tr>
<td>Low (5 g/L)</td>
<td>97.1% (35)</td>
<td>65.7% (35)</td>
<td>41.4% (29)</td>
</tr>
<tr>
<td>None (0 g/L)</td>
<td>100.0% (34)</td>
<td>71.4% (28)</td>
<td>7.4% (27)</td>
</tr>
</tbody>
</table>

Numbers in the brackets are the sample sizes.
Table 4.5. Duration of fertile period of fertile females (number of days that female produced viable eggs after copulation) after yeast supplementation.

| Yeast level | Week 1 | | Week 2 | | Week 3 |
|-------------|--------|--------|--------|--------|
|             | Mean   | 95% CI | Range  | N      | Mean   | 95% CI | Range  | N  | Mean   | 95% CI | Range  | N  |
| Full        | 6.5    | 5.8-7.2| 4-10   | 36     | 5.0    | 4.4-5.6| 1-8    | 34 | 3.1    | 2.6-3.7| 1-7    | 27 |
| Half        | 6.4    | 5.6-7.1| 2-10   | 33     | 4.9    | 4.3-5.6| 1-9    | 32 | 3.8    | 3.2-4.3| 1-6    | 25 |
| Low         | 5.5    | 4.7-6.4| 2-11   | 33     | 5.2    | 4.0-6.4| 2-12   | 23 | 4.7    | 2.9-6.4| 2-10   | 12 |
| No          | 6.0    | 5.4-6.6| 4-11   | 34     | 4.8    | 3.9-5.7| 2-9    | 20 | 2.5    | N/A*   | 2-3    | 2  |

*In the Week 3 No Yeast treatment, only two females produced hatched eggs.
Figure 4.1. Average daily oviposition (A), daily egg hatch (B), cumulative egg hatch (C), and daily egg-hatching rate (D) on five yeast levels after a single copulation until no more viable eggs were produced. We counted ‘ad libitum’ and Low treatments until day 32; Full and Half until day 46; and None until day 19. Average daily egg-hatching rate in the No Yeast treatment is plotted to day 6 due to the small sample size after that day.
Figure 4.2. Average daily oviposition (A-C) and average daily egg hatch (D-F) of singly-mated females after one week (A & D), two weeks (B & E) and three weeks of yeast restriction (C & F). *ad libitum*: female received surplus yeast since day 0. Average daily oviposition in the last four days of yeast retrieval might be subject to measurement error due to the small sample sizes.
Figure 4.3. Average cumulative egg hatch of singly-mated females after one week (A), two weeks (B) and three weeks of yeast restriction (C) (solid lines) in comparison with average cumulative egg hatch of singly-mated females constantly under yeast restriction (dashed lines).
Figure 4.4. Daily egg-hatching rate (percentage of hatched eggs over total eggs) after yeast supplementation in comparison with daily egg-hatching rate on yeast restriction at the same yeast concentration of the four base yeast levels (Full 30g/L (A), Half 15g/L (B), Low 5g/L (C), and None 0g/L (D)).
Figure 4.5. Estimated average sperm loss relative to the yeast concentration available to females. Sperm loss was calculated using the total number of hatched eggs in the maximum fertility treatment to subtract the total number of hatched eggs produced during and after yeast restriction on the four base yeast levels (Full 30g/L, Half 15g/L, Low 5g/L and None 0g/L).
Chapter 5: General Discussion

Thesis overview

Sperm senescence may result in reduced fertilization efficiency and increased offspring mutation load, and therefore impose a fitness cost to males and females. This may lead to numerous evolutionary consequences, such as the adaptation of both sexes to avoid using senesced sperm cells or to develop mechanisms to reduce the rate of sperm senescence. Exploring the evolutionary consequences of sperm senescence will advance our understanding of sexual selection, especially the mechanisms of post-copulatory sexual selection, such as sperm competition and cryptic female choice.

Although the existence of sperm senescence seems to have been well demonstrated in the literature (e.g. brown Norway rat, *Rattus norvegicus*: Serre and Robaire 1998; sandfly, *Lutzomyia longipalpis*: Jones et al. 2000; fruit flies, *Drosophila melanogaster*: Long and Pischedda 2005), two problems exist in previous research. First, few researchers have attempted to separate the confounding effects of male age and sperm age in the study of sperm senescence before copulation. The idea of eliminating the effects of sperm senescence while studying the effects of male ageing on sperm quality has been proposed in several review papers (Siva-Jothy 2000; Radwan 2003), but no standard protocol in any organism has been established. Therefore, studies often overlook the fact that different mechanisms may affect senescence at different stages of a sperm’s life: in the male germ-line before meiosis, in the male sperm storage organs after meiosis and before copulation, and in the female sperm storage organs after copulation. Second, previous investigations have been restricted to a small number of defined age classes that may not span the life of a male, or even include advanced ages where males show obvious signs of senescence (e.g. birds, Møller and Ninni 1998; Møller et al. 2009).
These two problems are resolved in this thesis. First, we provide a practical approach to standardize pre-copulatory sperm age and male mating history in young and old males in *Drosophila melanogaster* by depleting banked sperm in a standard number of matings and allowing for a fixed period of sperm recovery (Chapter 2). This approach may be applied to other animals in which males can achieve several matings in a quick succession to deplete their sperm store and their fertility can recover in relative short period after ejaculate exhaustion. Second, this thesis examined the proportion of fertile males every week (until the mean longevity of the base population) and concluded that four weeks of age was an appropriate age to study male reproductive senescence in the study fly population (*Ives*) because the majority of the cohort was still alive and fertile, yet exhibiting signs of senescence.

Applying the approach of standardizing pre-copulatory sperm age in two male age classes (four days old and four weeks old), this thesis aimed to address two questions: (1) to test the potential contributions of sperm senescence to male reproductive success and offspring fitness in *D. melanogaster* at three different stages of a sperm’s life: during spermatogenesis (mediated by male age), after spermatogenesis and before copulation (mediated by pre-copulatory sperm age), and after copulation (mediated by post-copulatory sperm age) and (2) to look for evidence consistent with nutritional costs of sperm storage in females, a factor that may be relevant to post-copulatory sperm senescence.

We found reduced male reproductive success and offspring fitness related to sperm senescence. Older males produced fewer offspring despite their larger sperm reservoir, suggesting that sperm senescence might occur before copulation (Chapter 2). In addition, post-copulatory sperm age had significant negative impacts on offspring fitness as estimated by fitness assays (Chapter 3), whereas male age and pre-copulatory sperm age did not influence offspring fitness of either sons or daughters. This result was surprising because it differs from what
previous studies suggested (Price and Hansen 1998; Long and Pischedda 2005). Unlike those studies, our results do not support the idea that male ageing affects the fitness of sons and daughters differentially.

Since our study provides evidence of post-copulatory sperm senescence, we geared our research toward female sperm storage and looked for evidence consistent with nutritional costs of sperm storage in females by manipulating the amount of dietary yeast in D. melanogaster (Chapter 4). This was tested by restricting dietary yeast in four successively declining yeast concentrations until no hatched eggs were produced, and for periods of one week, two weeks, or three weeks, followed by recovery on ad libitum yeast. We showed female daily oviposition, daily egg hatch, daily egg fertilization rate, and the duration of fertile period of the females (the duration that a female laid hatched eggs since copulation) were positively associated with the concentration of yeast in the food medium, and were negatively associated with the duration of yeast restriction. Also, they all increased after females received additional yeast. Although there is a potential for conflating sperm maintenance and egg quality-related effects, these results suggest that females in suboptimal nutritional condition might not be able to provide good nutrition or protection to their stored sperm, resulting in sperm death or loss. Therefore, we conclude that female sperm maintenance in D. melanogaster might carry a cost paid in components of dietary yeast.

**Evolutionary consequences of sperm senescence**

*Minimizing sperm senescence*

Sperm senescence imposes a fitness cost to males and females due to the decline in sperm fertilization success and reduction of the fitness of ensuing offspring. This thesis provides supporting evidence for deleterious impacts of sperm senescence on both aspects of sperm fitness.
in *D. melanogaster*. Our result of declined offspring production of old males indicates that pre-copulatory sperm senescence may curtail the fitness of the old males and their mates by reducing their fertilization success (Chapter 2). Our finding of a reduction in offspring fitness mediated by post-copulatory sperm ageing suggests that post-copulatory sperm senescence may reduce the fitness of the females who carry the sperm and the fitness of the males who provide the sperm (see Chapter 3). Since sperm senescence reduces the fitness of both sexes, we speculate that both sexes may have physiological and behavioural adaptations to reduce the speed of sperm senescence, especially in the species in which mating opportunities are limited. Two mechanisms listed below that may relate to the reduction in the speed of sperm senescence, including behavioural and morphological modifications of sperm cells and biochemical protection from male and female secretions.

First, sperm cells are often observed to have limited motility or are completely quiescent in the male or female sperm storage sites. For example, sperm in spiders are covered with a protein coat before copulation and only gain their motility in the female spermathecae (Foelix 1996). Sperm are embedded in the spermathecal epithelium in female salamanders and are immobile during storage (Boisseau and Joly 1975). Being quiescent reduces the requirement of metabolic fuel, and thus may reduce the intensity of oxidative stress. This idea has received support in the cricket *Gryllus bimaculatus*, in which stored sperm in females showed a 37% reduced metabolic rate, and a 42% reduced ROS production, compared with freshly ejaculated sperm from the same male (Ribou and Reinhardt 2012).

Second, both male and female secretions are also known to provide protection for sperm viability (Heifetz and Rivlin 2010). For example, male seminal fluid enhances sperm viability during female sperm storage in the leafcutter ant *Atta colombica* (den Boer *et al.* 2008) and the fruit fly *D. melanogaster* (Ravi Ram *et al.* 2005). Female secretions are important for sperm
survival in the marsupial mammals brushtail possum (*Trichosurus vulpecula*) and wallaby (*Macropus rufogriseus*) (Sidhu *et al.* 1999; Boere *et al.* 2011). Sperm viability can be enhanced by both nourishment and a reduction in senescence. For example, in honey bees, both male and female secretions have positive effects on sperm viability (den Boer *et al.* 2009). Both protein and non-protein components (*e.g.* amino acids and sugars) of the secretions of male accessory gland enhance sperm survival for the relative short period during ejaculation. The secretions of female spermathecal glands in honey bees also have positive effect on sperm viability during the long-term sperm storage likely due to antioxidant enzymes that protect sperm cells from oxidative stress (Weirich *et al.*, 2002). Functional and biochemical investigations of these fascinating sperm protection features would be an attractive direction for future research, and species with known male or female protective mechanisms for sperm viability would be good candidates for investigation of anti-sperm-senescence mechanisms.

If minimizing sperm senescence is beneficial to both sexes, then why do not females in all species develop a mechanism to limit the rate of sperm senescence during sperm storage? Our study of *D. melanogaster* provided several insights to answer this question. First, in species that the last male that mates with the female has sperm precedence, females can obtain fresh sperm by remating. Thus, their fitness gain may not extensively benefit from a reduced rate of sperm senescence. Second, if long-lived sperm are less effective in short-term sperm competition immediately after the copulation, which is where most fitness gains will accrue, then low sperm senescence rate may not be favored by selection. Third, sperm storage may impose a cost to females (Chapter 4). To fully understand sperm protection features and their evolutionary importance, future investigations should look for anti-sperm-senescence mechanisms, mating strategies, trade-offs between sperm longevity and other sperm characteristics, and the costs of maintaining sperm viability to the animal.
The avoidance of using senesced sperm cells

When the cost of sperm maintenance overrides the benefits of reducing the rate of sperm senescence, the process of senescence is inevitable. To avoid using senesced sperm cells, both sexes may choose their optimal timing of insemination (e.g. closer to female ovulation in the internal fertilizers) (Reinhardt 2007). On the male side, individuals may shift their reproduction earlier in life to avoid pre-meiotic sperm senescence (Reinhardt 2007), or possess physiological or behavioral traits that remove older sperm cells before insemination to eliminate the use of senesced sperm. Male autonomous sperm expulsion has been reported in a wide range of animals, such as crickets (Kumashiro et al. 2003), birds (Quay 1987), and humans (Baker and Bellis 1993). The exploration of the functional significance of this behaviour may benefit from considering the concept of sperm senescence. On the female side, they may have lower propensity to mate with older males to avoid low fertilization success (Beck and Promislow 2007). After matings, females may eject senesced sperm or avoid using them. The best example to date is probably the monogamous bird, the black-legged kittiwake *Rissa tridactyla*. Females adaptively use sperm from inseminations that have occurred soon before egg laying and eject the sperm from copulations that were performed a longer time before egg laying (White et al. 2008). This behavior coincides with our observed changes in behaviors and physiology in female *D. melanogaster* after mating. Oviposition of virgin females increased immediately after copulation, reached its peak on day 3, and declined thereafter (Chapter 4). This high egg laying rate within the first few days after copulation was coupled with high egg fertilization rate, which was likely due to high sperm usage efficiency. Since offspring fitness showed a significant decline by the fifth day post-copulation (Chapter 3), efficient usage of fresh sperm soon after copulation must be beneficial to both males and females. Indeed, female ovulation and egg deposition are
triggered by the molecules in the male seminal fluid, such as ovulin and sex peptide (reviewed in Ravi Ram and Wolfner 2007), indicating elevated female oviposition within the first few days after copulation is probably an adaptation of males to increase fitness. In addition, female animals may choose to remate to increase the proportion of younger sperm or the sperm of younger males in their sperm storage sites. This may provide at least a partial explanation for the finding that remating increases female fertility in insects (Arnqvist and Nilsson 2000). For example, in Drosophila, females are always found to remate before sperm from their previous matings are exhausted (Gromko et al. 1984) and to carry sperm from more than one male in both natural and laboratory populations (Milkman and Zeitler 1974, Harshman et al. 1988).

Post-copulatory sexual selection

In the scenario where there is a mixture of fresh sperm and senesced sperm in the female sperm storage sites after multiple matings, post-copulatory sexual selection may become another mechanism that reduces the use of senesced sperm for fertilization. On the male side, the outcomes of sperm competition between two males can be largely influenced by both pre-copulatory and post-copulatory sperm senescence. Under pre-copulatory sperm senescence, the proportion of live sperm present in a male’s ejaculate can be an important predictor of paternity (e.g. the cricket Teleogryllus oceanicus; García-González and Simmons 2005). Under post-copulatory sperm senescence, when there is a mixture of fresh and senesced sperm in the female reproductive tract, fresh sperm may outcompete or displace the senesced sperm. Therefore, I suggest that the measurements of sperm viability before and after copulations, like using live/dead viability/cytotoxicity stain (Holman 2009), are necessary for the understanding of the precise mechanisms of sperm competition. Observation of sperm competition in vivo, such as using transgenic D. melanogaster having red- or green-fluorescence-protein-labeled sperm to
distinguish sperm from young and old males or fresh and old sperm (Manier et al. 2010) may be of great help in understanding the impact of sperm senescence on sperm performance. On the female side, females may preferentially use younger sperm or the sperm of younger males, and thus control fertilization outcomes of the stored sperm. Research on sperm-age-mediated female preference may also benefit from transgenic techniques (Manier et al. 2010). By labeling sperm with fluorescent proteins in two different colors and examining the changes in fresh/old sperm ratio during the period of female sperm storage, one can identify whether a female preferentially uses younger sperm, or sperm from a younger male.

**Implications of sperm senescence on applied issues**

Studying sperm senescence not only sheds new light on understanding sperm evolution and sexual selection, but also has great value in applied sciences, such as long-term semen storage for artificial insemination. Apart from the use of cryopreservation, biotechnologists have not yet been able to develop methods of semen storage achieving > 20 years of storage duration. Deep-freezing technology has adverse effects on reduction of sperm viability and alternation of sperm chromatin structure, and thus impacts fertilization outcomes (Donnelly et al. 2001). The short fertilizable life of semen stored at ambient temperature (minutes or hours) is a limiting factor to increased usage of artificial insemination in human infertility treatments, livestock industry and conservation biology. If we could uncover the mechanisms behind the natural sperm storage processes in animal groups that have long-term sperm storage, thereby facilitating the long-term artificial storage of spermatozoa in an unfrozen state, there would be enormous medical and industrial benefits to human societies.
**Future directions**

Although growing interest has focused on the evolutionary and ecological repercussions of sperm senescence for gametic performance and offspring fitness, the evidence for it is rather fragmentary. Here, I discuss some areas that still need further exploration, suggest that existing biological methods are likely adequate to solve some of the problems, and encourage the development of new technologies in some areas.

First, research on the independent roles of pre- and post-meiotic sperm senescence on natural and sexual selection relies upon the measurement of sperm quality at both cellular and genetic levels. The traditional indices of the performance of sperm cells, such as sperm motility, velocity, and morphological abnormality, are mostly evaluated outside of males and females. A recently developed transgenic technique in *D. melanogaster* that enables direct observation of live sperm in the female reproductive tract and discriminates competing sperm of different males may help us to evaluate post-copulatory sperm performance *in vivo* (Manier *et al*. 2010). So far, this technique has only been applied to *Drosophila*. Application to other animal systems may be helpful to understand mechanisms of post-copulatory sexual selection. In addition to evaluating sperm performance, more accurate assessments of sperm quality at a cellular level that indicate the integrity of chromatin, mitochondria and membranes have been achieved by established techniques in cell biology, but need to be further incorporated into evolutionary analysis of sperm senescence.

Second, genomic and proteomic work, as well as the research on gene functions (reviewed by Dorus and Karr 2009) should be valuable to identify female molecules with potential roles in sperm storage (Prokupek *et al*. 2008). Since post-meiotic gene expression is extremely limited (Fuller 1993, reviewed in Chapter 1), genomic and proteomic work may help to reveal function of the expressed genes in controlling sperm activities, and therefore, to figure
out the relative importance of the genome of a male and the genome of his sperm with respect to sperm senescence. Today, we can create isogenic males that carry a transect of mutation accumulation in the germline (Mallet et al. 2011). By sequencing the sperm of these males and the control males, one can identify the nature of the mutations (e.g. the affected alleles and the locations on the chromosomes) and relate it to the fitness changes in the offspring.

Third, the association between sperm senescence and fertilizing efficiency has not been revealed with individual sperm cells. This area urgently needs the development of new technologies that can track the senescence of individual sperm cells and accurately distinguish viable and functional, viable but dysfunctional, and dead sperm.

Last, it seems to be the general consensus that sperm are susceptible to environmental changes, but it has so far received little attention. In external fertilizers, such as stickleback fish, it has been shown that water conditions, such as salinity and the presence of ovarian fluid, can drastically influence sperm longevity (Elofsson et al. 2003). In internal fertilizers, the biochemical environment and temperature around sperm cells might be associated with the female condition, and alter the patterns of sperm senescence. For instance, lowered body temperature and a reduced metabolic rate in females might contribute toward prolongation of the sperm life span (e.g. hibernating bats, Crichton and Krutzsch 2000; fruit flies, Bouléreau-Merle and Fouillet 2002). Future studies on sperm senescence at any stage of a sperm’s life should provide enough descriptions, as well as careful control in sperm living environments when sperm longevity is measured and the manifestation of sperm senescence is analyzed.

In conclusion, one cannot truly understand the reproductive biology and its relation to the ecology, behavior, and evolution of any animal taxon without considering the implications of sperm senescence. I hope this thesis raises more questions than it answers. We have much to learn.
References


