Causes and consequences of male reproductive senescence

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Abstract

Ageing, a universal phenomenon across all life forms, encompasses changes in a myriad of traits through organismal ontogeny. While senescence, the deterioration of traits with advancing age, is expected to be the most common outcome of ageing, there are exceptions, with some species exhibiting no change or even improvements in traits as they advance in age. Reproductive senescence is a result of declines in traits influencing mating and reproductive success. In my thesis, I address several major open questions pertaining to reproductive senescence: what is the diversity of reproductive senescence patterns across taxa?; what are the evolutionary and proximate causes of male reproductive senescence?; what processes might buffer reproductive senescence?; and what are the fitness consequences of male reproductive senescence? My thesis primarily focuses on males, which are under-represented in reproductive ageing literature compared to females. I use a combination of comparative approaches as well as experiments on the fruit fly *Drosophila melanogaster.* My thesis has five data chapters (chapters 2 to 6).

My second chapter compares the rates of reproductive senescence in ejaculate traits across non-human animals using meta-analytical approaches. My third chapter systematically reviews across animals, how advancing age influences seminal fluid proteins and oxidative stress. These two comparative chapters combined show that senescence at the level of the ejaculate is ejaculate trait-specific, with seminal fluid traits generally showing senescence, while ejaculate quantity traits rarely showing senescence, across taxa. In my fourth chapter, I use experiments in fruit flies to compare how age-related changes in sperm number and seminal fluid influences male fertility. These experimental results largely support conclusions from my comparative chapters (2 and 3), and suggest that male reproductive senescence is likely a consequence of decline in seminal fluid quality, not sperm number.

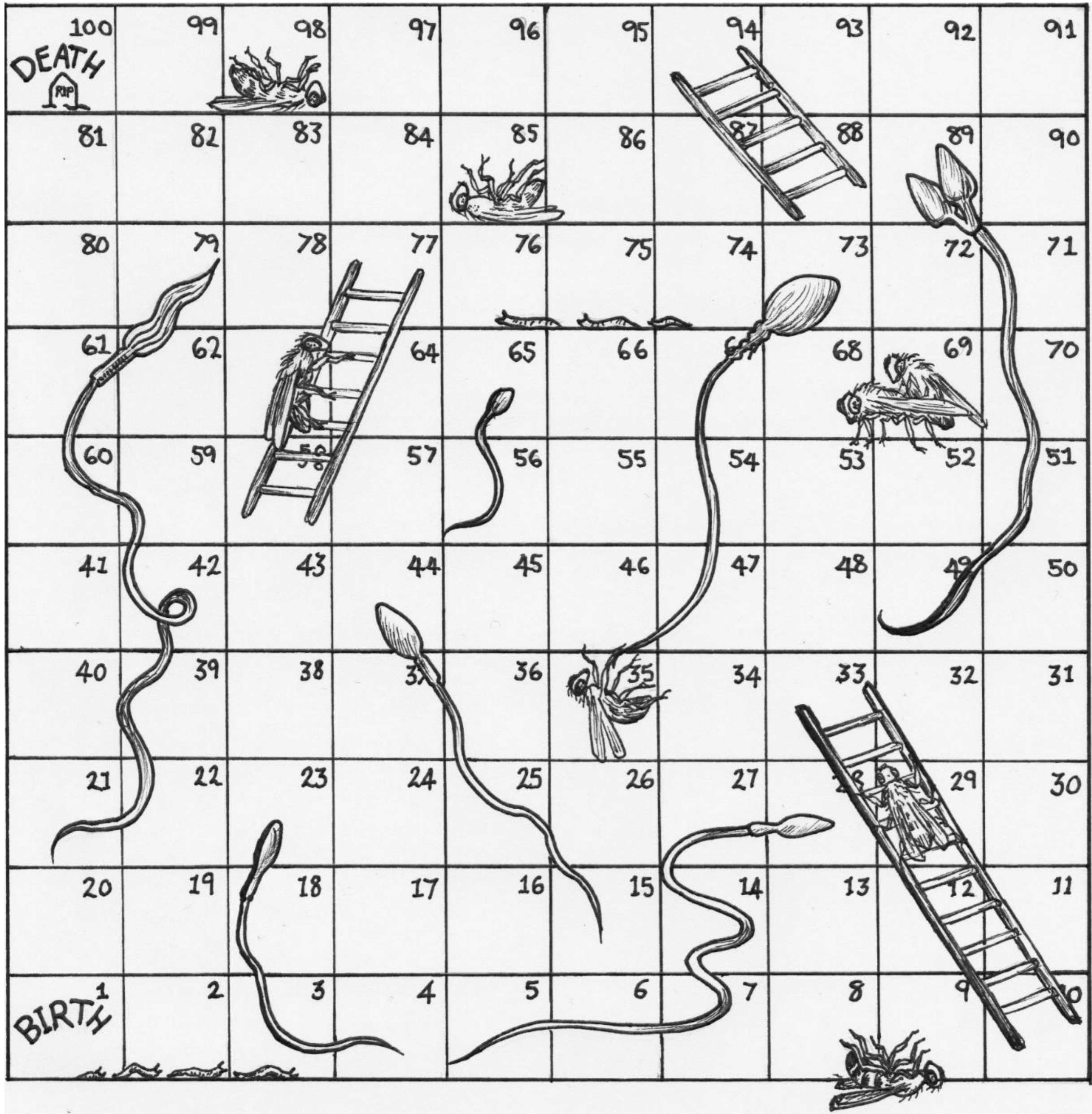
In my fifth and sixth chapters, I use fruit flies to study the inter-generational effects of advancing paternal age. Contrary to expectations, I find no evidence for advancing paternal age to affect offspring fitness deleteriously. Instead, my results indicate that advancing paternal age might influence offspring lifespans positively due to viability selection on old fathers. Additionally, I find that paternal age effects are confounded by the effects of paternal sperm storage duration, which studies rarely disentangle.

Overall, my thesis demonstrates that advancing male age can influence male fitness in complex ways. This complexity arises due to differential effects of male age on intra- and inter-generation fitness components, due to various demographic processes modulating senescence, and due to different traits that directly influence reproductive performance varying in their rates of senescence. My thesis has profound implications for reproductive health and understanding population dynamics because it dissects the evolutionary, mechanistic, and demographic causes and consequences of reproductive senescence.

Evolution

What's to come
Isn't always better
than what came.
If you thought that
The past has already gone,
Look within yourself,
And you'll find parts of it hidden.
What couldn't move
Can now leap.
What once remained silent
Now sings.
What came from the abyss
Now refuses to touch the ground.
Everything can be attained
If you know that you have to wait.
In here, lie the origins
Of love and deceit,
Of power and death.
And there continues an "us"
in all of "them",
A "me", in all of "you".

If I divide myself for long enough and separate,
I too may become something else.
I now know how to divide into a body and a body.
to be born and change,
Only to finally end.



-by Hannah King

Senescence

A symphony of whispers, echoes old,
As age unfolds its tale untold.
In hues of gold, we find our way,
Yet know, that time will have its say.
Each wrinkle a tale, each memory a sigh,
A canvas marked by days slipping by.
With each passing year, our burdens grow,
As age weaves its silent woe.
Like a legacy passed from hand to hand,
We inherit past flaws, in a double strand.
And in the end, what do we find?
A purpose lost, a fleeting mind.
So as the twilight hues descend,
Ageing prevents all amends,
And in the twilight's gentle glow,
We wonder what we'll pass on,
what we'll show.

Sexual selection

In nature's game of love and war,
The fittest rise to mate once more.
With colorful plumes and graceful dance,
The males show off their courtship stance.

The females watch with careful eyes,
Choosing the elaborate or the wise.
For strength and health are what they seek,
In a partner strong and unique.

But sometimes, it's not just brawn,
That catches a female's gaze and drawn.
It's the bright and flashy, bold and grand,
That wins the game, conquers the land.

And so the cycle spins anew,
As sexual selection seeps through.
Where those who are the sexiest,
Will pass on their genes to the next.

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Important note- Each chapter comes with its own reference list. Figure numbers within a chapter refer only to figures presented in that chapter. Chapters 2, 4, 5, and 6 have a supplementary information/appendix section.

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Declaration

I declare that the work presented in this thesis was composed by myself, and is my own, except when stated otherwise in the chapter contributions section below. The work has not been submitted for any other degree or professional qualifications.

Chapter contributions:

- Chapter 1: Thesis introduction
  - I am the sole author of this chapter, however significant feedback has been given by TP and IS.
- Chapter 2: **Sanghvi K**, Vega-Trejo R, Nakagawa S, Gascoigne S, Johnson S, Salguero-Gomez R, Pizzari T, Sepil I. Meta-analysis shows no consistent evidence for senescence in ejaculate traits across animals. *Nature Communications* 15, 558 (2024). https://doi.org/10.1038/s41467-024-44768-4
  - I am a first author on this chapter (shared equally with RVT). I conceptualised the idea with feedback from TP and IS. I screened papers and collected data from papers, and RVT and SG tested for repeatability of this process. Along with RVT (who led data analysis), I created the code required to analyse the data. I wrote the first and revised drafts of the paper, and RVT, IS, and TP provided inputs throughout its writing and commented on multiple drafts of the paper.
- Chapter 3: Fricke C, **Sanghvi K**, Emery M, Lindenbaum I, Wigby S, Ramm SA and Sepil I (2023) Timeless or tainted? The effects of male ageing on seminal fluid. *Frontiers in Ecology and Evolution.* 11:1066022. doi: 10.3389/fevo.2023.1066022
  - I am co-first author on this paper (equally shared with CF). CF, IS, SR, SW and I equally planned the project. I led the screening of studies with

contributions from all other authors. All authors collected data, however I summarised the data into tables and figures. CF led the writing of the paper, with my contribution towards writing being second most and equal to IS and SR.

- Chapter 4: **Sanghvi K**, Shandilya S, Brown A, Todorova B, Jahn M, Gascoigne SJL, Camilleri TL, Pizzari T, Sepil I. Reproductive success of old polygynous males is limited by seminal fluid, not sperm number. Target journal: *PNAS*
    - I am first author on this paper. I conceptualised the experiments with help from TP and IS. I led the experiments with help in the lab from other co-authors. I dissected and imaged all flies with help from MJ, who showed me how to use a confocal microscope. I analysed data and wrote all drafts of the paper with crucial inputs from all co-authors, especially IS and TP through their comments on multiple drafts.
- Chapter 5: **Sanghvi K**, Gascoigne SJL, Todorova B, Vega-Trejo R, Pizzari T, Sepil I. No evidence of paternal age effects in sons or daughters, when accounting for effects of paternal sperm storage. Target journal: *Functional ecology*
    - I am first author on this paper. I conceptualised the experiments with help from TP and IS. I led the experiments with help from IS, SG, BT, and RVT. I analysed data and wrote all drafts of the paper, with crucial inputs from all co-authors, especially IS and TP through their comments on multiple drafts.
- Chapter 6: **Sanghvi K**, Pizzari T, Sepil I. What doesn't kill you makes you stronger? Effects of paternal age at conception on fathers and sons. In press: *Evolution*.
    - I am first author on this paper. I conceptualised the experiments with help from TP and IS. I conducted the experiments with help from IS. I analysed

data and wrote all drafts of the paper with crucial inputs from IS and TP through their comments on multiple drafts.

- Chapter 7: Thesis discussion
    - I am the sole author of this chapter, however significant feedback has been given by TP and IS.

Chapter 1

Thesis introduction

Senescence

With passing time, organisms advance in age, a process that is ubiquitous to all life on earth. Advancing age can affect a whole suite of traits (Reichard, 2016), and the combined influence of these traits determines overall organismal fitness in complex ways. These traits range from physiological, such as immune function (Preshaw et al, 2017), to behavioural traits such as activity levels (Catry et al, 2011), sociality (Siracusa et al, 2022) and cognition (Heinen et al, 2021), to fitness-components such as survival and reproduction (Anderson and Apanius, 2003; Gaillard and Lemaitre, 2020). While organisms show a diversity of changes with advancing age (Jones et al, 2014), a pattern typically observed is senescence, the irreversible deterioration of traits with advancing age (Nussey et al, 2013). Here, two types of senescence have received considerable attention: actuarial senescence, which is calculated using mortality hazards and is the likelihood of instantaneous death at a given age or between two-time steps (Peron et al, 2019), and reproductive senescence, which is the decline in reproductive function with advancing age (Lemaitre and Gaillard, 2017). An example of actuarial senescence is human mortality hazard being high at birth due to high rates of infant death, reducing from childhood to sexual maturity, and then increasing from sexual maturity onwards until old age (Carnes et al, 2006; Engelman et al, 2017; Figure 1). Senescence however is not universal (Jones and Vaupel, 2017; Sanghvi et al 2024), and many taxa show no change in traits with advancing age, and others show improvements in survival probability and reproduction (Munne-Bosch, 2015).

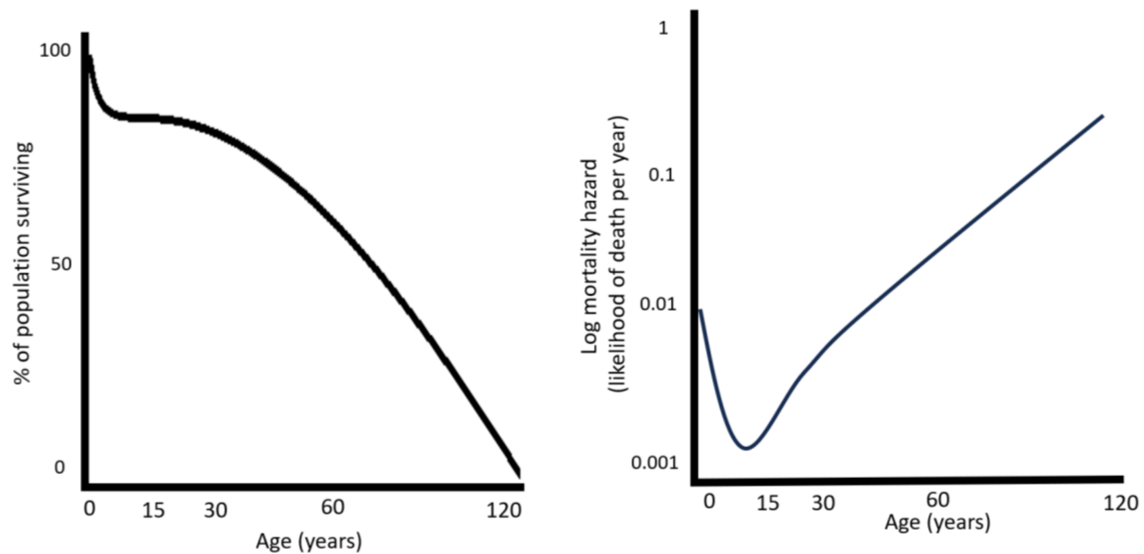

Figure 1: Example of survival probability (left) and mortality hazard (right), for human populations (adapted from Carnes et al, 2006; Engelman et al, 2017). Due to high mortality at birth, the hazard function is high, which then gradually reduces from birth until sexual maturity, due to very few deaths between these ages, and then the hazard function increases from sexual maturity until old age.

The puzzle of reproductive senescence

The focus of my thesis is reproductive senescence. Reproductive senescence is a consequence of age-dependent deterioration in an entire suite of traits that influence reproductive function (Naciri et al, 2022; Nussey et al, 2013). Reproductive senescence can thus result from fewer *quantities* of offspring being produced by old individuals (Vrtilek et al, 2022), due to the lower mating success of old individuals (Richard et al, 2005; Riecke et al, 2023) or fewer/poorer quality gametes in old individuals (Sanghvi et al, 2024). Reproductive senescence can also be the result of parental age effects, i.e. poorer *quality* offspring being produced by old individuals (Moorad and Nussey, 2016), because of lower gamete quality or lower provisioning ability (Beamonte-Barietos et al, 2010; Ivimey-Cook and Moorad, 2020) of old individuals.

Two questions regarding reproductive senescence have puzzled biologists for nearly a century. The first question is- why do organisms show a huge diversity in patterns of reproductive senescence? This question is motivated by the realisation that ageing does not

necessarily translate into reproductive senescence (Baudisch and Stott, 2019; Jones et al, 2014). For instance, taxa such as crustacea (Sanghvi et al, 2024), some fish species (Purchase et al, 2022), reptiles (Jones et al, 2014), and trees (Munne-Bosch, 2015), show no evidence for reproductive senescence. In contrast, other taxa such as most mammals and birds, typically show improvements in reproduction from early to mid-life, and then declines from mid- to late- life (Lemaitre et al, 2020; Vagasi et al, 2021; Figure 2).

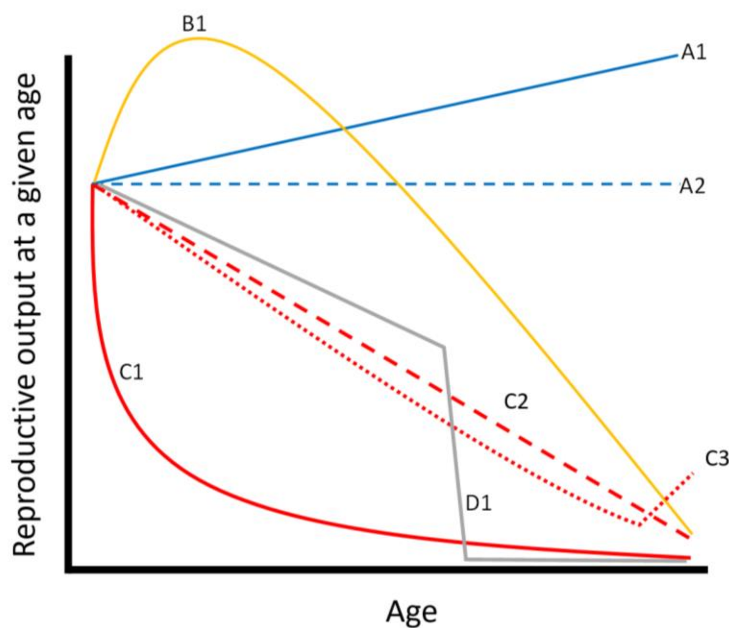

Figure 2: Some examples of change in reproductive output as a function of age, demonstrating different patterns of reproductive ageing. Different colours and dashes are used in the figure to aid visual differentiation between overlapping lines. A1: Linear improvement in reproductive output with age; A2: no reproductive senescence. A1 and A2 are expected in indeterminate growing taxa such as trees and crustacea (more below). B1: improvement in reproduction from birth until sexual maturity, and onset of senescence after sexual maturity. This pattern is typical in birds and mammals. C1: early onset of reproductive senescence, and steep rates of senescence in early life. Such a pattern is observed in semelparous species which reproduce early in life and die soon after. C2: Onset of senescence in early life, and a steady rate of senescence across life. C3: Onset of senescence in early life, and a steady rate of senescence until late-life, after which reproductive output sharply increases. Such a pattern is observed in terminally investing species. D1: Early onset of senescence and a steady rate of senescence until mid-life, after which senescence rate increases sharply and reproduction ceases. Such a pattern is seen in menopausal females.

The second puzzling question is- why does reproductive senescence occur in the first place? If natural selection maximizes the representation of genotypes in future gene pools, then all else being equal, individuals that reproduce effectively throughout their lives should be selected for and individuals that reproduce less effectively when older should be selected against (Gaillard and Lemaitre, 2017; Maklakov et al, 2015). Similarly, if individuals are able

to reproduce effectively in early life, they should also be able to do so in late life. Thus, reproductive senescence seems paradoxical. An extreme example of this apparent paradox is menopause, where females of some species continue to live far beyond an age where they can reproduce (Croft et al, 2015; Shanley et al, 2007). Many hypotheses in the field of life-history theory have been developed to address the underlying evolutionary causes of reproductive senescence.

Evolutionary causes of reproductive senescence

Selection shadow

The cumulative likelihood of individuals dying increases with age and leads to few individuals surviving to older ages (Flatt and Promislow, 2007), making individuals less likely to reproduce at an old age (Flatt and Promislow, 2007). Consequently, the reproductive value of older ages (i.e. the contribution to future gene pools) is in most cases, lower than that of younger age classes (Roper et al, 2021). Given that individuals are less likely to survive until, thus reproduce, later in life, selection is expected to be stronger on early-life reproductive performance (Partridge and Barton, 1993). Additionally, individuals are likely to die as they age due to extrinsic and stochastic reasons (Maklakov and Chapman, 2019). Extrinsic mortality leads to differential survival and reproduction at older ages, to be less likely due to heritable genetic differences, and more likely be due to chance. The combination of lower reproductive value of older ages and extrinsic mortality, leads selection to be weaker later in life than that at a younger age, creating a “selection shadow” at older ages (Hughes and Reynolds, 2005; Figure 3). The three major, interconnected hypotheses for the evolution of reproductive senescence- antagonistic pleiotropy, mutations accumulation, and disposable soma, are derived from the occurrence of a selection shadow (Baudisch and Stott, 2019; Johnson et al, 2019; Lemaitre et al, 2024; Maklakov and Chapman, 2019).

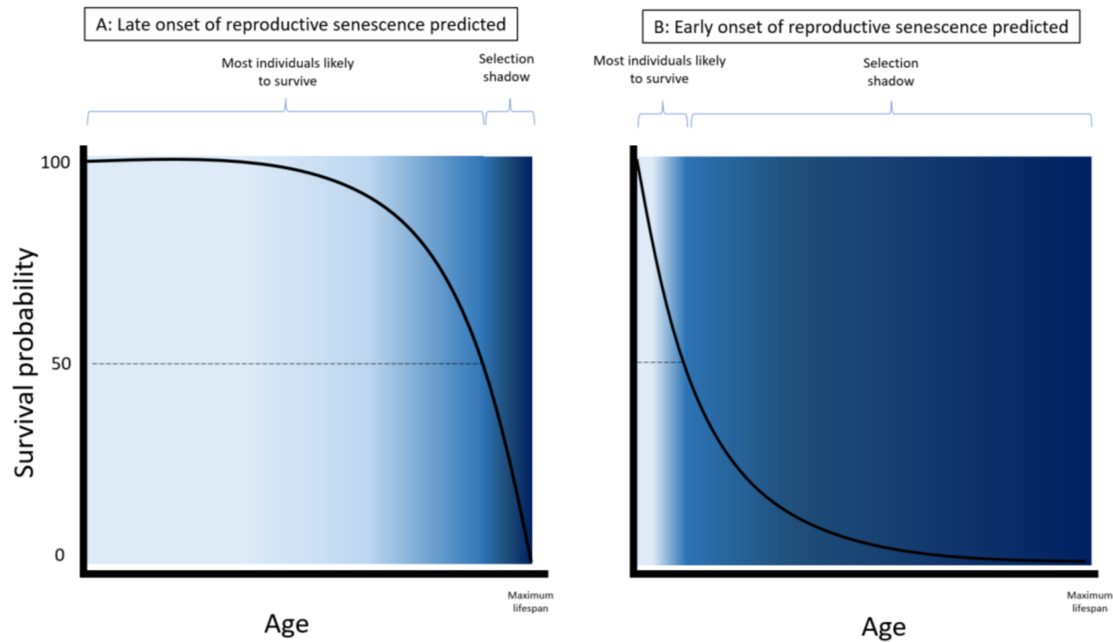

Figure 3: Examples of two hypothetical organisms, one with low mortality hazard in early- to mid-life, but high mortality hazard late in life (A); the other with high mortality hazard in early-life, but lower in late-life (B). The selection shadow region in A is apparent only late in life, whereas in B, the selection shadow becomes apparent from early life. In A, reproductive senescence is thus predicted to onset later in life than in B. Darkness of blue shows the magnitude of the selection shadow (i.e. the inverse of the strength of selection). Dotted line shows age at 50% survival probability.

Antagonistic pleiotropy

When alleles have pleiotropic effects, such that they are beneficial in early-life (when most individuals will survive and reproduce), but deleterious in late-life (when only few individuals will survive, thus reproduce), then natural selection will select for, and favour the spread of these pleiotropic alleles (Monaghan et al, 2008; Williams and Day, 2003). However, when alleles have the opposite pleiotropic effect such that they are deleterious in early-life but beneficial in late-life, selection will select against such alleles. This difference in selection occurs due to fewer individuals reproducing in late-life and the strength of selection being weaker in later-life (selection shadow) for most organisms (Flatt and Promislow, 2007). Reproductive senescence is manifested through such pleiotropic effects of alleles, which improve early-life reproductive success but reduce late-life reproductive success. There is

some evidence for such pleiotropic effects of genes, which are beneficial when expressed early in life, but are deleterious late in life (Rodriguez et al, 2017).

Mutation accumulation over generations

Mutations occur across an organism's lifespan and natural selection is predicted to select against mutations with deleterious fitness effects. Some mutations have immediate effects on fitness, while other mutations, even though acquired in early life, are expressed only in later-life. When effects of such mutations that are manifested only at later ages (when only few individuals survive until to reproduce) are deleterious, these mutations are unlikely to be effectively selected against, and accumulate with each generation (Lee and Chu, 2023; Rose and Charlesworth, 2002). This is because deleterious mutations that are acquired early in life but are not expressed until later in life, would not affect early-life reproduction. Therefore, individuals carrying such mutations would still reproduce effectively in early-life passing these mutations to the next generation (Aubier and Galipaud, 2024; Monaghan et al, 2008). Some evidence consistent with this hypothesis comes from Turan et al (2019), who show that genes expressed only late in life are more likely to be deleterious than genes expressed early in life.

The mutation accumulation and antagonistic pleiotropy hypotheses are the genetic evolutionary causes of reproductive senescence, where deleterious effects of alleles in late-life lead to poorer phenotypes of old individuals (Lemaitre et al, 2024).

Disposable soma and trade-offs

Resources that individuals can allocate towards growth, somatic maintenance, as well as to current and future reproduction, are limited (Aubier and Galipaud et al, 2024). Natural selection does not select for survival or growth unless increased survival and growth promote

individuals passing on their genes at higher rates than alternative genotypes (Kirkwood and Holliday, 1979). Thus, natural selection is predicted to cause organisms to invest into early-life reproduction than into survival or growth. Similarly, because fewer individuals are likely to survive to older ages, natural selection is predicted to cause organisms to invest more into early- than late-life reproduction (Lemaitre et al, 2015). Thus, energetic constraints lead to trade-offs between and differential investment in early- versus late-life reproduction manifested as reproductive senescence, and germline versus somatic traits. Some evidence for such differential investment and trade-offs comes from animals that invest more in early life reproduction, but suffer higher rates of late-life mortality (Lemaitre et al, 2015) and reproduce less effectively when old (Nussey et al, 2006).

Hallmarks of reproductive senescence

Older individuals have undergone more cell divisions and have produced more biomolecules than younger individuals. These processes lead to older individuals having more mutations (due to cumulatively more cell divisions) and higher levels of oxidative damage (due to cumulatively more biomolecule synthesis) than young individuals (Hughes and Reynolods, 2005; Monaghan et al, 2008; Monaghan and Metcalfe, 2019; Secomandi et al, 2022). Resources are limited and individuals are predicted to evolve allocating resources (e.g. DNA repair machinery and antioxidants) towards early life reproduction (see evolutionary hypotheses above). Therefore, older individuals are less able to repair damage in the germline than younger individuals (Aubier and Galipaud et al, 2024; Maklakov and Chapman, 2019). These proximate mechanisms of senescence are manifested as the hallmarks of: shortened telomeres, unstable genomes, epigenetic modifications, cellular damage and disfunction, impaired cellular signalling, and dysregulated nutrient sensing, in old individuals (Hernandex-Segura et al, 2018; Lemaitre et al, 2024).

Negligible reproductive senescence

While various hypotheses effectively explain why reproductive senescence occurs, reproductive senescence is not always observed across taxa (Sanghvi et al, 2024). This is exemplified in indeterminately growing taxa such as crustacea, trees, some reptiles and fish, where individuals grow throughout their lives, and reproductive performance does not decline with age (Depeux et al, 2020; Jones et al, 2014; Sanghvi et al, 2024) In indeterminate growers, individuals in older age classes have a lower mortality hazard and a higher likelihood of surviving and reproducing, due to being larger than younger individuals (Flatt and Partridge, 2018; Purchase et al, 2022). This prevents a selection shadow from occurring in late-life, because the contribution of older age classes to future generations is higher than that of younger age classes, allowing selection to effectively purge alleles with deleterious effects late in life (Charnov et al, 2001). Additionally, when gonads and other traits such as weapons, which determine reproductive success, grow allometrically with body size, older individuals have disproportionately higher reproductive success due to increased gamete production, mating success, and social competitiveness, than younger individuals (Flatt and Partridge, 2018; Purchase et al, 2022). In addition to indeterminate growth, other demographic processes can buffer reproductive senescence, and delay or mask its occurrence.

Demographic and within-individual processes

The effects of advancing age on organismal reproduction can be assessed by sampling individuals longitudinally and/or cross-sectionally (e.g. Blas et al, 2009). Longitudinal sampling occurs when the reproductive output of the same individual is assayed at multiple time points in the individual's life. Longitudinal sampling is informative about physiological changes occurring within an individual with advancing age, and can be particularly effective in controlling for individual differences in reproductive quality (McCleery et al, 2008;

Nussey et al, 2008). However, longitudinal sampling can have drawbacks due to its inability to separate the confounding effects of mating history from age (Aich et al, 2020). This is because as individuals age, they are likely to have accumulated more reproductive experience and have invested cumulatively more into reproduction when old, than when they were young (Aich et al, 2020, 2021, 2022; Johnson and Gemmell, 2012). Cross-sectional sampling (Figure 4) occurs when the reproductive performance at different ages is compared using different cohorts (i.e. one cohort is assayed at a young age, and a different cohort is assayed at an old age). Cross-sectional sampling is useful for understanding the demography of ageing populations. Furthermore, under experimental scenarios where matings can be controlled, cross-sectional sampling can uncouple effects of age from mating history. However, cross-sectional sampling is not informative of changes that occur within-individuals as they age (van de Pol and Verhulst, 2006).

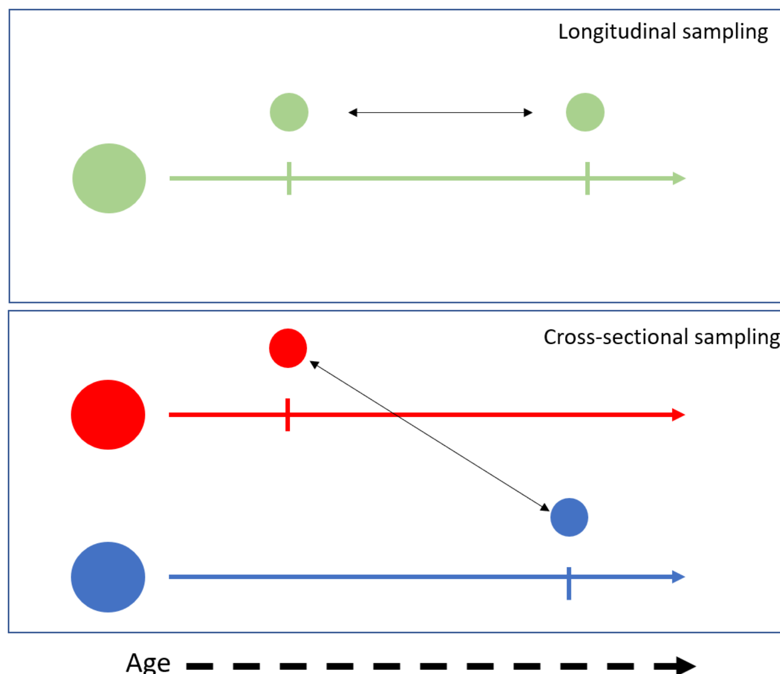

Figure 4: Differences between longitudinal and cross-sectional sampling approaches. Longitudinal sampling measures the reproductive output of the same individual repeatedly at different time points. Cross-sectional sampling compares reproductive output of different cohorts, each sampled at a single time point (i.e. old or young). Solid black arrows show the comparisons being made by each sampling method.

Depending on how individuals are sampled (longitudinal or cross-sectional), various processes (e.g. terminal investment, selective disappearance, viability selection) can mask reproductive senescence (Johnson and Gemmell, 2012). An example of these processes is terminal investment. Here, as the residual reproductive opportunities of individuals decreases, individuals may invest relatively more in current than future reproductive event (Foo et al, 2023). Terminal investment is predicted to occur either in old individual (who have fewer future opportunities to reproduce than young individuals), in individuals who are close to dying, or due to an interaction between age and proximity to death (Duffield et al, 2017; Foo et al, 2023; Velando et al, 2006). Terminal investment can lead to within-individual increases in reproductive output with age, therefore buffering reproductive senescence (Figure 2). Selective disappearance can also mask reproductive senescence (Figure 5). Selective disappearance occurs when lifespan and reproductive rate co-vary positively, such that individuals that survive to older ages (and are thus likely longer-lived), represent a non-random selection of individuals of higher reproductive quality (Bouwhuis et al, 2009). Viability selection, a process alike selective disappearance, can buffer reproductive senescence when measuring inter-generational effects of parental age. Here, individuals that reproduce when old on average have longer lifespans than individuals that reproduce when young (Johnson and Gemmell, 2012; Kokko, 1998). If lifespan is heritable, then individuals reproducing when older (and have undergone selection for viability) will pass on alleles conferring higher viability to their offspring, leading to longer-lived offspring. Selective disappearance (Figure 5) and viability selection (Figure 6) are typically observed under cross-sectional sampling where individuals have differential mortality, and those reproducing at an old age are a non-random cohort of surviving individuals.

These processes can lead to complex patterns of reproductive senescence (McCleery et al, 2008). Patterns of reproductive senescence can be further complicated by additional factors, such as differential effects of age on intra- versus inter-generational fitness-components (Moorad and Nussey, 2016), and by factors confounding the effects of age.

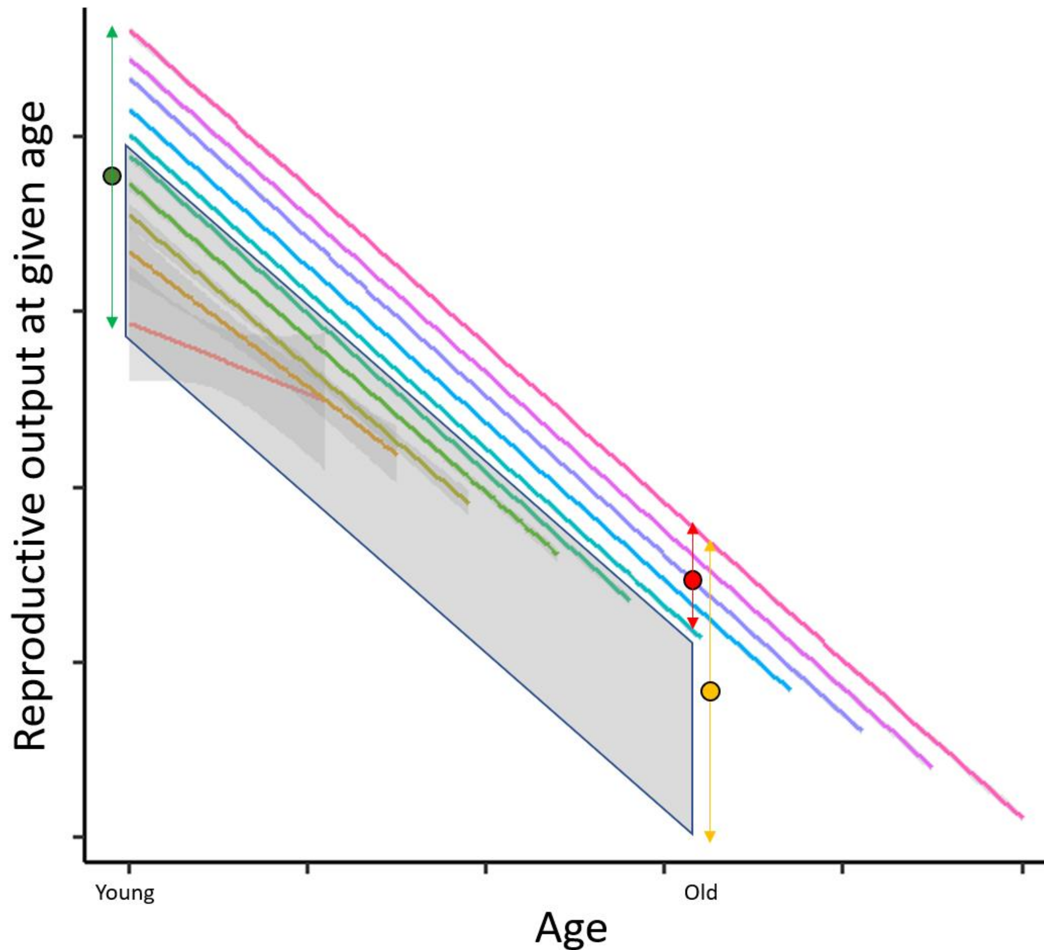

Figure 5: Hypothetical example of how selective disappearance buffers reproductive senescence. Each line represents one of 10 individuals. Each individual suffers a decline in reproductive output with advancing age. At a young age, all 10 individuals reproduce (mean represented by green point). However, some individuals die before they reach an old age (represented by grey polygon). If selective disappearance occurs such that reproductive performance covaries positively with survival, then older individuals will represent a non-random subset of high reproductive-quality individuals. Under selective disappearance, the mean reproductive performance of individuals surviving at an old age is represented by the red point. On the other hand, if there was no selective disappearance (i.e. random death) or no death, then the mean reproductive performance at an old age would be represented by the orange circle. Thus, selective disappearance buffers reproductive senescence.

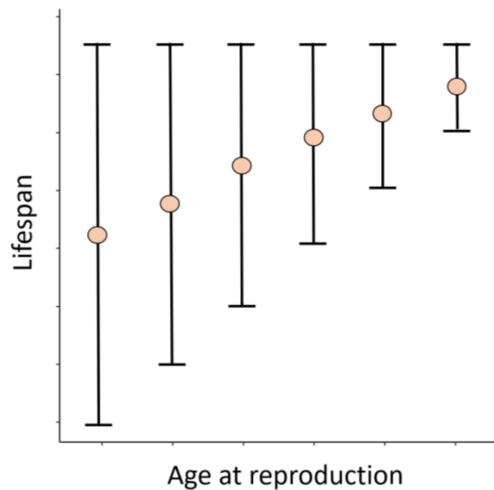

Figure 6: Hypothetical example of viability selection. Individuals that reproduce when older, on average, have longer lifespans than individuals that reproduce when younger. This differential lifespan arises because only individuals that survive to an old age will be able to reproduce at such age, whereas the fraction of the population reproduces at a young age comprises of individuals that will have a wider range of lifespans. If lifespan is heritable, this selection on viability with advancing age leads to offspring of older individuals living longer than those of younger individuals, on average. Hypothetical mean and range of lifespans shown.

Confounding factors

The apparent influence of advancing age on reproduction is not always causal and many factors can confound with age. These confounding factors can independently influence reproductive performance, which studies can misinterpret as being directly caused by age. My thesis focuses on two such factors- mating history (see above in demographic and within-individual processes section) and sexual rest. Sexual rest/post-meiotic gamete storage/sexual abstinence refers to the lag between production of a mature gametes and the use of those gamete for fertilization. When an individual's age covaries positively with the duration of post-meiotic gamete storage, it is difficult to disentangle the influence of age from gamete storage (Pizzari et al, 2008; Jones and Elgar, 2004). Such confounding effects are exemplified in females of species where all oocytes are produced at birth (Tan et al, 2013; Tarin, 2000). Here, whether reproductive senescence is due to ageing of the female or due to prolonged oocyte storage in older females, cannot be separated. Similarly in males, age can co-vary with the duration of sexual rest when old and young males are virgins (which is typically seen in experimental scenarios, Sanghvi et al, 2024), or when old males are less likely to acquire

matings than young males (Aich et al, 2020; Vega-Trejo et al, 2019). Separating confounding effects of mating history and sexual rest from age is crucial for understanding the evidence for reproductive senescence, yet studies rarely ever disentangle these. In addition, it is often difficult to distinguish between male- and female- driven mechanisms leading to senescence because studies on reproductive senescence have mostly focussed on females.

Reproductive senescence in males versus females

Reproductive senescence in females has been investigated considerably more than in males (Archer et al, 2022). This sex-bias in research is problematic because predictions from evolutionary and mechanistic theories of senescence to date, remain better tested in females than males. This sex-bias also risks theories being less attuned to male-specific patterns of ageing, causing uncertainty about whether ageing in males is consistent with predictions made by life-history theories. Understanding male reproductive senescence is especially crucial because males and females differ in their patterns of reproductive ageing (Bronikowski et al, 2022). Therefore, female-specific studies may not be informative about male-specific patterns of reproductive senescence. For instance, in species with only maternal care, females ae predicted to evolve investing energy into somatic traits that improve offspring provisioning rather than in gametes, potentially accelerating reproductive senescence in females compared to males (Lemaitre and Gaillard, 2017). In contrast, in species where intra- or inter-sexual selection has led to the evolution of exaggerated male traits (e.g. weapons or ornaments), males are predicted to evolve investing in these traits at the cost of lower investment in gametes (Hunt et al, 2004), therefore accelerating reproductive senescence in males. Additionally, sexual conflict can lead to opposing selection pressures on senescence patterns in males versus females, buffering senescence in one sex at the cost of accelerating senescence in the other sex (Bonduriansky et al, 2008). From a

genetic perspective, the heterogametic sex is predicted to have faster rates of senescence than the homogametic sex (Cayuela et al, 2022; Maklakov and Lummaa, 2013). Due to sex-differences in reproductive senescence and the under-representation of males, it is imperative that patterns of male reproductive senescence are documented, and the causes and consequences of male reproductive senescence are investigated. My thesis therefore focuses on male reproductive senescence.

Partitioning male reproductive senescence

Male reproductive senescence can be mediated through a variety of pathways and traits. For instance, traits typically assayed by studies on ageing include traits related to male mating success (e.g. courtship, attractiveness), male reproductive success (e.g. sperm quantity or quality, seminal fluid quantity or quality), or female behaviour (e.g. mate choice). However, the consequences of advancing male age are manifested not only intra-generationally, i.e. on offspring quantity (Vrtilek et al, 2022) or male mating success, but also inter-generationally, i.e. on offspring quality (Vuarin et al, 2021). Therefore, to comprehensively study male reproductive senescence, investigating the differential and combined influence of various traits, in influencing offspring quantity as well as quality is required. Such an investigation is especially crucial because different traits can senescence at different rates, and because offspring quantity versus quality can be influenced by each of these traits in contrasting ways (Johnson et al, 2018; Monaghan and Metcalfe, 2019). For example, some studies show senescence in male pre-copulatory but not ejaculate traits (Rutkowski et al, 2023), while others show senescence in ejaculate traits, but not in pre-copulatory traits or fertilisation success (Gasparini et al, 2010; Lifjeld et al, 2022). Studies also show ejaculate components-sperm versus seminal fluid, to senesce at different rates and have differential influence on male reproduction (Fricke and Koppik, 2019; Sepil et al, 2020). Other studies yet (Johnson et

al, 2018) show reproductive senescence being caused by declines in male fertility with advancing age, and not due to changes in offspring quality. Importantly, comparing the magnitude of senescence in different male traits can inform us about sexual conflict. For instance, if old males are better able to acquire matings but produce fewer or lower quality offspring compared to young males, this could exacerbate sexual conflict (Dean et al, 2010; Karl and Fischer, 2013; Perez-Staples et al, 2010). It is important that studies partition the separate and combined influence of age-related changes in various traits to assess the inter- and intra- generational consequences of advancing male age, thus the overall effect of ageing on fitness.

Knowledge gaps

There are still certain knowledge gaps in our understanding of male reproductive senescence, some of which are highlighted above. These knowledge gaps pertain to: which traits and pathways mediate male reproductive senescence; whether all evolutionary theories are applicable to male senescence; the direct versus indirect (via confounding factors) effects of advancing male age; the inter-generational consequences of advancing male age; the taxonomic generality of male reproductive senescence; and how demographic processes modulate the effects of advancing male age. Thus, an appraisal of male reproductive senescence needs to encompass evolutionary theories of senescence, within- and between-individual processes, separate confounding effects that co-vary with age, be applicable to and informed by a range of taxa, assess the consequences of male senescence over multiple generations, and partition the influence of various traits that contribute towards male reproductive senescence.

I address these knowledge gaps in my thesis using comparative approaches, and experiments on the tractable model organism- the fruit fly, *Drosophila melanogaster*.

The fly model organism

The fruit fly, *Drosophila melanogaster*, has been a historically important model organism for studying ageing and sexual selection. Some of the earliest and most important ideas in sexual selection (e.g. by Bateman, 1948; reviewed in Zuk et al, 2014) and ageing (e.g. Stearns, 1976, 1977; Piper and Partridge, 2018; Rose and Charlesworth, 1981; reviewed in Clancy et al, 2023), were developed using flies. The diversity of genetic tools available in the fly system (Hales et al, 2015), a deep understanding of fly development due to the simplicity yet generality of its biology, and the ease of maintaining flies, have made them suitable for ageing and sexual selection research. Specific to my thesis, flies have been an ideal and indispensable model organism for several additional reasons. First, they have short generation times, facilitating investigation of inter-generational effects of age, and assaying ages across the entire lifespan of individuals. Second, male flies have a pre- and post- copulatory sexually-selected phenotype, manifested in traits related to mate choice and courtship behaviour (Byrne and Rice, 2006), and ejaculate traits (Bjork and Pitnick, 2006), making flies an ideal organism to investigate sexual selection. Third, fruit fly reproductive biology has been studied extensively, for instance, the structure and function of many seminal fluid proteins are well quantified (Chapman and Davies, 2004; Ravi Ram and Wolfner, 2007) and we have an advanced understanding of fly spermatogenesis (Demarco et al, 2014). Fourth, transgenic lines developed in fruit flies, such as males with disrupted testes or with fluorescent sperm, have been crucial for addressing many of the aims of my thesis. Fifth, fruit flies have been crucial in shaping human biomedical research (Wangler et al, 2015), especially human ageing and reproduction research. This is due to similarities between the two species in many developmental pathways linked to senescence (Piper and Partridge, 2018), and similarities in human and fly reproductive biology (Wilson et al, 2017). Such biological similarities can potentially lead to translatable applications of my research.

Importance to humans

In addition to improving our fundamental understanding of evolutionary processes such as sexual selection (Bonduriansky et al, 2008), research on male reproductive senescence has crucial implications for human health and society. Modern society is faced with many recent demographic challenges: a growing population size (Lutz et al, 2002); increased life expectancy (Aburto et al, 2020); declining fertility (Aitken, 2022); later age at first reproduction (Bongaarts et al, 2017); decreasing sperm counts (Levine et al, 2023); high prevalence of male reproductive senescence (Johnson et al, 2015); and late-life acting diseases and disorders (Ferrucci et al, 2020). In humans, many of these demographic challenges have been linked to ageing males. For instance, several health and cognitive disorders in offspring are linked to advancing paternal age at conception (Chan and Robaire, 2022; Kaltsas, et al, 2023; Taylor et al, 2019). Similarly, reducing fertility in couples is primarily associated with ejaculate senescence (Johnson et al, 2015; Sharma et al, 2015) or long durations of sexual rest in men (Barbagallo et al, 2023; Sokol et al, 2021). Therefore, the study of male reproductive senescence is timely and urgent.

Aims

Across five data chapters, I address five broad overarching goals. These thesis goals focus on broad patterns that emerge from across my chapters, and are in addition to chapter-specific aims (described within each chapter). My first goal, using comparative approaches, is to compile and review the evidence for male reproductive senescence at the level of the ejaculate, and understand how ejaculates might mediate the effects of advancing age on male fertility across animals (goal 1a). Then, using inferences from these comparative approaches, I employ experiments to investigate the differential and combined effects of ejaculate components (sperm and seminal fluid) in mediating senescence in male fertility (goal 1b).

Second, I test the predictions of some hypotheses in life-history theory pertaining to the causes of senescence. Here, I specifically focus on the mutation accumulation, disposable soma, and life-history trade-offs hypotheses (goal 2). Third, I aim to understand the influence of within- and between- individual processes on reproductive senescence. Here, I focus on selective disappearance, viability selection, and terminal investment (goal 3). Fourth, I test the inter-generational effects (thus, long-term consequences) of advancing paternal age on offspring (goal 4). Fifth, I compare how advancing age differentially impacts various components of male reproduction (fertility versus offspring quality, mating success versus reproductive success, sperm versus seminal fluid) and the relative contribution of each component in driving overall male reproductive senescence (goal 5). A synopsis of my thesis data chapters (chapters 2 to 6) is as follows:

Chapter 2: Here, I conduct a meta-analysis across non-human animals, to first collate the evidence for senescence at the level of ejaculates (specifically sperm traits) in males (goal 1a). I then investigate the influence of various biological and methodological moderators in explaining heterogeneity in the rates of senescence.

Chapter 3: In this chapter, I conduct a systematic review across all animals to understand how advancing male age affects the non-sperm component of ejaculates- seminal fluid (goal 1a). Here, I specifically study age-related changes in two characteristics of seminal fluid- seminal fluid proteins and oxidative stress.

Chapter 4: Using inferences made from Chapters 2 and 3, I use experiments in the fruit fly, to investigate the relative contribution of sperm versus seminal fluid senescence, in mediating senescence in male fertility (goal 1b). I further mate-multiply males to infer whether male age

affects reproductive success and mating success differentially. Chapters 2, 3 and 4 collectively allow me to compare the rates of senescence in seminal fluid (produced by somatic tissue) versus sperm (produced by the germline) within the framework of predictions from the disposable soma theory for senescence (goal 2), and test the relative importance of each in driving male reproductive senescence (goal 5).

Chapter 5: I use experiments on fruit flies to test predictions from the mutation accumulation and life-history trade-offs hypotheses (goal 2), to understand the inter-generational effects of paternal age on offspring phenotypes (goal 4). Furthermore, here I separate the confounding effects of paternal sperm storage duration/sexual rest, to interpret the direct influence of paternal age on offspring fitness.

Chapter 6: I use fruit flies to investigate the effects of paternal age on paternal reproduction and the survival of sons (goal 4). Here, I employ a demographic framework to test how within-individual processes (terminal investment) and between individual processes (selective disappearance and viability selection), might influence the effects of male age on fitness (goal 3). Chapters 4, 5, and 6 collectively allow me to infer the relative importance of senescence in mating success versus reproductive success, and senescence in intra- versus inter-generational fitness components, in causing male reproductive senescence (goal 5).

References

Aburto, J.M., Villavicencio, F., Basellini, U., Kjærgaard, S., Vaupel, J.W., 2020. Dynamics of life expectancy and life span equality. Proc. Natl. Acad. Sci. U.S.A. 117, 5250–5259. https://doi.org/10.1073/pnas.1915884117

Aich, U., Bonnet, T., Head, M.L., Jennions, M.D., 2021. Disentangling the effects of male age and mating history: Contrasting effects of mating history on precopulatory mating behavior and paternity success. Evolution 75, 2867–2880. https://doi.org/10.1111/evo.14371

Aich, U., Chowdhury, S., Jennions, M.D., 2022. Separating the effects of paternal age and mating history: Evidence for sex-specific paternal effect in eastern mosquitofish. Evolution 76, 1565–1577. https://doi.org/10.1111/evo.14498

Aich, U., Jennions, M.D., Fox, R.J., 2020. An experimental test of the role of male mating history on paternal effects in the livebearer fish *Gambusia holbrooki*. Biol. Lett. 16, 20190945. https://doi.org/10.1098/rsbl.2019.0945

Aitken, R.J., 2022. The changing tide of human fertility. Human Reproduction 37, 629–638. https://doi.org/10.1093/humrep/deac011

Anderson, D.J., Apanius, V., 2003. Actuarial and reproductive senescence in a long-lived seabird: preliminary evidence. Experimental Gerontology 38, 757–760. https://doi.org/10.1016/S0531-5565(03)00104-9

Archer, R., Paniw, M., Vega-Trejo, R., Sepil, I., 2022. A sex skew in life-history research: the problem of missing males Proc. R. Soc. Proceedings of the Royal Society B: Biological Sciences 289. https://doi.org/10.1098/rspb.2022.1117

Aubier, T.G., Galipaud, M., 2024. Senescence evolution under the catastrophic accumulation of deleterious mutations. Evolution Letters 8, 212–221. https://doi.org/10.1093/evlett/qrad050

Barbagallo, F., Cannarella, R., Crafa, A., La Vignera, S., Condorelli, R.A., Manna, C., Calogero, A.E., 2023. The Impact of a Very Short Abstinence Period on Assisted Reproductive Technique Outcomes: A Systematic Review and Meta-Analysis. Antioxidants 12, 752. https://doi.org/10.3390/antiox12030752

Bateman, A.J., 1948. Intra-sexual selection in Drosophila. Heredity 2, 349–368. https://doi.org/10.1038/hdy.1948.21

Baudisch, A., Stott, I., 2019. A pace and shape perspective on fertility. Methods Ecol Evol 10, 1941–1951. https://doi.org/10.1111/2041-210X.13289

Beamonte-Barrientos, R., Velando, A., Drummond, H., Torres, R., 2010. Senescence of Maternal Effects: Aging Influences Egg Quality and Rearing Capacities of a Long-Lived Bird. The American Naturalist 175, 469–480. https://doi.org/10.1086/650726

Bjork, A., Pitnick, S., 2006. Intensity of sexual selection along the anisogamy–isogamy continuum. Nature 441, 742–745. https://doi.org/10.1038/nature04683

Blas, J., Sergio, F., Hiraldo, F., 2009. Age-related improvement in reproductive performance in a long-lived raptor: a cross-sectional and longitudinal study. Ecography 32, 647–657. https://doi.org/10.1111/j.1600-0587.2008.05700.x

Bonduriansky, R., Maklakov, A., Zajitschek, F., Brooks, R., 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. Functional Ecology 22, 443–453. https://doi.org/10.1111/j.1365-2435.2008.01417.x

Bongaarts, J., Mensch, B.S., Blanc, A.K., 2017. Trends in the age at reproductive transitions in the developing world: The role of education. Population Studies 71, 139–154. https://doi.org/10.1080/00324728.2017.1291986

Bouwhuis, S., Sheldon, B.C., Verhulst, S., Charmantier, A., 2009. Great tits growing old: selective disappearance and the partitioning of senescence to stages within the breeding cycle. Proc. R. Soc. B. 276, 2769–2777. https://doi.org/10.1098/rspb.2009.0457

Bronikowski, A.M., Meisel, R.P., Biga, P.R., Walters, J.R., Mank, J.E., Larschan, E., Wilkinson, G.S., Valenzuela, N., Conard, A.M., De Magalhães, J.P., Duan, J. (Ellie), Elias, A.E., Gamble, T., Graze, R.M., Gribble, K.E., Kreiling, J.A., Riddle, N.C., 2022. Sex-specific aging in animals: Perspective and future directions. Aging Cell 21, e13542. https://doi.org/10.1111/acel.13542

Byrne, P.G., Rice, W.R., 2006. Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. Proc. R. Soc. B. 273, 917–922. https://doi.org/10.1098/rspb.2005.3372

Carnes, B.A., Holden, L.R., Olshansky, S.J., Witten, M.T., Siegel, J.S., 2006. Mortality Partitions and their Relevance to Research on Senescence. Biogerontology 7, 183–198. https://doi.org/10.1007/s10522-006-9020-3

Catry, P., Granadeiro, J.P., Ramos, J., Phillips, R.A., Oliveira, P., 2011. Either taking it easy or feeling too tired: old Cory's Shearwaters display reduced activity levels while at sea. J Ornithol 152, 549–555. https://doi.org/10.1007/s10336-010-0616-7

Cayuela, H., Lemaître, J., Léna, J., Ronget, V., Martínez-Solano, I., Muths, E., Pilliod, D.S., Schmidt, B.R., Sánchez-Montes, G., Gutiérrez-Rodríguez, J., Pyke, G., Grossenbacher, K., Lenzi, O., Bosch, J., Beard, K.H., Woolbright, L.L., Lambert, B.A., Green, D.M., Jreidini, N., Garwood, J.M., Fisher, R.N., Matthews, K., Dudgeon, D., Lau, A., Speybroeck, J., Homan, R., Jehle, R., Başkale, E., Mori, E., Arntzen, J.W., Joly, P., Stiles, R.M., Lannoo, M.J., Maerz, J.C., Lowe, W.H., Valenzuela-Sánchez, A., Christiansen, D.G., Angelini, C., Thirion, J., Merilä, J., Colli, G.R., Vasconcellos, M.M., Boas, T.C.V., Arantes, Í.D.C., Levionnois, P., Reinke, B.A., Vieira, C., Marais, G.A.B., Gaillard, J., Miller, D.A.W., 2022. Sex-related differences in aging rate are associated with sex chromosome system in amphibians. Evolution 76, 346–356. https://doi.org/10.1111/evo.14410

Chan, P.T.K., Robaire, B., 2022. Advanced Paternal Age and Future Generations. Front. Endocrinol. 13, 897101. https://doi.org/10.3389/fendo.2022.897101

Chapman, T., Davies, S.J., 2004. Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies. Peptides 25, 1477–1490. https://doi.org/10.1016/j.peptides.2003.10.023

Charnov, E.L., Turner, T.F., Winemiller, K.O., 2001. Reproductive constraints and the evolution of life histories with indeterminate growth. Proc. Natl. Acad. Sci. U.S.A. 98, 9460–9464. https://doi.org/10.1073/pnas.161294498

Clancy, D., Chtarbanova, S., Broughton, S., 2023. Editorial: Model organisms in aging research: Drosophila melanogaster. Front. Aging 3, 1118299. https://doi.org/10.3389/fragi.2022.1118299

Croft, D.P., Brent, L.J.N., Franks, D.W., Cant, M.A., 2015. The evolution of prolonged life after reproduction. Trends in Ecology & Evolution 30, 407–416. https://doi.org/10.1016/j.tree.2015.04.011
Dean, R., Cornwallis, C.K., Løvlie, H., Worley, K., Richardson, D.S., Pizzari, T., 2010. Male Reproductive Senescence Causes Potential for Sexual Conflict over Mating. Current Biology 20, 1192–1196. https://doi.org/10.1016/j.cub.2010.04.059
Demarco, R.S., Eikenes, Å.H., Haglund, K., Jones, D.L., 2014. Investigating spermatogenesis in Drosophila melanogaster. Methods 68, 218–227. https://doi.org/10.1016/j.ymeth.2014.04.020
Depeux, C., Lemaître, J., Moreau, J., Dechaume-Moncharmont, F., Laverre, T., Pauhlac, H., Gaillard, J., Beltran-Bech, S., 2020. Reproductive senescence and parental effects in an indeterminate grower. J of Evolutionary Biology 33, 1256–1264. https://doi.org/10.1111/jeb.13667
Duffield, K.R., Bowers, E.K., Sakaluk, S.K., Sadd, B.M., 2017. A dynamic threshold model for terminal investment. Behav Ecol Sociobiol 71, 185. https://doi.org/10.1007/s00265-017-2416-z
Engelman, M., Seplaki, C.L., Varadhan, R., 2017. A Quiescent Phase in Human Mortality? Exploring the Ages of Least Vulnerability. Demography 54, 1097–1118. https://doi.org/10.1007/s13524-017-0569-z
Ferrucci, L., Gonzalez-Freire, M., Fabbri, E., Simonsick, E., Tanaka, T., Moore, Z., Salimi, S., Sierra, F., De Cabo, R., 2020. Measuring biological aging in humans: A quest. Aging Cell 19, e13080. https://doi.org/10.1111/acel.13080
Flatt, T., Partridge, L., 2018. Horizons in the evolution of aging. BMC Biol 16, 93. https://doi.org/10.1186/s12915-018-0562-z
Flatt, T., Promislow, D.E.L., 2007. Still Pondering an Age-Old Question. Science 318, 1255–1256. https://doi.org/10.1126/science.1147491
Foo, Y.Z., Lagisz, M., O'Dea, R.E., Nakagawa, S., 2023. The influence of immune challenges on the mean and variance in reproductive investment: a meta-analysis of the terminal investment hypothesis. BMC Biol 21, 107. https://doi.org/10.1186/s12915-023-01603-4
Fricke, C., Koppik, M., 2019. Male reproductive ageing: a tale of the whole ejaculate. Reproduction 158, R219–R229. https://doi.org/10.1530/REP-18-0579
Gaillard, J., Lemaître, J., 2020. An integrative view of senescence in nature. Functional Ecology 34, 4–16. https://doi.org/10.1111/1365-2435.13506
Gaillard, J.-M., Lemaître, J.-F., 2017. The Williams' legacy: A critical reappraisal of his nine predictions about the evolution of senescence: THE WILLIAMS' LEGACY. Evolution 71, 2768–2785. https://doi.org/10.1111/evo.13379
Gasparini, C., Marino, I.A.M., Boschetto, C., Pilastro, A., 2010. Effect of male age on sperm traits and sperm competition success in the guppy (*Poecilia reticulata*). Journal of Evolutionary Biology 23, 124–135. https://doi.org/10.1111/j.1420-9101.2009.01889.x
Hales, K.G., Korey, C.A., Larracuente, A.M., Roberts, D.M., 2015. Genetics on the Fly: A Primer on the *Drosophila* Model System. Genetics 201, 815–842. https://doi.org/10.1534/genetics.115.183392
Heinen, V.K., Pitera, A.M., Sonnenberg, B.R., Benedict, L.M., Branch, C.L., Bridge, E.S., Pravosudov, V.V., 2021. Specialized spatial cognition is associated with reduced cognitive senescence in a food-caching bird. Proc. R. Soc. B. 288, rspb.2020.3180, 20203180. https://doi.org/10.1098/rspb.2020.3180
Hernandez-Segura, A., Nehme, J., Demaria, M., 2018. Hallmarks of Cellular Senescence. Trends in Cell Biology 28, 436–453. https://doi.org/10.1016/j.tcb.2018.02.001
Hughes, K.A., Reynolds, R.M., 2005. EVOLUTIONARY AND MECHANISTIC THEORIES OF AGING. Annu. Rev. Entomol. 50, 421–445. https://doi.org/10.1146/annurev.ento.50.071803.130409
Hunt, J., Brooks, R., Jennions, M.D., Smith, M.J., Bentsen, C.L., Bussière, L.F., 2004. High-quality male field crickets invest heavily in sexual display but die young. Nature 432, 1024–1027. https://doi.org/10.1038/nature03084
Ivimey-Cook, E., Moorad, J., 2020. The diversity of maternal-age effects upon pre-adult survival across animal species. Proc. R. Soc. B. 287, 20200972. https://doi.org/10.1098/rspb.2020.0972
Johnson, A.A., Shokhirev, M.N., Shoshitaishvili, B., 2019. Revamping the evolutionary theories of aging. Ageing Research Reviews 55, 100947. https://doi.org/10.1016/j.arr.2019.100947
Johnson, S.L., Dunleavy, J., Gemmell, N.J., Nakagawa, S., 2015. Consistent age-dependent declines in human semen quality: A systematic review and meta-analysis. Ageing Research Reviews 19, 22–33. https://doi.org/10.1016/j.arr.2014.10.007
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 off-set costs related to fertility, or are we missing something else? BioEssays 34, 609–619. https://doi.org/10.1002/bies.201100157
Jones, O.R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C.G., Schaible, R., Casper, B.B., Dahlgren, J.P., Ehrlén, J., García, M.B., Menges, E.S., Quintana-Ascencio, P.F., Caswell, H., Baudisch, A., Vaupel, J.W., 2014. Diversity of ageing across the tree of life. Nature 505, 169–173. https://doi.org/10.1038/nature12789
Jones, O.R., Vaupel, J.W., 2017. Senescence is not inevitable. Biogerontology 18, 965–971. https://doi.org/10.1007/s10522-017-9727-3
Jones, T.M., Elgar, M.A., 2004. The role of male age, sperm age and mating history on fecundity and fertilization success in the hide beetle. Proc. R. Soc. Lond. B 271, 1311–1318. https://doi.org/10.1098/rspb.2004.2723
Kaltsas, A., Moustakli, E., Zikopoulos, A., Georgiou, I., Dimitriadis, F., Symeonidis, E.N., Markou, E., Michaelidis, T.M., Tien, D.M.B., Giannakis, I., Ioannidou, E.M., Papatsoris, A., Tsounapi, P., Takenaka, A., Sofikitis, N., Zachariou, A., 2023. Impact of Advanced Paternal Age on Fertility and Risks of Genetic Disorders in Offspring. Genes 14, 486. https://doi.org/10.3390/genes14020486
Karl, I., Fischer, K., 2013. Old male mating advantage results from sexual conflict in a butterfly. Animal Behaviour 85, 143–149. https://doi.org/10.1016/j.anbehav.2012.10.018
Kirkwood, T.B.L., Holliday, R., 1979. The evolution of ageing and longevity. Proc. R. Soc. Lond. B. 205, 531–546. https://doi.org/10.1098/rspb.1979.0083
Kokko, H., 1998. Good genes, old age and life-history trade-offs. Evolutionary Ecology 12, 739–750. https://doi.org/10.1023/A:1006541701002

Lee, R., Chu, C.Y.C., 2023. Theoretical perspectives on reproductive aging. Front. Ecol. Evol. 10, 934732. https://doi.org/10.3389/fevo.2022.934732
Lemaître, J., Gaillard, J., 2017. Reproductive senescence: new perspectives in the wild. Biological Reviews 92, 2182–2199. https://doi.org/10.1111/brv.12328
Lemaître, J.-F., Berger, V., Bonenfant, C., Douhard, M., Gamelon, M., Plard, F., Gaillard, J.-M., 2015. Early-late life trade-offs and the evolution of ageing in the wild. Proc. R. Soc. B. 282, 20150209. https://doi.org/10.1098/rspb.2015.0209
Lemaître, J.-F., Moorad, J., Gaillard, J.-M., Maklakov, A.A., Nussey, D.H., 2024. A unified framework for evolutionary genetic and physiological theories of aging. PLoS Biol 22, e3002513. https://doi.org/10.1371/journal.pbio.3002513
Lemaître, J.-F., Ronget, V., Gaillard, J.-M., 2020. Female reproductive senescence across mammals: A high diversity of patterns modulated by life history and mating traits. Mechanisms of Ageing and Development 192, 111377. https://doi.org/10.1016/j.mad.2020.111377
Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Jolles, M., Pinotti, R., Swan, S.H., 2023. Temporal trends in sperm count: a systematic review and meta-regression analysis of samples collected globally in the 20th and 21st centuries. Human Reproduction Update 29, 157–176. https://doi.org/10.1093/humupd/dmac035
Lifjeld, J.T., Kleven, O., Fossøy, F., Jacobsen, F., Laskemoen, T., Rudolfsen, G., Robertson, R.J., 2022. When Older Males Sire More Offspring—Increased Attractiveness or Higher Fertility? Behav Ecol Sociobiol 76, 61. https://doi.org/10.1007/s00265-022-03170-0
Lutz, W., Qiang, R., 2002. Determinants of human population growth. Phil. Trans. R. Soc. Lond. B 357, 1197–1210. https://doi.org/10.1098/rstb.2002.1121
Maklakov, A.A., Chapman, T., 2019. Evolution of ageing as a tangle of trade-offs: energy versus function. Proc. R. Soc. B. 286, 20191604. https://doi.org/10.1098/rspb.2019.1604
Maklakov, A.A., Lummaa, V., 2013. Evolution of sex differences in lifespan and aging: Causes and constraints. BioEssays 35, 717–724. https://doi.org/10.1002/bies.201300021
Maklakov, A.A., Rowe, L., Friberg, U., 2015. Why organisms age: Evolution of senescence under positive pleiotropy? BioEssays 37, 802–807. https://doi.org/10.1002/bies.201500025
McCleery, R.H., Perrins, C.M., Sheldon, B.C., Charmantier, A., 2008. Age-specific reproduction in a long-lived species: the combined effects of senescence and individual quality. Proc. R. Soc. B. 275, 963–970. https://doi.org/10.1098/rspb.2007.1418
Monaghan, P., Charmantier, A., Nussey, D.H., Ricklefs, R.E., 2008. The evolutionary ecology of senescence. Functional Ecology 22, 371–378. https://doi.org/10.1111/j.1365-2435.2008.01418.x
Monaghan, P., Metcalfe, N.B., 2019. The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. Proc. R. Soc. B. 286, 20192187. https://doi.org/10.1098/rspb.2019.2187
Moorad, J.A., Nussey, D.H., 2016. Evolution of maternal effect senescence. Proc. Natl. Acad. Sci. U.S.A. 113, 362–367. https://doi.org/10.1073/pnas.1520494113
Munné-Bosch, S., 2015. Senescence: Is It Universal or Not? Trends in Plant Science 20, 713–720. https://doi.org/10.1016/j.tplants.2015.07.009
Naciri, M., Aars, J., Blanchet, M.-A., Gimenez, O., Cubaynes, S., 2022. Reproductive senescence in polar bears in a variable environment. Front. Ecol. Evol. 10, 920481. https://doi.org/10.3389/fevo.2022.920481
Nussey, D.H., Coulson, T., Festa-Bianchet, M., Gaillard, J. -M., 2008. Measuring senescence in wild animal populations: towards a longitudinal approach. Functional Ecology 22, 393–406. https://doi.org/10.1111/j.1365-2435.2008.01408.x
Nussey, D.H., Froy, H., Lemaitre, J.-F., Gaillard, J.-M., Austad, S.N., 2013. Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. Ageing Research Reviews 12, 214–225. https://doi.org/10.1016/j.arr.2012.07.004
Nussey, D.H., Kruuk, L.E.B., Donald, A., Fowlie, M., Clutton-Brock, T.H., 2006. The rate of senescence in maternal performance increases with early-life fecundity in red deer. Ecology Letters 9, 1342–1350. https://doi.org/10.1111/j.1461-0248.2006.00989.x
Partridge, L., Barton, N.H., 1993. Optimally, mutation and the evolution of ageing. Nature 362, 305–311. https://doi.org/10.1038/362305a0
Pérez-Staples, D., Martínez-Hernández, M.G., Aluja, M., 2010. Male Age and Experience Increases Mating Success but Not Female Fitness in the Mexican Fruit Fly. Ethology 116, 778–786. https://doi.org/10.1111/j.1439-0310.2010.01790.x
Péron, G., Lemaître, J.-F., Ronget, V., Tidière, M., Gaillard, J.-M., 2019. Variation in actuarial senescence does not reflect life span variation across mammals. PLoS Biol 17, e3000432. https://doi.org/10.1371/journal.pbio.3000432
Piper, M.D.W., Partridge, L., 2018. Drosophila as a model for ageing. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1864, 2707–2717. https://doi.org/10.1016/j.bbadis.2017.09.016
Pizzari, T., Dean, R., Pacey, A., Moore, H., Bonsall, M.B., 2008. The evolutionary ecology of pre- and post-meiotic sperm senescence. Trends in Ecology & Evolution 23, 131–140. https://doi.org/10.1016/j.tree.2007.12.003
Preshaw, P.M., Henne, K., Taylor, J.J., Valentine, R.A., Conrads, G., 2017. Age-related changes in immune function (immune senescence) in caries and periodontal diseases: a systematic review. J Clinic Periodontology 44. https://doi.org/10.1111/jcpe.12675
Purchase, C.F., Rooke, A.C., Gaudry, M.J., Treberg, J.R., Mittell, E.A., Morrissey, M.B., Rennie, M.D., 2022. A synthesis of senescence predictions for indeterminate growth, and support from multiple tests in wild lake trout. Proc. R. Soc. B. 289, 20212146. https://doi.org/10.1098/rspb.2021.2146
Ravi Ram, K., Wolfner, M.F., 2007. Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. Integrative and Comparative Biology 47, 427–445. https://doi.org/10.1093/icb/icm046
Reichard, M., 2016. Evolutionary ecology of aging: time to reconcile field and laboratory research. Ecology and Evolution 6, 2988–3000. https://doi.org/10.1002/ece3.2093

Richard, M., Lecomte, J., De Fraipont, M., Clobert, J., 2005. Age-specific mating strategies and reproductive senescence. Molecular Ecology 14, 3147–3155. https://doi.org/10.1111/j.1365-294X.2005.02662.x
Riecke, T.V., Hegelbach, J., Schaub, M., 2023. Reproductive senescence and mating tactic interact and conflict to drive reproductive success in a passerine. Journal of Animal Ecology 92, 838–849. https://doi.org/10.1111/1365-2656.13893
Rodríguez, J.A., Marigorta, U.M., Hughes, D.A., Spataro, N., Bosch, E., Navarro, A., 2017. Antagonistic pleiotropy and mutation accumulation influence human senescence and disease. Nat Ecol Evol 1, 0055. https://doi.org/10.1038/s41559-016-0055
Roper, M., Capdevila, P., Salguero-Gómez, R., 2021. Senescence: why and where selection gradients might not decline with age. Proc. R. Soc. B. 288, 20210851. https://doi.org/10.1098/rspb.2021.0851
Rose, M., Charlesworth, B., 2002. A Test of Evolutionary Theories of Senescence. Sci. Aging Knowl. Environ. 2002. https://doi.org/10.1126/sageke.2002.37.cp17
Rose, M.R., Charlesworth, B., 1981. GENETICS OF LIFE HISTORY IN *DROSOPHILA MELANOGASTER* . II. EXPLORATORY SELECTION EXPERIMENTS. Genetics 97, 187–196. https://doi.org/10.1093/genetics/97.1.187
Rutkowski, N.-A.J., Foo, Y.Z., Jones, T.M., McNamara, K.B., 2023. Age, but not an immune challenge, triggers terminal investment in the Pacific field cricket, *Teleogryllus oceanicus*. Behavioral Ecology 34, 468–479. https://doi.org/10.1093/beheco/arad021
Secomandi, L., Borghesan, M., Velarde, M., Demaria, M., 2022. The role of cellular senescence in female reproductive aging and the potential for senotherapeutic interventions. Human Reproduction Update 28, 172–189. https://doi.org/10.1093/humupd/dmab038
Shanley, D.P., Sear, R., Mace, R., Kirkwood, T.B.L., 2007. Testing evolutionary theories of menopause. Proc. R. Soc. B. 274, 2943–2949. https://doi.org/10.1098/rspb.2007.1028
Sharma, R., Agarwal, A., Rohra, V.K., Assidi, M., Abu-Elmagd, M., Turki, R.F., 2015. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13, 35. https://doi.org/10.1186/s12958-015-0028-x
Siracusa, E.R., Higham, J.P., Snyder-Mackler, N., Brent, L.J.N., 2022. Social ageing: exploring the drivers of late-life changes in social behaviour in mammals. Biol. Lett. 18, 20210643. https://doi.org/10.1098/rsbl.2021.0643
Sokol, P., Drakopoulos, P., Polyzos, N.P., 2021. The Effect of Ejaculatory Abstinence Interval on Sperm Parameters and Clinical Outcome of ART. A Systematic Review of the Literature. JCM 10, 3213. https://doi.org/10.3390/jcm10153213
Stearns, S.C., 1977. The Evolution of Life History Traits: A Critique of the Theory and a Review of the Data. Annu. Rev. Ecol. Syst. 8, 145–171. https://doi.org/10.1146/annurev.es.08.110177.001045
Stearns, S.C., 1976. Life-History Tactics: A Review of the Ideas. The Quarterly Review of Biology 51, 3–47. https://doi.org/10.1086/409052
Tan, C.K.W., Pizzari, T., Wigby, S., 2013. PARENTAL AGE, GAMETIC AGE, AND INBREEDING INTERACT TO MODULATE OFFSPRING VIABILITY IN *DROSOPHILA MELANOGASTER*: BRIEF COMMUNICATION. Evolution n/a-n/a. https://doi.org/10.1111/evo.12131
Tarin, J.J., 2000. Consequences on offspring of abnormal function in ageing gametes. Human Reproduction Update 6, 532–549. https://doi.org/10.1093/humupd/6.6.532
Taylor, J.L., Debost, J.-C.P.G., Morton, S.U., Wigdor, E.M., Heyne, H.O., Lal, D., Howrigan, D.P., Bloemendal, A., Larsen, J.T., Kosmicki, J.A., Weiner, D.J., Homsy, J., Seidman, J.G., Seidman, C.E., Agerbo, E., McGrath, J.J., Mortensen, P.B., Petersen, L., Daly, M.J., Robinson, E.B., 2019. Paternal-age-related de novo mutations and risk for five disorders. Nat Commun 10, 3043. https://doi.org/10.1038/s41467-019-11039-6
Turan, Z.G., Parvizi, P., Dönertaş, H.M., Tung, J., Khaitovich, P., Somel, M., 2019. Molecular footprint of Medawar's mutation accumulation process in mammalian aging. Aging Cell 18, e12965. https://doi.org/10.1111/acel.12965
Vágási, C.I., Vincze, O., Lemaître, J.-F., Pap, P.L., Ronget, V., Gaillard, J.-M., 2021. Is degree of sociality associated with reproductive senescence? A comparative analysis across birds and mammals. Phil. Trans. R. Soc. B 376, 20190744. https://doi.org/10.1098/rstb.2019.0744
Van De Pol, M., Verhulst, S., 2006. Age-Dependent Traits: A New Statistical Model to Separate Within- and Between-Individual Effects. The American Naturalist 167, 766–773. https://doi.org/10.1086/503331
Vega-Trejo, R., Fox, R.J., Iglesias-Carrasco, M., Head, M.L., Jennions, M.D., 2019. The effects of male age, sperm age and mating history on ejaculate senescence. Functional Ecology 33, 1267–1279. https://doi.org/10.1111/1365-2435.13305
Velando, A., Drummond, H., Torres, R., 2006. Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. Proc. R. Soc. B. 273, 1443–1448. https://doi.org/10.1098/rspb.2006.3480
Vrtílek, M., Žák, J., Reichard, M., 2022. Evidence for reproductive senescence across ray-finned fishes: A review. Front. Ecol. Evol. 10, 982915. https://doi.org/10.3389/fevo.2022.982915
Wangler, M.F., Yamamoto, S., Bellen, H.J., 2015. Fruit Flies in Biomedical Research. Genetics 199, 639–653. https://doi.org/10.1534/genetics.114.171785
Williams, P.D., Day, T., 2003. ANTAGONISTIC PLEIOTROPY, MORTALITY SOURCE INTERACTIONS, AND THE EVOLUTIONARY THEORY OF SENESCENCE. Evolution 57, 1478–1488. https://doi.org/10.1111/j.0014-3820.2003.tb00356.x
Wilson, C., Leiblich, A., Goberdhan, D.C.I., Hamdy, F., 2017. The Drosophila Accessory Gland as a Model for Prostate Cancer and Other Pathologies, in: Current Topics in Developmental Biology. Elsevier, pp. 339–375. https://doi.org/10.1016/bs.ctdb.2016.06.001
Zuk, M., Garcia-Gonzalez, F., Herberstein, M.E., Simmons, L.W., 2014. Model Systems, Taxonomic Bias, and Sexual Selection: Beyond *Drosophila*. Annu. Rev. Entomol. 59, 321–338. https://doi.org/10.1146/annurev-ento-011613-162014

Chapter 2

Meta-analysis shows no consistent evidence for senescence in ejaculate traits across animals*

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Abstract

Male reproductive traits such as ejaculate size and quality, are expected to decline with advancing age due to senescence. It is however unclear whether this expectation is upheld across taxa. We perform a meta-analysis on 379 studies, to quantify the effects of advancing male age on ejaculate traits across 157 species of non-human animals. Contrary to predictions, we find no consistent pattern of age-dependent changes in ejaculate traits. This result partly reflects methodological limitations, such as studies sampling a low proportion of adult lifespan, or the inability of meta-analytical approaches to document non-linear ageing trajectories of ejaculate traits; which could potentially lead to an underestimation of senescence. Yet, we find taxon-specific differences in patterns of ejaculate senescence. For instance, older males produce less motile and slower sperm in ray-finned fishes, but larger ejaculates in insects, compared to younger males. Notably, lab rodents show senescence in most ejaculate traits measured. Our study challenges the notion of universal reproductive senescence, highlighting the need for controlled methodologies and a more nuanced understanding of reproductive senescence, cognisant of taxon-specific biology, experimental design, selection pressures, and life-history.

Keywords: ageing, life-history, reproduction, sexual conflict, sexual selection, sperm

Introduction

Senescence is the age-dependent irreversible deterioration of organismal function that leads to an increased risk of intrinsic mortality[1] and decline in reproductive output[2] with advancing age. While senescence has been reported in some taxa[3], it is unclear whether senescence is a general outcome of ageing[4-8]. Senescence is driven by a variety of proximate mechanisms, from excessive biosynthesis in late-life (hyperfunction theory[9]) and age-dependent deterioration of cellular repair[10], to the accumulation of mutations[11], oxidative damage[12], and telomere attrition[13]. From an evolutionary perspective, senescence is commonly hypothesized to be the result of relaxed selection against deleterious mutations in older organisms, as first proposed by Medawar's 'mutation accumulation theory'[14]. Other evolutionary explanations for senescence include selection for alleles, which increase performance early in life, but convey net costs later in life ('antagonistic pleiotropy'[15]), and trade-offs between investment in survival versus reproduction ('disposable soma'[16]). In contrast, some animals show an absence of reproductive senescence[4]. Negligible senescence is predicted in animals with indeterminate growth, like some fish[17,18], where individuals continue to grow post-maturity, thus improving their ability to reproduce throughout their lives due to age-dependent increases in gonad size[8,17]. The ability of some animals to maintain cellular repair and sustain homeostasis in reproductive tissues throughout life, might also lead to negligible senescence[19].

Reproductive senescence (i.e. the age dependent decline in reproductive success) has been relatively well documented in females[20,21]. Yet, patterns, causes, and consequences of male reproductive senescence are less understood[22]. Understanding male reproductive senescence is crucial for several reasons. Males typically face intense intra-sexual competition. Thus, age-dependent changes in male ejaculate traits can drive variation in male reproductive success[23,24], affecting sperm competition, cryptic female choice[25], and generate

potential for sexual conflict[26-28]. Additionally, sperm are potentially more vulnerable to organismal ageing than eggs[10,29], because male germlines have higher rates of cell divisions and mutation accumulation[30,31], but poorer DNA repair machinery[12,32], than female germlines. Such deterioration in the male germline can severely impact offspring phenotypes via paternal age effects, thus having important consequences for organismal health (reviewed in [33]).

Current evidence for senescence in male ejaculate traits is inconclusive. Several studies show that older males have lower ejaculate quantities[34] and poorer sperm quality[35-37] than younger males. However, other studies have reported improvements[38-41], or no significant changes in ejaculate traits with advancing male age[42-45]. The heterogeneity in these reported effects might be caused by various biological and methodological factors that modulate the effects of advancing male age on ejaculate traits[35, 46] (Table 1 and 2). A meta-analytical approach is thus crucial to understand the influence of these 'moderators' (Table 1 and 2), and to investigate the general effects of advancing male age on ejaculate traits. Yet, no study has done this systematically for non-human animals (see [35] for humans; [18] for a review in fish; [47] for effects of male age on seminal fluid).

Here, we conduct a meta-analysis to address three aims. First, we test whether advancing male age affects ejaculate traits across non-human animals (aim 1). Although reproductive senescence is not a ubiquitous outcome of ageing, it is commonly predicted to occur by classical theories of ageing. We thus predict that senescence in ejaculate traits will be observed commonly across species (see Table 1 and 2 for predictions as to how different ejaculate traits might be affected differently). Second, we investigate the role of biological and methodological moderators (see Table 1 and 2 for possible influence of each) in modulating the effects of male age on ejaculate traits (aim 2). Third, we quantify how advancing male age affects reproductive outcomes, such as male fertilisation success and

fecundity. Here, we also test whether effects of advancing male age on ejaculate traits differ from those on reproductive outcomes (aim 3). We find no consistent evidence for senescence in ejaculate traits overall, however find taxonomic class- and trait-specific patterns. We also find that studies sampling higher proportions of species' lifespans, show stronger evidence for senescence. Overall, we suggest methodological improvements and provide novel hypotheses for studying senescence. The research gaps highlighted by us will be key in aiding our understanding of male reproductive senescence.

Table 1: Possible influence of different biological moderators on male reproductive senescence at the level of ejaculate traits. Moderators marked with an asterisk were included in our meta-analysis because there were sufficient data across studies.

Biological moderators	Possible influence
Taxon-specific effects*	Phylogenetic history and taxa-specific biology (e.g. ecosystems, niches, metabolic rates, mating systems, mode of thermoregulation, degree of parental care) could influence how male age affects ejaculate traits[4,59].
Ejaculate traits*	Evidence for reproductive senescence can depend on the specific trait measured[61]. This can be due to trade-offs between different ejaculate traits[60] or different traits being under varying selection pressures[62].
Degree of sperm competition*	Species with increased levels of sperm competition have evolved increased investment in competitive ejaculate traits such as sperm number and velocity[95], which may reduce the rate of senescence in these traits[42]. However, high levels of sperm competition may also lead males to produce large, high quality ejaculates early in life, but exacerbate senescence in ejaculate traits at an old age[96].
Life-history strategies and mortality risk	Life-history strategies of animals, and pace of life of individuals determine the rate and onset of reproductive senescence[48]. Life-history strategies are affected by mortality risk in populations. For instance, animals may invest more in early-life reproduction when age-dependent mortality risk is high[97], and thus show higher reproductive senescence rates than animals facing lower age-dependent mortality risk[98]. Organisms that evolve in environments with high extrinsic mortality might show faster rates of senescence when old, due to deleterious late-life expressed alleles not being selected against[15].
Seminal fluid changes	Levels of antioxidants in seminal fluid[47] and abundance of seminal fluid proteins can change as males age[66], independent of changes in sperm. These age-dependent

	changes in seminal fluid can affect sperm phenotype over and above the direct effects of male age on sperm[47].
Ontogeny of secondary sexual traits	The ontogeny of secondary sexual traits can influence the evolution of male reproductive senescence rates[27]. For instance, in species where male traits such as weapons or ornaments improve with age, males are hypothesized to evolve lower rates of reproductive senescence, compared to species where these traits do not improve with age[40,51]
Parental care	Species with parental care might have evolved to allocate more energy/resources to caring for offspring and investing in current reproductive opportunities, at the cost of reduced allocation to future reproduction. This could accelerate reproductive senescence in species with parental care[2].

Table 2: Possible influence of different methodological moderators on male reproductive senescence at the level of ejaculate traits. Moderators marked with an asterisk were included in our meta-analysis because there were sufficient data across studies.

Methodological moderators	Possible influence
Proportion lifespan sampled*	A higher proportion of lifespan sampled will increase the probability of detecting reproductive senescence, as the onset of senescence usually occurs late in life[4,18,35,47].
Ejaculate collection method*	If males have control over ejaculation during ejaculate collection (e.g. natural mating or mating with dummy females), males might have the opportunity to strategically adjust ejaculate phenotypes[99]. This could cause age-independent changes in ejaculate traits, reducing the detectability of senescence. Additionally, when males have control over ejaculation, studies might obtain a smaller proportion of the sperm reserves available to a male, which may not be representative of a male's whole-ejaculate phenotype, compared to studies that use invasive methods to obtain ejaculates (e.g. dissection).
Population type*	Reproductive senescence rates can differ between males in captive versus wild populations[68,100]. Additionally, some domesticated animals are often culled prior to reaching ages where senescence can be detected[101]. Other domesticated animals have undergone generations of artificial selection for unusual life-histories (e.g. extremely short generation time in broiler chicken[102]). These factors could lead to patterns of senescence differing between domesticated and wild animals.
Cross-sectional versus longitudinal sampling*	Cross-sectional sampling of males makes reproductive senescence harder to detect, especially if low-quality males selectively disappear[55,56]. Cross-sectional studies might thus underestimate male reproductive senescence, compared to longitudinal sampling of the same males at different ages[103].

Manipulations*	Manipulated environments that are outside of what healthy organisms typically experience, such as environments with stressful conditions, can exacerbate reproductive senescence[104]. Thus, males exposed to manipulations such as thermal stress, poor diet, or toxins could be more likely to show reproductive senescence than males not subjected to these stressors. Other manipulations such as experimental inbreeding[105] or selection for deleterious mutations[106], may exacerbate reproductive senescence.
Mating history	High mating rates can exacerbate male reproductive senescence[23]. In studies where male mating history is not controlled for, old males often have more matings than young males. These studies might thus show stronger evidence for senescence in ejaculate traits. On the other hand, low mating rates (e.g. virgins) might cause old males to accumulate sperm for longer durations, thus produce larger ejaculates, than young males[66].
Post-meiotic sperm storage	Temporal changes in sperm traits can also occur due to post-meiotic storage of mature sperm in males before ejaculation, and in females following mating[54]. The duration of sexual rest in males can influence the amount of post-meiotic damage to sperm, such that for a given age, males with shorter sexual rest (e.g. high mating rate) will incur lower post-meiotic sperm damage[54]. Further, deleterious effects of post-meiotic sperm storage may be exacerbated in old males, if old males are less able to repair post-meiotic cellular damage in sperm[54].

Results

Using a systematic review, we identified 379 studies with relevant data on how advancing male age affects ejaculate traits (Supplementary Fig. 1). From these studies, we obtained 1814 effects sizes across 157 species of non-human animals. We then created a meta-analytical model, using Zr (Fischer's z-transformed correlation coefficient) as our effect size, to understand the overall effects of advancing male age on ejaculate traits. For all our meta-analytical models, we included effect size, cohort, study, species, and phylogenetic relatedness, as random effects. From included studies, we further collected data on various biological and methodological variables (moderators), to test their independent and additive influence on patterns of ageing in ejaculate traits, using meta-regressions. Importantly, for

four over-represented taxonomic classes (Mammalia, Insecta, Aves, Actinopterygii), we further conducted four separate meta-regressions to investigate the extent of senescence in ejaculate traits. Some studies also contained additional data on age-dependent changes in reproductive outcomes (e.g. fertilisation success, reproductive output, offspring traits). For these studies, we compared the effects of advancing male age on ejaculate traits and reproductive outcomes. Furthermore, we conducted several analyses to test for different forms of publication biases. Finally, we also conducted two sensitivity analyses to test whether evidence for senescence was sensitive to the proportion of lifespan of the associated species a study sampled, and the aims of a study.

Aim 1: Effects of advancing male age on ejaculate traits

We found no general effect of advancing male age on ejaculate traits (mean [95% confidence interval (C.I.)]: -0.006 [-0.486 to 0.474], $z = -0.025$, $P = 0.978$, Fig. 1A). Heterogeneity in our dataset was high ($I^2 = 95$ %), with 40% attributed to true differences between studies, 19% to differences between effect sizes, 0% to between-species differences, and 0.6% to differences between cohorts. Notably, phylogenetic relatedness (Supplementary Fig. 2) explained 35.4% of heterogeneity, suggesting a phylogenetic signal on male reproductive senescence.

Aim 2: Role of biological and methodological moderators

We did not find a significant general effect of advancing male age on ejaculate traits in our full model (which included all moderators with data for >75% of effect sizes; mean [95% C.I.]: -0.197 [-1.496 to 1.103]). However, the included moderators explained a significant proportion of the total heterogeneity in our data ($R^2 = 12.17\%$, $Q_M = 99.606$, $Q_E = 15299.075$, $P < 0.001$, $DF = 36$).

We did not find evidence for age-dependent changes in ejaculates in any taxonomic class (Fig. 1B for four major classes, Supplementary Fig. 3 for all classes), except in Malacostraca (which showed improvement with advancing male age), when effects were averaged across all ejaculate traits. However, taxonomic class explained a significant proportion of heterogeneity (R^2 = 8.26%, Q_M = 26.082, P = 0.025, DF = 14). Similarly, when averaged across all taxa, we did not find evidence for advancing male age to affect any individual ejaculate trait significantly. Yet, ejaculate trait explained a small but significant proportion of heterogeneity (R^2 = 1.72%; Q_M = 51.287; P < 0.001, DF = 13, Fig. 2A).

We detected taxonomic class-specific effects of advancing male age on individual ejaculate traits. For insects (Insecta, k = 258), ejaculate size, quantity of sperm (corrected for body or testis size), number of sperm, and sperm viability, improved with advancing male age (Fig. 2B). For ray-finned fish (Actinopterygii, k = 174), sperm motility and velocity decreased, whereas ejaculate size increased, with advancing male age (Supplementary Fig. 4A). However, we found no significant effect of advancing male age on individual ejaculate traits in birds (Aves, k = 318; Supplementary Fig. 4B) or mammals (Mammalia, k = 990; Supplementary Fig. 4C).

We also observed species-specific effects of advancing male age on individual ejaculate traits. For lab rodents, *Rattus norvegicus* and *Mus musculus* (k = 373, combined), most traits (i.e. sperm viability, number, motility, percent of sperm with morphological defects, sperm concentration, sperm mitochondrial function, sperm DNA and oxidative damage) showed senescence (Fig. 2C; Supplementary Fig. 5A). For bulls (*Bos taurus*, k = 173), ejaculate size increased with advancing male age (Supplementary Fig. 5B). For *Gallus spp.* (domestic chicken and red junglefowl combined, k = 183), number of sperm and ejaculate size showed senescence (Supplementary Fig. 5C; See Fig. 3 for a summary of all taxa- and species-specific effects). The male gonadosomatic index of a species (GSI: i.e. ratio

of testes to body mass, used as a proxy for degree of sperm competition) did not modulate how advancing male age affected ejaculate traits ($R^2 = 0.26\%$, $Q_M = 0.786$, $P = 0.375$, DF = 1, Supplementary Fig. 6). Finally, using linear mixed-effects models, we detected some evidence for a quadratic effect of advancing male age on the percent of morphologically normal sperm, viable sperm, and motile sperm (Supplementary Fig. 7).

Studies sampling a higher proportion of maximum adult lifespan of a species provided stronger evidence for senescence in ejaculate traits ($R^2_{all} = 0.57\%$, $Q_M = 4.838$, $P = 0.028$, DF = 1, Fig. 4A; see Supplementary Fig. 8 for distribution of lifespans sampled across taxa). This result was supported mainly in captive and lab populations, but not wild and domestic populations ($R^2_{captive} = 32.43\%$, $R^2_{lab} = 1.24\%$, $R^2_{wild} = 0.52\%$, $R^2_{domestic} = 0.36\%$; Fig. 4B-E). The stage of an organism's ontogeny (Supplementary Fig. 9) at which it was sampled, significantly influenced the evidence for senescence. Specifically, studies that sampled a higher youngest or oldest age of the associated species (as a proportion of a species' maximum adult lifespan), reported stronger evidence for senescence in ejaculate traits (youngest: $P = 0.032$, $R^2_{all} = 0.64\%$, Supplementary Fig. 10; oldest: $P = 0.009$, $R^2_{all} = 0.97\%$, Supplementary Fig. 11). We did not find evidence for reproductive senescence in ejaculate traits, irrespective of the method used to collect ejaculates from males (e.g. electroejaculation, dissection, natural matings). However, ejaculate collection method explained significant heterogeneity in the data ($R^2 = 1.36\%$; $Q_M = 7.52$, $P = 0.023$, DF = 2, Supplementary Fig. 12). Population type ($R^2 = 1.12\%$; $Q_M = 2.724$, $P = 0.605$, DF = 4, Supplementary Fig. 13) or male sampling method (i.e. longitudinal or cross-sectional; $R^2 = 0.08\%$, $Q_M = 0.639$, $P = 0.887$, DF = 3, Supplementary Fig. 14), did not modulate the effect of advancing male age on ejaculate traits. We also tested whether males that experienced unnatural manipulations (i.e. conditions outside of their typical range, compared to a well-defined control in the study), showed more senescence than males who did not undergo

unnatural manipulations. We detected no senescence or improvement in ejaculate traits irrespective of whether males underwent unnatural manipulations (e.g. heat stress) or not (R^2 = 0 %, Q_M = 0.021, P = 0.989, DF = 2, Supplementary Fig. 15A, 15B), or find significant differences in effects sizes between manipulated and unmanipulated males (P = 0.885).

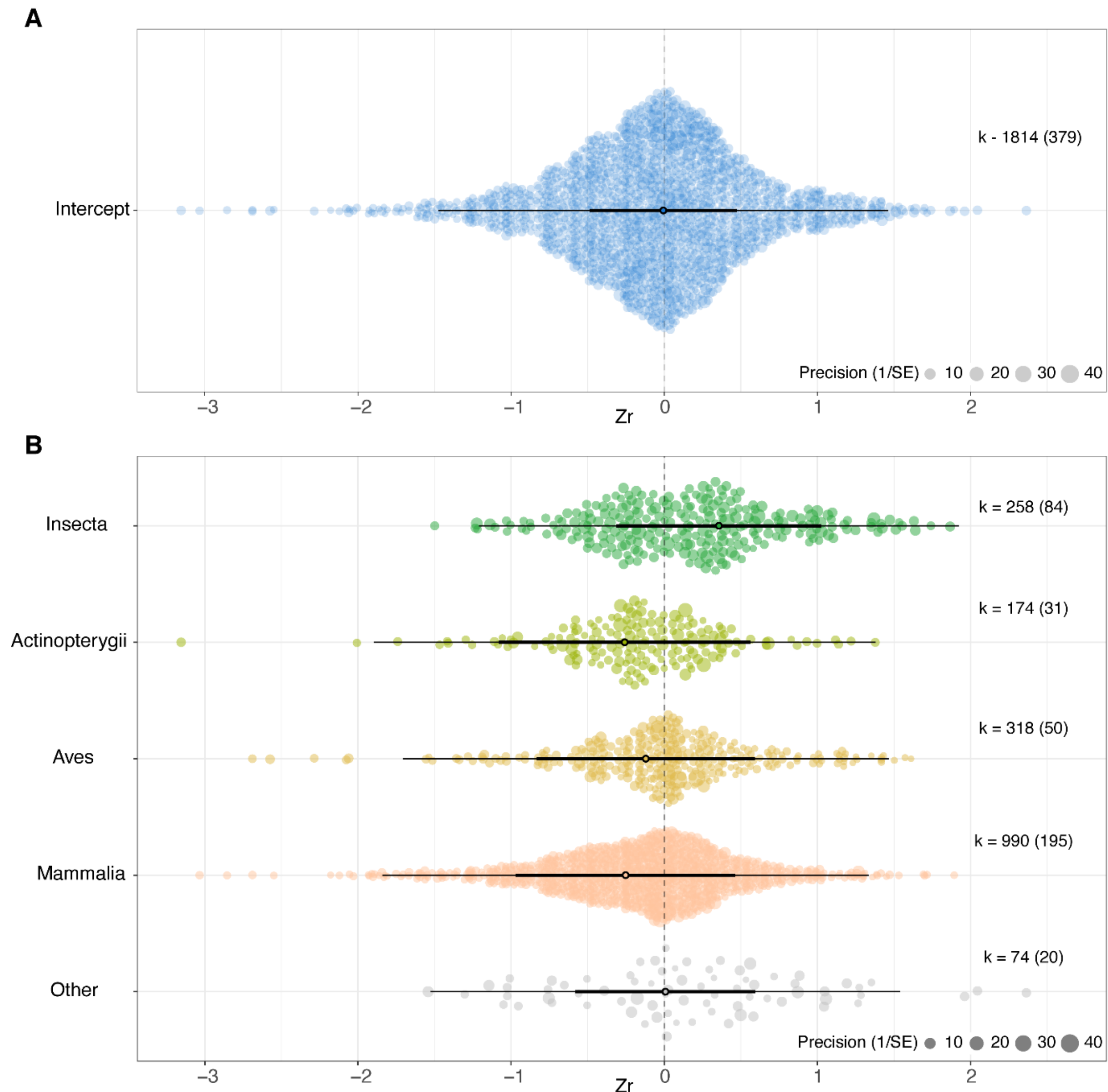


Figure 1: No consistent evidence for senescence in ejaculate traits, irrespective of the study population type or taxonomic class. A. Meta-analytical model of the overall effect of advancing male age on ejaculate traits. B. Effect of advancing male age on ejaculate traits for each taxonomic class (note that animal classes with less than 25 effect sizes were grouped together in ‘Other’). The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher’s z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes, and black dot shows mean effect size. Sample sizes reported

as: k = number of effect sizes (in brackets: number of studies). Source data provided as a source data file.

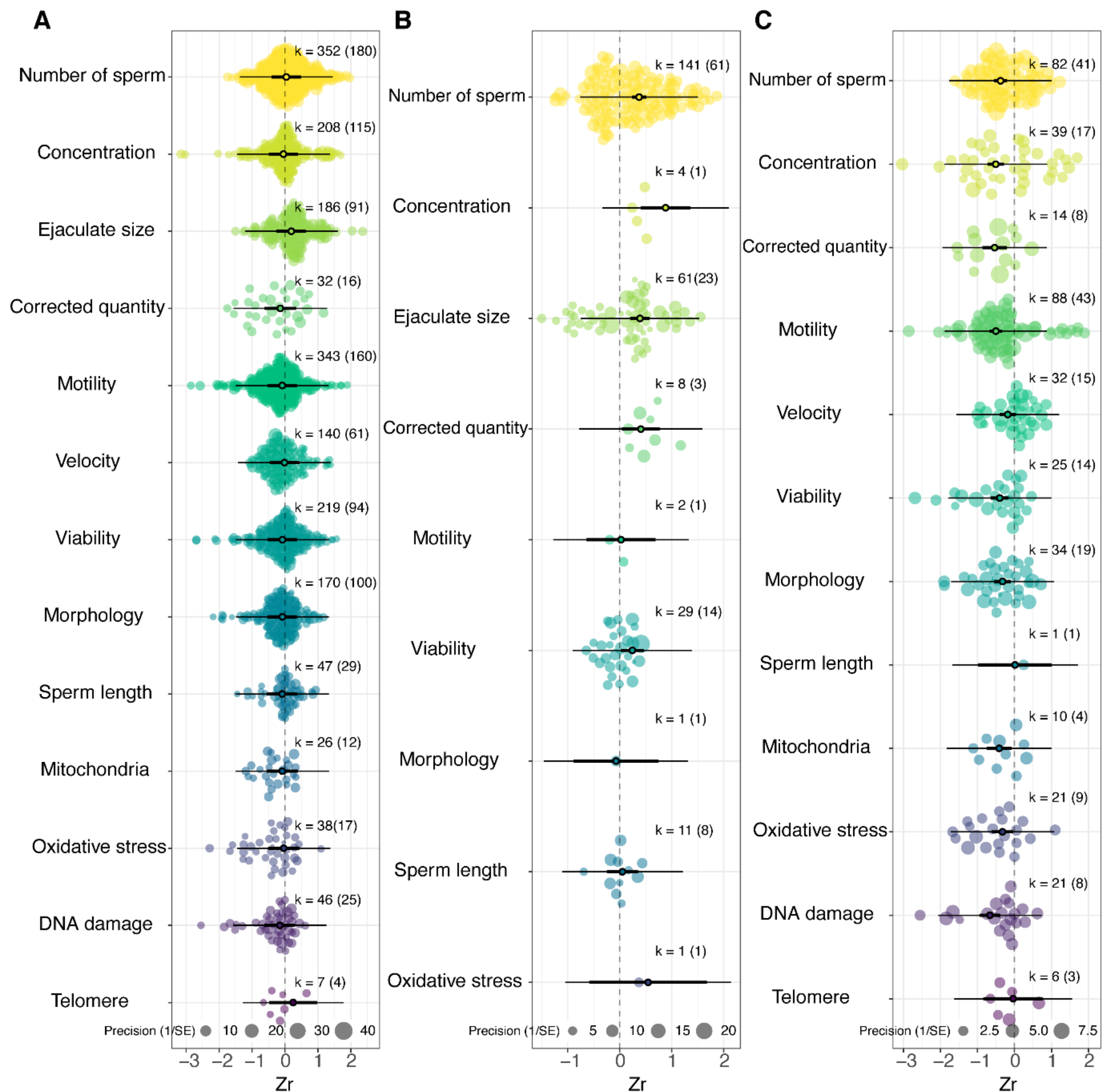


Figure 2: No consistent evidence for senescence in ejaculate traits when all taxa were considered, but some ejaculate traits improve with advancing age (in insects), while other traits decline (in lab rodents). A. Effect of advancing male age on individual ejaculate traits across all 157 species in the dataset. B. Effect of advancing male age on individual ejaculate traits in the class- Insecta. C. Effect of advancing male age on individual ejaculate traits for the two most over-represented species combined (lab rodents): *Mus musculus* and *Rattus norvegicus*. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies). Bold error bars (95% C.I) show whether overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% P.I. of effect sizes, black dot shows mean effect sizes. Source data provided as a source data file.

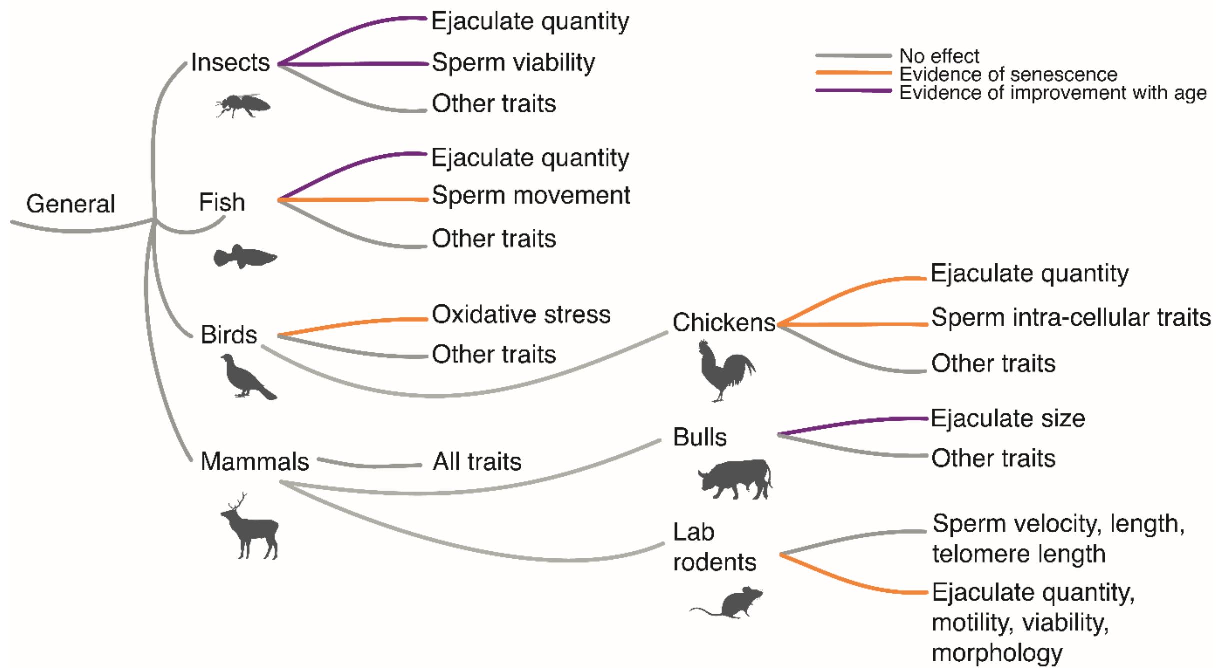


Figure 3: Specific ejaculate traits and taxonomy interacted to affect the evidence for senescence. Summary of results for how advancing male age affects different ejaculate traits across various taxa in our meta-analysis. "Chickens" refers to domestic chickens and red junglefowl combined. Species icons from PhyloPics, with artist credits and copyright: Kamil S. Jaron (CC0 1.0), Emma Moffett (CC0 1.0), T. Michael Keesey (PDM 1.0), Steven Traver (CC0 1.0), Georgios Lyras (CC0 1.0).

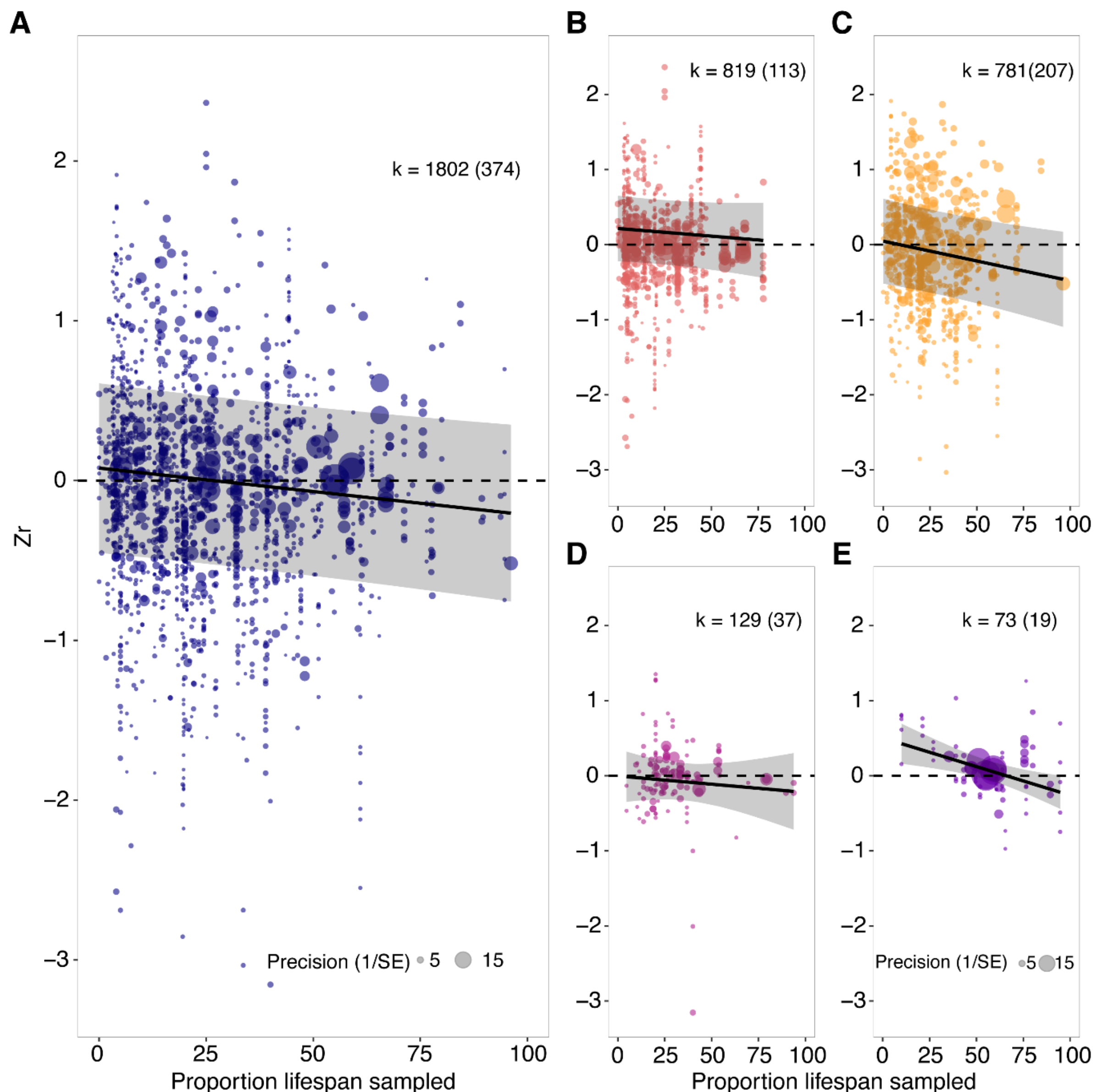


Figure 4: Increasing the proportion of maximum adult lifespan sampled, increased the likelihood of finding senescence. Effect of proportion of maximum adult lifespan sampled (X axis) on the effect size i.e. Fisher's z transformed r (Y axis) across the entire dataset (A), and broken down for domestic (B), laboratory (C), wild (D), and captive animals (E). The size of each data point represents the precision of the effect size (1/SE). The dark line with shaded bars represents the overall effect of lifespan sampled on effect sizes and its 95% C.I., respectively, black line shows mean regression line. Negative values depict senescence in ejaculate traits with advancing age while positive values show improvement in ejaculate traits with advancing male age. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies). Source data provided as a source data file.

Aim 3: Effects of advancing male age on reproductive outcomes

We found that male reproductive outcomes (i.e. measures of male fertilisation success, reproductive output, or offspring quality) did not improve or decline with advancing male age overall (Supplementary Fig. 16A). However, reproductive outcomes were less likely to deteriorate with advancing male age, than ejaculate traits (R^2 = 1.76%, Q_M = 9.783, P = 0.002, DF = 1; Supplementary Fig. 16B).

Publication bias

We found no statistical evidence for publication bias, except for a time-lag bias, with more recent studies being more likely to show senescence in ejaculate traits (Supplementary Fig. 17, 18, 19).

Other sensitivity analyses

We obtained no significant evidence for senescence in ejaculate traits, even when only studies that sampled >10% of maximum adult lifespan of species were analysed (mean [95% confidence interval (C.I.)]: -0.020 [-0.549 to 0.509], z = -0.075, P = 0.940, Supplementary Fig. 20). Results from our taxonomic class-specific models, which again only included studies that sampled >10% of maximum adult lifespan, were qualitatively similar to results from models that included all studies (Supplementary Fig. 21).

We additionally objectively categorised study aims as explicitly interested in senescence (i.e. studies using “ageing”, “aging”, “senescence”, “senescent”, or “senescing” in their abstracts or titles, N = 101 studies) or not (N = 273 studies). We did not find significant evidence for overall senescence in ejaculate traits, even when we only analysed studies whose aims were categorised as interested in senescence (mean [95% confidence interval (C.I.)]: -0.294 [-0.760 to 0.172], z = -1.238, P = 0.216, Supplementary Fig. 22). Study aim

however, explained a significant proportion of heterogeneity in effect sizes (R^2 = 5.08%, Q_M = 36.287, $P < 0.001$, DF = 2; Supplementary Fig. 22). Furthermore, studies that were interested in senescence sampled a higher proportion of maximum adult lifespan of the associated species (34%) than studies not interested in senescence (20%, Supplementary Fig. 23).

Discussion

Senescence is central to our understanding of ecology[48], evolution[48], life history[16], and society[49]. Senescence in male ejaculate traits can influence sexual selection[50,51], sexual conflict[26,36], and offspring health[13]. We thus cannot fully understand organismal biology without understanding the evidence for, and consequences of, male reproductive senescence at the level of ejaculates. Our meta-analysis reviews the effects of advancing male age on ejaculate traits across animals in order to test for senescence, and highlights key gaps in knowledge that will facilitate a better understanding of ageing.

Contrary to expectations, we detected no consistent evidence for senescence in ejaculate traits across studies (aim 1). Our results contrast those of a meta-analysis in humans[35], which found senescence across most ejaculate traits in men. These differences in results possibly reflect stronger selection pressures in non-human animals, to maintain sperm function across all ages compared to men. In our dataset, the phylogenetically closest relative to humans were rodents (exemplified by lab rodents), which like humans[35], showed evidence for senescence in most ejaculate traits. Current human longevity is much higher than what it was just few centuries ago[52]. Such recent increases in human longevity could lead to men living much beyond the age at which sperm function can be maintained, leading to greater senescence in ejaculates of men compared to other animals.

We suggest several potential non-mutually exclusive reasons for the lack of senescence in our meta-analysis. While we discovered that increasing the proportion of lifespan sampled by a study yielded greater evidence for senescence (also shown by [18,35,47]), studies in our meta-analysis tended to sample a low proportion of maximum adult lifespan (median = ~25%, Supplementary Fig. 9), which could have underestimated senescence. Another reason could be because many of the studies included in our analysis were not explicitly testing for senescence. To account for this, we conducted an analysis only on studies that were explicitly interested in senescence. These studies sampled a higher proportion of lifespan of the associated species, yet did not provide evidence for senescence in ejaculate traits overall. However, a reason for this lack of evidence could be that study aims are difficult to quantify, and our classification of aims might have excluded relevant studies. Curvilinear patterns of ageing could also have led us to underestimate senescence. This is because age-dependent changes in ejaculate traits were analysed as a linear function (effect sizes), however ageing is often curvilinear[4,53]. Our test of quadratic effects showed some evidence in support of this. Thus, if ejaculate traits improve from early to mid-adult life (i.e. maturation) and deteriorate (i.e. senesce) later in life, the positive part of the function would be disproportionately represented against the negative part of the function[54]. Our results overall highlight the need for meta-analysts to develop techniques to calculate and analyse non-linear effect sizes to investigate such patterns.

Selective disappearance of poor-quality males with increasing age could also underestimate senescence[55,56]. Comparing means of age groups in longitudinal studies (like in our meta-analysis) can only account for selective disappearance if all individuals are sampled at all ages, which was rarely done across studies. To account for selective disappearance in cases where not all males survive to be sampled at all ages, we would need to analyse individual-level longitudinal data [rarely reported] for each male in each study, rather than

comparing the means of different age groups[57]. Age-dependent improvement or negligible senescence in ejaculate traits could also reflect a true biological pattern, with senescence not being an inevitable outcome of ageing for many ejaculate traits and species[4,58]. For instance, taxa with indeterminate growth or slow life-histories might show negligible senescence[8,17].

We suggest corollary methodological improvements for more rigorous testing of male reproductive senescence. Specifically, studies could: sample higher proportions of a male's maximum lifespan and report survival curves of the studied populations; test for curvilinear effects of age by measuring at least three age cohorts in early-, mid-, and late-adult life; separate confounding effects of male mating history and age by comparing virgin versus frequently mated old and young males; test for selective disappearance by sampling males longitudinally and report individual-level data for each male; be explicit about which theories of ageing are being tested and their corollary predictions; and sample equal number of males in all age classes. Overall, we conclude that senescence is likely occurring in the taxon-specific ejaculate traits where we found supporting evidence. However, we could have underestimated the extent of senescence where supporting evidence was lacking, due to some aforementioned limitations.

Some biological and methodological moderators were important in explaining the observed heterogeneity in effect sizes (aim 2). However, as the effects of these moderators were tested individually, our results could possibly be explained by other moderators not simultaneously included in the analysis. Thus, our results should only be treated as hypothesis-generating rather than evidence of causation. Taxonomic class and ejaculate trait explained a significant proportion of heterogeneity. This heterogeneity could be attributed to differences in ecologies, niches, behaviours, life-history strategies, metabolisms, and evolutionary histories of animals[4,59]. Heterogeneity explained by ejaculate traits could be due to: covariances between different ejaculate traits[60]; some traits being more sensitive to age-

dependent deterioration than others[61]; or different traits being under varying selection pressures[62]. Additionally, some ejaculate traits are more likely to influence fertilisation success than others[60]. It is thus possible for traits that are more important determinants of fertilisation success to evolve slower rates of senescence than less important traits[63], which future studies could test.

We discovered some taxonomic class-specific evidence for age-dependent changes in individual ejaculate traits. Insects showed an increase in all sperm and ejaculate quantity traits. This increase could be associated with their mating status, as most studies (>75%) on insects in our meta-analysis kept males as virgins. Specifically, in species with life-long spermatogenesis and low rates of sperm loss (such as some insects[64,65]), low mating rates can result in old males accumulating more sperm and producing larger ejaculates than young males[66]. Ray-finned fish (Actinopterygii) showed evidence for senescence in sperm velocity and motility, but also age-dependent increases in ejaculate size. This result could be due to old males producing larger ejaculates to compensate for senescence in sperm performance. Age-dependent increases in the ejaculate of fish and improvements in ejaculate traits with age in Malacostraca, could also reflect continuous post-maturity growth in these indeterminately growing taxa[17,18,67]. Indeterminate growth can lead to older males having larger gonads and reduces reproductive senescence[4,17]. We did not find consistent evidence for senescence in ejaculate traits in mammals or birds.

We detected several species-specific patterns of senescence. Specifically, most ejaculate traits in lab rodents (*Mus musculus* and *Rattus norvegicus* combined) showed senescence, even when only control/wild type genetic strains were analysed (e.g. C57 for mice, Brown Norway and Sprague Dawley for rats). This could be due to studies on lab rodents usually having equal sample sizes of males in each age cohort, thus possibly limiting bias towards weighting of the positive part (early- to mid-life) of the curvilinear ageing

function. Consistent evidence for senescence in lab rodents could further be associated with lab rodent strains being inbred, which might exacerbate senescence. Consistent evidence in lab rodents could also be associated with senescence being exaggerated in lab adapted populations[68]. For a more nuanced understanding of such trait by taxon interactions, we suggest that future studies: account for age-dependent changes in body and testes size (as covariates); test for post-meiotic senescence of sperm during storage in males; record whether studied species exhibit continuous spermatogenesis and sperm reabsorption; and measure multiple ejaculate traits simultaneously (i.e. sperm quantity, performance/viability), because sperm quantity versus performance traits might be affected by age in different ways.

Extending the proportion of maximum adult lifespan sampled increased the evidence for senescence in ejaculate traits for a species. This result suggests that the onset of reproductive senescence usually occurs late in life[4,69], and senescence will more likely be detected if studies sample a larger proportion of lifespan. However, this may be biased by the population sampled, as this association was strong in captive and lab animals, but not in wild and domestic animals. We did not find evidence for senescence in any levels of other methodological moderators (aim 2). This result could be due to effects of methodological moderators being taxon-specific, or being revealed only under interactions with other methodological or biological moderators. The lack of an effect of study methodologies might also be explained by moderators that we did not include in our analyses (Table 2).

We detected no consistent evidence for overall improvement or senescence in reproductive outcomes of males (i.e. measures of fertilisation success, egg/offspring number/viability/quality; aim 3). Our meta-analysis used data on reproductive outcomes only from studies that also measured ejaculate traits, which possibly represents a biased subset of studies on ageing of reproductive outcomes. However, we found that reproductive outcomes were less likely to exhibit age-dependent deterioration than ejaculate traits. This difference

could be due to not all ejaculate traits being key determinants of reproductive success (e.g. fertilisation success[70]), and deterioration in some ejaculate traits having little consequence for a male's reproductive outcome[71]. Lower rates of age-dependent declines in male reproductive outcomes could also be due to female-driven effects (e.g. cryptic female choice, reproductive compensation), which might provide a buffer against low-quality ejaculates of old males. For instance, females might be able to eject poor quality sperm via cryptic female choice[72], or females mated to older males might compensate by investing more resources into provisioning[73]. Additionally, viability selection in old males could purge low quality male genotypes, leading to old males having higher means and lower variances for reproductive outcomes than young males[46,74]. These results suggest that age-dependent changes in ejaculate traits may not accurately reflect changes in reproductive outcomes. We emphasize that studies should ideally measure ejaculate traits, male reproductive success, and offspring phenotypes, to elucidate the fitness consequences of advancing male age.

Methods

We followed the PRISMA-EcoEvo guidelines for our meta-analysis[75] and conducted statistical analyses in R[76] v 4.1.2. Supplementary figures (1-24) and Supplementary notes (1-12) are provided in the "Supplementary Information" file. Data, model outputs, metadata, code, PRISMA checklist, and pre-registration have all been deposited at OSF (https://osf.io/dk8sq/).

Search protocol

We conducted a literature search using search strings on SCOPUS and Web of Science on 21st January and 27th March 2021, respectively (see Supplementary notes 1 for specific search strings). In addition, we conducted a backward and forward search using seven relevant

papers related to the topic of our meta-analysis[11, 13, 22, 24, 43, 54, 77]. We additionally conducted a search for unpublished research using the Bielefeld Academy Search Engine[78]. Finally, we contacted 56 researchers who study the ecology and evolution of male reproductive senescence to ask for unpublished data. Our search resulted in a total of 9412 unique abstracts from published sources and 271 abstracts from unpublished sources (PRISMA diagram: Supplementary Fig. 1). We screened these abstracts in Rayyan[79] and abstrackr[80] using pre-defined selection criteria (see below). We ensured that the screening process was highly repeatable (Supplementary notes 2).

Inclusion criteria

For a study to be included in our analysis, some selection criteria had to be fulfilled during the abstract and full-text screening stages. When screening abstracts, the study had to be a research article (not a review, meta-analysis, or case study), written in English, on non-human animals, and quantify ejaculate traits in males of different ages. When screening full-texts, the study needed to contain: data on the effects of male age on ejaculate traits, non-overlapping age groups of males, and appropriate data for calculation of effect sizes. We only included studies where at least two age groups of adult males could be compared (see Supplementary notes 3 for our definition of "adults"). We deemed a total of 379 studies (374 from published, and five from unpublished sources) appropriate for data extraction based on our selection criteria, and included them in our meta-analysis (PRISMA diagram in Supplementary Fig. 1). These studies represented 157 species.

Data collection

To quantify the evidence for or against male reproductive senescence (aim 1), we collected data on: means, standard deviations (SD) or standard errors (SE), the number of males in

each age group, and the number of unique males in the study, wherever reported (see Supplementary notes 4 for formulae used to calculate SD). If we could not obtain means and SD/SE, we noted the "test statistic" (e.g. t from t-tests or R^2 values) reported in the study from which effect sizes can be easily obtained. We ensured that the data extraction process was highly repeatable (Supplementary notes 2).

To understand how biological moderators affect patterns of senescence (aim 2), we recorded information on various biological variables from the 379 studies included in the meta-analysis. We recorded the species and taxonomic class of the study organism, and the ejaculate traits measured in the study (see Supplementary notes 5 for definitions of each trait). The ejaculate traits were either measures of sperm/ejaculate quantity (e.g. sperm concentration, sperm number, and ejaculate volume), sperm performance (e.g. sperm motility, velocity, viability), or intra-cellular measures of sperm quality (e.g. oxidative stress in sperm, DNA damage to sperm, sperm telomere length). Finally, we recorded the gonadosomatic index (GSI, i.e. ratio of testis mass to body mass, as a proxy for sperm competition[82]) for each species, wherever possible (see Supplementary notes 6; meta-data on OSF https://osf.io/dk8sq/).

To understand how methodological moderators affect patterns of senescence in ejaculate traits (aim 2), we collected data on various methodological variables from included studies (see Supplementary notes 7). Initially, we recorded the maximum lifespan (male-specific whenever possible, or species-specific when male-specific data were not available), and age at adulthood of the species studied (see Supplementary notes 6). Data on maximum lifespan and age at adulthood, as well as sources of these data, can be found at OSF (https://osf.io/dk8sq/). We then calculated the proportion of maximum adult lifespan sampled for a species in each study (converted to years). Some of the data on maximum adult lifespans (especially for vertebrates) were obtained from large databases/datasets (that often

only reported species-level lifespans without reporting the sex of the measured individuals). Thus, these data may not always accurately reflect the maximum male lifespans of the specific populations included in our meta-analysis.

We also recorded: method of sperm extraction (e.g. electro-ejaculation, natural mating); population type (whether males belonged to wild, domestic, captive or laboratory populations (see Supplementary notes 8 for definitions)); method for measuring male age (i.e. whether male age was known directly or indirectly estimated from a measure of phenotype); whether the ejaculate was stored in cold conditions (<5°C, irrespective of the duration of storage) before analysis of sperm performance; and whether the study was experimental or not[18]. In some studies, males underwent "unnatural manipulations" (see Supplementary notes 9 for detailed definitions). Here, we also recorded whether the data were obtained from males that underwent these "unnatural" manipulations (i.e. males that experienced conditions outside of their typical range, that were compared to a well-defined control in the study), or from males that were used as controls in the same study.

We investigated whether advancing male age affects male reproductive outcomes (aim 3), and whether the effects of male age on reproductive outcomes (see Supplementary notes 5 for definitions) differ from those on ejaculate traits. For this, we collected data on how advancing male age affects: male fertilization success; number of eggs produced by the mated females; number of offspring produced by the mated females; egg viability and hatchability; offspring viability; offspring developmental rate; and offspring body condition, whenever available in a study (53 studies in total).

Calculating effect sizes

We used Fisher's z transformed correlation coefficient (Zr) as the effect size in our meta-analysis[83]. Each effect size was calculated from either: standardized mean differences (when

two age groups were compared), or simulations (when multiple age groups were compared), or test statistics (see Supplementary notes 10 for formulae used). Effect sizes from these three calculation methods were not significantly different from each other (Supplementary notes 10; Supplementary Fig. 24), thus all effect sizes, irrespective of their calculation methods, were analysed together in our models. We corrected all calculated effect sizes (Zr) by a multiplier to obtain the final effect sizes to be used in the analyses (See Supplementary notes 10), so that negative effect sizes indicated senescence, while positive effect sizes indicated improvement in ejaculate traits with advancing male age.

Data analysis

We first created a meta-analytical model (i.e. null model) to test for the general overall effect of advancing male age on ejaculate traits (aim 1), using the rma.mv function in the metafor package[84]. We included the effect size (Zr) as our response variable in the null model, and random effects of: effect size ID (which represents the residual within-study variance), cohort ID, study ID, and species name to control for non-independence of effect sizes[85]. We also added a correlation matrix quantifying the phylogenetic relatedness of species in our dataset, to control for non-independence arising due to shared phylogenetic history and test for a phylogenetic signal[86]. The phylogenetic tree (Supplementary Fig. 2) was built using the packages ape[87] and rotl[88], which use data from the OpenTreeOfLife[89]. We quantified the total heterogeneity[90] not due to sampling error as I^2, which can range from 0-100. We quantified partial heterogeneity explained by each random effect using the function i2_ml from the orchard package[91].

We created meta-regressions to investigate how moderators modulated the effects of advancing male age on ejaculate traits (aim 2). In all meta-regressions, we included the same random effects and phylogenetic matrix as in our null model, and effect size (Zr) as our

response variable. We first conducted a meta regression with all moderators for which data were available for >75% of effect sizes and studies ("full" model). This full model was used to estimate the proportion of heterogeneity explained by moderators[92], while accounting for the confounding effects of other moderators. The full model included moderators of: taxonomic class, ejaculate trait, proportion of maximum adult lifespan sampled, whether or not males had control over ejaculation, population type, sampling method of males, method of age estimation, whether or not a study was experimental, and whether or not males underwent "unnatural" manipulations. We then built several meta-regressions to explore individually, the effects of each methodological and biological moderator (See Table 1 and 2, Supplementary notes 7, most of which had been pre-registered at OSF: https://osf.io/dk8sq/). Here, we also tested how the youngest and oldest ages sampled of the associated species (as proportion of the maximum lifespan of the species), affected the evidence for senescence. We further tested the influence of the gonadosomatic index of species (GSI), which was not included in the full model, as it only had data for <75% of studies and was not pre-registered.

For each meta-regression model, we calculated the total heterogeneity (Q_M) and the proportion of total heterogeneity explained by moderators (marginal R^2), with the function r2_ml using the orchard package[91]. P values ($\alpha = 0.05$) indicate whether the heterogeneity explained was significant or not[90]. We created models without an intercept to test whether each level of a moderator showed evidence for senescence or improvement in ejaculate traits with age. However, for moderators with two levels, we were additionally interested in comparing effect sizes in one level to those in the other level. In such cases, we created a model with one level of the moderator as the intercept (here, a P value expressed whether one level of the moderator was different from the other level).

Taxonomic classes of Insecta, Actinopterygii, Aves, and Mammalia were over-represented classes in our dataset, each with >150 effect sizes from >30 studies

(Supplementary Fig. 2). We thus created four separate meta-regressions for each class, with ejaculate trait as a moderator. Moreover, four species: lab mice (*Mus musculus*), lab rats (*Rattus norvegicus*), chicken/red junglefowl (*Gallus spp*.), and bulls (*Bos taurus*) were over-represented in our dataset (each species had >150 effect sizes across >20 studies; Supplementary Fig. 2). For these species, we created separate meta-regression models with ejaculate trait as a moderator.

Shapes of reproductive ageing are often curvilinear, characterised by an initial period of maturation, where performance increases from early- to mid-adult life, and subsequently decreases (i.e. senescence) in late-adult life[4,53]. To test whether the effects of male age on ejaculate traits were curvilinear, we used linear mixed-effects models[93] (Supplementary notes 11). These analyses were limited to traits which were measured on the same scale and units across studies/taxa.

We also used data from studies that measured age-dependent changes in both ejaculate traits and reproductive outcomes. Then, we ran a meta-regression using type of trait (reproductive outcome or ejaculate trait) as a moderator (aim 3).

Publication bias

We conducted a sensitivity analysis of our null model, by replacing the random effects terms of cohort and effect size ID, with a variance covariance matrix[94]. We also performed various publication bias tests[94] (funnel plot, trim and fill, multi-level meta-regression, and selection model; Supplementary notes 12). These analyses were done to test for biased sampling of effect sizes in our study, based on their precision, magnitude, publication year, and sample size.

Other sensitivity analysis

We conducted two additional sensitivity analyses. First, we accounted for the low proportions of maximum adult lifespans sampled by studies in our meta-analysis. Here, we re-ran our null model and models for the taxonomic classes of Insecta, Mammalia, Aves, and Actinopterygii, only using data from studies that sampled >10% of the maximum adult lifespan of the species. Second, we classified study aims as being explicitly interested in senescence or not. Studies that mentioned "ageing", "aging", "senescence", "senescent", or "senescing" in their abstracts or titles, were classified as explicitly interested in senescence. We then created a meta-regression with study aim (i.e. interested in senescence or not) as our moderator, to test whether studies that were interested in senescence showed senescence in ejaculate traits overall.

Data availability

The data generated in this study have been deposited in the Open science framework database under accession code 10.17605/OSF.IO/DK8SQ [https://osf.io/dk8sq/]. The data are available without any restricted access. The raw data are available under the file name "raw_data.csv". The processed data are available under the file name "spermFinalAllData.csv". The data used to produce the manuscript figures are provided in the Source Data file. Source data are provided with this paper. All associated code can be found at OSF https://osf.io/dk8sq/ under accession code 10.17605/OSF.IO/DK8SQ.

References

1. Reinke, B. A. *et al.* Diverse aging rates in ectothermic tetrapods provide insights for the evolution of aging and longevity. *Science* **376**, 1459–1466 (2022).
2. Lemaître, J.-F. & Gaillard, J.-M. Reproductive senescence: new perspectives in the wild: Reproductive senescence in the wild. *Biol Rev* **92**, 2182–2199 (2017).
3. Nussey, D. H., Froy, H., Lemaitre, J.-F., Gaillard, J.-M. & Austad, S. N. Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. *Ageing Research Reviews* **12**, 214–225 (2013).
4. Jones, O. R. *et al.* Diversity of ageing across the tree of life. *Nature* **505**, 169–173 (2014).
5. Jones, O. R. & Vaupel, J. W. Senescence is not inevitable. *Biogerontology* **18**, 965–971 (2017).
6. Monaghan, P., Charmantier, A., Nussey, D. H. & Ricklefs, R. E. The evolutionary ecology of senescence. *Functional Ecology* **22**, 371–378 (2008).
7. Roper, M., Capdevila, P. & Salguero-Gómez, R. Senescence: why and where selection gradients might not decline with age. *Proc. R. Soc. B.* **288**, 20210851 (2021).
8. Vaupel, J., Baudisch, A., Dölling, M., A. Roach, D. & Gampe, J. The case for negative senescence. *Theoretical Population Biology* **65**, 339–351 (2004).
9. Blagosklonny, M. V. The hyperfunction theory of aging: three common misconceptions. *Oncoscience* **8**, 103–107 (2021).
10. Paul, C. & Robaire, B. Ageing of the male germ line. *Nat Rev Urol* **10**, 227–234 (2013).
11. Radwan, J. Male age, germline mutations and the benefits of polyandry: Germline mutations, ageing and polyandry. *Ecology Letters* **6**, 581–586 (2003).
12. Mannucci, A. *et al.* The Impact of Oxidative Stress in Male Infertility. *Front. Mol. Biosci.* **8**, 799294 (2022).
13. Monaghan, P. & Metcalfe, N. B. The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. *Proc. R. Soc. B.* **286**, 20192187 (2019).
14. Medawar, P. *An unsolved problem of biology*. (1952).
15. Williams, P. D. & Day, T. Antagonistic pleiotropy, mortality source interactions, and the evolutionary theory of senescence. *Evolution* **57**, 1478–1488 (2003).
16. Rose, M. & Charlesworth, B. A test of evolutionary theories of senescence. *Nature* **287**, 141–142 (1980).
17. Purchase, C. F. *et al.* A synthesis of senescence predictions for indeterminate growth, and support from multiple tests in wild lake trout. *Proc. R. Soc. B.* **289**, 20212146 (2022).
18. Vrtílek, M., Žák, J. & Reichard, M. Evidence for reproductive senescence across ray-finned fishes: A review. *Front. Ecol. Evol.* **10**, 982915 (2022).
19. Bodnar, A. G. Cellular and molecular mechanisms of negligible senescence: insight from the sea urchin. *Invertebrate Reproduction & Development* **59**, 23–27 (2015).
20. Archer, R., Paniw, M., Vega-Trejo, R. & Sepil, I. A sex skew in life-history research: the problem of missing males Proc. R. Soc. *Proceedings of the Royal Society B: Biological Sciences* **289**, (2022).
21. Lemaître, J.-F., Ronget, V. & Gaillard, J.-M. Female reproductive senescence across mammals: A high diversity of patterns modulated by life history and mating traits. *Mechanisms of Ageing and Development* **192**, 111377 (2020).
22. Fricke, C. & Koppik, M. Male reproductive ageing: a tale of the whole ejaculate. *Reproduction* **158**, R219–R229 (2019).
23. Aich, U., Head, M. L., Fox, R. J. & Jennions, M. D. Male age alone predicts paternity success under sperm competition when effects of age and past mating effort are experimentally separated. *Proc. R. Soc. B.* **288**, 20210979 (2021).
24. Gasparini, C., Marino, I. A. M., Boschetto, C. & Pilastro, A. Effect of male age on sperm traits and sperm competition success in the guppy (*Poecilia reticulata*). *Journal of Evolutionary Biology* **23**, 124–135 (2010).
25. Jones, A. G. The evolution of alternative cryptic female choice strategies in age-structured populations. *Evolution* **56**, 2530–2536 (2002).
26. Adler, M. I. & Bonduriansky, R. Sexual Conflict, Life Span, and Aging. *Cold Spring Harbor Perspectives in Biology* **6**, a017566–a017566 (2014).
27. Bonduriansky, R., Maklakov, A., Zajitschek, F. & Brooks, R. Sexual selection, sexual conflict and the evolution of ageing and life span. *Functional Ecology* **22**, 443–453 (2008).
28. Parker, G. A. Sexual conflict over mating and fertilization: an overview. *Phil. Trans. R. Soc. B* **361**, 235–259 (2006).
29. Bronikowski, A. M. *et al.* Sex-specific aging in animals: Perspective and future directions. *Aging Cell* **21**, (2022).
30. Bergeron, L. A. *et al.* Evolution of the germline mutation rate across vertebrates. *Nature* (2023) doi:10.1038/s41586-023-05752-y.
31. Crow, J. F. The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* **1**, 40–47 (2000).
32. Reinhardt, K. & Turnell, B. Sperm ageing: A complex business. *Funct Ecol* **33**, 1188–1189 (2019).
33. Sharma, R. *et al.* Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reprod Biol Endocrinol* **13**, 35 (2015).
34. Cornwallis, C. K., Dean, R. & Pizzari, T. Sex-Specific Patterns of Aging in Sexual Ornaments and Gametes. *The American Naturalist* **184**, E66–E78 (2014).
35. Johnson, S. L., Dunleavy, J., Gemmell, N. J. & Nakagawa, S. Consistent age-dependent declines in human semen quality: A systematic review and meta-analysis. *Ageing Research Reviews* **19**, 22–33 (2015).

36. Dean, R. *et al.* Male Reproductive Senescence Causes Potential for Sexual Conflict over Mating. *Current Biology* **20**, 1192–1196 (2010).
37. Gasparini, C., Devigili, A. & Pilastro, A. Sexual selection and ageing: interplay between pre- and post-copulatory traits senescence in the guppy. *Proc. R. Soc. B.* **286**, 20182873 (2019).
38. Decanini, D. P., Wong, B. B. M. & Dowling, D. K. Context-dependent expression of sperm quality in the fruitfly. *Biol. Lett.* **9**, 20130736 (2013).
39. Girndt, A. *et al.* Male age and its association with reproductive traits in captive and wild house sparrows. *J Evol Biol* **32**, 1432–1443 (2019).
40. Lifjeld, J. T. *et al.* When Older Males Sire More Offspring—Increased Attractiveness or Higher Fertility? *Behav Ecol Sociobiol* **76**, 61 (2022).
41. Wedell, N. & Ritchie, M. G. Male age, mating status and nuptial gift quality in a bushcricket. *Animal Behaviour* **67**, 1059–1065 (2004).
42. Delbarco-Trillo, J., Tourmente, M., Varea-Sánchez, M. & Roldan, E. R. S. Is male reproductive senescence minimized in Mus species with high levels of sperm competition? *Biological Journal of the Linnean Society* **123**, 463–470 (2018).
43. Vega-Trejo, R., Fox, R. J., Iglesias-Carrasco, M., Head, M. L. & Jennions, M. D. The effects of male age, sperm age and mating history on ejaculate senescence. *Funct Ecol* **33**, 1267–1279 (2019).
44. Mickova, K. *et al.* Age-Related Changes in Sperm Traits and Evidence for Ageing Costs of Sperm Production in a Sexually Promiscuous Passerine. *Frontiers in Ecology and Evolution* **11**, (2023).
45. Sandfoss, M. R., Reichling, S. & Roberts, B. M. Sperm morphology and forward motility are indicators of reproductive success and are not age- or condition-dependent in a captive breeding population of endangered snake. *PLoS ONE* **18**, e0282845 (2023).
46. Johnson, S. L. & Gemmell, N. J. Are old males still good males and can females tell the difference?: Do hidden advantages of mating with old males off-set costs related to fertility, or are we missing something else? *Bioessays* **34**, 609–619 (2012).
47. Fricke, C. *et al.* Timeless or tainted? The effects of male ageing on seminal fluid. *Front. Ecol. Evol.* **11**, 1066022 (2023).
48. Healy, K., Ezard, T. H. G., Jones, O. R., Salguero-Gómez, R. & Buckley, Y. M. Animal life history is shaped by the pace of life and the distribution of age-specific mortality and reproduction. *Nat Ecol Evol* **3**, 1217–1224 (2019).
49. Kirkwood, T. B. L. The origins of human ageing. *Phil. Trans. R. Soc. Lond. B* **352**, 1765–1772 (1997).
50. Promislow, D. Mate Choice, Sexual Conflict, and Evolution of Senescence. *Behavior Genetics* **33**, 191–201 (2003).
51. Archer, C. R. & Hunt, J. Understanding the link between sexual selection, sexual conflict and aging using crickets as a model. *Experimental Gerontology* **71**, 4–13 (2015).
52. Oeppen, J. & Vaupel, J. W. Broken Limits to Life Expectancy. *Science* **296**, 1029–1031 (2002).
53. Baudisch, A. & Stott, I. A pace and shape perspective on fertility. *Methods Ecol Evol* **10**, 1941–1951 (2019).
54. Pizzari, T., Dean, R., Pacey, A., Moore, H. & Bonsall, M. B. The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends in Ecology & Evolution* **23**, 131–140 (2008).
55. Hämäläinen, A. *et al.* Senescence or selective disappearance? Age trajectories of body mass in wild and captive populations of a small-bodied primate. *Proc. R. Soc. B.* **281**, 20140830 (2014).
56. Sanghvi, K., Iglesias-Carrasco, M., Zajitschek, F., Kruuk, L. E. B. & Head, M. L. Effects of developmental and adult environments on ageing. *Evolution* **76**, 1868–1882 (2022).
57. van de Pol, M. & Verhulst, S. Age-Dependent Traits: A New Statistical Model to Separate Within- and Between-Individual Effects. *The American Naturalist* **167**, 766–773 (2006).
58. Finch, C. E. Update on Slow Aging and Negligible Senescence – A Mini-Review. *Gerontology* **55**, 307–313 (2009).
59. Jones, O. R. *et al.* Senescence rates are determined by ranking on the fast-slow life-history continuum. *Ecology Letters* **11**, 664–673 (2008).
60. Snook, R. Sperm in competition: not playing by the numbers. *Trends in Ecology & Evolution* **20**, 46–53 (2005).
61. Naciri, M., Aars, J., Blanchet, M.-A., Gimenez, O. & Cubaynes, S. Reproductive senescence in polar bears in a variable environment. *Front. Ecol. Evol.* **10**, 920481 (2022).
62. Schluter, D., Price, T. & Rowe, L. Conflicting selection pressures and life history trade-offs. *Proc. R. Soc. Lond. B* **246**, 11–17 (1991).
63. Moorad, J. A. & Ravindran, S. Natural Selection and the Evolution of Asynchronous Aging. *The American Naturalist* **199**, 551–563 (2022).
64. Kehl, T., Karl, I. & Fischer, K. Old-male paternity advantage is a function of accumulating sperm and last-male precedence in a butterfly. *Mol Ecol* **22**, 4289–4297 (2013).
65. Reinhardt, K., Naylor, R. & Siva-Jothy, M. T. Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, Cimex lectularius. *PLoS ONE* **6**, e22082 (2011).
66. Sepil, I. *et al.* Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 17094–17103 (2020).
67. Patnaik, B. K., Mahapatro, N. & Jena, B. S. Ageing in Fishes. *Gerontology* **40**, 113–132 (1994).
68. Zajitschek, F., Zajitschek, S. & Bonduriansky, R. Senescence in wild insects: Key questions and challenges. *Funct Ecol* **34**, 26–37 (2020).
69. Gaillard, J. & Lemaître, J. An integrative view of senescence in nature. *Funct Ecol* **34**, 4–16 (2020).
70. Smith, C. C. Opposing effects of sperm viability and velocity on the outcome of sperm competition. *Behavioral Ecology* **23**, 820–826 (2012).

71. Gage, M. J. G. *et al.* Spermatozoal Traits and Sperm Competition in Atlantic Salmon. *Current Biology* **14**, 44–47 (2004).
72. Wagner, R. H., Helfenstein, F. & Danchin, E. Female choice of young sperm in a genetically monogamous bird. *Proc. R. Soc. Lond. B* **271**, (2004).
73. Harris, W. E. & Uller, T. Reproductive investment when mate quality varies: differential allocation versus reproductive compensation. *Phil. Trans. R. Soc. B* **364**, 1039–1048 (2009).
74. Brooks, R. Can older males deliver the good genes? *Trends in Ecology & Evolution* **16**, 308–313 (2001).
75. O'Dea, R. E. *et al.* Preferred reporting items for systematic reviews and meta-analyses in ecology and evolutionary biology: a PRISMA extension. *Biological Reviews* **96**, 1695–1722 (2021).
76. R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. (2022)
77. Jones, T. M. & Elgar, M. A. The role of male age, sperm age and mating history on fecundity and fertilization success in the hide beetle. *Proc. R. Soc. Lond. B* **271**, 1311–1318 (2004).
78. Pieper, D. & Summann, F. Bielefeld Academic Search Engine (BASE): An end-user oriented institutional repository search service. *Library Hi Tech* **24**, 614–619 (2006).
79. Ouzzani, M., Hammady, H., Fedorowicz, Z. & Elmagarmid, A. Rayyan—a web and mobile app for systematic reviews. *Syst Rev* **5**, 210 (2016).
80. Rathbone, J., Hoffmann, T. & Glasziou, P. Faster title and abstract screening? Evaluating Abstrackr, a semi-automated online screening program for systematic reviewers. *Syst Rev* **4**, 80 (2015).
81. Polanin, J. R. & Snilstveit, B. Converting between effect sizes. *Campbell Systematic Reviews* **12**, 1–13 (2016).
82. Parker, G. A., Ramm, S. A., Lehtonen, J. & Henshaw, J. M. The evolution of gonad expenditure and gonadosomatic index (GSI) in male and female broadcast-spawning invertebrates: GSI in broadcast spawning invertertebrates. *Biol Rev* **93**, 693–753 (2018).
83. Nakagawa, S. & Cuthill, I. C. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biological Reviews* **82**, 591–605 (2007).
84. Viechtbauer, W. Conducting Meta-Analyses in *R* with the **metafor** Package. *J. Stat. Soft.* **36**, (2010).
85. Noble, D. W. A., Lagisz, M., O'dea, R. E. & Nakagawa, S. Nonindependence and sensitivity analyses in ecological and evolutionary meta-analyses. *Mol Ecol* **26**, 2410–2425 (2017).
86. Cinar, O., Nakagawa, S. & Viechtbauer, W. Phylogenetic multilevel meta-analysis: A simulation study on the importance of modelling the phylogeny. *Methods Ecol Evol* **13**, 383–395 (2022).
87. Paradis, E., Claude, J. & Strimmer, K. APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* **20**, 289–290 (2004).
88. Michonneau, F., Brown, J. W. & Winter, D. J. rotl: an R package to interact with the Open Tree of Life data. *Methods Ecol Evol* **7**, 1476–1481 (2016).
89. Hinchliff, C. E. *et al.* Synthesis of phylogeny and taxonomy into a comprehensive tree of life. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 12764–12769 (2015).
90. Higgins, J. P. T. & Thompson, S. G. Quantifying heterogeneity in a meta-analysis. *Statist. Med.* **21**, 1539–1558 (2002).
91. Nakagawa, S. *et al.* orchaRd: An R package for drawing 'orchard'plots (and 'caterpillars' plots) from meta-analyses and meta-regressions with categorical moderators. Preprint at EcoEvoRxiv. https://doi.org/10.32942/X2QC7K (2020).
92. Senior, A. M. *et al.* Heterogeneity in ecological and evolutionary meta-analyses: its magnitude and implications. *Ecology* **97**, 3293–3299 (2016).
93. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models using lme4. doi:10.48550/ARXIV.1406.5823. (2014)
94. Nakagawa, S. *et al.* Methods for testing publication bias in ecological and evolutionary meta-analyses. *Methods Ecol Evol* **13**, 4–21 (2022).
95. Lüpold, S., de Boer, R. A., Evans, J. P., Tomkins, J. L. & Fitzpatrick, J. L. How sperm competition shapes the evolution of testes and sperm: a meta-analysis. *Phil. Trans. R. Soc. B* **375**, 20200064 (2020).
96. Lemaître, J.-F. *et al.* Early-late life trade-offs and the evolution of ageing in the wild. *Proc. R. Soc. B.* **282**, 20150209 (2015).
97. Hechinger, R. F. Mortality affects adaptive allocation to growth and reproduction: field evidence from a guild of body snatchers. *BMC Evol Biol* **10**, 136 (2010).
98. Reznick, D. N., Bryant, M. J., Roff, D., Ghalambor, C. K. & Ghalambor, D. E. Effect of extrinsic mortality on the evolution of senescence in guppies. *Nature* **431**, 1095–1099 (2004).
99. Kelly, C. D. & Jennions, M. D. Sexual selection and sperm quantity: meta-analyses of strategic ejaculation. *Biological Reviews* **86**, 863–884 (2011).
100. Reichard, M. Evolutionary ecology of aging: time to reconcile field and laboratory research. *Ecol Evol* **6**, 2988–3000 (2016).
101. Wondatir Workie, Z., Gibson, J. P. & van der Werf, J. H. J. Analysis of culling reasons and age at culling in Australian dairy cattle. *Anim. Prod. Sci.* **61**, 680 (2021).
102. Bennett, C. E. *et al.* The broiler chicken as a signal of a human reconfigured biosphere. *R. Soc. open sci.* **5**, 180325 (2018).
103. Nussey, D. H., Coulson, T., Festa-Bianchet, M. & Gaillard, J.-M. Measuring senescence in wild animal populations: towards a longitudinal approach. *Functional Ecology* **22**, 393–406 (2008).
104. Cooper, E. B. & Kruuk, L. E. B. Ageing with a silver-spoon: A meta-analysis of the effect of developmental environment on senescence. *Evolution Letters* **2**, 460–471 (2018).

105. Keller, L. F., Reid, J. M. & Arcese, P. Testing evolutionary models of senescence in a natural population: age and inbreeding effects on fitness components in song sparrows. *Proc. R. Soc. B.* **275**, 597–604 (2008).
106. Yousefzadeh, M. J. *et al.* Mouse Models of Accelerated Cellular Senescence. *Methods Mol Biol* **1896**, 203–230 (2019).

Supplementary information

Supplementary notes 1: Search string
We first conducted a scoping search on Google Scholar. Our scoping search was done using the keywords "male age sperm -human", and we used the first 48 papers (i.e. all papers from the first 5 pages out of 11800 pages of the search) to build a word cloud to discern the most common words used in these studies. We used the most commonly occurring relevant words from this word cloud to create keywords for our search string.
The table below shows the most frequent words from our scoping search collected from 48 relevant abstracts generated through a word cloud. Words used in our search string are highlighted in bold and italics. The selection of words was made such that they were not specific to a single species, nor biologically broad.

frequency	word	frequency	word
305	***sperm***	25	size
191	***male***	24	fluid
151	***age***	24	transfer
104	mating	22	fitness
75	reproductive	21	offspring
66	female	21	proteins
60	females	20	competitive
58	success	20	ejaculates
53	competition	20	fertilization
47	***ejaculate***	20	mated
45	***old***	20	selection
45	***young***	20	transferred
41	quality	19	history
36	traits	19	Sperm
33	older	18	viability
32	seminal	17	associated
31	species	17	rate
30	***ageing***	16	Drosophila
29	fertility	16	paternity
29	sexual	16	production
28	***senescence***	**16**	***semen***
27	mate	**14**	***aging***
25	ability	14	cells

Distinct Boolean characters used for the search strings in each database.

Search string used for SCOPUS:

"(TITLE-ABS-KEY (male) AND TITLE-ABS-KEY (age) OR TITLE-ABS-KEY (ageing) OR TITLE-ABS-KEY (aging) OR TITLE-ABS-KEY (senesc*) OR TITLE-ABS-KEY (old) OR TITLE-ABS-KEY (young) AND TITLE-ABS-KEY (sperm) OR TITLE-ABS-

KEY (ejaculate) OR TITLE-ABS-KEY (semen) AND NOT TITLE-ABS-KEY (human) OR TITLE-ABS-KEY (men)) "

<u>Search string used for Web of Science:</u>

"AB= (male AND (age OR ageing OR senesc* OR aging OR old OR young) AND (sperm OR ejaculate OR semen) NOT (human OR men)) OR TI= (male AND (age OR ageing OR senesc* OR aging OR old OR young) AND (sperm OR ejaculate OR semen) NOT (human OR men))"

<u>Search string used for BASE:</u>

"male age sperm"

Supplementary notes 2: Repeatability

Screening process

To ensure repeatability of the screening process, two analysts screened abstracts and full-texts independently and checked for agreement on included versus excluded studies. Analyst 1 screened all abstracts to check the suitability of each study (n = 9683) while analyst 2 checked for repeatability by screening ~50% of abstracts (n = 4918). Analyst 1 and 2 agreed on the inclusion or exclusion (i.e. suitability) of 91% of the studies when screening abstracts (Kappa = 0.56; Kappa calculates inter-rater reliability for qualitative items, Koricheva et al, 2013). Analyst 1 then screened all full texts that passed the abstract screening stage (n = 1003 studies from published sources and four from unpublished sources) to determine whether a study was suitable to be included in the meta-analysis, while analyst 2 screened ~10% of full texts to test for repeatability (n = 100). Analyst 1 and 2 agreed on the inclusion or exclusion of 98% of the studies when screening full-texts (Kappa = 0.96).

Data extraction

To assure repeatability of data extraction, two analysts checked data obtained from ~5% of papers (*i.e.* 19 studies and 75 effect sizes). To do this, analyst 1 and 2 first independently extracted data and calculated effect sizes (Fisher's z-transformed correlation coefficient) for each of the 75 rows of the data collected. Then a coefficient of determination between the effect sizes obtained by analyst 1 and 2 was calculated ($R^2 = 0.96$, $P<0.001$), indicating strong repeatability.

Supplementary notes 3: Definition of adult

Groups that were described as "[pre-] pubertal", "adolescent", "juvenile", or "immature" in the study were not considered as adults and thus not included in the analysis, because we were only interested in age-dependent changes in adult individuals. For arthropods, we included all post-eclosion/last-moult ages in the analysis because this is when arthropods are considered adults[1]. If all age groups in a study had immature (non-adult) males, we excluded these studies. On the other hand, if at least two age groups of males were adults, we included the study, and we used only the data from adult males.

Supplementary notes 4: calculation of SD

We collected data on means, SD/SE, and sample sizes of males in each age group from: text in the Results section, figures using WebPlotDigitizer[2] and MetaDigitize[3], the supplementary information or raw data provided with the paper, a data repository, or by directly contacting the authors of the paper, in that order. We converted standard errors to standard deviations (SD) using the formula[4] SD = SE* sqrt (N). When medians and inter-quartile ranges were reported, we converted these to SD[5].

Supplementary notes 5: Ejaculate traits and reproductive outcomes definitions

For all studies, we collected data on ejaculate traits as reported by the original paper. When a study reported data for multiple traits, data on all traits were collected. Although, when a study reported data on a whole trait (e.g. % of total motile sperm; % sperm with morphological defects) as well as sub traits (e.g. % progressively motile sperm, % sperm with mid-piece defects), only data from the whole trait was recorded. Due to differences in terminology when describing similar traits between different studies, we created a broad category to describe different types of traits. The categorization of these traits is described below.

Ejaculate traits

In meta-analysis	In paper
Concentration	Sperm density, concentration, count per mL/ µL, spermatocrit
DNA damage	Sperm chromatin damage, sperm DNA damage, sperm chromatin instability, sperm DNA fragmentation, sperm chromatin structure damage
Ejaculate size	Spermatophore size, ejaculate mass, ejaculate volume, area of seminal vesicle filled with ejaculate
Corrected quantity	Sperm concentration or number as proportion of body mass/testis mass/epididymis mass
Mitochondria	Sperm ATP, sperm metabolic activity, sperm mitochondrial function, sperm mitochondrial activity, sperm mitochondrial membrane potential
Morphology	Normal/abnormal sperm morphology, tail/head/midpiece morphological defects, cytoplasmic droplets on sperm
Motility	Percent progressive motility, percent non-progressive motility, percent total motility, sperm vigor, sperm mass motility, motility (on a numerical subjective scale)
Number of sperm	Number of apyrene sperm, number of eupyrene sperm, number/count of sperm, number of spermatophores, number of sperm bundles
Oxidative stress	Reactive oxygen species in sperm, sperm oxidant or antioxidant levels, sperm lipid peroxidation, sperm glutathione peroxidase, sperm superoxidase dismutase
Sperm length	Total sperm length, sperm tail length
Telomere	Sperm telomere length

Viability	Sperm acrosome integrity, sperm viability, % sperm alive/dead, sperm vitality, sperm membrane integrity
Velocity	Average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL) (in that order of preference, when more than one reported)

Reproductive outcomes

In meta-analysis	In paper
Fertilization success	Percent eggs fertilized, Percent eggs sired under sperm competition, fertilization rate, fertilization success, PVL hole number, paternity share
Number/viability/quality of egg/offspring	Egg hatchability, egg mass, egg viability, male or female fecundity, hatching success, number of eggs laid, female lifetime reproductive success, number of offspring, number of fetuses, offspring developmental rate, offspring survival, offspring body mass/ size

Supplementary notes 6: Collection of data on sperm competition levels, adult lifespan and age of sexual maturity for different species.
We recorded the level of sperm competition faced by a species by collecting data on the Gonadosomatic index (i.e. testis weight as a percentage of body weight), which has been shown to be a reliable predictor of sperm competition. This was done by searching for "testes weight" or "gonadosomatic index" of each species in our dataset on google scholar as well as collecting data from comparative studies on this topic (OSF https://osf.io/dk8sq/ for raw data). If the study did not report the maximum adult lifespan or age of sexual maturity/adulthood of the species/population being studied, these were collected from other sources, and then used to calculate the percentage of maximum adult lifespan sampled for a given species in the study. Data on maximum adult lifespan of a given species, as well as age of sexual maturity were then used to calculate the proportion of maximum adult lifespan sampled for a given species in a study as follows:

$$Proportion\ of\ maximum\ adult\ lifespan\ sampled = \frac{\left((Max\ age\ sampled - age\ of\ maturity) - (Min\ age\ sampled - age\ of\ maturity)\right)}{(Maximum\ lifespan\ of\ species - age\ of\ adulthood)} * 100\%$$

To collect data on maximum lifespans (MAL) and age of sexual maturity/adulthood (AoSM) on a given species or population, we first prioritized using the data on lifespan reported directly in the study. If the study did not report MAL or AoSM, we collected this data from multiple sources. These included large publicly available databases such as Animal Diversity Web (https://animaldiversity.org/), AnAge[6], and Pantheria[7] as well as from data in published articles that contained supplementary datasets on MAL and AoSM estimates of species included in our study. There were very few databases that contained MAL and AoSM for animals such as insects. As a result, peer reviewed, published articles in scientific journals were used for data on MAL and AoSM for such species. When values were reported as a survival curve, the maximum age of the curve where individuals were alive was used. When data was not available from any of these sources, we also contacted the corresponding authors of the papers in our meta-analysis for maximum lifespan estimates on the species. We prioritized collecting data for MAL and AoSM on males. When male-specific data was not available, species-specific data was collected (this was the case for many vertebrates whose data were obtained from large datasets/databases). We never used female-specific data for any species. If a species had data on MAL and AoSM from multiple sources, these estimates were averaged, and the average was then used as the maximum lifespan of the species. This averaging was done so that exceptionally large values that might be unusual for a species (e.g. red junglefowl maximum lifespan is reported as 30 years on AnAge, which is quite unusual), did not bias our dataset. Additionally, when data from multiple sources was available, we ensured that we used data from males which most closely matched the rearing conditions and morphs of the species/study males in our meta-analysis. For insects, age of adulthood was considered as the age of eclosion/ last moult. Note that for species which had domesticated as well as wild-derived populations in our dataset (e.g. red junglefowl vs chicken, both of which were called *Gallus gallus* in our phylogeny), we collected their lifespan and sexual maturity data separately. We tested the association between maximum and average lifespans collected for each species. This was high (R sq. = 0.85). Thus, maximum lifespan was used because it was available for a greater number of species than average lifespan, and less affected by juvenile mortality.

Supplementary notes 7: Moderators tested in our meta-regression models and their ranges/levels

Moderator	Levels
Proportion maximum adult lifespan sampled	0 to 100%
Ejaculate collection method	Male has control: males mated to female and female dissected/weighed post-insemination, male masturbated via dummy female, natural spawning Male does not have control: Catheter, Males dissected, electroejaculation, males massaged with abdominal pressure
Taxonomic Class	Reptilia, Prosomapoda, Monogononta, Mammalia, Malacostraca, Insecta, Hexanauplia, Gastropoda, Clitellata, Chromadorea, Aves, Arachnida, Amphibia, Actinopterygii
Population (i.e. Setting)	Laboratory, Domestic, Captive, Wild
Method of age estimation	Direct/ Indirect (i.e. inferred from body condition)
Trait	Concentration, DNA damage, Ejaculate size, Corrected quantity, Mitochondria, Morphology, Motility, Number of sperm, Oxidative stress, Length, Velocity, Viability
Longitudinal sampling	Yes/Semi (when only a subset of males were measured repeatedly)/No
Experimental	Yes: manipulated something in addition to male age, or assigned males to specific age groups at the start of the experiment to a target age class No: observational sampling of the available age class distributions opportunistically
Gonadosomatic index	0 to 100 %
"Unnatural" manipulations	Yes/No
Cold storage of ejaculates (i.e. stored at <5°C)	Yes /No (only for sperm motility, viability, and velocity)

Supplementary notes 8: Definitions of population type

Captive: Managed study population which lives in an enclosed environment, and used in a captive breeding program, captive breeding centre, wildlife conservation research centre, or zoo. The webpage of the reported institutes where the study was conducted was checked when unsure, to ascertain whether it fell into this category.

Lab: Study population maintained under or adapted to lab conditions (for at least their adult life), living in an enclosed environment (even if collected in the wild pre-adulthood), but not used for a captive breeding program.

Domestic: Study population descended from a line of deliberately artificially selected individuals (inferred), or raised on commercial farms whose purpose is using animals for direct human consumption (e.g. as food, clothing, protection).

Wild: Was born in the wild/lives in natural unenclosed environments and was caught from the wild (post-adulthood) for the study.

Supplementary notes 9: "Unnatural" manipulations

"Unnatural" manipulations were defined as conditions experienced by males outside their physiological range (as defined in the study) and as conditions not typically experienced by healthy individuals (in the study populations). These manipulations also had a well-defined control in the study. These are as follows: pharmacological interventions such as toxins, chemicals, or medicines (Control: no pharmacological intervention); temperature manipulations (Control: standard temperature as defined in the study); radiation (Control: no radiation); genetic mutations/mutant lines with specific knocked out genes (Control: Wild type, or control lines as defined in the study); 24 hour dark or 24 hour light circadian durations (Control: 12:12 hour light durations); infection/disease (Control: no infection or disease); inbreeding (Control: Outbreeding); dietary or protein restriction (Control: Standard diet as defined in the study).

Other types of manipulations, such as sperm storage durations, mating history of males, seasons, male social status, or female age were not considered to be outside the typical range of conditions experienced by males, nor did they have an easy to define control, thus were not defined as "unnatural".

Supplementary notes 10: Calculation of effect sizes

We calculated Fisher's z transformed correlation coefficient (Zr) values from correlation coefficients (r) using the formula[4]: Zr = 0.5*(log(1+ r) - log(1-r)). Because r values were rarely ever directly reported in studies, we had to calculate these indirectly. For studies with two age groups, we calculated correlation coefficients (r) using standardized mean differences (SMD). When there were more than two age groups, we calculated correlation coefficients using a simulation. For studies where only test statistics were reported, we calculated correlation coefficients using test-specific formulae

A. Two-age groups

To calculate correlation coefficients from studies which reported comparisons between two age groups, we first calculated a standardized mean difference (SMD). SMD here, was calculated using the package *metafor* in R[8] with the function *escalc.* SMD provides the true strength of an effect by dividing the difference in means between two groups, by their pooled standard deviation. We used the following formula:

SMD = $\frac{(Mean\ (old) - Mean\ (young))}{S\ pooled}$

$$S_{pooled} = \sqrt{\frac{(n_{old} - 1)S_{old}^2 + (n_{young} - 1)S_{young}^2}{n_{old} + n_{young} - 2}}$$

where Mean (o) and (y) and means of the old and young age groups respectively, and S pooled is the pooled standard deviation. These SMD values were then converted to a correlation coefficient using the function *convert_d2r* in the package *meta*[9].

B. Multiple (>2) age groups

To calculate effect sizes from studies which reported means and standard deviations from more than two age groups, we used a simulation (with 1000 iterations). This simulation resulted in a correlation coefficient between the age of males and their means at each age, while weighting the means by their standard deviations. To test for consistency between effect size outcomes from the simulation and the outcomes from SMD, we additionally calculated correlation coefficient for outcomes with only two-groups using the simulation. There was a very strong agreement between r values obtained from these two methods (i.e. SMD and simulation) (R sq.> 0.95).

C. Test statistics[10,11]

1. For converting "t" from independent t-test into *r*

$$r = \frac{t}{\sqrt{(t^2 + degrees\ of\ freedom)}}$$

2. For converting F from ANOVA or ANCOVA with 1 degree of freedom to *r*

$$r = \sqrt{(\frac{F}{F + N - 2})}$$

Where N is the sample size of unique number of males

3. For converting spearman's rho to *r*

$$r = 2 * \sin((\pi * \rho)/6)$$

4. From Mann Whitney-U to *r*

$$r = \frac{1 - (2 * U)}{n1 * n2}$$

Where n1 and n2 are sample sizes of the younger and older age groups respectively

5. From z-score to *r*

$$r = \frac{z}{\sqrt{N}}$$

Where N is the sample size of unique number of males

6. For Converting T from Seigel's T test, and converting P values from Mann Whitney U test, to *r*, the Campbell Collaboration website was used (https://www.campbellcollaboration.org/research-resources/effect-size-calculator.html)
7. For converting R squared and adjusted R squared values to *r*

$$r = \sqrt{R}^2$$

8. For converting Chisq. values from Chisq. test with one degree of freedom, to *r*

$$r = \sqrt{(\frac{Chi\ sq.}{N})}$$

9. For converting Pearson's or Spearman's correlation coefficient (r) to *r*

$$r = \text{r}$$

Multiplier and signs

If an increase in a trait signified a deleterious effect with increasing age, for example, an increase in sperm abnormal morphology, or sperm DNA damage, we assigned it a multiplier of "-1", whereas if increase in a trait suggested improvement with age, we assigned it a positive multiplier, i.e. "+1". Similarly, when a test statistic was reported (e.g. R sq., correlation coefficient. F values from ANOVA), where older males had worse sperm or ejaculates than younger males, we assigned it a negative multiplier of "-1". Conversely, if older males had better sperm or ejaculates than younger males, we assigned it a multiplier of "+1". Thus, for all the effect sizes in our models, a negative sign indicated reproductive senescence with increasing age, while a positive sign indicated reproductive improvement with increasing age.

Comparing calculation methods

To ensure that the three different effect size calculation methods did not affect the overall outcome in our meta-analysis, we compared the meta-analytical mean obtained from each method (*i.e.* SMD, simulation, and test-statistics). These did not differ from each other (see Supplementary Fig. 24), hence we analysed effect sizes calculated from SMD, simulation, and test-statistics together in subsequent models.

Supplementary notes 11: Quadratic effects of age
Our meta-analyses used an effect size (Zr), which assumes a linear relationship between independent (age) and dependent (ejaculate traits) variables[12]. Thus, to test whether the effects of advancing male age on ejaculate traits are curvilinear in shape, we created linear

mixed models (LMM) in the package *lme4*[13] and *lmerTest*[14], because meta-regression models, to our knowledge, cannot model non-linear effect sizes.
Our LMM was conducted on three traits (percent motile, percent morphologically normal, and percent viable sperm), which were measured on the same scale across studies and species. These three traits were thus already "standardized" thus could be compared across studies the way an effect size is. We standardized each of the different ages at which males were sampled at, as the proportion of maximum adult lifespan of the species (independent variable). We standardized the traits (response variable) by calculating the proportion of: morphologically normal sperm (85 studies), viable sperm (137 studies), and motile sperm (81 studies), and analyzed them in three separate models.
We included linear and quadratic effects of [standardized] age (covariates), with population type (i.e, lab, captive, domestic, wild) and taxonomic class as fixed effects. We also used the sample size of males within each age class (log transformed) as weights in the LMM. We included effect size ID, cohort ID, and study ID as random effects. Our models met assumptions of homoscedasticity and normality of residuals, checked using the *stats* package[15].

We found significant quadratic effects of [standardised] male age on [standardised] ejaculate traits (Supplementary Fig. 7). Specifically, we found significant quadratic effects for age-dependent changes in: percent morphologically normal sperm ($t = -3.023$, $P = 0.003$, $DF = 346$); percent motile sperm ($t = 2.296$, $P = 0.022$, $DF = 589$); and percent viable sperm ($t = -3.909$, $P < 0.001$, $DF = 437$).

Supplementary notes 12: Sensitivity analysis and publication bias

Sensitivity analysis

To test for robustness of our full and null models, we created a variance-covariance matrix (VCV) of correlation values between effect size ID and cohort ID that replaced the variance argument[16] in both, null and full models. The results obtained for the null model or full model were not different between the model that used the VCV matrix versus the one that did not, thus in the main text, we present the model without the VCV matrix throughout (See "metaA HTML" file for model outputs).

Publication bias

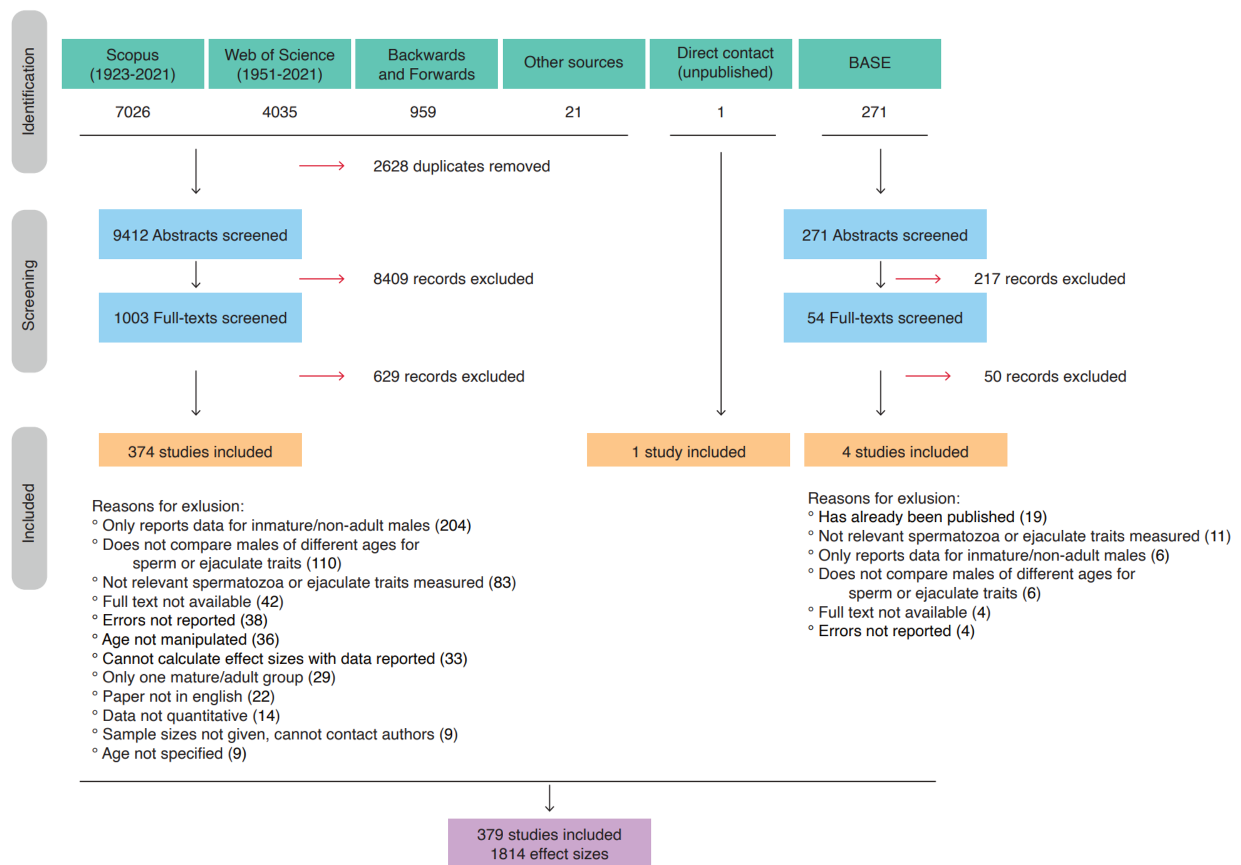
We tested for publication bias using various methods[16,17]. We first visually evaluated symmetry in a funnel plot with effect sizes (Zr) on the X axis, and the inverse of the standard error (1/SE) of the effect size (i.e. precision) on the Y axis (note that for Zr, precision is proportional to sample sizes). Second, we conducted a multi-level meta-regression to evaluate whether the size of a study and its year of publication influences effect sizes (i.e. small study bias and time-lag bias) by including both, standard error of effect sizes and year of publication as moderators, and effect size ID, cohort ID, study ID, species name, and phylogeny as random effects. Third, we created a funnel plot with the average of effect sizes from each study (X axis) against precision (Y axis), and tested for funnel asymmetry using a trim-and-fill method[16]. Finally, we created a selection model to test whether the probability of selecting a study depended on the significance of its effect size[18].

We found little evidence for publication bias. Visual inspection of funnel plots did not indicate any asymmetrical distribution of effect sizes around zero, indicating no evidence for publication bias (Supplementary Fig. 17 below, test for funnel plot asymmetry: $P = 0.992$). Our multi-level meta-regression publication bias test indicated no evidence for a small study bias ($t = 7.21$, $P = 0.721$, DF = 1810; Supplementary Fig. 18) but significant evidence for a time-lag bias, with more recent studies being more likely to show reproductive senescence ($t = -2.34$, $P = 0.019$, DF = 1810, Supplementary Fig. 18). We found no evidence for missing studies (missing studies on right= 0, SE = 10.94) in our trim-and-fill model that used one averaged effect size from each study ($n = k = 379$). Finally, we did not find a bias toward more significant effect sizes in our selection model (Supplementary Fig. 19).

Supplementary references

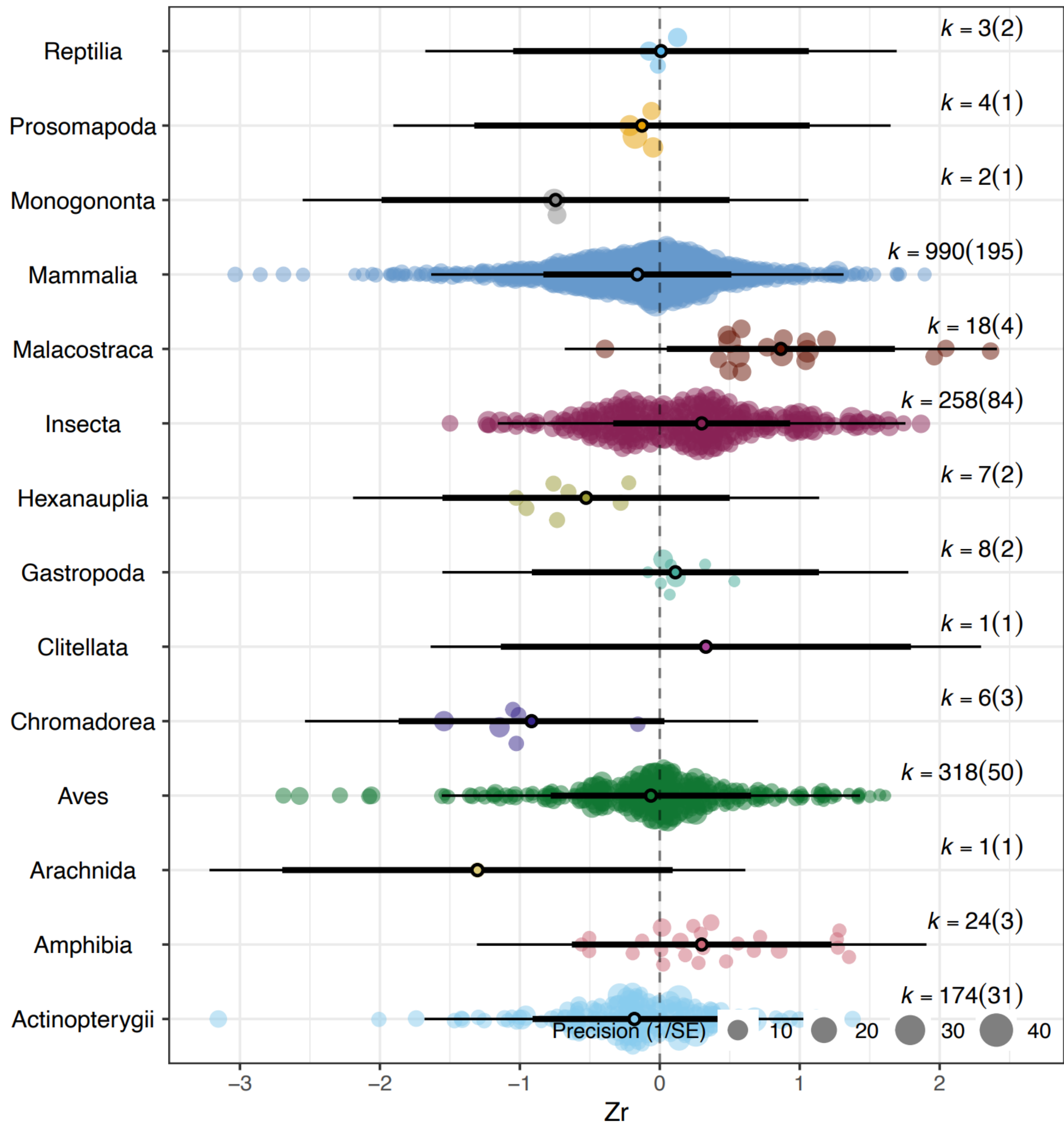
1. Rolff, J., Johnston, P.R., Reynolds, S., 2019. Complete metamorphosis of insects. Phil. Trans. R. Soc. B 374, 20190063. https://doi.org/10.1098/rstb.2019.0063
2. Rohtagi, A., 2014. WebPlotDigitizer user manual version 3.4. URL http://arohatgi. info/WebPlotDigitizer/app.
3. Pick, J.L., Nakagawa, S., Noble, D.W.A., 2019. Reproducible, flexible and high-throughput data extraction from primary literature: The METADIGITISE R package. Methods Ecol Evol 10, 426–431. https://doi.org/10.1111/2041-210X.13118
4. Nakagawa, S., Cuthill, I.C., 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biological Reviews 82, 591–605. https://doi.org/10.1111/j.1469-185X.2007.00027.x
5. Wan, X., Wang, W., Liu, J., Tong, T., 2014. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. BMC Med Res Methodol 14, 135. https://doi.org/10.1186/1471-2288-14-135
6. De Magalhães, J.P., Costa, J., 2009. A database of vertebrate longevity records and their relation to other life-history traits. Journal of Evolutionary Biology 22, 1770–1774. https://doi.org/10.1111/j.1420-9101.2009.01783.x
7. Jones, K.E., Bielby, J., Cardillo, M., Fritz, S.A., O'Dell, J., Orme, C.D.L., Safi, K., Sechrest, W., Boakes, E.H., Carbone, C., Connolly, C., Cutts, M.J., Foster, J.K., Grenyer, R., Habib, M., Plaster, C.A., Price, S.A., Rigby, E.A., Rist, J., Teacher, A., Bininda-Emonds, O.R.P., Gittleman, J.L., Mace, G.M., Purvis, A., 2009. PanTHERIA: a species-level database of life history, ecology, and geography of extant and recently extinct mammals: Ecological Archives E090-184. Ecology 90, 2648–2648. https://doi.org/10.1890/08-1494.1
8. Viechtbauer, W., 2010. Conducting Meta-Analyses in *R* with the metafor Package. J. Stat. Soft. 36. https://doi.org/10.18637/jss.v036.i03
9. Schwarzer, G. and Schwarzer, M.G., 2012. Package 'meta'. *The R foundation for statistical computing*, *9*, p.27.
10. Koricheva, J., Gurevitch, J., Mengersen, K. (Eds.), 2013. Handbook of meta-analysis in ecology and evolution. Princeton University Press, Princeton.
11. Polanin, J.R., Snilstveit, B., 2016. Converting between effect sizes. Campbell Systematic Reviews 12, 1–13. https://doi.org/10.4073/cmpn.2016.3
12. Baker, W.L., Michael White, C., Cappelleri, J.C., Kluger, J., Coleman, C.I., From the Health Outcomes, Policy, and Economics (HOPE) Collaborative Group, 2009. Understanding heterogeneity in meta-analysis: the role of meta-regression. International Journal of Clinical Practice 63, 1426–1434. https://doi.org/10.1111/j.1742-1241.2009.02168.x
13. Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting Linear Mixed-Effects Models using lme4. https://doi.org/10.48550/ARXIV.1406.5823
14. Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest Package: Tests in Linear Mixed Effects Models. J. Stat. Soft. 82. https://doi.org/10.18637/jss.v082.i13
15. R Development Core Team. (2022) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
16. Nakagawa, S., Lagisz, M., Jennions, M.D., Koricheva, J., Noble, D.W.A., Parker, T.H., Sánchez-Tójar, A., Yang, Y., O'Dea, R.E., 2022. Methods for testing publication bias in ecological and evolutionary meta-analyses. Methods Ecol Evol 13, 4–21. https://doi.org/10.1111/2041-210X.13724
17. Nakagawa, S., Santos, E.S.A., 2012. Methodological issues and advances in biological meta-analysis. Evol Ecol 26, 1253–1274. https://doi.org/10.1007/s10682-012-9555-5
18. Marks-Anglin, A., Chen, Y., 2020. A historical review of publication bias. Res Syn Meth 11, 725–742. https://doi.org/10.1002/jrsm.1452

Supplementary figures

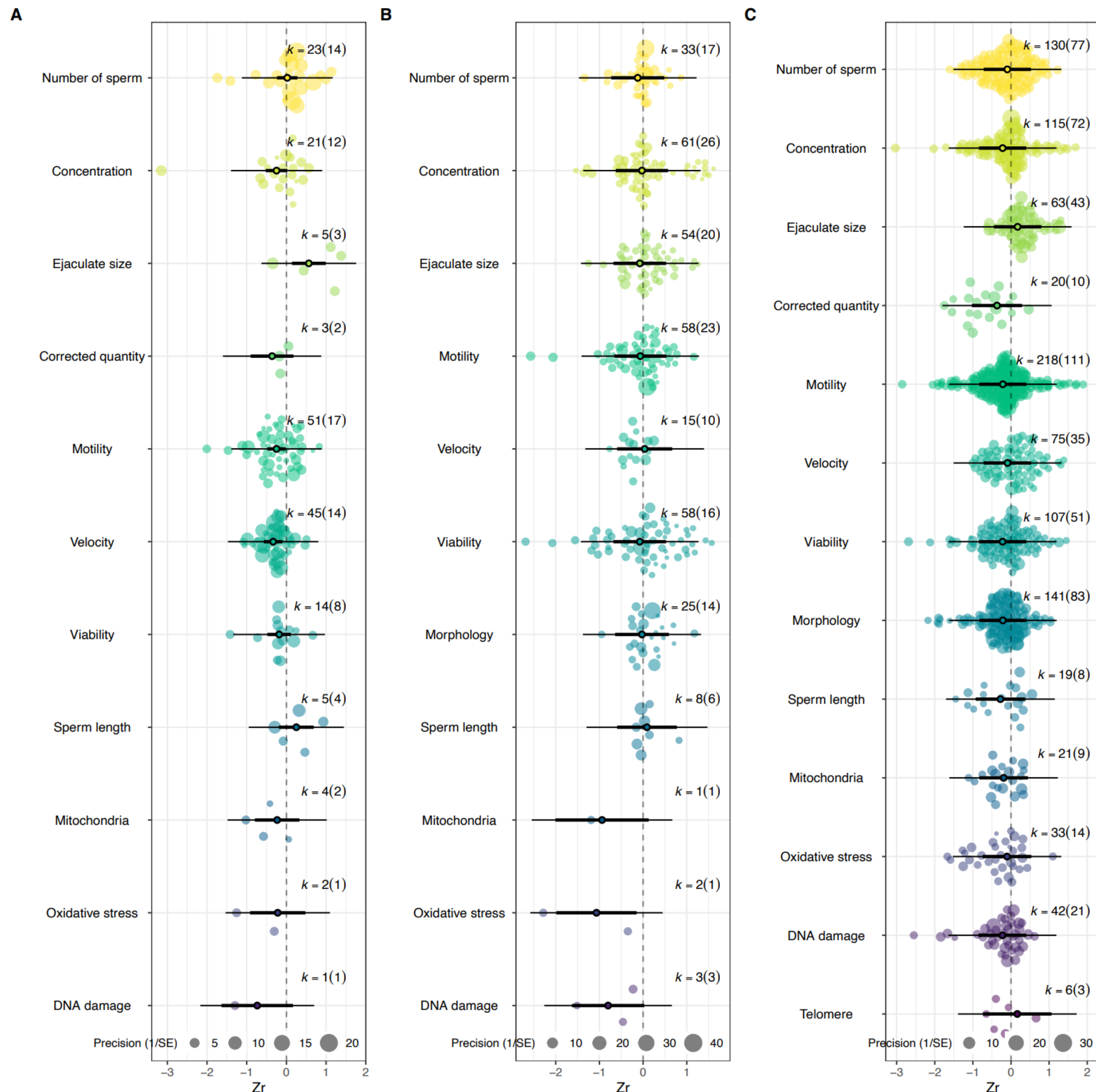


Supplementary Fig.1: PRISMA diagram describing the search results in different search engines and the different steps of selecting articles for inclusion in the meta-analysis. Depicted are the number of studies excluded at each stage and then those extracted, screened, and included in the meta-analysis.

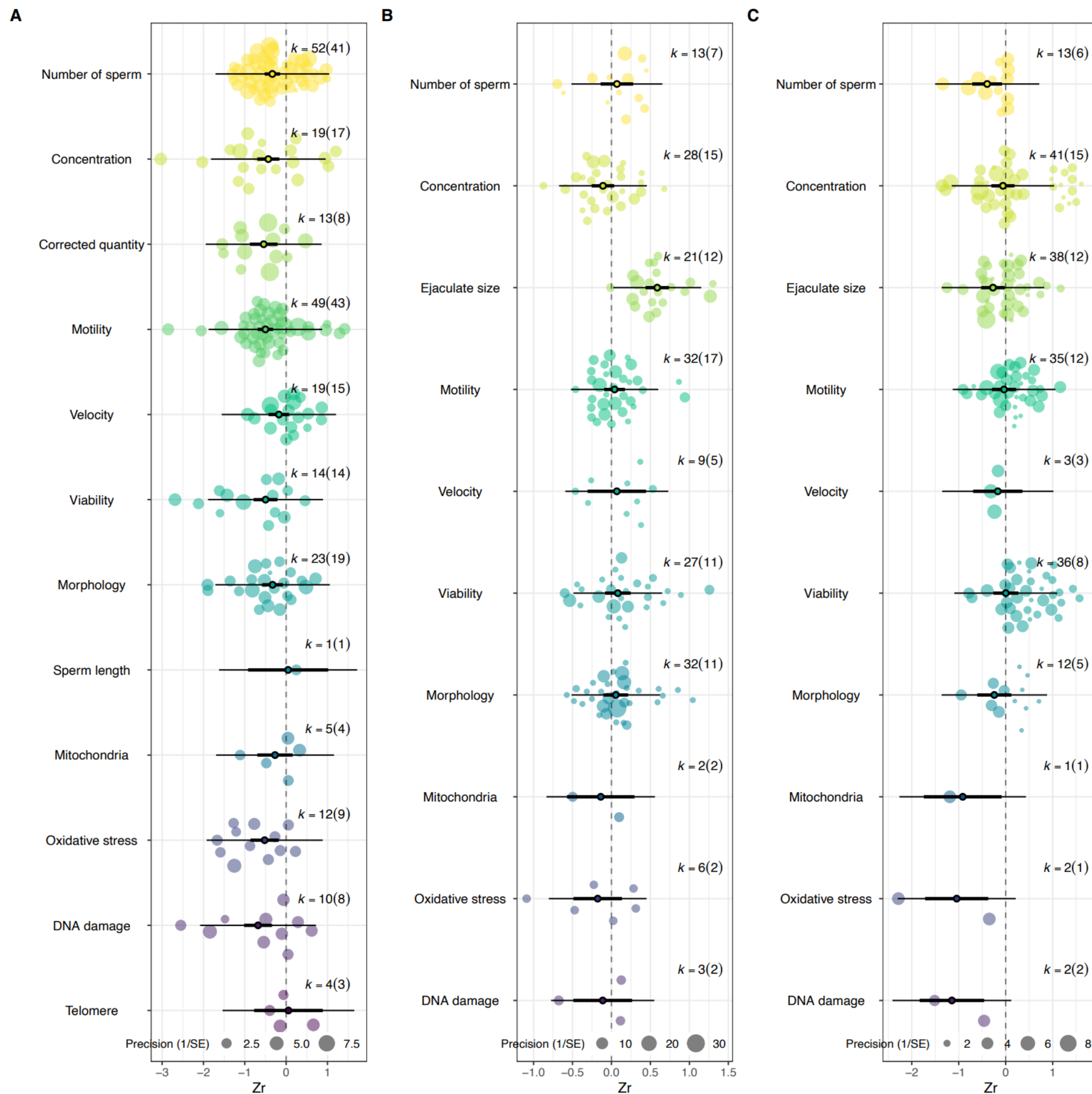
Supplementary Fig.2: Phylogenetic relatedness explained significant variance in our data (I^2 = 35.40%). Phylogenetic tree of all species (157) included in our meta-analysis, along with the number (N) of studies and effect sizes represented by each species. Overall mean effect size for each species showed as Fisher's *z*-transformed correlation coefficient (Zr) with negative values representing senescence with increasing age and positive values representing improvement in ejaculates with increasing age. Species icons from PhyloPic www.phylopic.org, with all associated images under the CC0 1.0 or PDM 1.0 copyright.



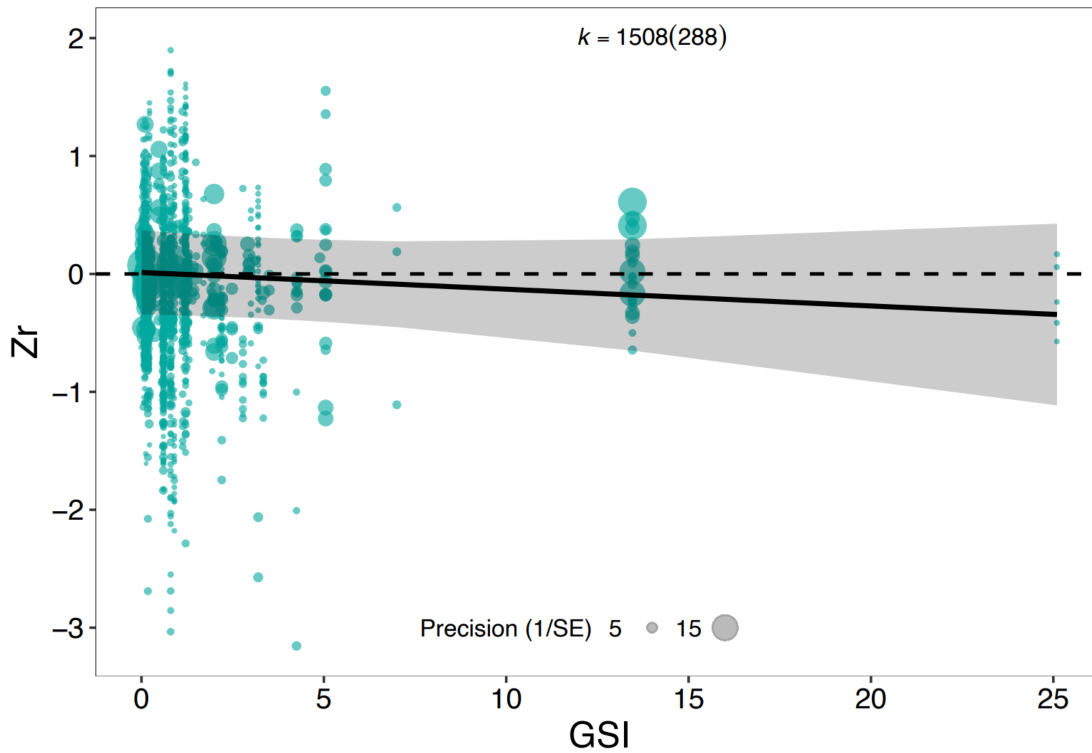
Supplementary Fig.3: Effect of male age on ejaculates for each class. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



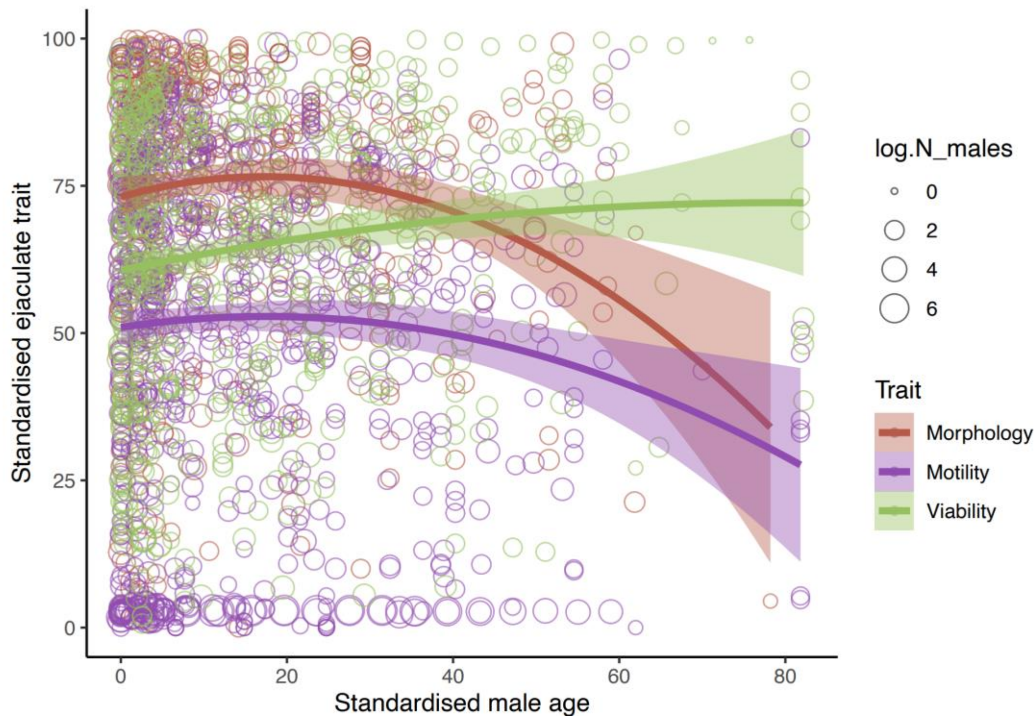
Supplementary Fig.4: Effect of advancing male age on various ejaculate traits for A. Fish, B. Birds, C. Mammals. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



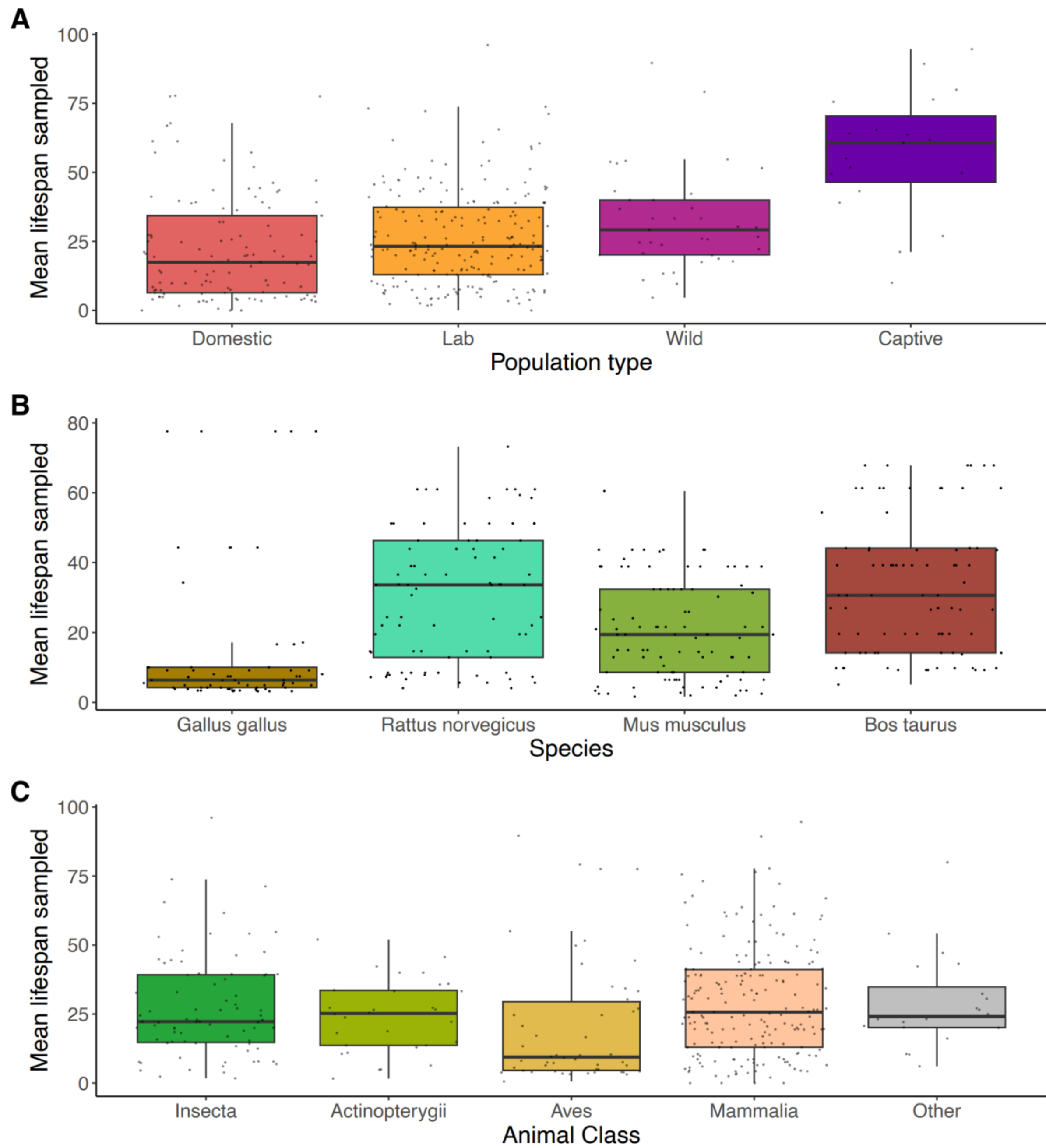
Supplementary Fig.5: Effect of advancing male age on various ejaculate traits for A. Rodents (*Mus musculus* and *Rattus norvegicus*) that did not go through any manipulation. B. Bulls (*Bos taurus*), C. Chickens (*Gallus gallus*). The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



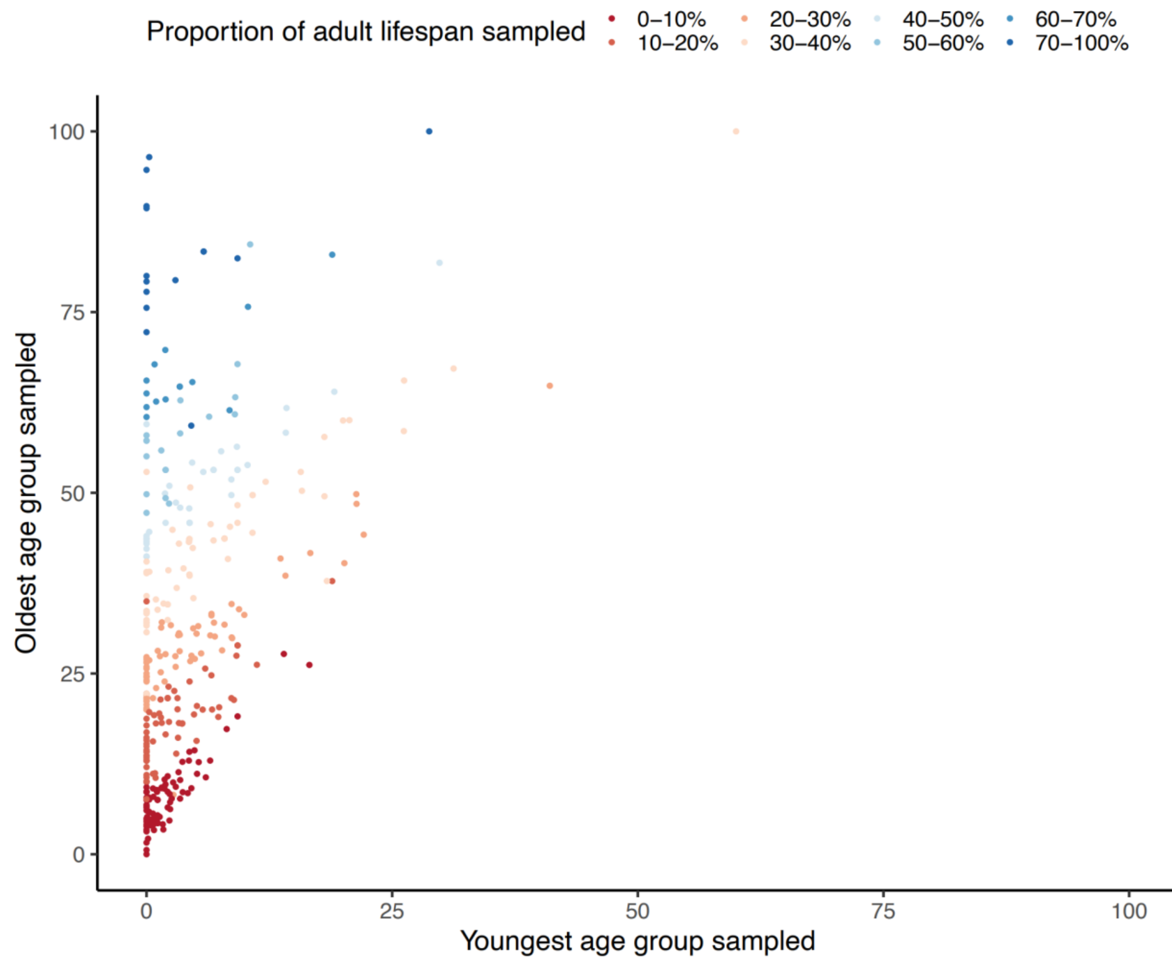
Supplementary Fig.6: Relationship between gonadosomatic index (GSI) and effect sizes for ejaculate senescence. Grey lines indicate 95% C.I.



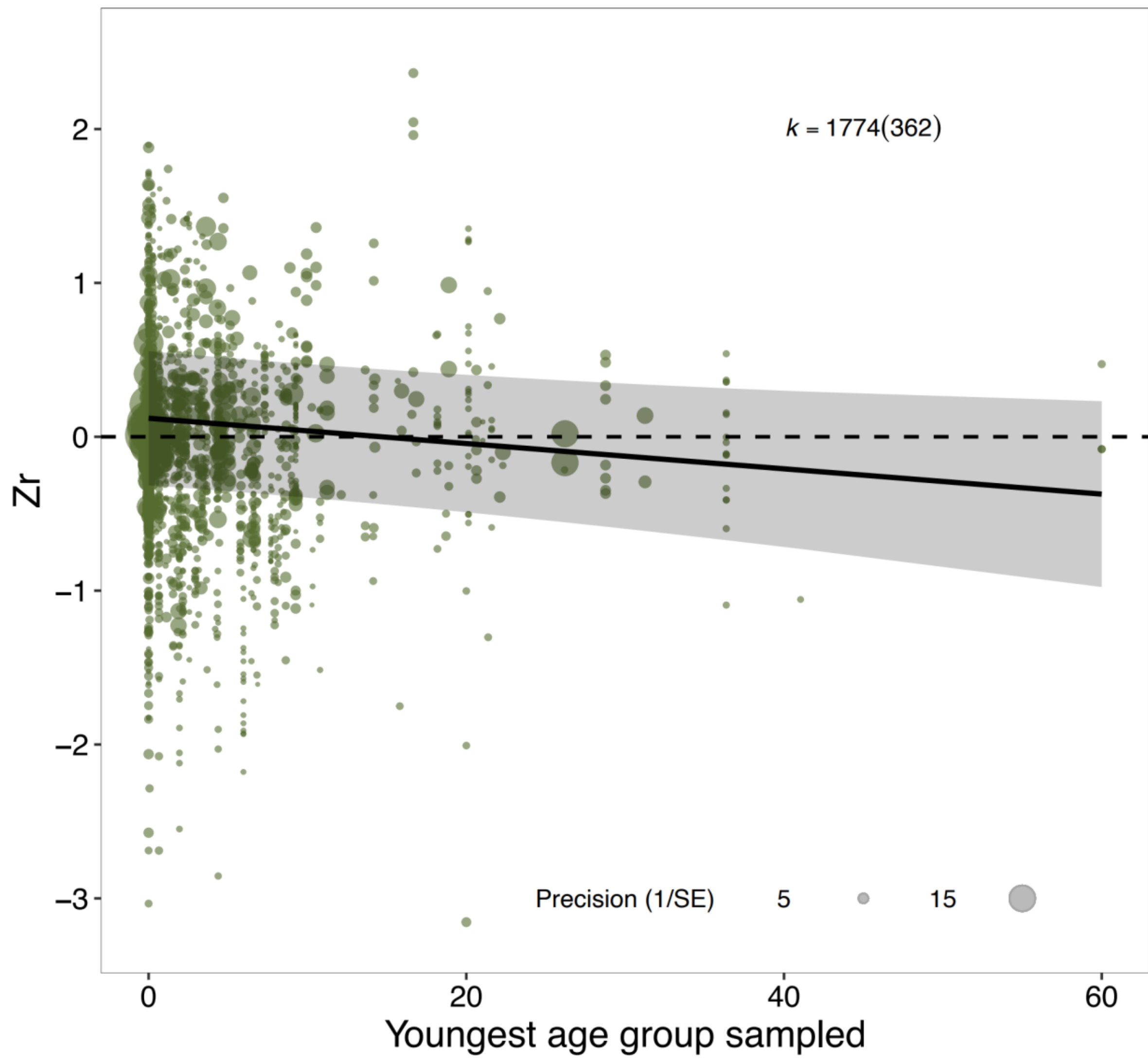
Supplementary Fig.7: Effects of standardised male age (X axis) on standardised ejaculate trait values (Y axis). These are separately shown for % morphologically normal sperm, % motile sperm, and % viable sperm). Standardised male age was calculated as the proportion of maximum adult lifespan represented by the specific age class. Standardised trait value was calculated as percentage of morphologically normal sperm (N= 85 studies, k= 153 effect sizes), percentage of motile sperm (N= 137 studies, k= 294 effect sizes), or percentage of viable sperm (N= 81 studies, k= 193 effect sizes). Shaded lines indicate 95% C.I.



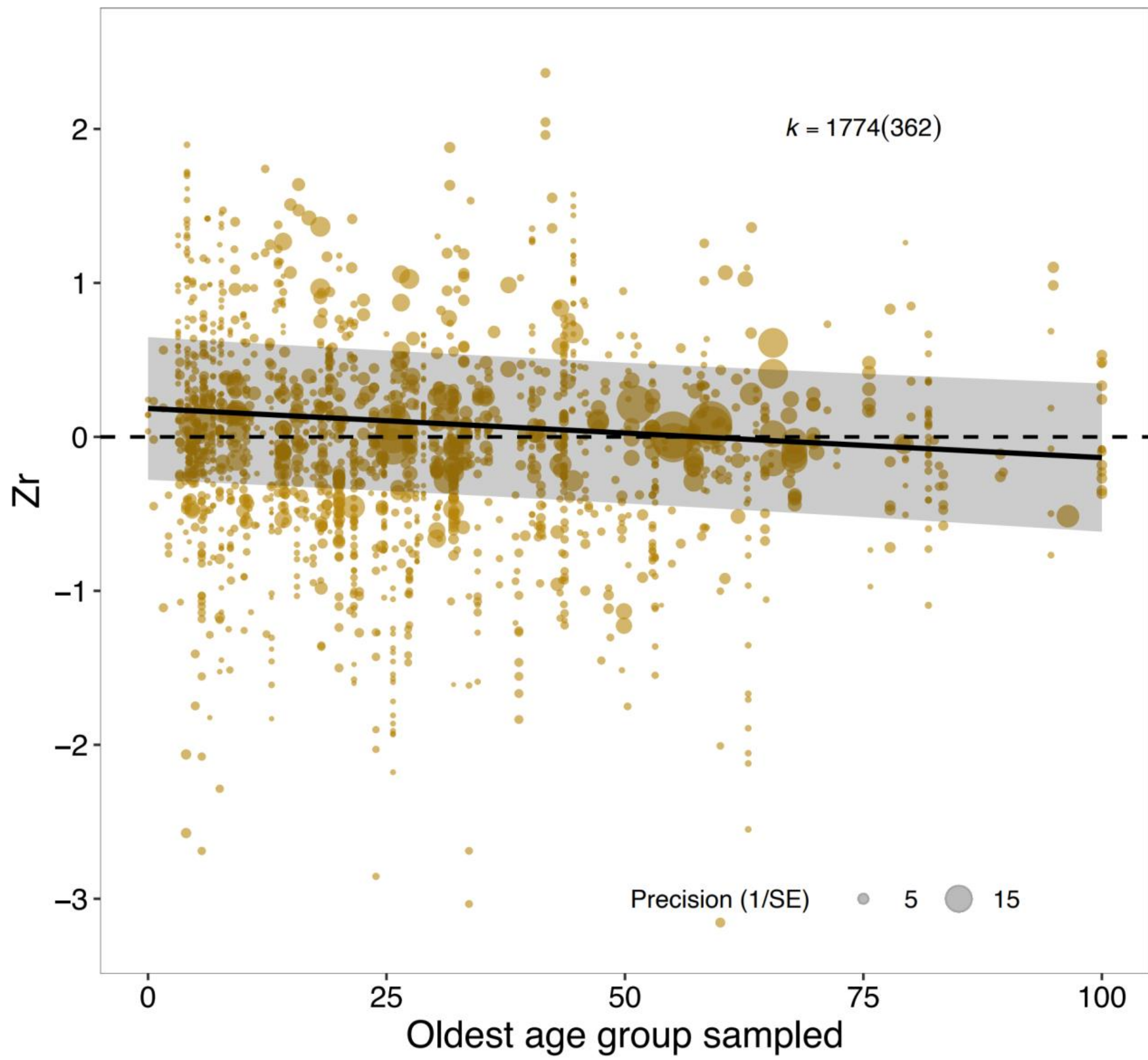
Supplementary Fig 8: Medians (50%) and interquartile ranges (5%, 25%, 75%, 95%) of proportion of maximum adult lifespan sampled for A. Different population types, B. Four most common species in our dataset, and C. Different animal classes (note that animal classes with less than 25 effect sizes were grouped together in 'Other'). Each point depicts the average lifespan sampled from a study.



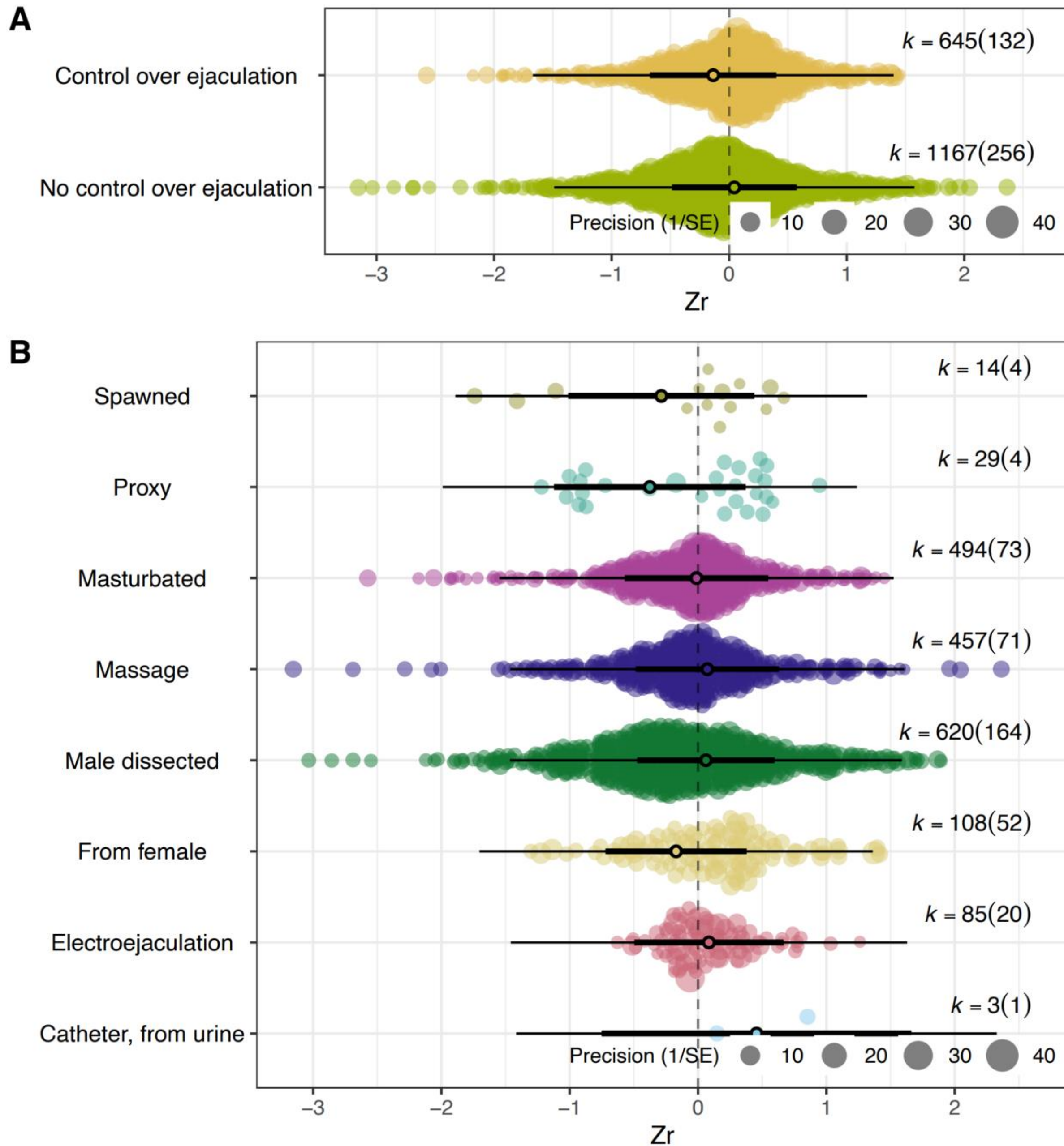
Supplementary Fig.9: Relationship between the youngest and oldest age sampled (as proportion of adult lifespan sampled) for each study (n = 362). Colours indicate the proportion of maximum adult lifespan sampled



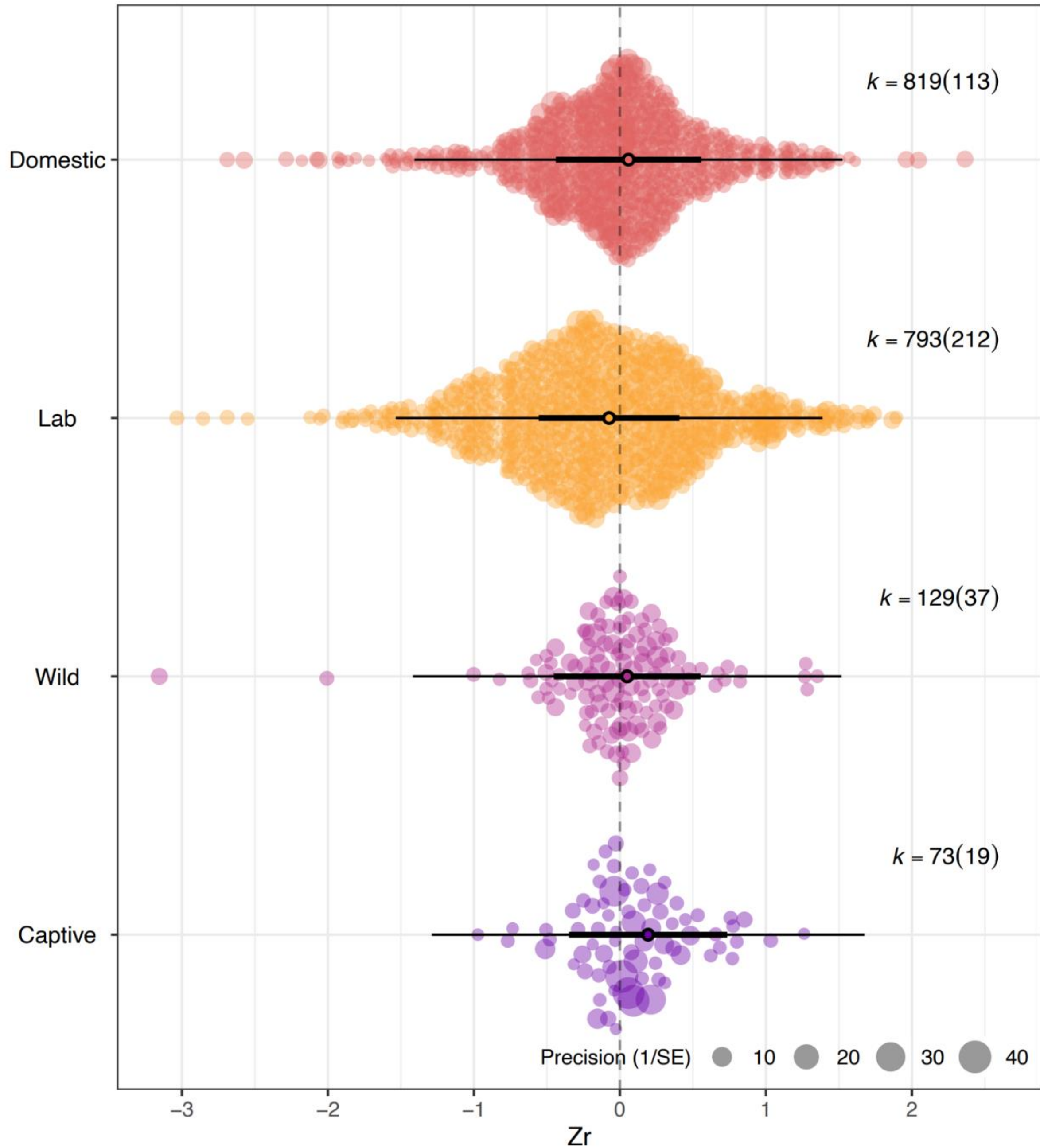
Supplementary Fig.10: Relationship between the youngest age sampled (as proportion of maximum lifespan sampled) and effect sizes for ejaculate senescence



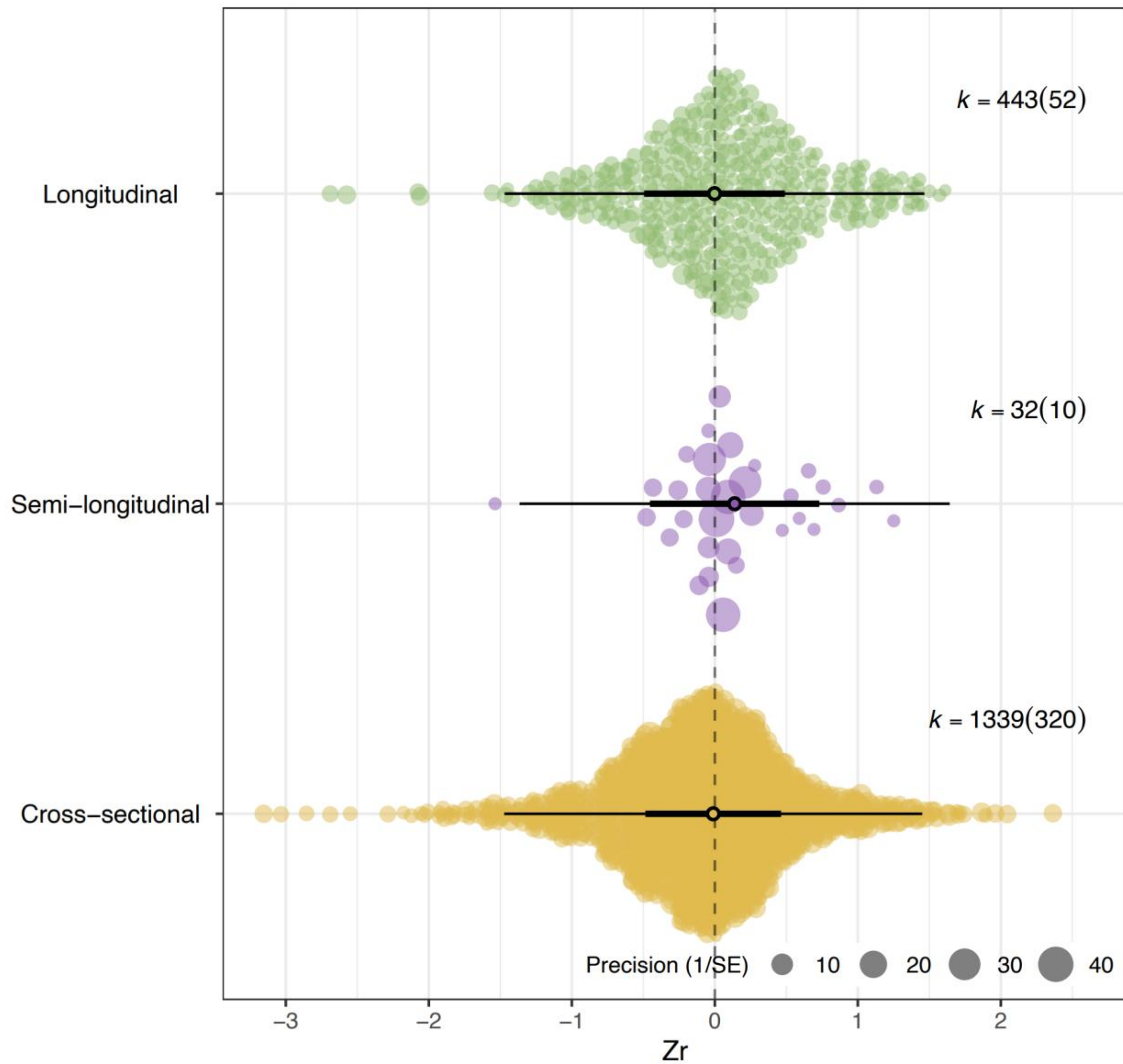
Supplementary Fig.11: Relationship between the oldest age sampled (as proportion of maximum lifespan sampled) and effect sizes for ejaculate senescence



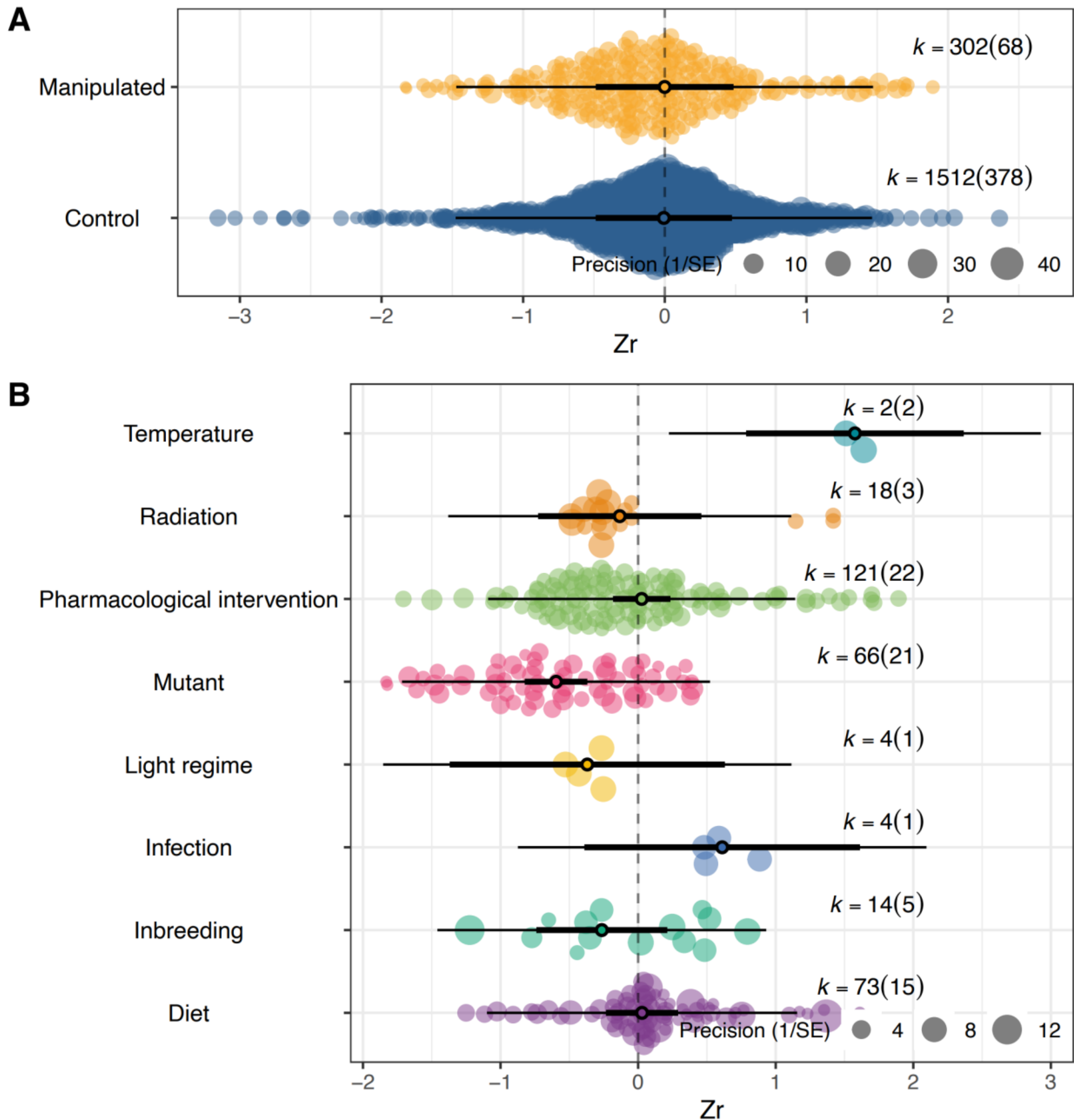
Supplementary Fig.12: A. Effect of male's 'control' over ejaculation on advancing male age. See Supplementary section 7 for definitions. B. Effect of advancing male age on ejaculates for each type of ejaculate collection method. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



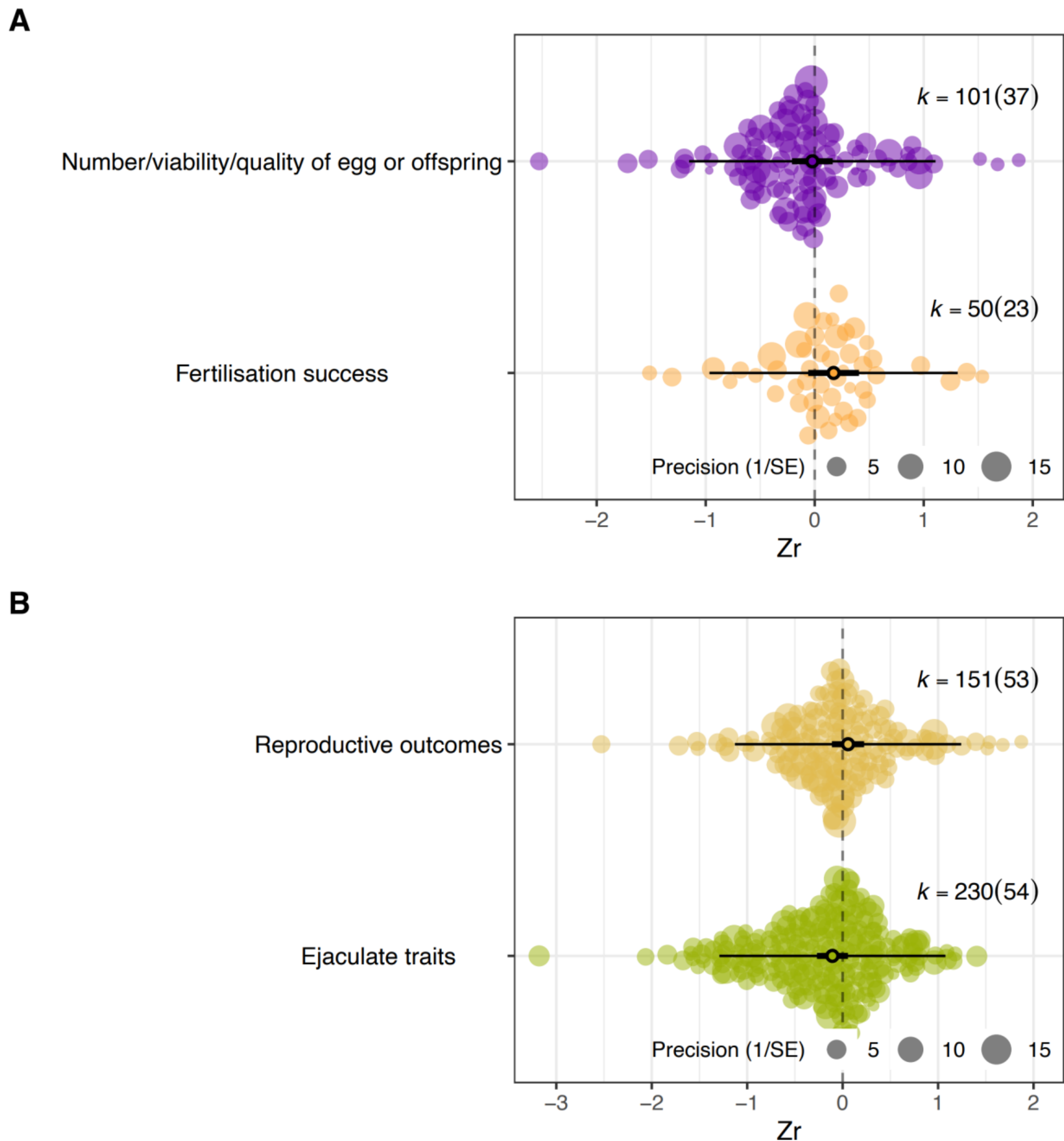
Supplementary Fig.13: Effect of advancing male age on ejaculate traits for different types of population. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



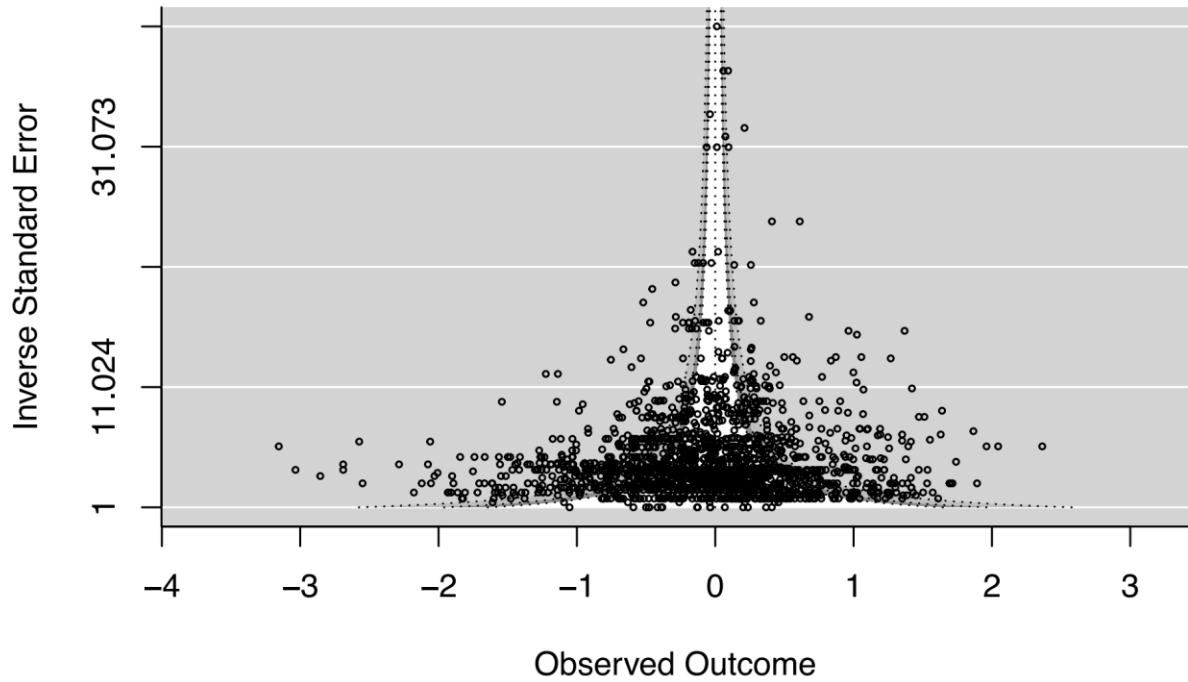
Supplementary Fig.14: Effect of advancing male age on ejaculates for studies with longitudinal vs cross-sectional sampling on males' ejaculates. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



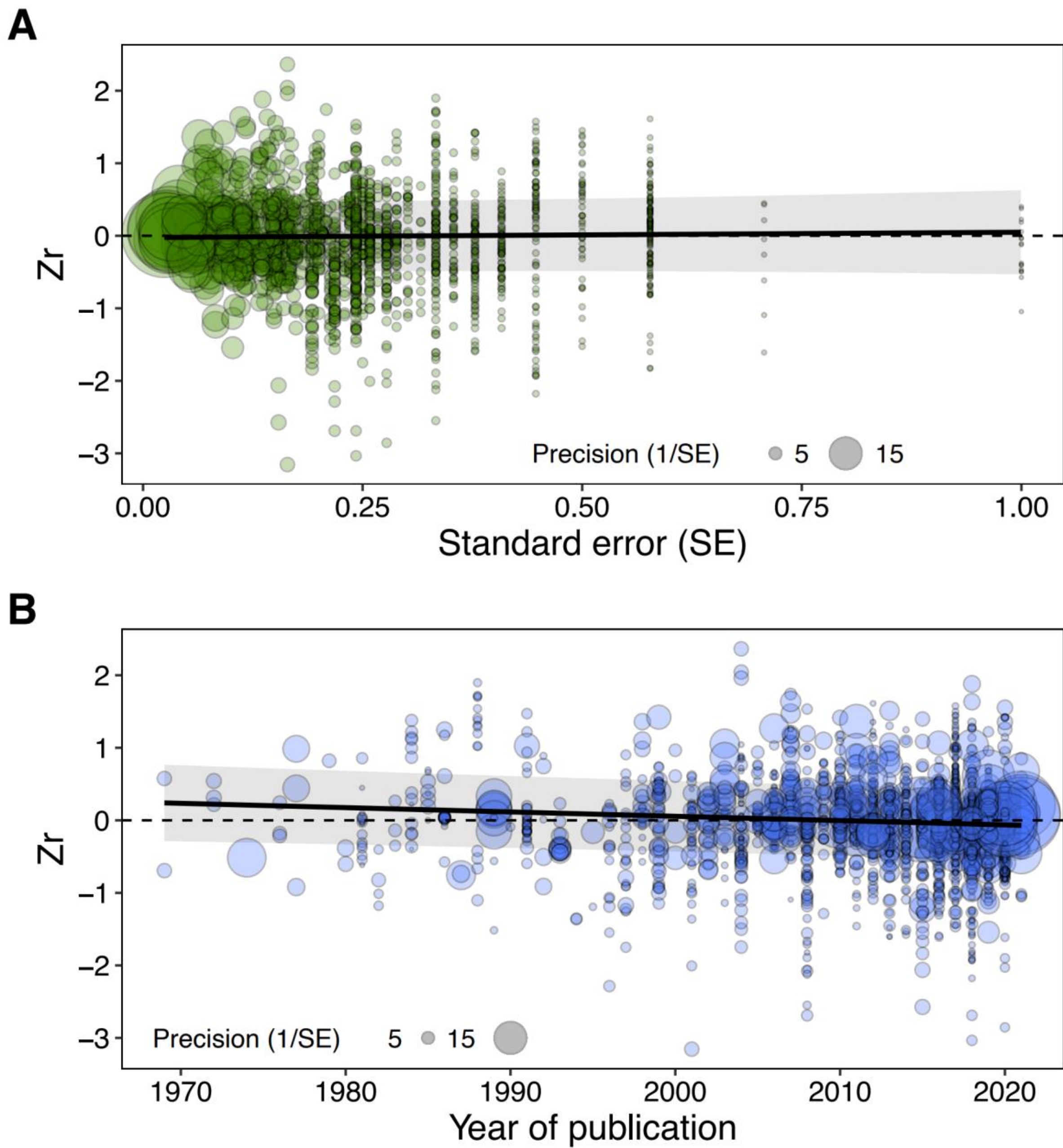
Supplementary Fig.15: A. Effect of advancing male age on ejaculates for studies with 'control' vs 'manipulated' males (see Supplementary section 9 for definitions), B. Effects of advancing male age on ejaculates for each type of manipulation/treatment including manipulated males only. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



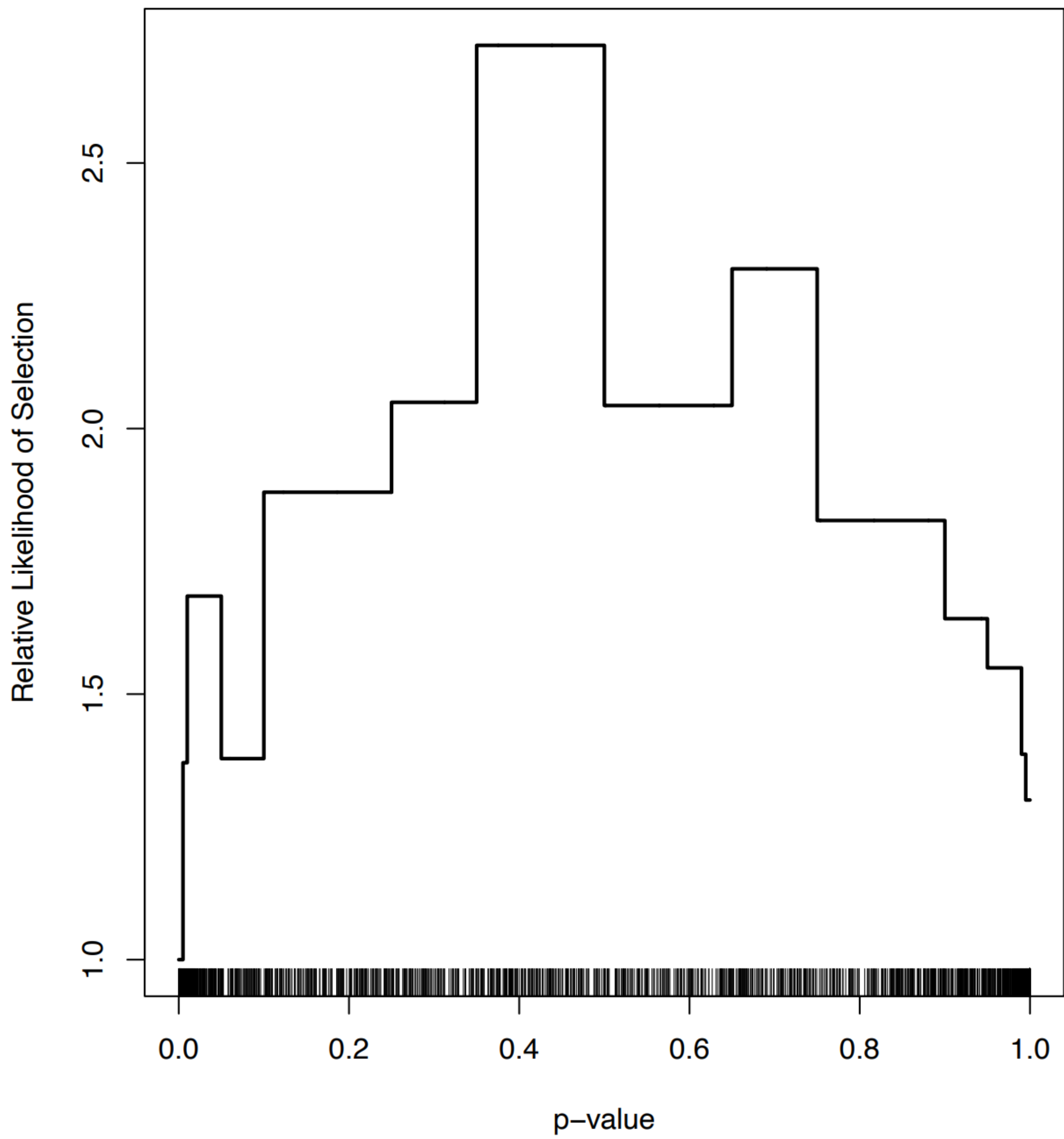
Supplementary Fig.16: A. Effect of advancing male age on the type of fitness trait measured in unmanipulated males. B. Effects of advancing male age on reproductive output and on ejaculates from studies which measure both traits. See Supplementary section 9 for definitions. The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



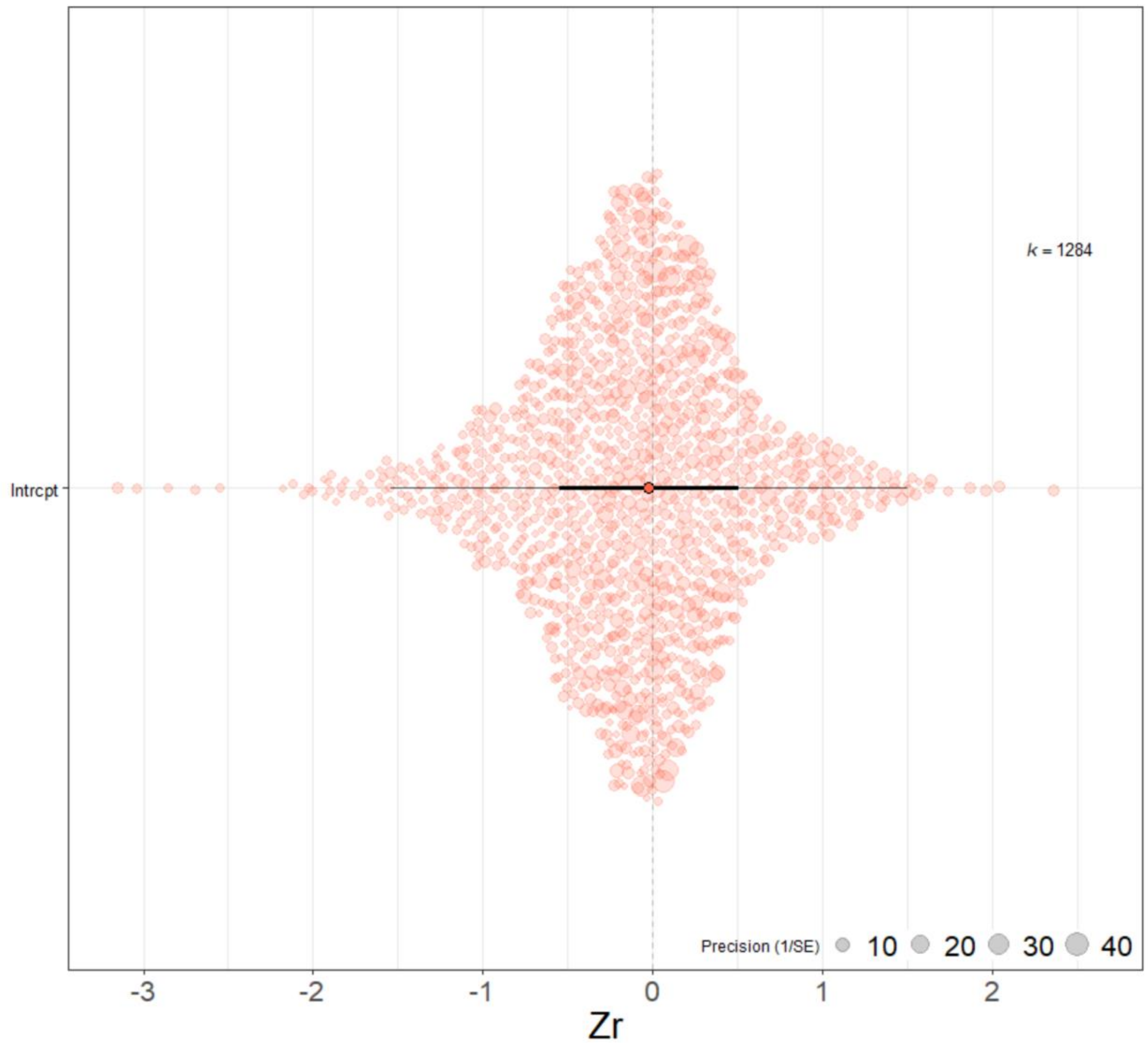
Supplementary Fig.17: Funnel plot of the precision (1/SE) and the residual effect (Observed outcome) from the null model to explore the existence of outliers and publication bias in the dataset.



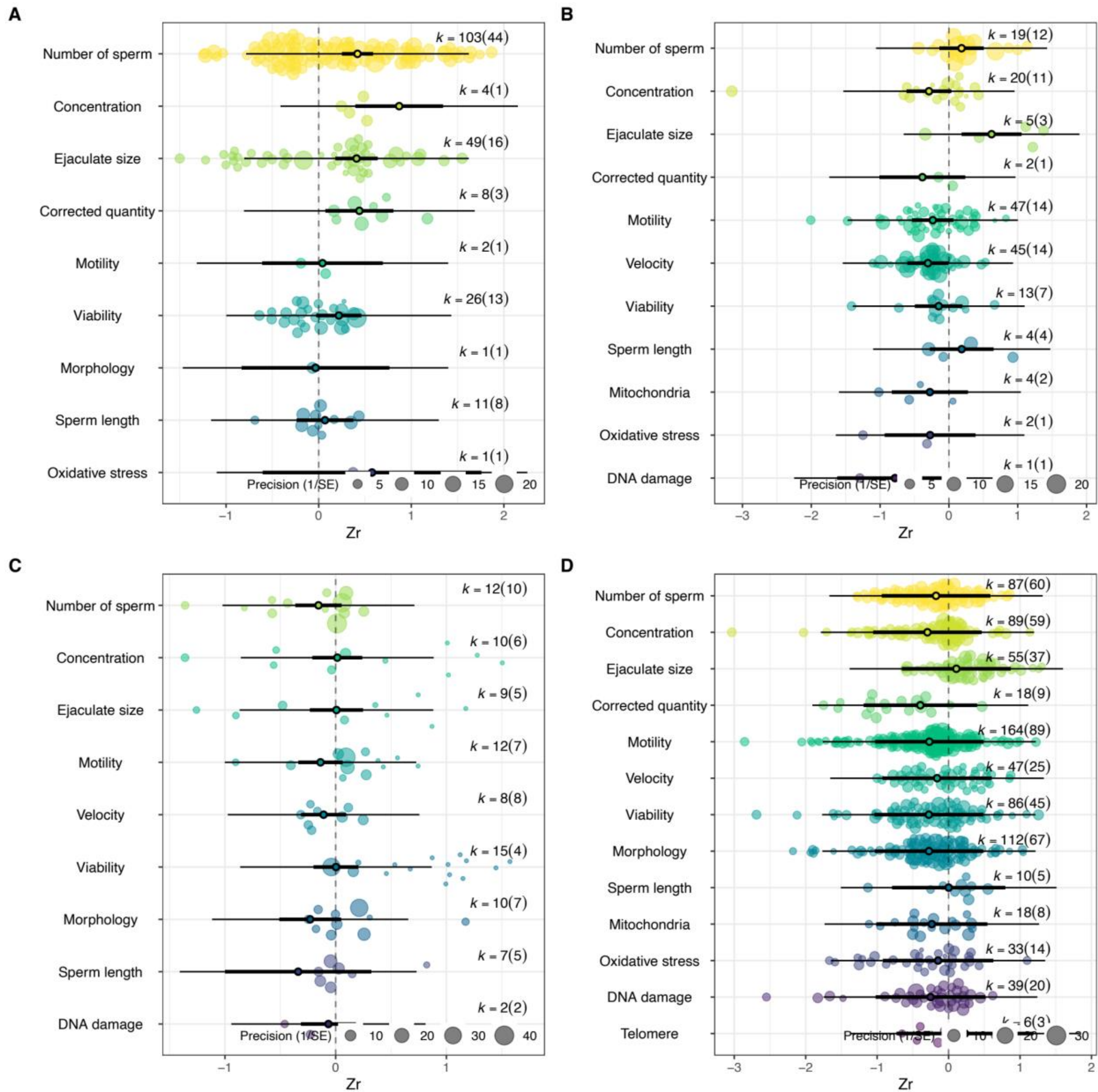
Supplementary Fig.18: Relationship between A. Standard error and effect size estimates (Zr), and B. Year of publication and effect size estimates (Zr). The points are scaled according to the inverse of their variance, so that larger points are given greater weight in the model and represent more reliable estimates. Shaded lines indicate 95% C.I.



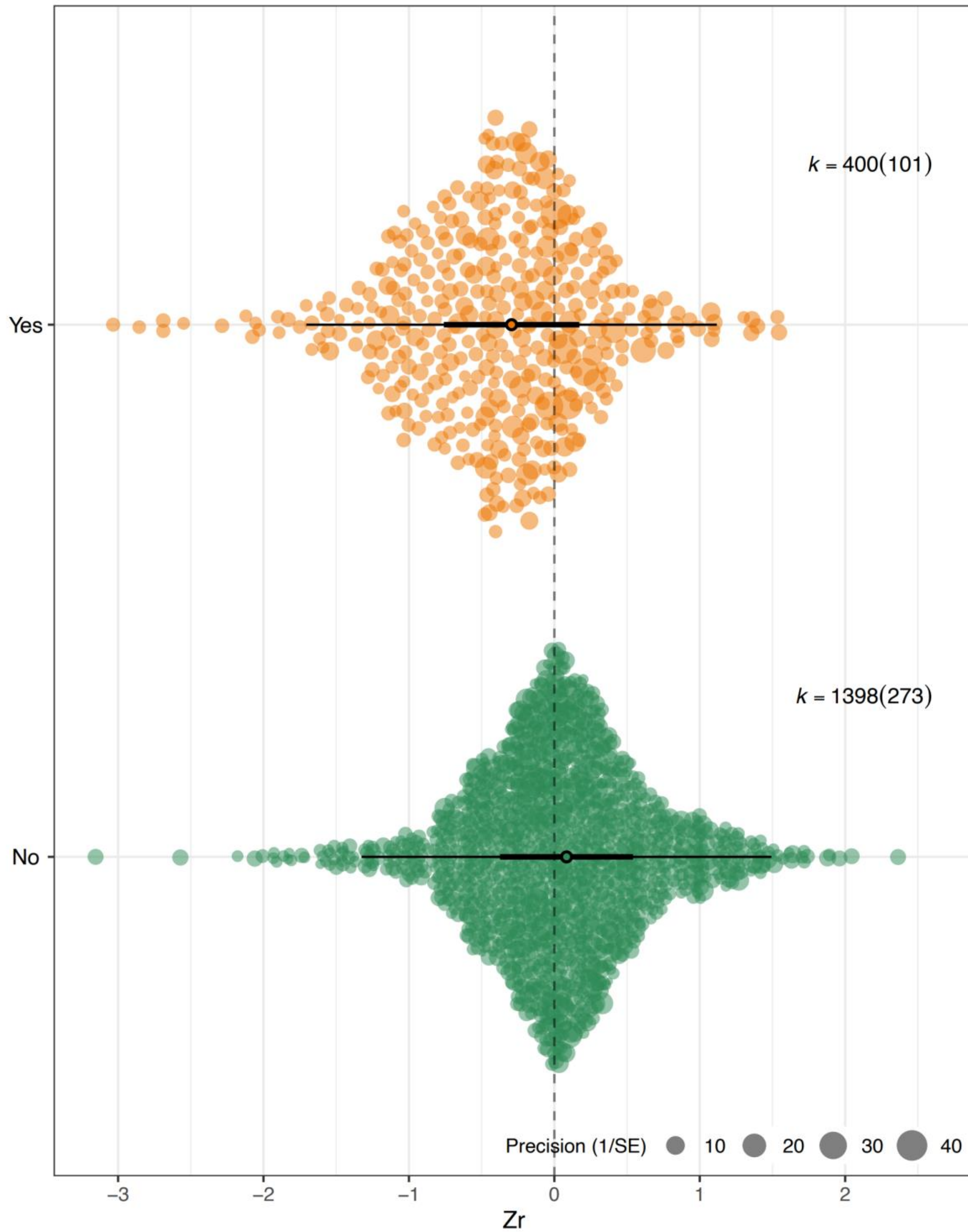
Supplementary Fig.19: Results of a step function selection model based on several cut-points for a model without any moderators.



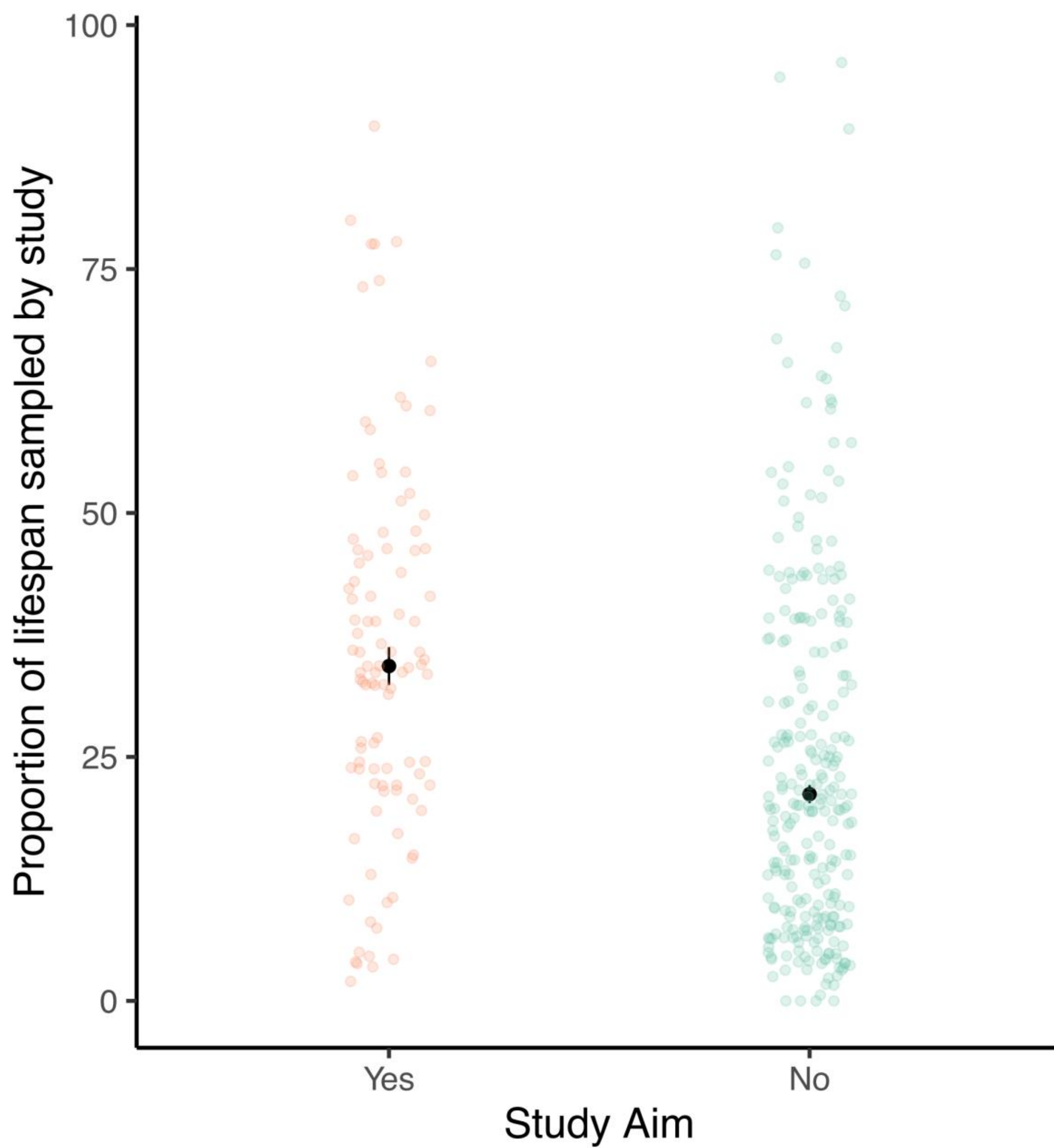
Supplementary Fig.20: The overall effect of advancing male age on ejaculates for studies with >10% of lifespan sampled.



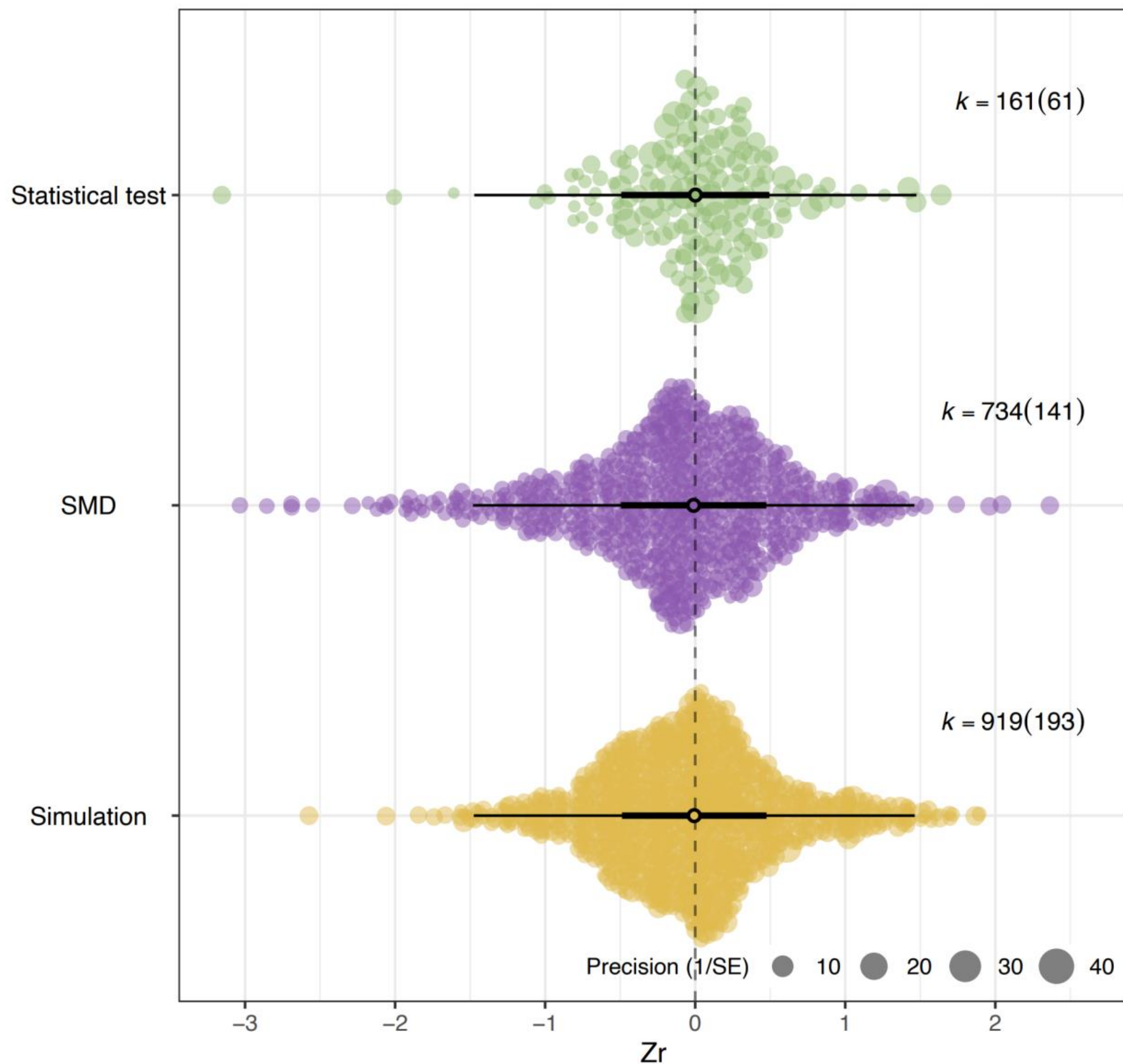
Supplementary Fig.21: Effect of advancing male age on various ejaculate traits when >10% of lifespan was sampled for A. Insects, B. Fish, C. Birds, D. Mammals. The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).



Supplementary Fig.22: Effect of advancing male age on ejaculates when studies explicitly test for an effect of ageing (Yes; i.e. - studies that used the words - ageing, aging, senescence, senescent, or senescing in their abstracts or titles and determined to be interested in senescence) vs studies that do not explicitly test for senescence (No).



Supplementary Fig.23: Means and 95% CI of lifespan sampled for studies that explicitly test for senescence (Yes; i.e. - studies that used the words - ageing, aging, senescence, senescent, or senescing in their abstracts or titles and determined to be interested in senescence) vs studies that do not explicitly test for senescence (No). Each point refers to the average lifespan sampled from a study.



Supplementary Fig.24: Effect of method used to calculate effect sizes. Effect sizes were calculated from statistical tests (Test stat), standardized mean difference (SMD - when only two age groups were available) or through a simulation (when more than two age groups were available). The size of each data point represents the precision of the effect size (1/SE). The X axis represents values of effect sizes as Fisher's z-transformed correlation coefficient (Zr), while the Y axis shows the density distribution of effect sizes. The position of the overall effect is shown by the dark circle, with negative values depicting senescence in ejaculate traits and positive values showing improvement in ejaculate traits with advancing male age. Bold error bars (95% C.I) show whether the overall effect size is significantly different from zero (i.e. not overlapping zero), while light error bars show the 95% prediction interval (P.I.) of effect sizes. Sample sizes reported as: k = number of effect sizes (in brackets: number of studies).

Chapter 3

Timeless or tainted? The effects of male ageing on seminal fluid*

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Abstract

Reproductive senescence can occur due to the deterioration of both the soma and germline. In males, it has mostly been studied with respect to age-related changes in sperm. However, the somatic component of the ejaculate, seminal fluid, is also essential for maintaining reproductive function and can senesce independently of sperm. Whilst we know that seminal fluid proteins (SFPs) are required for male reproductive success across diverse taxa, age-related changes in SFP quantity, quality, and composition are little understood. Additionally, only few studies have explored the reproductive senescence of the tissues that produce SFPs, and the resulting reproductive outcomes. Here we provide a systematic review of studies addressing how advancing male age affects the characteristics of seminal fluid, in particular SFPs and oxidative stress, highlighting open questions and generating new avenues for further research. We additionally discuss how declines in function of different characteristics of seminal fluid, such as SFPs and antioxidants, could contribute to age-related loss of reproductive ability. Overall, we find evidence that advancing age results in increased oxidative stress in seminal fluid and a decrease in the abundance of various SFPs. These results suggest that seminal fluid contributes towards important age-related changes influencing male reproduction. Thus, it is essential to study this mostly ignored component of the ejaculate, seminal fluid, to understand male reproductive ageing, and its consequences for sexual selection and paternal age effects on offspring.

Keywords: senescence, reproduction, ejaculates, germline, oxidative damage, SFP

Introduction

Senescence is the time-dependent decline of an organism's biological function (Monaghan et al., 2008), leading to reduced physiological abilities and ultimately death. Senescence results in numerous biological changes that include telomere shortening, accumulation of mutations, loss of proteostasis, mitochondrial dysfunction, and disruption of nutrient sensing pathways (Charlesworth, 1993; Kirkwood, 2005; López-Otín et al., 2013). Organisms also tend to have a lower reproductive output at older compared to younger ages. However, the onset and rate of decline in fertility varies considerably across taxa, depending on life-history strategies and ecologies of species (e.g. Lemaître et al., 2020b; Campos et al., 2022), making it difficult to generalise patterns of ageing across the tree of life (Jones et al., 2014).

Onset and rate of age-related biological decline and impaired reproductive function varies between males and females (Bronikowski et al., 2022). There has been a long-standing focus on females in life-history research, and studies have only recently begun to consider male reproductive ageing (e.g. Fricke and Koppik, 2019; Comizzoli and Ottinger, 2021; Archer et al., 2022). Evidence suggests that advancing male age can affect male fertilising ability (Paul and Robaire, 2013; Aich et al., 2021), influence female behavior (Dean et al., 2010; Vuarin et al., 2019), and lead to paternal effects on offspring (Daxinger and Whitelaw, 2012). Male houbara bustards (*Chlamydotis undulata*), for example, produce fewer progeny as they age, and sons of old fathers have greatly reduced sperm numbers (Vuarin et al., 2019, 2021). Other studies show that advancing male age can lead to lower sperm quality (Gasparini et al., 2010, 2014; Velando et al., 2011; Cornwallis et al., 2014; Selvaratnam and Robaire, 2016; Monaghan and Metcalfe, 2019; Vega-Trejo et al., 2019; Turnell and Reinhardt, 2020), and quantity (Johnson et al., 2015; Sepil et al., 2020). Additionally, sperm from older males have lower success in sperm competition and fertilize fewer eggs than sperm from younger males, as seen in guppies (*Poecilia reticulata*, Gasparini et al., 2019),

zebra fish (*Danio rerio*, Kanuga et al., 2011) and crickets (*Acheta domesticus*, Reinhardt and Siva-Jothy, 2005). Senescence in sperm can also affect the quality of offspring (Gasparini et al., 2017), characterized by offspring lifespan (Xie et al., 2018; Wylde et al., 2019), telomere length (Bouwhuis et al., 2018; Noguera et al., 2018; Bauch et al., 2019), development (Preston et al., 2015), reproduction (Bouwhuis et al., 2015; Vuarin et al., 2021), and viability (Tan et al., 2013). While most studies on male ageing have focused on sperm traits (reviewed in Sanghvi et al, 2024), only few have tested for changes in the quality and quantity of seminal fluid with age, and its resultant fitness outcomes. Therefore, whether the reported effects of male age on male fertility are also driven by changes in seminal fluid rather than just sperm are yet unknown.

Ejaculated sperm are usually surrounded by a cocktail of substances collectively called the seminal fluid (Poiani, 2006; Hopkins et al., 2017). These consist of somatic cells such as immune cells; macromolecules such as carbohydrates, vitamins, minerals; hormones; and seminal fluid proteins (SFPs). Seminal fluid (SF) in most species is made in specialized somatic tissues such as accessory glands, the prostate, seminal vesicle, bulbourethral, and ampullary glands (McGraw et al., 2015). Within seminal fluid, SFPs have been shown to be especially crucial in influencing male and female reproduction; these SFPs belong to a range of molecular classes such as antioxidants, lipases, lectins, proteases, and protease inhibitors and have been shown to have a diverse set of functions (Chapman, 2001; Avila et al., 2011; Perry et al., 2013; Ramm, 2020). For instance, SFPs facilitate normal sperm function (Wolfner, 1997), aid sperm storage and male sperm competitiveness (Fiumera et al., 2005, 2007; Goenaga et al., 2015; Patlar et al., 2020), maintain sperm viability (den Boer et al., 2008, 2009; King et al., 2011) and regulate sperm capacitation (Manjunath and Thérien, 2002). But SFPs can also act on attributes beyond sperm, for example, by affecting female reproductive behavior (Bath et al., 2017; Chapman et al., 2003; Liu and Kubli, 2003). Indeed,

seminal fluid has been shown to affect female immunity (Short and Lazzaro, 2010), female investment in the current mating (Nakadera et al., 2014), female egg-laying behaviour (Chapman et al., 2003; Liu and Kubli, 2003), and female re-mating rates (Stockley et al., 2020).

The germline is predicted to receive higher protection than somatic tissue (Maklakov and Immler, 2016), and both tissues are separated early in development. However, seminal fluid being produced by somatic tissue and directly interacting with germ cells, could play an important role in facilitating interactions between somatic tissue and the germline. This could have effects across the Weismann barrier (i.e. despite the germline and somatic tissue being separated early in development, changes in the soma could affect the germline or the next generation) (Sciamanna et al., 2019; Bline et al., 2020). Knowing how seminal fluid changes with age and how this can influence sperm, offspring, and female physiological and behavioural responses to mating, in addition to understanding age-related changes in sperm, is essential to gain a complete picture of male reproductive senescence. Here, we first conduct a systematic review on how advancing male age influences the non-sperm component of the ejaculate (i.e. seminal fluid) across animals, and then discuss the impacts this might have on age-specific reproductive success. While the effects of advancing male age on sperm have been reviewed elsewhere (e.g. Reinhardt, 2007; Pizzari et al., 2008; Monaghan and Metcalfe, 2019; Sanghvi et al, 2024), to our knowledge, this is the first systematic review of how advancing male age affects seminal fluid. Studies differ greatly in their biological and methodological factors, which can modulate or confound male ageing effects. Thus, we also compile how some of these factors differ across studies to highlight gaps in current research.

Systematic review

<u>Literature search and data collection</u>

To understand how male age affects seminal fluid, we conducted a literature search following PRISMA eco-evo guidelines (O'Dea et al., 2021). We used a search string for abstracts, titles, and keywords "*(sfp* OR seminal fluid OR seminal plasma) AND (ageing OR age OR aging OR senescence)"* to identify studies which test how advancing male age affects seminal fluid, using two search engines: SCOPUS and Web of Science (WoS), on December 14th 2021, accessed through the University of Oxford server. The searches returned a total of 738 hits from WoS (year range: 1991 to 2021) and 620 from SCOPUS (year range: 1941 to 2021). After duplicate deletion, which was done using Rayyan (Ouzzani et al., 2016), we obtained a total of 970 unique papers. We then screened the abstracts of these papers using pre-defined inclusion and exclusion criteria (see below), before screening the full-texts to obtain a final list of papers from which relevant data was extracted.

To be retained for full-text screening, the paper had to be a research article (not a review or meta-analysis) on any animal, and measure a seminal fluid trait for males of different ages, judged from its abstract. We excluded studies during abstract screening if they were on the wrong topic, did not compare males of different ages, did not have clear ageing data, only covered a small proportion of lifespan (e.g. only included young males), did not measure seminal fluid traits, or only measured seminal fluid during maturation of males (i.e. during juvenile or pubertal stages). The initial screening of abstracts produced a total of 94 studies whose full texts were considered in more detail.

When assessing full texts, to be included in our analysis review, a study needed to: compare males of non-overlapping age groups, compare non-sperm components of the ejaculate (like seminal fluid oxidative stress enzymes, proteins, hormones, lipids, and macro- or micronutrients), report sample sizes of males in each age group and exact ages (or range

of ages) to which males in each age group belonged. We excluded studies whose full texts were not available (two studies), or which were not in English (three studies). We additionally conducted a scoping search on Google Scholar to obtain additional papers which might have been missed in our systematic screening and search. This was done by using the keywords “seminal fluid protein + aging + ageing + senescence” for each of the following taxa: “bulls”, “insects”, “pigs”, “rodents”, “humans”, “birds”, “mammals”, “fish”, and searching the first five search result pages for relevant studies.

From all studies which fulfilled our inclusion criteria, we collected information on how male age affected various non-sperm characteristics of the ejaculate, as described in the paper. Additionally, we collected data on factors which could modulate the influence of seminal fluid ageing. These factors were: male mating history (i.e. whether males were held as virgin or not prior to testing), at which ages males were sampled, what fraction of average lifespan was covered and sampling methodology. The fraction of average lifespan covered is likely to influence whether seminal fluid ageing is detected in a study because ageing trajectories are expected to follow a non-linear pattern, with senescence being more prominent in late-adult life (e.g. Jones et al., 2014; Lemaître et al., 2020b).

Male mating history could influence the ageing of seminal fluid, such that if males are kept virgins, old males would have stored seminal fluid for longer durations, thus have more degraded SFPs and a higher accumulation of oxidative damage in SF, than mated old males or virgin young males. On the other hand, old virgin males would accumulate higher quantities of SF than younger virgin males (Koppik et al., 2018; Sepil et al., 2020). If previously mated males are tested, the quantity of seminal fluid produced would depend on the timing of the last mating, number of times the male mated in succession and its rate of replenishment, given that the abundance of SFPs within accessory tissues/glands decreases significantly immediately after a mating event (Hopkins et al., 2019a; Sepil et al., 2019).

Furthermore, if mating history is not controlled for, then older males would have mated more times over their life (e.g. Aich et al, 2021), and thus have undergone more rounds of SFP replenishment and thus potentially a higher cell-division turnover of the glandular tissue producing the SFP than young males.

Male sampling methodology (if samples are collected longitudinally or cross-sectionally) can also have a large impact on the study outcome. Cross-sectional sampling of males makes individual-level deterioration in ejaculate traits with advancing age harder to detect (Nussey et al, 2008), especially if low-quality males selectively disappear (Bouwhuis et al, 2009; Hamalainen et al, 2014). This non-random age-dependent mortality could lead to biased sampling of males, where younger age classes would have higher variance and might bias estimates of averages in seminal fluid traits compared to old age classes. Thus, cross-sectional studies might underestimate male reproductive senescence, compared to longitudinal sampling measuring the same individuals at different ages.

Results

Overall, we obtained data from 27 papers through our systematic searches, and 7 additional papers from Google Scholar. Out of these 34 studies, 14 reported how male age affected SFPs (see table 1), however some of these studies reported changes in total seminal fluid protein content, while others, changes in abundance of specific SFPs only. 10 studies reported data on oxidative damage levels or antioxidants present in seminal fluid (henceforth collectively called “oxidative stress”, see table 2). Apart from these two components of the seminal fluid, a smaller fraction of studies assessed the concentration of lipids or lipoproteins (4 studies), minerals/vitamins content (4 studies), sugar content (2 studies), or hormone concentrations (4 studies) in the seminal plasma/ejaculate. In our review, we restricted our discussion to studies that tested for male age-related changes in SFPs and oxidative stress

response, as these aspects of the seminal fluid were represented by a more adequate sample size compared to other seminal fluid components. The low number of studies dedicated to male age-related changes in the seminal fluid was also reflected in the limited taxonomic breadth, with a strong focus on mammals (see Fig. 1). Within mammals, studies were conducted on farm animals, humans, and laboratory rodents (see Figure 1). For most studies, males were sampled up to around 80% of their average adult lifespan and 50% of their maximum adult lifespan (tables 1 and 2).

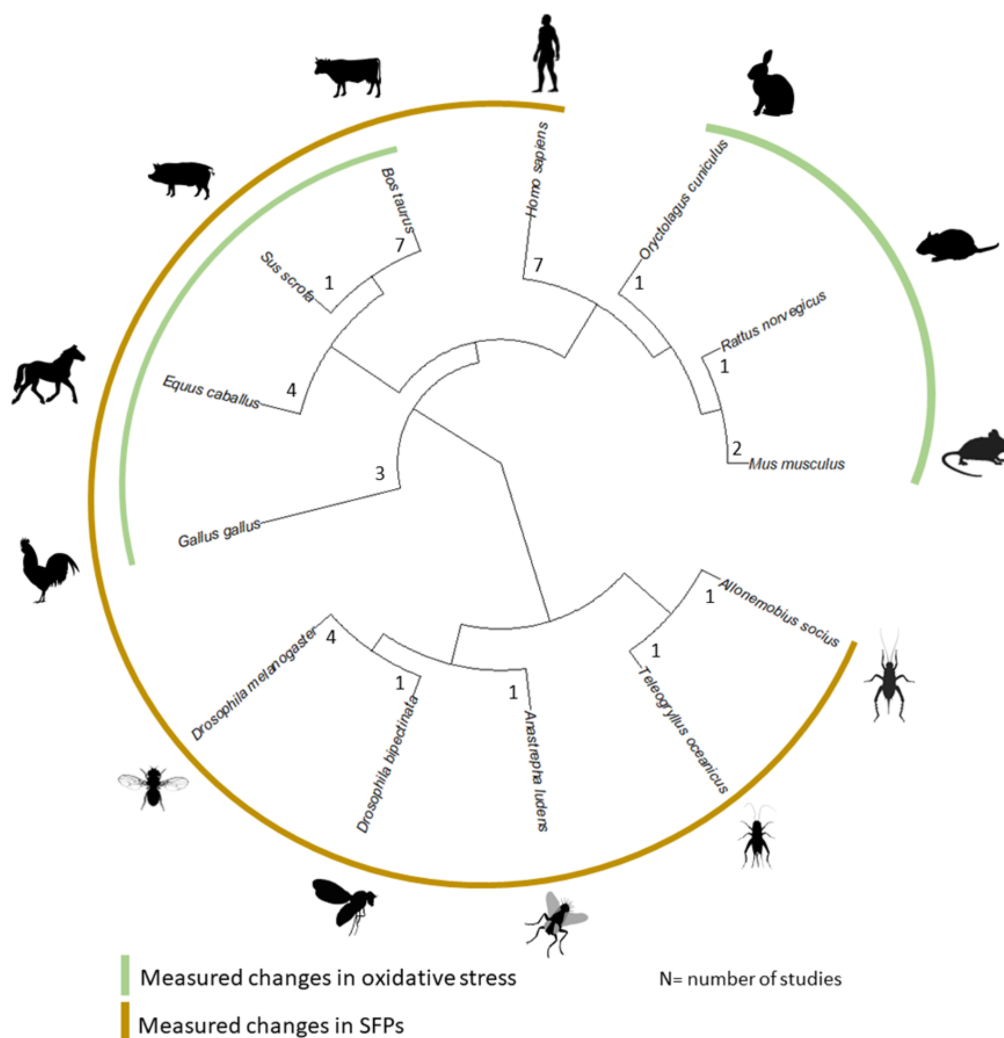


Figure 1: Phylogenetic distribution of all species in our review across 34 studies along with the number of studies on each. Species which had data reported for age-dependent changes in SFPs and oxidative stress are marked.

Seminal fluid proteins

Some studies that measured total seminal fluid protein content found an overall decline in SF quantity with male age (Rezaei et al., 2015; Fraser et al., 2016; table 1). However, studies that quantified individual SFPs or SFP compositional changes, found more inconsistent patterns for how individual SFPs changed with age. Here, some SFPs increased (Santhosh and Krishna, 2013; Simmons et al., 2014; Borziak et al., 2016; Inyawilert et al., 2019; Kant et al., 2019; Westfalewicz et al., 2021), while others decreased (Marshall et al., 2009; Rezaei et al., 2015; Koppik and Fricke, 2017; Herrera-Cruz et al., 2018; Ruhmann et al., 2018; Sepil et al., 2020; Westfalewicz et al., 2021) with male age. Furthermore, in studies which analysed the full proteome of the seminal fluid, only a small proportion of SFPs changed with age (e.g. Borziak et al, 2016; Sepil et al., 2020).

Methodologies differed widely between studies, ranging from estimating changes in overall SFP content to reporting individual protein changes. Generally, studies that tended to report increases in SFPs with age (e.g. in *Homo sapiens*, *Bos taurus* and *Teleogryllus oceanicus*) sampled <50% of the average lifespan of the species (e.g. Simmons et al., 2014; Kant et al., 2019; Santosh and Krishna, 2013; Westfalewicz et al., 2021; Table 1). This result suggests that extending sampling to cover the entire average lifespan is crucial and seminal fluid ageing trajectories might follow a hump-shaped pattern, with seminal fluid quantity peaking in mid-life and senescence being more prominent in late-adult life (also shown by Sanghvi et al, 2024, for sperm ageing).

Many studies on non-human mammals did not report male mating history (virgin or mated) prior to testing. In farm animal studies, older males are likely to have been mated as part of a breeding program, although this was not always explicitly stated. For studies on insects, males were primarily kept virgin prior to testing. It is known that in *D. melanogaster*, age-related changes in SFPs depend on male mating history (Koppik and Fricke, 2017;

Koppik et al., 2018; Sepil et al., 2020). For instance, old unmated males transfer a lower abundance of SFPs in a first mating relative to young males, despite having a higher abundance of SFPs in storage, whereas old frequently mated males show no change in either transfer or storage (Sepil et al., 2020). Thus, mating history has the potential to influence the results reported in studies which do not control for it. We suggest future studies should adopt a fully factorial design to test for effects of mating history on seminal fluid ageing and use young and old males both as virgin and mated males, and ideally control for mating number.

In most studies, samples were acquired from the male directly (e.g. via dissection or masturbation), but whether this accurately represents what would be transferred to females in a natural ejaculate is uncertain, especially when males have the potential for strategic ejaculation (Wedell et al., 2002). Most studies were cross-sectional and thus future studies to employ a longitudinal approach would be promising if males are not needed to be sacrificed to extract an ejaculate/ the seminal fluid and a large cohort of males can be followed across their lifetime.

Table 1: Summary of studies testing the effect of male age on seminal fluid proteins across different taxa as found in the systematic search. Proportion lifespan (LS) sampled is given in relation to reported average lifespan (avg) or maximum (max) recorded lifespan for each species.

Study	Species	Proportion LS sampled	Sample sizes	Changes observed in SFPs	Sampling	Mating history
Borziak et al, 2016	*Gallus gallus*	1 to 7 years out of 5.5 (avg in wild) and 18 (max)	16 total	Total of 1141 SFPs identified, out of which 9 change with age*velocity, and 4 with age only. Protein tyrosine phosphatase type IVA 1 was present in old males only. Young males had more of SPARC precursor, acetyl-CoA acetyltransferase cytosolic, and ras-related protein Rab-11B compared to old males.	Cross-sectional	Mated but sexually rested
Inyawilert et al, 2019	*Gallus gallus domesticus*	7 to 24 months out of 60 (avg) and 112 (max) months	18 total	Proteins with light (72kDa) molecular weights decreased with increasing age. Mid-weight proteins (90 kDa) increased with increasing age. Heavy proteins (140 kDa) showed no significant change.	Cross-sectional	Unreported
Abou-Ahmed et al, 1993	*Equus caballus*	7 to 25 years out of 25 (avg) and 47 (max) years	53 total	Total seminal fluid protein content was highest in middle aged males, and lowest in the youngest and oldest age groups	Cross-sectional	Mated
Westfalewicz et al, 2021	*Bos taurus*	2 to 4 years out of 10 (avg) and 25 (max)	6 total	Seventeen SFPs differed between young and old males. Older bulls had higher proteins of: glutathione; S-transferase omega 2 (GSTO2); PRDX5; PARK7; superoxide dismutase (SODC), compared to younger males. Younger bulls had higher amounts of: keratin, type II cytoskeletal 59 kDa, component IV (K2C4); outer dense fiber protein 2 (ODF2); tektin-5 (TEKT5) and TBB2B compared to older bulls	Longitudinal	Unreported
Fraser et al, 2016	*Sus scrofa*	19 to 42 months out of 66 (avg) and 264 (max) months	4 total	Overall content of seminal fluid proteins decline with age. Did not identify specific SFPs.	Longitudinal	Unreported
Kant et al, 2019	*Homo spaiens*	20 to 40 years out of 72 (avg) and 120 (max) years	6 per age group	Seventeen protein spots and 10 proteins differed between young and old groups (humans are known to contain ~3000 SFPs). Glutaredoxin domain containing cysteine-rich protein-2, clusterin, serum albumin, translation initiation factor IF-2 like, ecto-ADP-ribosyltransferase 4, CB1 cannabinoid receptor-interacting protein 1, serotransferrin were found in higher abundance in older males compared to younger males. Alternative protein RRT-34 and protein Unc-119 homolog A were found in lower abundance in older age samples compared younger males.	Cross-sectional	Mated

Simmons et al, 2014	*Teleogryllus oceanicus*	4 to 20 days out of 74 (avg) and 135 (max) days	57 total	Total of 27 distinct SFPs identified. Total protein content did not vary with age. ToSfp014, ToSfp025, ToSfp007 (Trypsin-like serine protease), ToSfp017, ToSfp011, ToSfp026, ToSfp005 (Dipeptidase), ToSfp027 (apyrase), ToSfp001, ToSfp024 (carbonic anhydrase) increased with age. Other SFPs did not change significantly with age.	Cross-sectional	Virgins
Koppik and Fricke, 2017	*Drosophila melanogaster*	7 to 42 days out of 45 (avg) and 110 (max) days	10 per age group	All 5 SFP genes tested, decreased in expression with age: Acp26Aa, Acp29AB, Acp36DE, SP and Acp62F.	Cross-sectional	Mated and unmated treatments
Sepil et al, 2020	*D. melanogaster*	7 to 35 days out of 45 (avg) and 110 (max) days	80 per age group	117 SFPs identified, out of which 40 changed with age. Focused on 6 functionally important SFPs. Acp62F, Semp1, and Acp26Aa decreased with age. Acp70A [sex peptide], Acp36DE, and CG9997 showed no change with age. Age-related accumulation of SFPs in unmated males, but reduced transfer. No change in SFP abundance or transfer with age in frequently-mating males Evidence of age related Post-translational modifications in some SFPs	Cross-sectional	Mated and unmated treatments
Rezaei et al, 2015	*D. melanogaster*	2 to 53 days out of 45 (avg) and 110 (max) days	20 per age group	Overall seminal fluid amount decreases with age. Did not measure specific SFPs.	Cross-sectional	Virgins
Ruhmann et al, 2018	*D. melanogaster*	4 d - 42 d out of 45 (avg) and 110 (max) days	18 per age group	Measure two SFPs: sex peptide and ovulin. Sex peptide decreased in old males, ovulin levels did not change with age.	Cross-sectional	Mated
Herrera-Cruz et al, 2018	*Anastrepha ludens*	8 d to 72 days out of 50 days (avg), 1 year (max)	20 per age group	Old males had lower overall protein content in their testis (but not accessory glands) compared to young males.	Cross-sectional	Virgins
Marshall et al, 2009	*Allonemobius socius*	5 to 40 days out of 35 days (avg) and 100 days (max)	42 total	Protein X (trypsin like serine protein) reduces with male age.	Cross-sectional	Virgins
Santhosh and Krishna, 2013	*Drosophila bipectinata*	2 to 47 days out of 58 days (avg) and 200 days (max)	50 per age group	Overall SFP quantity increases with male age	Cross-sectional	Virgins

Seminal fluid oxidative stress

Overall, the antioxidant enzymes involved in protecting against oxidative damage decreased significantly in the seminal fluid with advancing male age (see table 2). All studies that measured antioxidant content in the seminal fluid (e.g. TSOD, MnSOD, CuZnSOD, TGSH, CAT) consistently reported a decline in older males compared to younger or middle-aged males. Additionally, oxidative stress markers and reactive oxygen species were found in higher quantities in older male seminal fluid compared to younger males (e.g. El-Gindy and Zeweil, 2017; Kara et al., 2019). Notably, all studies on seminal fluid oxidative stress used mammals, so we cannot ascertain whether this is a pattern also seen in other animal groups. None of these studies reported the mating history of the males, and only one study sampled males longitudinally (Fraser et al., 2016).

Table 2: Summary of studies found in the systematic literature search that focus on male-age dependent changes in antioxidants, oxidative stress biomarkers and reactive oxygen species in male ejaculates/ seminal plasma. Proportion lifespan (LS) sampled is given in relation to reported average lifespan (avg) or maximum (max) recorded lifespan for each species.

Study	Species	Proportion LS sampled	Sample sizes	Changes observed in oxidative stress	Sampling	Mating history
Vince et al., 2018	*Bos taurus*	2 to 10 years out of 10 (avg) and 25 (max)	9 young, 9 old	Antioxidants such as TSOD, MnSOD, CuZnSOD, TGSH, CAT all higher in young males. Oxidative stress was higher in old males.	Cross-sectional	Unreported
Ahmad et al., 2020	*Bos taurus*	3 to 10 years out of 10 (avg) and 25 (max)	6 young 6 old	Younger bulls have higher total antioxidants. For catalase and malondialdehyde, there is no sig. difference.	Cross-sectional	Unreported
Majić Balić et al., 2012	*Bos taurus*	2 to 10 years out of 10 (avg) and 25 (max)	9 young, 10 old	Seasonal dependent changes in antioxidants: For total glutathione peroxidase (T-GSH-Px), young bulls have more in all seasons. For glutathione peroxidase (Se-GSH-Px), protein carbonyl content (PCC), young males have more in 3/4 seasons.	Cross-sectional	Unreported
Kelso et al., 1997	*Bos taurus*	2 to 9 years out of 10 (avg) and 25 (max)	4 in each of the three classes	For both antioxidants measured, Glutathione peroxidase and Superoxide dismutase, younger males had more than older males	Cross-sectional	Unreported
Noguera et al., 2012	*Gallus gallus*	1 to 4 years out of 5.5 (avg in wild) and 18 (max)	6 young, 15 old	Decrease in antioxidants such as -SH group of proteins, uric acid, vitA, vit C, vit E in old males	Cross-sectional	Unreported
El-Gindy et al, 2017	*Oryctolagus cuniculus*	9 to 42 months out of 24 months (avg) and 150 months (max)	18 young, 18 old	Aspartate transaminase showed no significant change with age Antioxidants decreased in old males Oxidative stress marker Malondialdehyde increased sig in old males	Cross-sectional	Unreported
Kara et al, 2019	*Mus musculus*	3 to 24 months out of 24 (avg) and 48 (max) months	14 young, 21 old	Antioxidants glutathione peroxidase and reductive glutathione decreased in older males. Oxidative stress marker malondialdehyde increased in old males	Cross-sectional	Unreported

Fraser et al, 2016	*Sus scrofa*	19 to 42 months out of 66 (avg) and 264 (max) months	4 in total	Antiperoxidant activity lower in older animals. Antioxidant L-glutathione concentration peaks at mid age, and declines in older animals	Longitudinal	Unreported
Waheed et al., 2013	*Equus caballus*	4 to 22 years out of 25 (avg) and 47 (max) years	6 in each age group	Antioxidant Glutathioneperoxidase highest in middle aged males, and lower in oldest and youngest males	Cross-sectional	Unreported
Takemura et al., 2014	*Rattus norvegicus*	15 to 75 weeks out of 124 (avg) and 187 (max) weeks	4 to 5 in each group	DJ-1 antioxidant decreased with age. Cu/ZnSOD antioxidant decreased with age	Cross-sectional	Unreported

Discussion

Here, we systematically reviewed how the non-sperm components of the ejaculate (i.e. seminal fluid) changed with male age. Sperm ageing has been a major focus of previous studies, while seminal fluid has not been studied nearly as extensively. This is highlighted by the limited number of studies and taxa found in our systematic review, with the majority of studies either investigating age-related changes in SFPs or oxidative stress. Below, we discuss how the age-dependent changes in seminal fluid components found in our systematic review might influence male reproductive ageing, and discuss why the omission of seminal fluid and its associated somatic tissue is an important oversight in evolutionary and ecological research.

Seminal fluid protein

We found some heterogeneity between studies in age-related SFP changes. While total seminal fluid protein content and the abundance of many SFPs declined with age, some specific SFPs also increased in abundance with age. This disparity could be due to specific proteins responding differently to age based on their function or tissue-of-origin (Borziak et al., 2016; Sepil et al., 2020), or due to a reporting bias, or due to studies not sampling males to late-life.

While studies in our review rarely directly discussed the functional importance of the changes in observed SFPs, below we highlight potential fitness consequences of our obtained results, based on known functions of proteins that declined with age. Overall protein content can influence male fertilisation success as well as a variety of female responses. For instance, older male *D. melanogaster* are less able to delay female remating and stimulate egg laying compared to younger males due to reduced overall SFP content (Chapman et al., 2003; Koppik and Fricke, 2017; Ruhmann et al., 2018; Sepil et al., 2020). Similarly in *Aedes*

aegypti mosquitoes, older males are less able to prevent female remating due to reduced SFP content (Agudelo et al., 2021). Therefore, a consistent reduction observed in overall protein content in old males, could indicate reduced egg-laying in females mated to old males.

Sepil et al. (2020) found a significant age-related increase in SFP abundances in the accessory glands of unmated males, but no change in SFP abundances in the accessory glands of frequently-mated males. Yet, they also found that female egg laying behaviour and remating affinity changed as a function of male age following matings with sperm-less (but seminal fluid producing) males, demonstrating seminal fluid alone contributed to the decline in reproductive function with male age. This disparity in results in Sepil et al (2020) might be explained by SFPs that males transfer to females, and by declines in SFP quality rather than abundance. Specifically, while Sepil et al. (2020) found no age-related decline in SFP abundances in the accessory glands, old unmated males transferred a lower quantity of SFPs to females compared to younger unmated males. Additionally, Sepil et al (2020) found that SFPs in old males were more degraded (in western blots) than in young males, making it likely that changes in SFP quality and not just quantity explain reproductive senescence.

Apart from affecting male ability to induce female post-mating responses, age-related changes in seminal fluid might also affect sperm traits. For example, in the jungle fowl *Gallus gallus*, age-related changes in proteins which affect sperm velocity were detected (Borziak et al., 2016). Thus, the decreased ability of older males to gain paternity under sperm competition and fertilise eggs may be driven by changes in SFPs influencing sperm performance, rather than direct age-related changes in sperm per se.

It is becoming increasingly possible to manipulate the expression of individual SFPs to better understand how particular SFPs affect female post-mating behaviour and sperm competition. For instance, using a combination of proteomics and RNAi knockout assays, Marshall et al. (2009) identified a single accessory gland-derived ejaculate protein in the

ground cricket *Allonemobius socius* that influences female egg-laying and declines in expression with male age. Age-related declines in this protein (and after it being knocked out) explained the waning ability of males to induce female egg laying as the male ages. A limitation of such knockout studies however is that they consider only one or a few seminal fluid proteins, whereas in reality the seminal fluid proteome is a highly integrated unit whose individual components co-vary in their expression (Mohorianu et al., 2018; Patlar et al., 2019).

Generally, the studies in our review suggest that senescence in male fertility due to senescence in seminal fluid proteins, might occur due to the following pathways: SFPs influencing female oviposition, ovulation, and remating; and SFPs influencing sperm performance. Our review also suggests that declines in SFP quality with advancing male age due to proteostasis, rather than solely the diminishing abundance of SFPs per se, might be important in mediating male reproductive senescence. A loss of protein homeostasis – proteostasis – is a well-known feature of ageing, characterised by a failure of chaperones and protein degradation machinery, and protein misfolding (Labbadia and Morimoto, 2014), and we suggest that SFP proteostasis with advancing age be investigated more thoroughly.

While our systematic review showed general age-related declines in SFPs, whether the somatic tissues that produce SFPs are affected by advancing age, remains unclear. Generally, the size of prostate/accessory glands (where SF is produced) tends to increase as males grow older (Jin et al., 1996; Atalan et al., 1999; Mubenga et al., 2020; Rezaei et al., 2015; Reyes-Hernández and Pérez-Staples, 2017), although shrinkage with age is also reported in a few studies (Mazeed and Mohanny, 2010; Santhosh and Krishna, 2013). However, the overall size of the seminal fluid-producing organ does not necessarily predict protein content, as found in *A. ludens* (Herrera-Cruz et al., 2018) and *D. bipectinata* (Santhosh and Krishna, 2013). In humans, the increase in prostate size is known as benign

prostatic hyperplasia (Berges and Oelke, 2011; Zhang et al., 2013). Some theory predicts that the enlargement of the prostate is a side-effect of cellular hyperfunction (Blagosklonny, 2021) where suboptimal nutrient-sensing molecular signalling in late-life causes excessive biosynthesis (Lind et al., 2019). Future studies can compare changes in seminal-fluid producing organs against the quality, quantity, and composition of seminal fluid proteins produced, to test whether changes in SFP quantity is merely a consequence of changes in the size of organs that produce SFP.

Seminal fluid oxidative stress

The studies we reviewed consistently found that antioxidant quantity in the seminal fluid decreases with advancing male age, while oxidative stress markers tended to increase in the seminal fluid as males aged. Reactive oxygen species (ROS) are unstable, free radical compounds and are required for vital cellular processes (Finkel and Holbrook, 2000; Hajam et al., 2022), but can also be deleterious to cells in high concentrations. ROS play a role in sperm activation and influencing sperm motility (Aitken et al., 2022) therefore ROS has an important function for male reproduction (Mannucci et al., 2022). ROS however, in excess quantities, can deteriorate sperm homeostasis and reduce male fertility (Mannucci et al., 2022). Antioxidants play a key role in stabilising free radicals generated as part of cellular processes (Hood et al., 2019), and an imbalance between antioxidants and ROS causes oxidative stress. Oxidative stress in seminal fluid can cause sperm DNA damage and negatively influence the activation of various sperm transcription factors (Aitken and Baker, 2006; Sabeti et al., 2016; Aitken, 2017). Our review suggest that older males have lower antioxidant levels but higher oxidative stress markers and ROS in their seminal fluid, and thus likely have higher overall oxidative stress than young males. The excessive ROS in seminal fluid of old males could be due to higher ROS production in seminal-fluid producing

tissue of old males, or due to ROS in sperm of old males "leaking" into seminal fluid or somatic cells. Future studies could investigate what mechanisms lead to higher ROS in seminal fluid of old males, and what the fitness consequences of this is.

Inter-generational effects of seminal fluid ageing

The impact of male age is not limited to his own and his mates' reproductive success, but can potentially influence the fitness of offspring through epigenetic mechanisms (Curley et al., 2011; Crean and Bonduriansky, 2014). Advanced paternal age has been shown to shorten offspring lifespan, exacerbate ageing-related pathology and to alter offspring social behaviour (Kong et al., 2012; Brenman-Suttner et al., 2018; Xie et al., 2018). These inter-generational paternal effects were believed to be primarily due to the accumulation of de novo mutations in ageing germ cells. However, recent work suggests that paternal effects on offspring could be mediated via seminal fluid (Watkins et al., 2018; Evans et al., 2019; Simmons and Lovegrove, 2019, 2020; Kekäläinen et al., 2020). These inter-generational effects of seminal fluid on offspring could be due to: seminal fluid influencing female investment in embryos; seminal fluid acting as a medium for extracellular vesicles carrying epigenetic information to sperm and eggs; and antioxidants in seminal fluid aiding in sperm DNA repair (Watkins et al., 2018; Evans et al., 2019; Simmons and Lovegrove, 2019, 2020; Kekäläinen et al., 2020). Inter-generational effects mediated via seminal fluid are yet to be investigated in the context of male age, providing an important avenue for future research.

Conclusion

Our review highlights the gaps in our knowledge about seminal fluid ageing, and our relative ignorance about how male age influences seminal fluid compared to sperm. For instance, we emphasize that the current literature on seminal fluid ageing has a large taxonomic bias (insects and farm animals over-represented), sampling bias (i.e. ejaculates obtained from males and using cross-sectional sampling), only few studies focus on the complete seminal fluid proteome or on characteristics other than SFPs and oxidative stress. Our review generates some hypotheses for how ageing of seminal fluid might affect male and female fitness, and shows that male age consistently impacts the level of oxidative stress in the seminal fluid, and to some extent the abundance and quality of SFPs in the ejaculate. We link these results of age-dependent changes observed in the seminal fluid profile with male fitness. These changes in SFPs might be linked with changes in female post-mating behaviour (Koppik and Fricke, 2017; Sepil et al., 2020) and oviposition rate (Marshall et al., 2009), as well as male sperm competitiveness (Ruhmann et al., 2018; Sepil et al., 2020) and sperm oxidative stress (Kant et al., 2019; Westfalewicz et al., 2021). Understanding reproductive ageing patterns of sperm as well as seminal fluid, and the tissues producing them, can provide a better understanding of male reproductive senescence.

References

Agudelo, J., Alfonso-Parra, C., and Avila, F. W. (2021). Male Age Influences Re-mating Incidence and Sperm Use in Females of the Dengue Vector Aedes aegypti. *Front. Physiol.* 12, 691221. doi: 10.3389/fphys.2021.691221.

Ahmad, I., Ahmad, I., Ahmad, N., Qureshi, Z., Jamil, H., and Rahman, Z. (2020). Determination of physiological biomarkers in the semen and peripheral blood of sahiwal bulls of two age groups. *Pak. J. Agric. Sci.* 57, 263–268.

Aich, U., Head, M. L., Fox, R. J., and Jennions, M. D. (2021). Male age alone predicts paternity success under sperm competition when effects of age and past mating effort are experimentally separated. *Proc. R. Soc. B Biol. Sci.* 288, 20210979. doi: 10.1098/rspb.2021.0979.

Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Mol. Reprod. Dev.* 84, 1039–1052. doi: 10.1002/mrd.22871.

Aitken, R. J., and Baker, M. A. (2006). Oxidative stress, sperm survival and fertility control. *Mol. Cell. Endocrinol.* 250, 66–69. doi: 10.1016/j.mce.2005.12.026.

Aitken, R. J., Drevet, J. R., Moazamian, A., and Gharagozloo, P. (2022). Male Infertility and Oxidative Stress: A Focus on the Underlying Mechanisms. *Antioxidants* 11, 306. doi: 10.3390/antiox11020306.

Archer, C. R., Paniw, M., Vega-Trejo, R., and Sepil, I. (2022). A sex skew in life-history research: the problem of missing males. *Proc. R. Soc. B Biol. Sci.* 289, 20221117. doi: 10.1098/rspb.2022.1117.

Atalan, G., Holt, P. E., and Barr, F. J. (1999). Ultrasonographic estimation of prostate size in normal dogs and relationship to bodyweight and age. *J. Small Anim. Pract.* 40, 119–122.

Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D., and Wolfner, M. F. (2011). Insect Seminal Fluid Proteins: Identification and Function. *Annu. Rev. Entomol.* 56, 21–40. doi: 10.1146/annurev-ento-120709-144823.

Bath, E., Bowden, S., Peters, C., Reddy, A., Tobias, J. A., Easton-Calabria, E., et al. (2017). Sperm and sex peptide stimulate aggression in female Drosophila. *Nat. Ecol. Evol.* 1, 0154. doi: 10.1038/s41559-017-0154.

Bauch, C., Boonekamp, J. J., Korsten, P., Mulder, E., and Verhulst, S. (2019). Epigenetic inheritance of telomere length in wild birds. *PLOS Genet.* 15, e1007827. doi: 10.1371/journal.pgen.1007827.

Berges, R., and Oelke, M. (2011). Age-stratified normal values for prostate volume, PSA, maximum urinary flow rate, IPSS, and other LUTS/BPH indicators in the German male community-dwelling population aged 50 years or older. *World J. Urol.* 29, 171–178. doi: 10.1007/s00345-010-0638-z.

Blagosklonny, M. V. (2021). The hyperfunction theory of aging: three common misconceptions. *Oncoscience* 8, 103–107. doi: 10.18632/oncoscience.545.

Bline, A. P., Le Goff, A., and Allard, P. (2020). What Is Lost in the Weismann Barrier? *J. Dev. Biol.* 8, 35. doi: 10.3390/jdb8040035.

Borziak, K., Álvarez-Fernández, A., L. Karr, T., Pizzari, T., and Dorus, S. (2016). The Seminal fluid proteome of the polyandrous Red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication. *Sci. Rep.* 6, 35864. doi: 10.1038/srep35864.

Bouwhuis, S., Vedder, O., and Becker, P. H. (2015). Sex-specific pathways of parental age effects on offspring lifetime reproductive success in a long-lived seabird: SEX-SPECIFIC PARENTAL EFFECTS ON OFFSPRING FITNESS. *Evolution* 69, 1760–1771. doi: 10.1111/evo.12692.

Bouwhuis, S., Verhulst, S., Bauch, C., and Vedder, O. (2018). Reduced telomere length in offspring of old fathers in a long-lived seabird. *Biol. Lett.* 14, 20180213. doi: 10.1098/rsbl.2018.0213.

Brenman-Suttner, D. B., Long, S. Q., Kamesan, V., de Belle, J. N., Yost, R. T., Kanippayoor, R. L., et al. (2018). Progeny of old parents have increased social space in Drosophila melanogaster. *Sci. Rep.* 8, 3673. doi: 10.1038/s41598-018-21731-0.

Bronikowski, A. M., Meisel, R. P., Biga, P. R., Walters, J. R., Mank, J. E., Larschan, E., et al. (2022). Sex-specific aging in animals: Perspective and future directions. *Aging Cell* 21. doi: 10.1111/acel.13542.

Campos, F. A., Altmann, J., Cords, M., Fedigan, L. M., Lawler, R., Lonsdorf, E. V., et al. (2022). Female reproductive aging in seven primate species: Patterns and consequences. *Proc. Natl. Acad. Sci.* 119, e2117669119. doi: 10.1073/pnas.2117669119.

Chapman, T. (2001). Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity* 87, 511–521.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F., et al. (2003). The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proc. Natl. Acad. Sci. U. S. A.* 100, 9923–9928.

Charlesworth, B. (1993). Evolutionary mechanisms of senescence. *Genetica* 91, 11–19. doi: 10.1007/BF01435984.

Comizzoli, P., and Ottinger, M. A. (2021). Understanding Reproductive Aging in Wildlife to Improve Animal Conservation and Human Reproductive Health. *Front. Cell Dev. Biol.* 9, 1–8. doi: 10.3389/fcell.2021.680471.

Cornwallis, C. K., Dean, R., and Pizzari, T. (2014). Sex-specific patterns of aging in sexual ornaments and gametes. *Am. Nat.* 184, E66–E78.

Crean, A. J., and Bonduriansky, R. (2014). What is a paternal effect? *Trends Ecol. Evol.* 29, 554–559. doi: 10.1016/j.tree.2014.07.009.

Curley, J. P., Mashoodh, R., and Champagne, F. A. (2011). Epigenetics and the origins of paternal effects. *Horm. Behav.* 59, 306–314. doi: 10.1016/j.yhbeh.2010.06.018.

Daxinger, L., and Whitelaw, E. (2012). Understanding transgenerational epigenetic inheritance via the gametes in mammals. *Nat. Rev. Genet.* 13, 153–162. doi: 10.1038/nrg3188.

Dean, R., Cornwallis, C. K., Løvlie, H., Worley, K., Richardson, D. S., and Pizzari, T. (2010). Male reproductive senescence causes potential for sexual conflict over mating. *Curr. Biol.* 20, 1192–1196. doi: 10.1016/j.cub.2010.04.059.

den Boer, S. P. A., Boomsma, J. J., and Baer, B. (2008). Seminal fluid enhances sperm viability in the leafcutter ant *Atta colombica*. *Behav. Ecol. Sociobiol.* 62, 1843–1849.

den Boer, S. P. A., Boomsma, J. J., and Baer, B. (2009). Honey bee males and queens use glandular secretions to enhance sperm viability before and after storage. *J. Insect Physiol.* 55, 538–543.
El-Gindy, Y. M., and Zeweil, H. S. (2017). Effects of parsley supplementation on the seminal quality, blood lipid profile and oxidant status of young and old male rabbits. *World Rabbit Sci.* 25, 215. doi: 10.4995/wrs.2017.6532.
Evans, J. P., Wilson, A. J., Pilastro, A., and Garcia-Gonzalez, F. (2019). Ejaculate-mediated paternal effects: evidence, mechanisms and evolutionary implications. *Reproduction* 157, R109–R126. doi: 10.1530/REP-18-0524.
Finkel, T., and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239–247. doi: 10.1038/35041687.
Fiumera, A. C., Dumont, B. L., and Clark, A. G. (2005). Sperm competitive ability in *Drosophila melanogaster* associated with variation in male reproductive proteins. *Genetics* 169, 243–257.
Fiumera, A. C., Dumont, B. L., and Clark, A. G. (2007). Associations between sperm competition and natural variation in male reproductive genes on the third chromosome of *Drosophila melanogaster*. *Genetics* 176, 1245–1260.
Fraser, L., Strzeżek, J., Filipowicz, K., Mogielnicka-Brzozowska, M., and Zasiadczyk, L. (2016). Age and seasonal-dependent variations in the biochemical composition of boar semen. *Theriogenology* 86, 806–816. doi: 10.1016/j.theriogenology.2016.02.035.
Fricke, C., and Koppik, M. (2019). Male reproductive ageing: A tale of the whole ejaculate. *Reproduction* 158, R219–R229. doi: 10.1530/REP-18-0579.
Gasparini, C., Devigili, A., and Pilastro, A. (2019). Sexual selection and ageing: interplay between pre- and post-copulatory traits senescence in the guppy. *Proc. R. Soc. B Biol. Sci.* 286, 20182873–20182873. doi: 10.1098/rspb.2018.2873.
Gasparini, C., Dosselli, R., and Evans, J. P. (2017). Sperm storage by males causes changes in sperm phenotype and influences the reproductive fitness of males and their sons. *Evol. Lett.* 1, 16–25.
Gasparini, C., Kelley, J. L., and Evans, J. P. (2014). Male sperm storage compromises sperm motility in guppies. *Biol. Lett.* 10, 20140681.
Gasparini, C., Marino, I. A. M., Boschetto, C., and Pilastro, A. (2010). Effect of male age on sperm traits and sperm competition success in the guppy (Poecilia reticulata). *J. Evol. Biol.* 23, 124–135. doi: 10.1111/j.1420-9101.2009.01889.x.
Goenaga, J., Yamane, T., Rönn, J., and Arnqvist, G. (2015). Within-species divergence in the seminal fluid proteome and its effect on male and female reproduction in a beetle. *BMC Evol. Biol.* 15, 266. doi: 10.1186/s12862-015-0547-2.
Hajam, Y. A., Rani, R., Ganie, S. Y., Sheikh, T. A., Javaid, D., Qadri, S. S., et al. (2022). Oxidative Stress in Human Pathology and Aging: Molecular Mechanisms and Perspectives. *Cells* 11, 552. doi: 10.3390/cells11030552.
Herrera-Cruz, M., Abraham, S., Nuñez-Beverido, N., Flores-Estévez, N., Reyes-Hernández, M., Alvarado, M., et al. (2018). Male age and strain affect ejaculate quality in the Mexican fruit fly: Age and strain affect Mexican fly ejaculates. *Insect Sci.* 25, 703–711. doi: 10.1111/1744-7917.12446.
Hood, W. R., Williams, A. S., and Hill, G. E. (2019). An Ecologist's Guide to Mitochondrial DNA Mutations and Senescence. *Integr. Comp. Biol.* 59, 970–982. doi: 10.1093/icb/icz097.
Hopkins, B. R., Sepil, I., Bonham, S., Miller, T., Charles, P. D., Fischer, R., et al. (2019a). BMP signaling inhibition in *Drosophila* secondary cells remodels the seminal proteome and self and rival ejaculate functions. *Proc. Natl. Acad. Sci. U. S. A.* 116, 24719–24728.
Hopkins, B. R., Sepil, I., Thézénas, M.-L., Craig, J. F., Miller, T., Charles, P. D., et al. (2019b). Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* 116, 17925–17933. doi: 10.1073/pnas.1906149116.
Hopkins, B. R., Sepil, I., and Wigby, S. (2017). Seminal fluid. *Curr. Biol.* 27, R404–R405.
Inyawilert, W., Rungruangsak, J., Chanthi, S., Liao, Y., Phinyo, M., Tang, P., et al. (2019). Age-related difference changes semen quality and seminal plasma protein patterns of Thai native rooster. *Int. J. Agric. Technol.* 15, 287–296.
Jin, B., Turner, L., Crawford, B., Birrell, A., and Handelsman, D. J. (1996). The development of the Baboon prostate: ultrasound methodology, modelling, and natural history. *J. Androl.* 17, 342–352.
Johnson, S. L., Dunleavy, J., Gemmell, N. J., and Nakagawa, S. (2015). Consistent age-dependent declines in human semen quality: A systematic review and meta-analysis. *Ageing Res. Rev.* 19, 22–33. doi: 10.1016/j.arr.2014.10.007.
Jones, O. R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C. G., Schaible, R., Casper, B. B., et al. (2014). Diversity of ageing across the tree of life. *Nature* 505, 169–173. doi: 10.1038/nature12789.
Kant, K., Tomar, A. K., Singh, S., and Yadav, S. (2019). Ageing associated proteomic variations in seminal plasma of Indian men. *J. Proteins Proteomics* 10, 83–89. doi: 10.1007/s42485-019-00013-x.
Kanuga, M. K., Benner, M. J., Doble, J. A., Wilson-Leedy, J. G., Robison, B. D., and Ingermann, R. L. (2011). Effect of aging on male reproduction in zebrafish (Danio rerio). *J. Exp. Zool. Part Ecol. Genet. Physiol.* 315A, 156–161. doi: 10.1002/jez.661.
Kara, H., Orem, A., Yulug, E., Yucesan, F. B., Kerimoglu, G., Yaman, S. O., et al. (2019). Hazelnut consumption improves testicular antioxidant function and semen quality in young and old male rats. *Food Chem.* 294, 1–8. doi: 10.1016/j.foodchem.2019.04.087.
Kekäläinen, J., Jokiniemi, A., Janhunen, M., and Huuskonen, H. (2020). Offspring phenotype is shaped by the nonsperm fraction of semen. *J. Evol. Biol.* 33, 584–594. doi: 10.1111/jeb.13592.
Kelso, K. A., Redpath, A., Noble, R. C., and Speake, B. K. (1997). Lipid and antioxidant changes in spermatozoa and seminal plasma throughout the reproductive period of bulls. *Reproduction* 109, 1–6. doi: 10.1530/jrf.0.1090001.
King, M., Eubel, H., Millar, A. H., and Baer, B. (2011). Proteins within the seminal fluid are crucial to keep sperm viable in the honeybee *Apis mellifera*. *J. Insect Physiol.* 57, 409–414.
Kirkwood, T. B. L. (2005). Understanding the Odd Science of Aging. *Cell* 120, 437–447. doi: 10.1016/j.cell.2005.01.027.
Kong, A., Frigge, M. L., Masson, G., Besenbacher, S., Sulem, P., Magnusson, G., et al. (2012). Rate of de novo mutations and the importance of father's age to disease risk. *Nature* 488, 471–475. doi: 10.1038/nature11396.

Koppik, M., and Fricke, C. (2017). Gene expression changes in male accessory glands during ageing are accompanied by reproductive decline in *Drosophila melanogaster*. *Mol. Ecol.* 26, 6704–6716. doi: 10.1111/mec.14384.

Koppik, M., Ruhmann, H., and Fricke, C. (2018). The effect of mating history on male reproductive ageing in Drosophila melanogaster. *J. Insect Physiol.* 111, 16–24. doi: 10.1016/j.jinsphys.2018.10.003.

Labbadia, J., and Morimoto, R. I. (2014). Proteostasis and longevity: when does aging really begin? *F1000Prime Rep.* 6. doi: 10.12703/P6-07.

Lemaître, J.-F., Ronget, V., and Gaillard, J.-M. (2020b). Female reproductive senescence across mammals: A high diversity of patterns modulated by life history and mating traits. *Mech. Ageing Dev.* 192, 111377. doi: 10.1016/j.mad.2020.111377.

Lind, M. I., Ravindran, S., Sekajova, Z., Carlsson, H., Hinas, A., and Maklakov, A. A. (2019). Experimentally reduced insulin/IGF-1 signaling in adulthood extends lifespan of parents and improves Darwinian fitness of their offspring. *Evol. Lett.* 3, 207–2016.

Liu, H., and Kubli, E. (2003). Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 9929–9933.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The Hallmarks of Aging. *Cell* 153, 1194–1217. doi: 10.1016/j.cell.2013.05.039.

Majić Balić, I., Milinković-Tur, S., Samardžija, M., and Vince, S. (2012). Effect of age and environmental factors on semen quality, glutathione peroxidase activity and oxidative parameters in simmental bulls. *Theriogenology* 78, 423–431. doi: 10.1016/j.theriogenology.2012.02.022.

Maklakov, A. A., and Immler, S. (2016). The Expensive Germline and the Evolution of Ageing. *Curr. Biol.* 26, R577–R586. doi: 10.1016/j.cub.2016.04.012.

Manjunath, P., and Thérien, I. (2002). Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. *J. Reprod. Immunol.* 53, 109–119. doi: 10.1016/S0165-0378(01)00098-5.

Mannucci, A., Argento, F. R., Fini, E., Coccia, M. E., Taddei, N., Becatti, M., et al. (2022). The Impact of Oxidative Stress in Male Infertility. *Front. Mol. Biosci.* 8, 799294. doi: 10.3389/fmolb.2021.799294.

Marshall, J. L., Huestis, D. L., Hiromasa, Y., Wheeler, S., Oppert, C., Marshall, S. A., et al. (2009). Identification, RNAi Knockdown, and Functional Analysis of an Ejaculate Protein that Mediates a Postmating, Prezygotic Phenotype in a Cricket. *PLoS ONE* 4, e7537. doi: 10.1371/journal.pone.0007537.

Mazeed, A. M., and Mohanny, K. M. (2010). Some reproductive characteristics of honeybee drones in relation to their ages: Reproductive characters of honeybee drones. *Entomol. Res.* 40, 245–250. doi: 10.1111/j.1748-5967.2010.00297.x.

McGraw, L. A., Suarez, S. S., and Wolfner, M. F. (2015). On a matter of seminal importance: Insights & Perspectives. *BioEssays* 37, 142–147. doi: 10.1002/bies.201400117.

Mohorianu, I., Fowler, E. K., Dalmay, T., and Chapman, T. (2018). Control of seminal fluid protein expression via regulatory hubs in *Drosophila melanogaster*. *Proc. R. Soc. B Biol. Sci.* 285, 20181681. doi: 10.1098/rspb.2018.1681.

Monaghan, P., Charmantier, A., Nussey, D. H., and Ricklefs, R. E. (2008). The evolutionary ecology of senescence. *Funct. Ecol.* 22, 371–378. doi: 10.1111/j.1365-2435.2008.01418.x.

Monaghan, P., and Metcalfe, N. B. (2019). The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. *Proc. R. Soc. B Biol. Sci.* 286, 20192187. doi: 10.1098/rspb.2019.2187.

Mubenga, L. E., Hermans, M. P., Chimanuka, D., Muhindo, L., Bwenge, E., and Tombal, B. (2020). Prostate volume and its relationship with anthropometric variables among different ethnic groups of South-Kivu, DR Congo. *Afr. J. Urol.* 26, 32. doi: 10.1186/s12301-020-00040-x.

Nakadera, Y., Swart, E. M., Hoffer, J. N. A., den Boon, O., Ellers, J., and Koene, J. M. (2014). Receipt of Seminal Fluid Proteins Causes Reduction of Male Investment in a Simultaneous Hermaphrodite. *Curr. Biol.* 24, 859–862. doi: 10.1016/j.cub.2014.02.052.

Noguera, J. C., Dean, R., Isaksson, C., Velando, A., and Pizzari, T. (2012). Age-specific oxidative status and the expression of pre- and postcopulatory sexually selected traits in male red junglefowl, Gallus gallus. *Ecol. Evol.* 2, 2155–2167. doi: 10.1002/ece3.300.

Noguera, J. C., Metcalfe, N. B., and Monaghan, P. (2018). Experimental demonstration that offspring fathered by old males have shorter telomeres and reduced lifespans. *Proc. R. Soc. B Biol. Sci.* 285, 20180268. doi: 10.1098/rspb.2018.0268.

Nussey, D. H., Froy, H., Lemaitre, J.-F., Gaillard, J.-M., and Austad, S. N. (2013). Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. *Ageing Res. Rev.* 12, 214–225. doi: 10.1016/j.arr.2012.07.004.

O'Dea, R. E., Lagisz, M., Jennions, M. D., Koricheva, J., Noble, D. W. A., Parker, T. H., et al. (2021). Preferred reporting items for systematic reviews and meta-analyses in ecology and evolutionary biology: a PRISMA extension. *Biol. Rev.* 96, 1695–1722. doi: 10.1111/brv.12721.

Ouzzani, M., Hammady, H., Fedorowicz, Z., and Elmagarmid, A. (2016). Rayyan—a web and mobile app for systematic reviews. *Syst. Rev.* 5, 210. doi: 10.1186/s13643-016-0384-4.

Patlar, B., Weber, M., and Ramm, S. A. (2019). Genetic and environmental variation in transcriptional expression of seminal fluid proteins. *Heredity* 122, 595–611. doi: 10.1038/s41437-018-0160-4.

Patlar, B., Weber, M., Temizyürek, T., and Ramm, S. A. (2020). Seminal Fluid-Mediated Manipulation of Post-mating Behavior in a Simultaneous Hermaphrodite. *Curr. Biol.* 30, 143-149.e4. doi: 10.1016/j.cub.2019.11.018.

Paul, C., and Robaire, B. (2013). Ageing of the male germ line. *Nat. Rev. Urol.* 10, 227–234. doi: 10.1038/nrurol.2013.18.

Perry, J. C., Sirot, L., and Wigby, S. (2013). The seminal symphony: how to compose an ejaculate. *Trends Ecol. Evol.* 28, 414–422. doi: 10.1016/j.tree.2013.03.005.

Pizzari, T., Dean, R., Pacey, A., Moore, H., and Bonsall, M. B. (2008). The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends Ecol. Evol.* 23, 131–140. doi: 10.1016/j.tree.2007.12.003.

Poiani, A. (2006). Complexity of seminal fluid: a review. *Behav. Ecol. Sociobiol.* 60, 289–310. doi: 10.1007/s00265-006-0178-0.

Preston, B. T., Saint Jalme, M., Hingrat, Y., Lacroix, F., and Sorci, G. (2015). The sperm of aging male bustards retards their offspring's development. *Nat. Commun.* 6, 6146. doi: 10.1038/ncomms7146.

Ramm, S. A. (2020). Seminal fluid and accessory male investment in sperm competition. *Philos. Trans. R. Soc. B Biol. Sci.* 375, 20200068. doi: 10.1098/rstb.2020.0068.

Reinhardt, K. (2007). Evolutionary Consequences of Sperm Cell Aging. *Q. Rev. Biol.* 82, 375–393. doi: 10.1086/522811.

Reinhardt, K., and Siva-Jothy, M. T. (2005). An Advantage for Young Sperm in the House Cricket *Acheta domesticus*. *Am. Nat.* 165, 718–723. doi: 10.1086/430010.

Reyes-Hernández, M., and Pérez-Staples, D. (2017). Mating senescence and male reproductive organ size in the Mexican fruit fly: Age effects on mating in Mexican fruit fly. *Physiol. Entomol.* 42, 26–35. doi: 10.1111/phen.12160.

Rezaei, A., Krishna, M. S., and Santhosh, H. T. (2015). Male Age Affects Female Mate Preference, Quantity of Accessory Gland Proteins, and Sperm Traits and Female Fitness in D. melanogaster. *Zoolog. Sci.* 32, 16. doi: 10.2108/zs140121.

Ruhmann, H., Koppik, M., Wolfner, M. F., and Fricke, C. (2018). The impact of ageing on male reproductive success in Drosophila melanogaster. *Exp. Gerontol.* 103, 1–10. doi: 10.1016/j.exger.2017.12.013.

Sabeti, P., Pourmasumi, S., Rahiminia, T., Akyash, F., and Talebi, A. R. (2016). Etiologies of sperm oxidative stress. *Int. J. Reprod. Biomed.* 14, 231–240.

Santhosh, H. T., and Krishna, M. S. (2013). Relationship between male age, accessory gland, sperm transferred, and fitness traits in *Drosophila bipectinata*. *J. Insect Sci.* 13, 159.

Sciamanna, I., Serafino, A., Shapiro, J. A., and Spadafora, C. (2019). The active role of spermatozoa in transgenerational inheritance. *Proc. R. Soc. B Biol. Sci.* 286, 20191263. doi: 10.1098/rspb.2019.1263.

Selvaratnam, J., and Robaire, B. (2016). Overexpression of catalase in mice reduces age-related oxidative stress and maintains sperm production. *Exp. Gerontol.* 84, 12–20. doi: 10.1016/j.exger.2016.08.012.

Sepil, I., Hopkins, B. R., Dean, R., Bath, E., Friedman, S., Swanson, B., et al. (2019). Ejaculate deterioration with male age, and its amelioration in *Drosophila*. Evolutionary Biology doi: 10.1101/624734.

Sepil, I., Hopkins, B. R., Dean, R., Bath, E., Friedman, S., Swanson, B., et al. (2020). Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. *Proc. Natl. Acad. Sci.* 117, 17094–17103. doi: 10.1073/pnas.2009053117.

Short, S. M., and Lazzaro, B. P. (2010). Female and male genetic contributins to post-mating immune defence in female *Drosophila melanogaster*. *Proc. R. Soc. Lond. B* 277, 3649–3657.

Simmons, L. W. (2011). Allocation of maternal- and ejaculate-derived proteins to reproduction in female crickets, Teleogryllus oceanicus: Allocation of maternal- and ejaculate-derived proteins. *J. Evol. Biol.* 24, 132–138. doi: 10.1111/j.1420-9101.2010.02158.x.

Simmons, L. W., Beveridge, M., Li, L., Tan, Y., and Millar, A. H. (2014). Ontogenetic changes in seminal fluid gene expression and the protein composition of cricket seminal fluid. *Evol. Dev.* 16, 101–109. doi: 10.1111/ede.12068.

Simmons, L. W., and Lovegrove, M. (2019). Nongenetic paternal effects via seminal fluid. *Evol. Lett.* 3, 403–411. doi: 10.1002/evl3.124.

Simmons, L. W., and Lovegrove, M. (2020). Can paternal effects via seminal fluid contribute to the evolution of polyandry? *Biol. Lett.* 16, 20200680. doi: 10.1098/rsbl.2020.0680.

Stockley, P., Franco, C., Claydon, A. J., Davidson, A., Hammond, D. E., Brownridge, P. J., et al. (2020). Revealing mechanisms of mating plug function under sexual selection. *Proc. Natl. Acad. Sci.* 117, 27465–27473. doi: 10.1073/pnas.1920526117.

Takemura, S., Ichikawa, H., Naito, Y., Takagi, T., Yoshikawa, T., and Minamiyama, Y. (2014). S-allyl cysteine ameliorates the quality of sperm and provides protection from age-related sperm dysfunction and oxidative stress in rats. *J. Clin. Biochem. Nutr.* 55, 155–161. doi: 10.3164/jcbn.14-39.

Tan, C. K. W., Pizzari, T., and Wigby, S. (2013). PARENTAL AGE, GAMETIC AGE, AND INBREEDING INTERACT TO MODULATE OFFSPRING VIABILITY IN *DROSOPHILA MELANOGASTER*: BRIEF COMMUNICATION. *Evolution*, n/a-n/a. doi: 10.1111/evo.12131.

Turnell, B. R., and Reinhardt, K. (2020). Metablic rate and oxygen radical levels increase but radical generation rate decreases with male age in *Drosophila melanogaster* sperm. *J. Gerontol. Biol. Sci.*, glaa078.

Vega-Trejo, R., Fox, R. J., Iglesias-Carrasco, M., Head, M. L., and Jennions, M. D. (2019). The effects of male age, sperm age and mating history on ejaculate senescence. *Funct. Ecol.* in press, 1–13.

Vince, S., Zaja, I. Z., Samardzija, M., Balic, I. M., Vilic, M., Duricic, D., et al. (2018). Age-related differences of semen quality, seminal plasma, and spermatozoa antioxidative and oxidative stress variables in bulls during cold and warm periods of the year. *ANIMAL* 12, 559–568. doi: 10.1017/S1751731117001811.

Vuarin, P., Bouchard, A., Lesobre, L., Levêque, G., Chalah, T., Jalme, M. S., et al. (2019). Post-copulatory sexual selection allows females to alleviate the fitness costs incurred when mating with senescing males. *Proc. Biol. Sci.* 286, 20191675–20191675. doi: 10.1098/rspb.2019.1675.

Vuarin, P., Lesobre, L., Levêque, G., Saint Jalme, M., Lacroix, F., Hingrat, Y., et al. (2021). Paternal age negatively affects sperm production of the progeny. *Ecol. Lett.*, 1–9. doi: 10.1111/ele.13696.

Waheed, M. M., El-Bahr, S. M., and Al-haider, A. K. (2013). Influence of Seminal Plasma Antioxidants and Osteopontin on Fertility of the Arabian Horse. *J. Equine Vet. Sci.* 33, 705–709. doi: 10.1016/j.jevs.2012.11.006.

Watkins, A. J., Dias, I., Tsuro, H., Allen, D., Emes, R. D., Moreton, J., et al. (2018). Paternal diet programs offspring health through sperm- and seminal plasma-specific pathways in mice. *Proc. Natl. Acad. Sci.* 115, 10064–10069. doi: 10.1073/pnas.1806333115.

Wedell, N., Gage, M. J. G., and Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends Ecol. Evol.* 17, 313–320.

Westfalewicz, B., Słowińska, M., Judycka, S., Ciereszko, A., and Dietrich, M. A. (2021). Comparative Proteomic Analysis of Young and Adult Bull (Bos taurus) Cryopreserved Semen. *Animals* 11, 2013. doi: 10.3390/ani11072013.

Wolfner, M. F. (1997). Tokens of love: Functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem. Mol. Biol.* 27, 179–192.

Wylde, Z., Spagopoulou, F., Hooper, A. K., Maklakov, A. A., and Bonduriansky, R. (2019). Parental breeding age effects on descendants' longevity interact over 2 generations in matrilines and patrilines. *PLOS Biol.* 17, e3000556. doi: 10.1371/journal.pbio.3000556.

Xie, K., Ryan, D. P., Pearson, B. L., Henzel, K. S., Neff, F., Vidal, R. O., et al. (2018). Epigenetic alterations in longevity regulators, reduced life span, and exacerbated aging-related pathology in old father offspring mice. *Proc. Natl. Acad. Sci.* 115. doi: 10.1073/pnas.1707337115.

Zhang, S.-J., Qian, H.-N., Zhao, Y., Sun, K., Wang, H.-Q., Liang, G.-Q., et al. (2013). Relationship between age and prostate size. *Asian J. Androl.* 15, 116–120. doi: 10.1038/aja.2012.127.

Chapter 4

Reproductive success of old polygynous males is limited by seminal fluid, not sperm number

Abstract

Advancing male age can lead to reproductive senescence, which in males is thought to be largely driven by declines in the numbers of sperm transferred by old males. This decline is predicted to be particularly pronounced in polygynous species, where male sperm reserves can deplete over a series of successive matings. However, males also transfer seminal fluid to females, and little is known about the differential contribution of sperm and seminal fluid to constrain the reproductive success of young and old multiply-mating males. Here, we investigated whether age-related variation in male reproductive success is driven by differential limitation of sperm and seminal fluid, in *Drosophila melanogaster*, over a series of experimental matings. Consistent with reproductive senescence, old males produced fewer offspring than young males. Surprisingly however, old males had more sperm in their seminal vesicles and transferred similar numbers of sperm to females, compared to young males. However, females that mated to old males stored fewer sperm than females mated to young males. Furthermore, the size of the seminal fluid-producing accessory glands declined more steeply over the mating sequence of old males. The reproductive decline suffered by old males was alleviated when a female received extra seminal fluid from a young male prior to her mating with focal old males. Collectively, our results indicate that reproductive success of old and of multiply-mating males is likely limited by seminal fluid quality and composition, rather than sperm number. While other factors such as differential sperm viability and female post-mating responses could have also influenced our results, our study highlights the under-appreciated role of seminal fluid in mediating male reproductive senescence in polygynous species.

Keywords: ageing, senescence, ejaculate, sexual conflict, sexual selection

Introduction

Advancing age, often (Lemaitre and Gaillard, 2017; Monaghan et al, 2008), but not always (Jones et al, 2014; Sanghvi et al, 2024), leads to irreversible declines in fecundity and fertility (Vrtilek et al, 2023), a pattern known as reproductive senescence (Monaghan and Metcalfe, 2019). In males, reproductive senescence is primarily attributed to age-dependent deterioration in mating success (Amin et al, 2012; Rezaei et al, 2015), reduced sperm quality (Dean et al, 2010; Gasparini et al, 2019; Johnson et al, 2015; Vuarin et al, 2019), quantity (Cornwallis et al, 2014; Sasson et al, 2012), and performance (Aich et al, 2021; Gasparini et al, 2010). However, male reproductive senescence can also occur due to age-dependent deterioration in the non-sperm component of the ejaculate, seminal fluid (Borziak, 2016; Fricke and Koppik, 2019; Koppik and Fricke, 2017; Sepil et al, 2020).

Understanding male reproductive senescence requires an integrated study of both, sperm- and seminal fluid-mediated age-related changes in male reproductive success (Koppik and Fricke, 2017; Sepil et al, 2020). In many species, seminal fluid is crucial for ensuring fertilization (Chapman and Davies, 2004), maintaining sperm performance (den Boer et al, 2010; Ramm, 2020), promoting female oviposition (Heifetz et al, 2005; Poiani, 2006; Sirot et al, 2009b), and facilitating long-term sperm storage (Avila et al, 2011). Ejaculates produced by old males often have an altered seminal fluid protein (SFP) composition (Koppik and Fricke, 2017, Sepil et al, 2020), lower quantities of seminal fluid (Ruhmann et al, 2018), more degraded SFPs (Fricke et al, 2023), and higher levels of oxidative stress (Noguera et al, 2012) than ejaculates of young males. Thus, age-related changes in seminal fluid can play an important role in constraining the reproductive success of old males (Fricke et al, 2023; Sepil et al, 2020). Measuring changes caused by both ejaculate components is crucial because each can senesce at different rates (Reinhardt et al, 2011; Sepil et al, 2020). In line with the disposable-soma theory of ageing (Maklakov and Immler, 2017), a higher rate of senescence

in seminal fluid (SF) than sperm is hypothesized due to SF being produced by somatic tissue, which might be less protected from age-related damage than the germline (Fricke et al, 2023; Milholland et al, 2017). Understanding senescence in the whole ejaculate, and not only sperm, will lead to more effective strategies for alleviating senescence in fertility.

Male reproductive senescence might be particularly relevant in polygynous species, where males can mate with a series of multiple females in quick succession over a 'mating sequence'. Ejaculate production is costly (Dowling and Simmons, 2012; Olsson et al, 1997), and males in polygynous species can become depleted of sperm (Gerofotis et al, 2015; Pitnick and Markow, 1994; Rubolini et al, 2007) and seminal fluid (Hopkins et al 2019b; Linklater et al, 2007; Reinhardt et al, 2011; Sirot et al, 2009a) when mating-multiply. Such ejaculate limitation can decrease the reproductive output of polygynous males as they progress through series of successive matings (Abe, 2019; Lewis, 2004; Loyau et al, 2012; Macartney et al, 2020; Swierk et al, 2015). In such scenarios, the effects of male reproductive senescence might become more pronounced later in a mating sequence if old males suffer steeper rates of ejaculate depletion (Bressac et al, 2008, 2009, Appendix 1). For instance, terminal investment could lead old males to invest relatively more in early matings of a mating sequence, at the cost of lower investment in later matings (Duffield et al, 2017; Part et al, 1992), leading to a steeper decline in reproductive output for old males through a mating sequence. Such hypotheses (see Appendix 1) can only be tested by investigating age-specific patterns of sperm and seminal fluid depletion over a mating sequence. Surprisingly however, little is known about such patterns.

Here we use the fruit fly, *Drosophila melanogaster*, to investigate changes in the reproductive success of old and young males over a mating sequence, and subsequently investigate the ejaculate-mediated mechanisms underpinning these patterns. Fruit flies are an ideal system for our study because they have been used extensively for reproductive ageing

research. Previous studies show that old males have lower quality and quantity of sperm (Sepil et al, 2020; Turnell and Reinhardt, 2020) and seminal fluid (reviewed in Fricke et al, 2023), and lower reproductive success (Sanghvi et al, 2023; Snoke and Promislow, 2003) than young males. Male fruit flies are polygynous (Hopkins et al, 2019b), and due to a low sperm to egg number ratio (Bjork and Pitnick, 2006), can become ejaculate depleted after few matings (Pitnick and Markow, 1994; Hopkins et al, 2019b).

We conduct three experiments. First, we compare the reproductive success of old and young males exposed to 10 females in a mating sequence (experiment A). Second, we investigate the role of seminal fluid in explaining age differences in reproductive success (experiment B). Finally, we test whether sperm number limitation in males and females explains differences in reproductive success of old and young males (experiment C). We test predictions form three non-mutually exclusive hypotheses (H1-H3). First, old males might be terminally investing in reproduction (i.e. steeper slopes for old males through a mating sequence), or be more prudent in their ejaculate allocation than young males (i.e. shallower slopes for old males through a mating sequence). In line with these hypotheses, we predict male age to interact with female order in a mating sequence, to affect male reproductive output and ejaculate allocation (experiments A and C; H1; see Appendix 1). Second, males might show higher rates of senescence in somatic tissue than the germline. This would cause old males to have lower reproductive output due to senescence in seminal fluid, rather than sperm. Thus, we predict that when females receive seminal fluid from young males before mating with focal males, the reproductive output of old and young focal males will be similar (experiment B; H2). Third, lower reproductive output of old compared to young males might be due to sperm number limitation. If so, we predict old males to have smaller sperm reserves and transfer fewer sperm to females, compared to young males (experiment C; H3).

Method

Stock maintenance

All flies in our experiments were maintained at a 12:12hr light cycle at a constant temperature of 25°C and 45% relative humidity, under which, flies have an egg-to-adult developmental time of 10 days. For experiments A and B, we used males of the wildtype Dahomey strain (henceforth, "*dah"*) that has been maintained since the 1970's in the lab. Experiment B additionally used *son-of-tudor* (henceforth, "*sot"*) males that are infertile (i.e. sperm-less) but transfer seminal fluid (Boswell and Mahowald, 1985; Hopkins et al, 2019a; Kalb et al, 1993; Sepil et al, 2020; see Appendix 2). For experiment C, we used the *Ub-GFP* line (henceforth, "*gfp"*) where transgenes expressing green fluorescent proteins enable the visualization of sperm heads (Manier et al, 2010). All females used in our experiments were young (3-4 days old) and of *dah* background. Across the three experiments, male flies between 3 to 11 days of age were considered young, and between 37 to 46 days as old, based on previous studies (Aguilar et al, 2023; Sanghvi et al, 2023; Sepil et al, 2020; Snoke and Promislow, 2003; Ruhmann et al, 2018; Turnell and Reinhardt, 2020).

Experimental flies

To generate experimental males of each line (*dah*, *sot* and *gfp*), we collected eggs from our stock populations with a standardised egg density of ~150 flies per bottle (Clancy and Kennington, 2001). To ensure virginity, we collected flies within six hours of emergence, using ice anaesthesia (Clancy and Kennington, 2001). All experimental males and females were fed with Lewis medium supplemented with molasses and *ad libitum* live yeast (Lewis, 1960). All experimental flies were kept as virgins in single-sex vials of 10 individuals, until being used for the experimental assays (more below). Experimental virgin males were transferred onto new food once a week. Virgin males in our lab usually have a median and

maximum lifespan of 50 and 90 days respectively (Sanghvi et al, 2023; Sepil et al 2020; also see Figure S6). Sample sizes across the three experiments are provided in table S1.

Mating experiments

Experiment A

We first compared changes in the reproductive output of young (9-10 days old) and old (44-46 days old) males in a multiple-mating sequence. On the day of the mating assay, 60 old and 60 young experimental males were haphazardly chosen to successively mate with a maximum of 10 females, over a duration of 9 hours. Each male was moved into a vial containing a single virgin young *dah* female, and observed. Each male remained with a female until the pair mated, and if males did not mate until the end of the 9 hour assay with any female, that female was not included in our analysis. Once mated, the male was immediately transferred into another vial containing a new, young virgin female. Mated females were given 24 hours to oviposit in the same vial, after which females were discarded. The flies (i.e. offspring) in these vials were given 14 days to develop and eclose, after which these vials were frozen at -20°C and the number of eclosed (adult) offspring counted. Experiment A was conducted across two replicates (blocks).

Experiment B

We tested whether seminal fluid limitation modulates the reproductive output of old and young, multiply-mate males. Specifically, we investigated whether seminal fluid obtained by a female from her first mating (with a young *sot* male), impacts the reproductive output of old and young *dah* males, who subsequently mate with these females. To do this, we generated young (4 to 11 days old) virgin *sot* males which lack sperm but produce seminal fluid (see Appendix 2). We mated ~200 young (3 to 4 days old) virgin *dah* females, each with a young

virgin *sot* male, and observed their matings. On the same day, we then conducted a multiple-mating assay using these *sot*-mated *dah* females, and experimental young (9-10 days old) and old (44-46 days old) *dah* males. Specifically, we chose 115 old and 80 young experimental *dah* males haphazardly to successively mate with a up to 10 *sot*-mated *dah* females, over a duration of 9 hours. More old males were used because old males were less likely to mate with females. Each young or old experimental *dah* male was moved into a vial containing a single *sot*-mated *dah* female, and observed, as described above for experiment A. Once mated, experimental *dah* males were immediately transferred into a new vial containing a different, young *sot*-mated *dah* female. Females who mated with experimental *dah* males were given 24 hours to oviposit in the same vial, after which females were discarded. These vials were frozen 14 days after the oviposition period at -20°C, and the number of eclosed (adult) offspring in the vials were later counted. Experiment B was conducted across two replicates (blocks).

Experiment C

Finally, we investigated whether sperm limitation drove differences in reproductive output of old and young mate-multiplying males. Specifically, we compared the number of sperm transferred to, and stored by, females mated to old and young mate-multiplying males, as well as sperm reserves and accessory gland size of mate-multiplying males. For this, we first generated experimental old (37 to 41 days old) and young (3 to 7 days old) *gfp* males. We then haphazardly chose 50 old and 28 young males to successively mate with a maximum of 10 *dah* females, over a duration of 9 hours. Old males are less likely to mate thus more were used. Similar to experiments A and B, each male was moved into a vial containing a single virgin *dah* female and following a mating, males were immediately transferred into a new vial containing a different virgin *dah* female. Following mating, odd-numbered females in a

male's mating sequence (i.e. 1st, 3rd, 5th, 7th, and 9th female), were given 24 hours to oviposit in the same vial, after which the female was frozen at -20°C. These vials were frozen 14 days after oviposition, and the number of eclosed (adult) offspring in these vials were counted. All even-numbered females in a male's mating sequence (i.e. the 2nd, 4th, 6th, 8th, and 10th female) were frozen within 30 minutes of mating, at -20°C. Additionally, all mated *gfp* males as well as four old and nine young virgin *gfp* males not exposed to females, were frozen at -20°C after the mating assay. Reproductive tracts of frozen females (bursa, seminal receptacle, and spermathecae) and males (seminal vesicle and accessory glands) were dissected in PBS under a Leica M80 dissection microscope (Appendix 3, 4, 5). Male accessory glands (the primary site of seminal fluid production in flies) and the sperm stored in odd-numbered females were imaged using a Nikon E600 fluorescence microscope. Sperm stored in even-numbered females and in male seminal vesicles were imaged using a Zeiss LSM880 confocal microscope (Appendix 3, 4, 5). Data from images was obtained using FIJI (Schindelin et al, 2012; Appendix 4, 5). Experiment C took place across three replicates (blocks).

Data analysis

Male reproductive output

We analysed data on male reproductive output through a mating sequence from each of the three experiments separately, using the package glmmTMB (Brooks et al, 2017) in R v4.2 (R core team, 2012). In each of these analyses, the numbers of offspring produced by each female over 24 hours of oviposition, was our dependent variable. We included male age (young or old), female order (henceforth, 'female number', i.e. the 1st to 10th female a male mates with) in a mating sequence, their two-way interaction (i.e. male age x female number), and replicate, as fixed effects, with male ID as a random effect across all models. Additionally, we included observation-level (i.e. row) as a random effect to control for

overdispersion in the data (Harrison, 2014), which was assessed using DHARMa (Hartig, 2017). Each analysis involved two steps. First, we ascertained whether including a zero-inflation term improved model fit. We did this by comparing a model with Poisson against one with a zero-inflated Poisson error distribution, and chose the model with the lowest AIC (henceforth, best-fit distribution). Second, using our best-fit distribution model, we compared three different fixed effect structures pertaining to male age and female number, to understand whether changes in male reproductive output though a mating sequence was linear or not. These model comparisons were done using a likelihood ratio test with the function *anova* in the package *base* (R core team, 2012). These fixed effect structures were: a model where male age interacted with only the linear term of female number (age * female number); one where male age interacted separately with both, the linear and quadratic term for female number ((age * female number) + (age * I(female number^2))); and one with male age, a linear term for female number, their two-way interaction, and a separate quadratic term for female number ((age * female number) + I(female number^2)). Importantly, males that did not copulate with a single female were excluded from these analyses.

Sperm transfer and storage in females

For experiment C, we additionally analysed data on the number of sperm transferred to odd-numbered females, and the number of sperm stored by even-numbered females after 24 hours of egg laying, in two separate models. To test how male age affected the number of sperm transferred to odd-numbered females, we modelled sperm number as our dependent variable. Here, sperm numbers were estimated automatically from images of female bursa, seminal receptacle, and spermathecae, using the 'find maxima' plugin on FIJI win32 (see Appendix 4). To test how male age affected the number of sperm stored by even-numbered females, we modelled sperm number as our dependent variable. Here, sperm numbers in female

spermathecae and seminal receptacle were counted manually using the cell counter plugin on FIJI win32 (see Appendix 4). For both models on sperm number in females, we modelled male age, female number in a mating sequence, their two-way interaction, and replicate, as fixed effects, with male ID and an observation-level as random effects. Our model selection procedure for both models was the same as described above (see data analysis: male reproductive output). Specifically, we first determined the best-fit error distribution for our models, and then determined the best fixed effects pertaining to female number being linear or quadratic.

Male sperm reserves and accessory glands

For experiment C, we further compared the number of sperm in the seminal vesicles of old and young males. Here, we included male age, male mating success (i.e. total number of females a male mated with), their two-way interaction, and replicate, as fixed effects. We modelled sperm counts (estimated using the 'find maxima' plugin on FIJI, Appendix 4) as our dependent variable. Similar to the models on reproductive output described above, for our model on sperm numbers in males, we first determined the best-fit error distribution. We then chose the most suitable fixed effects terms pertaining to male mating success being included as a linear or quadratic term. For experiment C, we also analysed data on the area of accessory glands (which are the primary site of SF production) on a subset on males. For this, we created a linear model with Gaussian error distribution in the *lme4* (Bates et al, 2014) package, and included male accessory gland area (one value per male, which was the average area of the two accessory glands) as our dependent variable. Male age, male mating success (i.e. total number of females a male mated with), their two-way interaction, and replicate, were modelled as fixed effects. All LMMs were checked for normality of residuals and homoscedasticity, using the *stats* package (R core team, 2012).

For all our models, only when the two-way interactions between male age and female number or male mating success were non-significant, we created a main-effects model to interpret the independent influence of fixed effects (Engqvist, 2005). For all our models, we conducted post-hoc pairwise comparisons between old and young males using effect sizes (Hedge's g, with $\alpha = 0.05$) for each female number in a mating sequence. For final models, we calculated the variance explained by fixed effects as $R^2_{marginal}$. Final model structures are described in Table S2.

Results

Male reproductive output

In experiment A, we mated *dah* males to *dah* females, to compare the reproductive output of old and young males over a mating sequence. Variation in the number of offspring produced by mated females was explained by a significant two-way interaction between male age and female number in a mating sequence ($z = -2.24$; $P = 0.025$; Figure 1A, Table S3). Post-hoc tests revealed that young males produced significantly more offspring compared to old males, earlier in a mating sequence. Overall, male reproductive output declined through a mating sequence, however this decline was steeper for young than old males (Figure S7).

In experiment B, we first mated *dah* females to young, virgin *sot* males (which transfer SF but not sperm), and then re-mated these females to either young or old *dah* males. We found no significant effect of male age ($z = 1.65$, $P = 0.099$), female number in a mating sequence ($z = 1.3$, $P = 0.193$), or an interaction between male age and female number to influence the number of offspring produced ($z = -1.46$, $P = 0.145$, Figure 1B, Figure S8; Table S4). When females previously received SF from *sot* males, the reproductive success of old and young *dah* males was statistically similar (Figure 1B), and male reproductive output did not decrease through a mating sequence.

In experiment C, we mated *gfp* males to *dah* females to compare the reproductive output of old and young males over a mating sequence. There was no significant interaction between male age and female number in the mating sequence, on the number of offspring produced ($z = -1.14$, $P = 0.254$, Figure 1C, Table S5). However, variation in the number of offspring produced by individual females was explained by significant main-effects of male age ($z = 2.01$, $P = 0.044$) and female number ($z = -3.23$, $P = 0.001$). Specifically, old males produced fewer offspring than young males, and males produced fewer offspring with females later in a mating sequence (Figure 1C, Figure S9, Table S5). In all three experiments, old males mated with fewer females (i.e. had lower mating success) compared to young males (Table S1b), which could be associated with longer mating latencies of old males (Appendix 7, Figure S10).

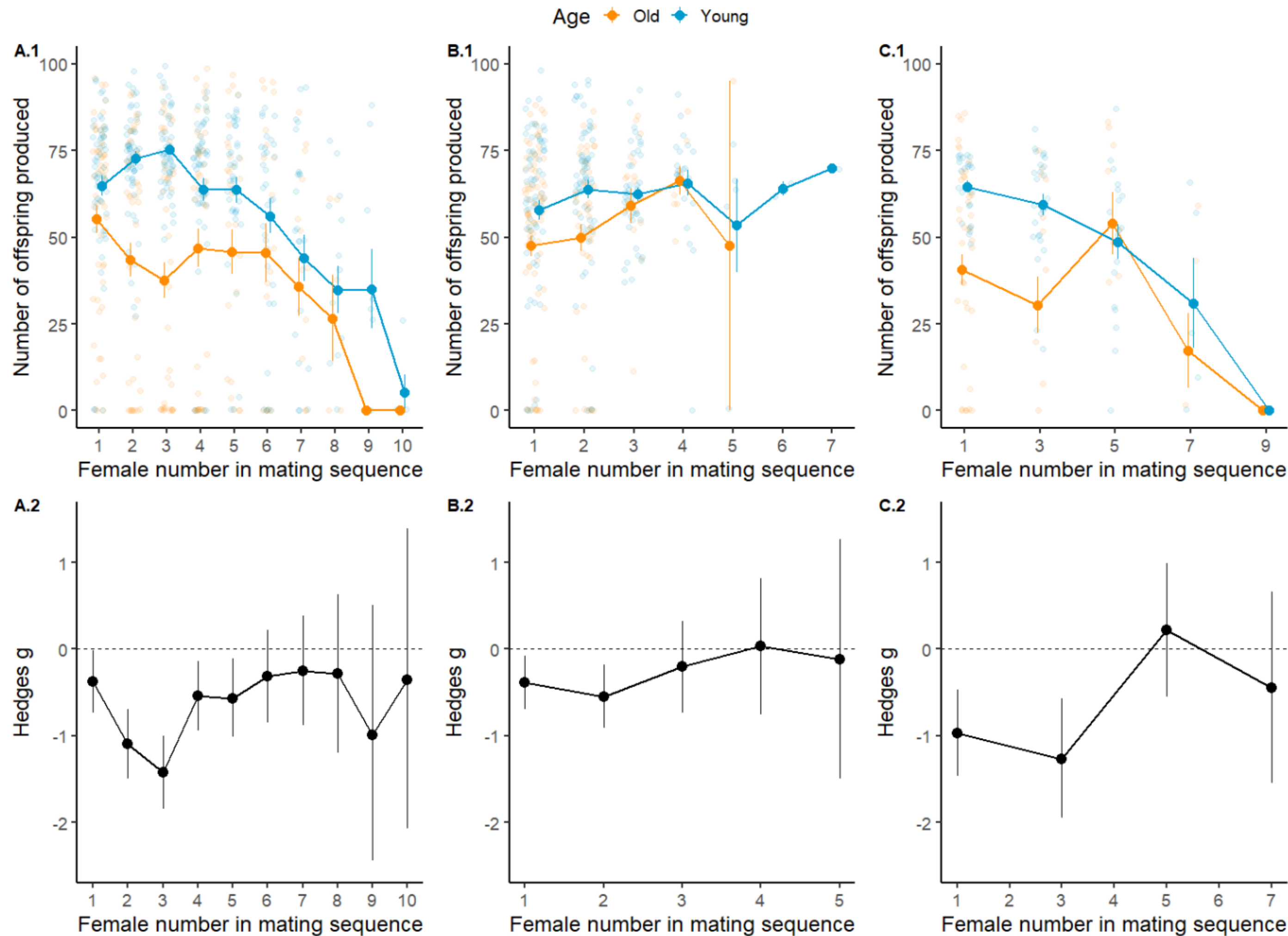


Figure 1: Reproductive output of old and young males in a mating sequence. **A.1, B.1, C.1**- Effect of male age and female number in a male's mating sequence, on the number of offspring produced by mated females, in experiments A, B, and C respectively. Means and SE shown. **A.2, B.2, C.2**- Post-hoc pair-wise comparisons between young and old males using effect sizes (Hedges' g) of reproductive output with each female in a mating sequence, for experiments A, B, and C respectively. Means and 95% C.I. shown. When C.I. does not overlap with zero, the effect is significant for $\alpha = 0.05$. Negative values indicate that old males have lower reproductive output than young males. Young males were 3-11 days old, old males between 37-46 days old.

Sperm transfer and storage in females

In experiment C, we further compared the number of sperm transferred to, and stored by, *dah* females mated to old or young *gfp* males in a mating sequence. We found a significant two-way interaction between male age and female number in a mating sequence, to affect the number of sperm transferred to females ($z = -2.905$, $P = 0.004$, Table S6). Specifically, old and young males transferred similar numbers of sperm to females early in their mating sequence. However, old males transferred more sperm than young males, to females later in the mating sequence (Figure 2A, 2D, Figure S11). When comparing the number of sperm stored in females after 24 hours, we found a significant two-way interaction between male age and female number ($z = -2.030$, $P = 0.042$, Table S7). Specifically, early in a mating sequence, females mated to young males stored more sperm than females mated to old males (Figure 2B, 2E, Figure S12). Compared to young males, old males showed shallower rates of decline through a mating sequence, in the number of sperm transferred to, or stored by females (Figure S11, S12A). Visual inspection revealed an asymptotic relationship between the number of sperm stored by odd-numbered females after 24 hours, and the number of offspring produced by these females over the first 24 hours after mating (Figure S12B).

Sperm and seminal fluid in males

In experiment C, we additionally compared the number of sperm present in the seminal vesicles (SV) of young and old males. We found a significant interaction between male age and mating success (i.e. total number of females a male mated with), on the number of sperm present in a male's SV ($z = -4.63$, $P < 0.001$, Figure 2C, 2F, Table S8). Specifically, old males had consistently more sperm present in their SV than young males, and this difference was greatest for males with intermediate mating success (Figure 2F). Finally, we compared the area of accessory glands (AG) of old and young males used in experiment C, as a proxy for

the quantity of seminal fluid in males. We found a significant interaction between male age and mating success ($z = 6.577$, $P < 0.001$, Table S9) on AG size. Old virgin males had larger AG than young males, however the rate of decline in AG size was steeper for old than young males (Figure 3A) such that later in a mating sequence, AG size did not differ between old and young males. Generally, virgin old males had a higher ratio of sperm to AG size than virgin young males (Figure 3B), and this difference between old and young males became more exaggerated in males with higher mating success.

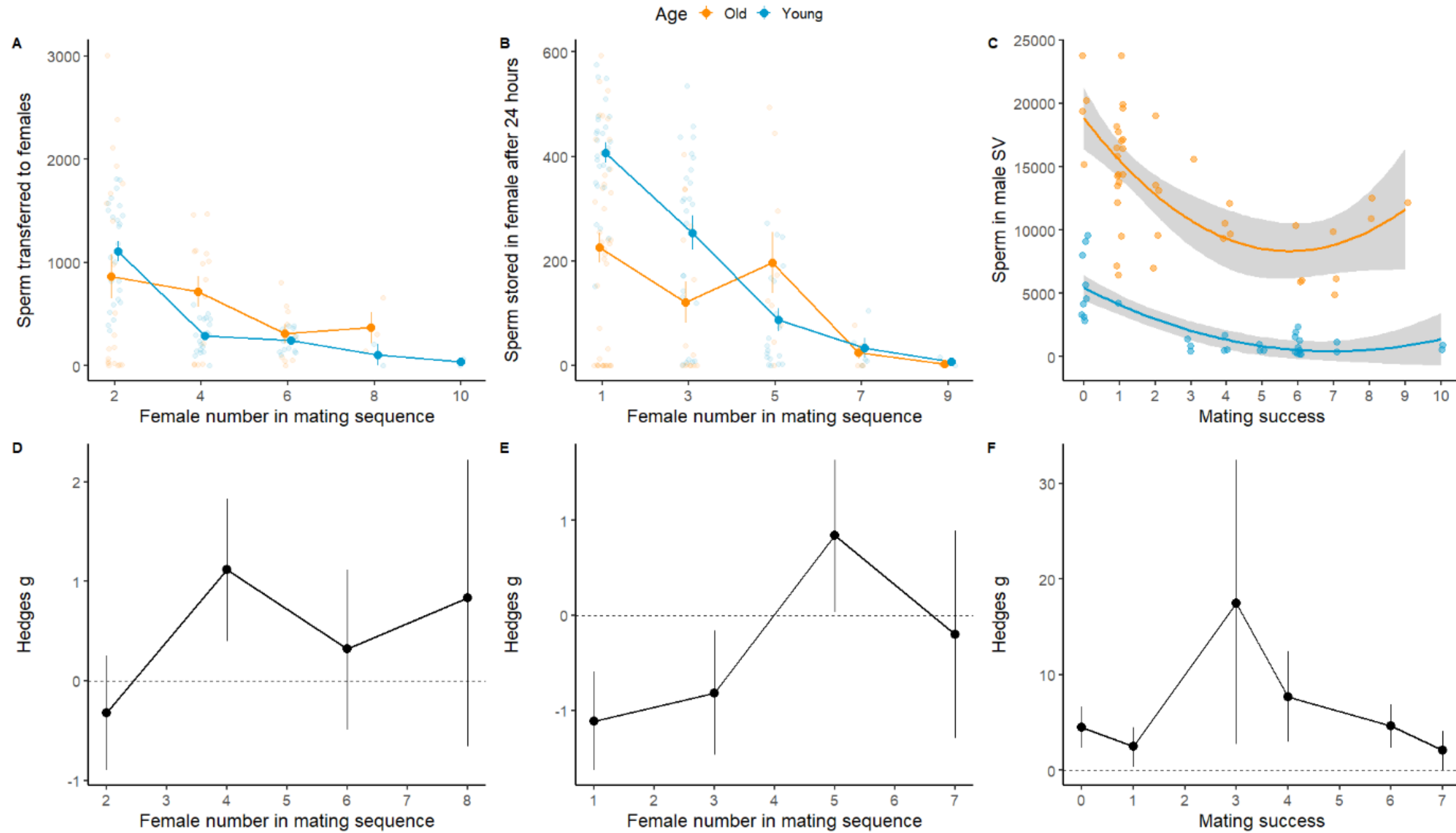


Figure 2: Sperm numbers in females and males. A- Sperm transferred to females by young and old males in a mating sequence, B- Sperm stored by females mated to young and old males, after 24 hours. C- Number of sperm in male seminal vesicles (SV) for old and young males with different mating success. Means and SE shown for panels A, B, C. Panels D, E, F- associated effect sizes when comparing young and old males in panels A, B, and C respectively. Means and C.I. shown for panels D, E, and F. For panels D, E, and F, when C.I. does not overlap with zero, the effect is significant for $\alpha = 0.05$; negative values indicate that old males have lower sperm numbers than young males. Young males were between 3-7 days, old males 37-42 days old.

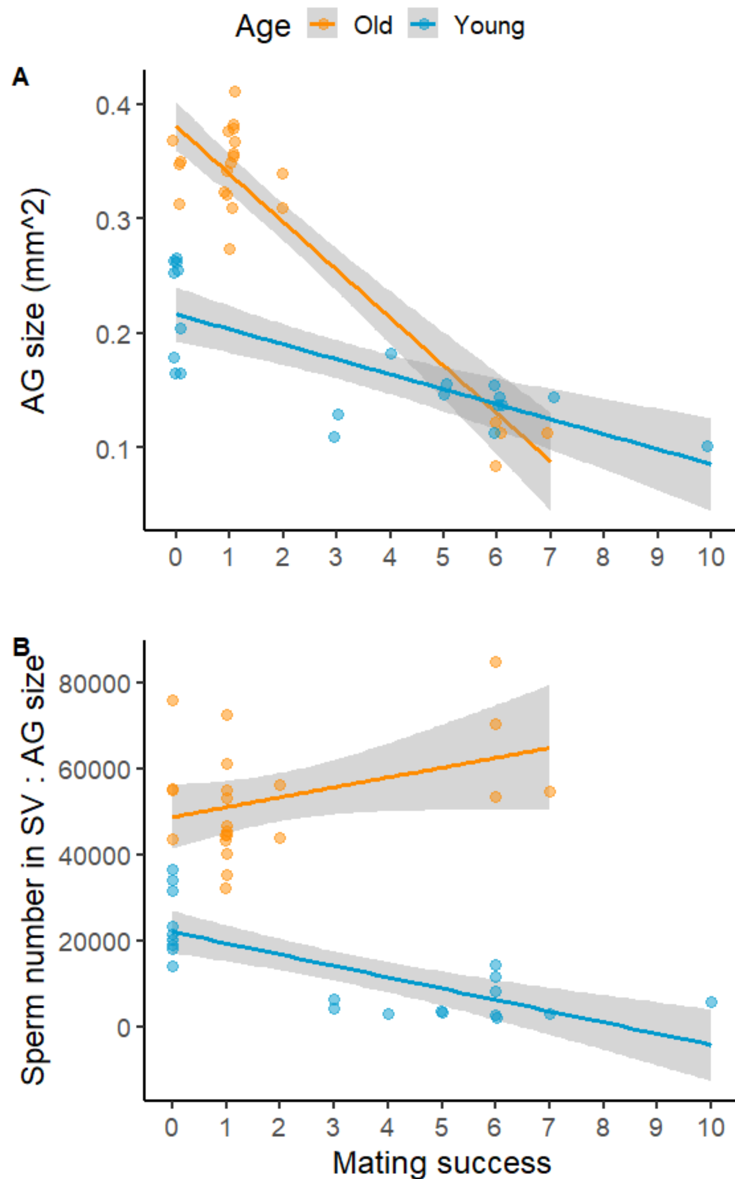


Figure 3: Changes in accessory gland in old and young males with different mating success. A. In experiment C, there was a significant interaction between male age and male mating success, to affect the size of male accessory glands (mm^2). Old males, despite having larger AGs when virgins, showed steeper declines in AG size than young males through a mating sequence. Means and 95% C.I. shown. B. Ratio of sperm reserves in male SV to accessory gland size ($count/mm^2$). Virgin old males have more sperm in their SV, than SF in their AGs, compared to virgin young males. Old males however allocate relatively more seminal fluid than sperm per mating while young males do the opposite, through a mating sequence. Young males were 3-7 days old, old males 37-42 days old.

Discussion

Male reproductive senescence has been demonstrated across taxa, in studies that mate males with an individual female (reviewed in Monaghan and Metcalfe, 2019; Sanghvi et al, 2024). However, these studies are not informative about reproductive senescence in polygynous species (Bressac et al, 2008, 2009), where males are exposed to multiple partners and male reproductive success can be modulated by female order/number in a male's mating sequence (Abe, 2019; Lewis, 2004; Macartney et al, 2020; Appendix 1). Additionally, the traits and pathways contributing towards male reproductive senescence can be diverse, and have been difficult to pinpoint. These include age-dependent declines in quality and quantity of sperm (Sanghvi et al, 2024) and seminal fluid (Fricke et al, 2023), changes in male behaviour and mating success (Dean et al, 2010; Hayes et al, 2013) as well as ejaculate allocation (Win et al, 2013). Furthermore, females might contribute to male reproductive senescence via preference for young males pre- or post-copulation (Radwan, 2003; Vuarin et al, 2019). Our study bridges these knowledge gaps by investigating age-related changes in male reproductive output using multiple-mating assays, to understand the contribution of various pathways causing male reproductive senescence.

Using three experiments, we investigated whether old and young males differed in their reproductive output through a mating sequence (H1). We then explored whether observed patterns are explained by depletion of seminal fluid (H2) or sperm limitation in old males (H3). Overall, we found that old males had a lower reproductive output than young males (H1). In general, male reproductive output declined through a mating sequence, and males produced fewer offspring with, and transferred fewer sperm to, females later in a mating sequence. However, contrary to predictions, this decline was steeper for young rather than old males (H1, Appendix 1). We also discovered that when a female received extra seminal fluid (SF) from a young male prior to her focal mating, differences in reproductive

output between old and young focal males diminished (H2). This 'extra' seminal fluid further alleviated declines in male reproductive output through a mating sequence. We found old males to have larger accessory glands (AGs), the main site of SF production and storage compared to young males. Yet, old males experienced steeper rates of decline in AG size (thus SF reserves) through a mating sequence, relative to young males (H2). Overall, females mated to old males stored fewer sperm compared to females mated to young males. Surprisingly, this result emerged despite old males having larger sperm reserves, and transferring similar or greater numbers of sperm to females, than young males (H3). Collectively, our results suggest that male reproductive senescence is less apparent later in a male's mating sequence. Furthermore, our findings indicate that male reproductive senescence may be largely driven by senescence in seminal fluid or sperm *quality*, or by female preference for young males, rather than senescence in sperm *quantity*.

Changes with male age

We found that old males had higher numbers of sperm stored in their seminal vesicles than young males (also shown by Decanini et al, 2013; Kehl et al, 2013, 2015; Reinhardt et al, 2011). Sanghvi et al (2024) in a meta-analysis found a similar pattern across insects, and attribute this to experimental males typically being maintained as virgin, which can lead to longer periods of sperm accumulation in old than young males. Consistent with this, at low mating rates, old males often have larger ejaculates than young males, but at high mating rates this pattern is reversed (Aich et al, 2021; Bressac et al, 2009; Sepil et al, 2020). In our study, we maintained males as virgins until the mating assay, and old males might have accumulated more sperm than young males because spermatogenesis occurs throughout adult life in fruit flies with relatively low levels of sperm loss (Bjork et al, 2007; Demarco et al, 2013; Santos et al, 2023; Sepil et al, 2020). Old virgin males had a higher ratio of sperm

reserves to AG size (thus SF reserves), compared to young virgin males, suggesting faster rates of sperm than SF accumulation, with age.

Despite having larger sperm reserves and transferring more or a similar number of sperm to females, females stored fewer sperm from old males, and old males produced fewer offspring, than young males. There could be several non-mutually exclusive explanations for these puzzling patterns. First, the prior receipt of seminal fluid resulted in similar number of offspring produced by old and young males. In fruit flies, seminal fluid proteins (SFPs), especially those produced in accessory glands (Acps), play an important role in fertilisation, female sperm uptake and storage, and ovulation (Avila et al, 2011; Bloch Qazi and Wolfner, 2003; Chapman and Davies, 2004; Heifetz et al, 2005; Poiani, 2006; Ravi Ram and Wolfner, 2007). Old *D. melanogaster* males lack important Acps such as ovulin and sex peptide, and have more degraded SFPs compared to young males (Fricke et al, 2023; Ruhmann et al, 2018; Sepil et al, 2020). Old *dah* males might have benefitted from females receiving seminal fluid from young *sot* males, explaining our results. Second, old males might have less viable or motile sperm than young males (e.g. Sepil et al, 2020; Sturup et al, 2013), leading fewer sperm to be stored by females and consequently reduce fertilisation rates for old males, despite old males transferring similar numbers of sperm to females, compared to young males. Third, females mated to old males might have ejected greater proportions of sperm than females mated to young males, via cryptic female choice (Snook and Hosken, 2004; Vuarin et al, 2019; Wagner et al, 2004). Future experiments could use transgenic females that do not eject sperm, to disentangle whether sperm ejection or low sperm viability explain our results.

Our result of old males transferring similar sperm numbers as young males, but producing fewer offspring, is unlikely to be explained by variation in overall *quantity* of seminal fluid. This is because old males had larger accessory glands, thus likely transferred

higher volumes of seminal fluid than young males (also shown by Rezaei et al, 2015- who importantly show that young males have smaller AGs but transfer more SFPs to females). We suggest that future studies could manipulate the age of *sot* (SF-donor) males, to disentangle whether quantity, composition, or quality of SF explains our results better. Future studies could also compare the quantity and composition of SFPs *transferred* by males to females. Our results have important implications for sexual selection and conflict because they demonstrate that males who mate second might indirectly benefit from the SF of males who mate first with a female (Alonzo and Pizzari, 2010; Holman et al, 2009; Nguyen and Moehring, 2018). While not a direct test of the disposable-soma hypothesis (Maklakov and Immler, 2016), our results indicate higher rates of senescence in seminal fluid quality/composition than sperm number, suggesting males might be investing more in maintaining the germline than soma.

Methodological factors might also explain why old males produced fewer offspring despite transferring similar or more numbers of sperm to females. For instance, offspring from old fathers might have lower egg-to-adult survival (e.g. Preston et al, 2015; Ruhmann et al, 2018) due to deleterious paternal age effects (Monaghan et al, 2020). This could have biased our measurement of the number of eclosing offspring toward those of young males, even if old and young males fertilised equal numbers of eggs. Selective disappearance of males (Bouwhuis et al, 2009; Sanghvi et al, 2022) with low sperm production rates, especially in our *gfp* line that experienced high mortality (Figure S6), could have biased the cohort of old *gfp* males. Additionally, post-meiotic sperm damage (Pizzari et al, 2008; Wagner et al, 2004; White et al, 2008) could reduce the reproductive output of old males, due to old virgin males likely storing sperm for longer durations before ejaculation than young virgin males (Pizzari et al, 2008).

Changes through male mating sequence

We found that males produced fewer offspring as they progressed through a mating sequence in experiments A and C. Studies have previously shown that males, when multiply-mating, can become depleted of sperm and seminal fluid (Gerofotis et al, 2015; Pitnick and Markow, 1994; Rubolini et al, 2007; Sirot et al, 2009a), producing fewer offspring with females encountered later in a mating sequence (Abe, 2019; Lewis, 2004; Loyau et al, 2012; Macartney et al, 2020; Swierk et al, 2015). The asymptotic relationship between sperm stored in females, and offspring produced by females in our study, indicates that reproductive output through a mating sequence is likely limited by seminal fluid, not sperm numbers (Hopkins et al 2019b; Linklater et al, 2007; Reinhardt et al, 2011; Sirot et al, 2009a). Here, even if males transfer more sperm, offspring production would not increase. This could either be due to a ceiling on how many eggs a female can lay, or because female ovulation and fertilisation rates are constrained by insufficient quantities of seminal fluid (Bloch Qazi et al, 2003; Chapman, 2001). Results from experiment B support this. Here, females were provided with SF from a previous mating before the focal mating took place, and focal male reproductive output did not decline through the mating sequence. Fruit fly males become depleted of seminal fluid at faster rates than sperm when multiply-mating (Reinhardt et al, 2011; Linklater et al, 2007), which is consistent with results of our study.

A lack of decline in reproductive output through a mating sequence in experiment B might also be explained by male ejaculate plasticity. Females in experiment B were first mated to *sot* males before being used to mate with focal *dah* males. Male flies can detect female mating status using olfactory cues (Friberg, 2006; Lupold et al, 2011) to infer levels of sperm competition. Cues of high sperm competition risk (i.e. many rival males) lead male flies to transfer larger ejaculates to females (Bretman et al, 2009; Hopkins et al, 2019b; Price et al, 2012; Thomas and Simmons, 2009; Wedell and Cook, 1999) and even alter their SFP

composition (Ramm et al, 2015; Wigby et al, 2009). In experiment B, focal *dah* males might have perceived higher sperm competition levels and increased their ejaculate investment in each mating across the mating sequence, compared to experiments A and C. This could have alleviated a male's reproductive decline through his mating sequence (possibly at the cost of fewer overall matings in experiment B).

Male age x mating sequence interactions

We discovered old males to have shallower declines in offspring production and sperm number through a mating sequence than young males. Contrary to predictions however (i.e. H1, see Appendix 1), this result was not due to prudent sperm allocation by old males which would also require old males to be sperm limited (Appendix 1). Our results do not support the terminal investment hypothesis either. This is because if old males were terminally investing (Duffield et al, 2017; Froy et al, 2013), we predicted them to have steeper declines in reproductive output through a mating sequence than young males. Generally, our results indicate that a female would benefit from mating with a young male only when she encounters him early in his mating sequence.

Conclusions

Male reproductive senescence is multifaceted and can be either exacerbated or buffered under polygyny, depending on patterns of ejaculate accumulation (in male extra-gonadal reserves) and allocation to females, as well as the reproductive trait being measured. In polygynous species with low anisogamy, ejaculate depletion can have severe consequences for male and female reproductive and mating success, and we show that different components of the ejaculate- sperm versus seminal fluid, decline at different rates with age and through a mating sequence. We show that old males have lower reproductive output than young males, which is likely driven by either senescence in seminal fluid quality/composition or sperm viability, or via female-mediated effects, rather than senescence in sperm number. These results have important consequences for sexual conflict and sexual selection (Adler et al, 2014; Damiens and Boivin, 2006; Carazo et al, 2011; see Appendix 7), whereby female fitness is not only determined by a male's mating history (e.g. Wedell et al, 2002) but also its interaction with male age. Our results show that declines in male reproductive output are not permanent, and can be reversed by the presence of sufficient seminal fluid from a young male. This interplay between sperm and seminal fluid could have crucial implications for alleviating declines in human and animal fertility (Eini et al, 2021).

Data availability

All data from our experiment and the R code used for analysis can be found at OSF: 10.17605/OSF.IO/5Z7M3. Images of dissected *gfp* males' SV, AGs, and images for sperm numbers transferred to and stored by females, can be found on figshare: 10.6084/m9.figshare.25407223, , 10.6084/m9.figshare.25407298, 10.6084/m9.figshare.25407253, 10.6084/m9.figshare.25407382.

References

Abe, J., 2019. Sperm-limited males continue to mate, but females cannot detect the male state in a parasitoid wasp. Behav Ecol Sociobiol 73, 52. https://doi.org/10.1007/s00265-019-2653-4

Adler, M.I., Bonduriansky, R., 2014. Sexual Conflict, Life Span, and Aging. Cold Spring Harbor Perspectives in Biology 6, a017566–a017566. https://doi.org/10.1101/cshperspect.a017566

Aguilar, P., Dag, B., Carazo, P., Sultanova, Z., 2023. Sex-specific paternal age effects on offspring quality in *Drosophila melanogaster*. J of Evolutionary Biology jeb.14167. https://doi.org/10.1111/jeb.14167

Aich, U., Head, M.L., Fox, R.J., Jennions, M.D., 2021. Male age alone predicts paternity success under sperm competition when effects of age and past mating effort are experimentally separated. Proc. R. Soc. B. 288, 20210979. https://doi.org/10.1098/rspb.2021.0979

Alonzo, S.H., Pizzari, T., 2010. Male Fecundity Stimulation: Conflict and Cooperation Within and Between the Sexes: Model Analyses and Coevolutionary Dynamics. The American Naturalist 175, 174–185. https://doi.org/10.1086/649596

Amin, M.R., Bussière, L.F., Goulson, D., 2012. Effects of Male age and Size on Mating Success in the Bumblebee Bombus terrestris. J Insect Behav 25, 362–374. https://doi.org/10.1007/s10905-011-9306-4

Anthes, N., Häderer, I.K., Michiels, N.K., Janicke, T., 2017. Measuring and interpreting sexual selection metrics: evaluation and guidelines. Methods Ecol Evol 8, 918–931. https://doi.org/10.1111/2041-210X.12707

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., Wolfner, M.F., 2011. Insect Seminal Fluid Proteins: Identification and Function. Annu. Rev. Entomol. 56, 21–40. https://doi.org/10.1146/annurev-ento-120709-144823

Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting Linear Mixed-Effects Models using lme4. https://doi.org/10.48550/ARXIV.1406.5823

Bjork, A., Dallai, R., Pitnick, S., 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca* , a species with giant sperm. Biol. Lett. 3, 517–519. https://doi.org/10.1098/rsbl.2007.0219

Bjork, A., Pitnick, S., 2006. Intensity of sexual selection along the anisogamy–isogamy continuum. Nature 441, 742–745. https://doi.org/10.1038/nature04683

Bloch Qazi, M.C., Heifetz, Y., Wolfner, M.F., 2003. The developments between gametogenesis and fertilization: ovulation and female sperm storage in drosophila melanogaster. Developmental Biology 256, 195–211. https://doi.org/10.1016/S0012-1606(02)00125-2

Bloch Qazi, M.C., Wolfner, M.F., 2003. An early role for the *Drosophila melanogaster* male seminal protein Acp36DE in female sperm storage. Journal of Experimental Biology 206, 3521–3528. https://doi.org/10.1242/jeb.00585

Bouwhuis, S., Sheldon, B.C., Verhulst, S., Charmantier, A., 2009. Great tits growing old: selective disappearance and the partitioning of senescence to stages within the breeding cycle. Proc. R. Soc. B. 276, 2769–2777. https://doi.org/10.1098/rspb.2009.0457

Borziak, K., Álvarez-Fernández, A., L. Karr, T., Pizzari, T., Dorus, S., 2016. The Seminal fluid proteome of the polyandrous Red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication. Sci Rep 6, 35864. https://doi.org/10.1038/srep35864

Boswell, R., Mahowald, 1985. tudor, a gene required for assembly of the germ plasm in Drosophila melanogaster. Cell 43, 97–104. https://doi.org/10.1016/0092-8674(85)90015-7

Bressac, C., Damiens, D., Chevrier, C., 2008. Sperm stock and mating of males in a parasitoid wasp. J. Exp. Zool. 310B, 160–166. https://doi.org/10.1002/jez.b.21168

Bressac, C., Thi Khanh, H.D., Chevrier, C., 2009. Effects of age and repeated mating on male sperm supply and paternity in a parasitoid wasp. Entomologia Experimentalis et Applicata 130, 207–213. https://doi.org/10.1111/j.1570-7458.2008.00810.x

Bretman, A., Fricke, C., Chapman, T., 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. Proc. R. Soc. B. 276, 1705–1711. https://doi.org/10.1098/rspb.2008.1878

Brooks, M.E., Kristensen, K., Van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Mächler, M., Bolker, B.M., 2017. Modeling zero-inflated count data with glmmTMB (preprint). Ecology. https://doi.org/10.1101/132753

Carazo, P., Molina-Vila, P., Font, E., 2011. Male reproductive senescence as a potential source of sexual conflict in a beetle. Behavioral Ecology 22, 192–198. https://doi.org/10.1093/beheco/arq187

Chapman, T., Davies, S.J., 2004. Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies. Peptides 25, 1477–1490. https://doi.org/10.1016/j.peptides.2003.10.023

Chapman, T., 2001. Seminal fluid-mediated fitness traits in Drosophila. Heredity 87, 511–521. https://doi.org/10.1046/j.1365-2540.2001.00961.x

Clancy, Kennington, 2001. A simple method to achieve consistent larval density in bottle cultures. Dros. Inf. Serv. 84.

Cornwallis, C.K., Dean, R., Pizzari, T., 2014. Sex-Specific Patterns of Aging in Sexual Ornaments and Gametes. The American Naturalist 184, E66–E78. https://doi.org/10.1086/677385

Crudgington, H.S., Fellows, S., Badcock, N.S., Snook, R.R., 2009. EXPERIMENTAL MANIPULATION OF SEXUAL SELECTION PROMOTES GREATER MALE MATING CAPACITY BUT DOES NOT ALTER SPERM INVESTMENT. Evolution 63, 926–938. https://doi.org/10.1111/j.1558-5646.2008.00601.x

Damiens, D., Boivin, G., 2006. Why do sperm-depleted parasitoid males continue to mate? Behavioral Ecology 17, 138–143. https://doi.org/10.1093/beheco/arj009

Dean, R., Cornwallis, C.K., Løvlie, H., Worley, K., Richardson, D.S., Pizzari, T., 2010. Male Reproductive Senescence Causes Potential for Sexual Conflict over Mating. Current Biology 20, 1192–1196. https://doi.org/10.1016/j.cub.2010.04.059

Decanini, D.P., Wong, B.B.M., Dowling, D.K., 2013. Context-dependent expression of sperm quality in the fruitfly. Biol. Lett. 9, 20130736. https://doi.org/10.1098/rsbl.2013.0736

Demarco, R.S., Eikenes, Å.H., Haglund, K., Jones, D.L., 2014. Investigating spermatogenesis in Drosophila melanogaster. Methods 68, 218–227. https://doi.org/10.1016/j.ymeth.2014.04.020

den Boer, S.P.A., Baer, B., Boomsma, J.J., 2010. Seminal Fluid Mediates Ejaculate Competition in Social Insects. Science 327, 1506–1509. https://doi.org/10.1126/science.1184709

Dowling, D.K., Simmons, L.W., 2012. Ejaculate Economics: Testing the Effects of Male Sexual History on the Trade-Off between Sperm and Immune Function in Australian Crickets. PLoS ONE 7, e30172. https://doi.org/10.1371/journal.pone.0030172
Duffield, K.R., Bowers, E.K., Sakaluk, S.K., Sadd, B.M., 2017. A dynamic threshold model for terminal investment. Behav Ecol Sociobiol 71, 185. https://doi.org/10.1007/s00265-017-2416-z
Eini, F., Kutenaei, M.A., Shirzeyli, M.H., Dastjerdi, Z.S., Omidi, M., Novin, M.G., 2021. Normal seminal plasma could preserve human spermatozoa against cryopreservation damages in Oligozoospermic patients. BMC Mol and Cell Biol 22, 50. https://doi.org/10.1186/s12860-021-00390-6
Engqvist, L., 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. Animal Behaviour 70, 967–971. https://doi.org/10.1016/j.anbehav.2005.01.016
Friberg, U., 2006. Male perception of female mating status: its effect on copulation duration, sperm defence and female fitness. Animal Behaviour 72, 1259–1268. https://doi.org/10.1016/j.anbehav.2006.03.021
Fricke, C., Koppik, M., 2019. Male reproductive ageing: a tale of the whole ejaculate. Reproduction 158, R219–R229. https://doi.org/10.1530/REP-18-0579
Fricke, C., Sanghvi, K., Emery, M., Lindenbaum, I., Wigby, S., Ramm, S.A., Sepil, I., 2023. Timeless or tainted? The effects of male ageing on seminal fluid. Front. Ecol. Evol. 11, 1066022. https://doi.org/10.3389/fevo.2023.1066022
Froy, H., Phillips, R.A., Wood, A.G., Nussey, D.H., Lewis, S., 2013. Age-related variation in reproductive traits in the wandering albatross: evidence for terminal improvement following senescence. Ecol Lett 16, 642–649. https://doi.org/10.1111/ele.12092
Gasparini, C., Devigili, A., Pilastro, A., 2019. Sexual selection and ageing: interplay between pre- and post-copulatory traits senescence in the guppy. Proc. R. Soc. B. 286, 20182873. https://doi.org/10.1098/rspb.2018.2873
Gasparini, C., Marino, I.A.M., Boschetto, C., Pilastro, A., 2010. Effect of male age on sperm traits and sperm competition success in the guppy (*Poecilia reticulata*). Journal of Evolutionary Biology 23, 124–135. https://doi.org/10.1111/j.1420-9101.2009.01889.x
Gerofotis, C.D., Yuval, B., Ioannou, C.S., Nakas, C.T., Papadopoulos, N.T., 2015. Polygyny in the olive fly—effects on male and female fitness. Behav Ecol Sociobiol 69, 1323–1332. https://doi.org/10.1007/s00265-015-1945-6
Harrison, X.A., 2014. Using observation-level random effects to model overdispersion in count data in ecology and evolution. PeerJ 2, e616. https://doi.org/10.7717/peerj.616
Hartig, F., Hartig, M.F., 2017. Package 'DHARMa'. R package. https://cran.r-hub.io/web/packages/DHARMa/DHARMa.pdf
Hayes, C.L., Booksmythe, I., Jennions, M.D., Backwell, P.R.Y., 2013. Does male reproductive effort increase with age? Courtship in fiddler crabs. Biol. Lett. 9, 20121078. https://doi.org/10.1098/rsbl.2012.1078
Heifetz, Y., Vandenberg, L.N., Cohn, H.I., Wolfner, M.F., 2005. Two cleavage products of the *Drosophila* accessory gland protein ovulin can independently induce ovulation. Proc. Natl. Acad. Sci. U.S.A. 102, 743–748. https://doi.org/10.1073/pnas.0407692102
Holman, L., 2009. *Drosophila melanogaster* seminal fluid can protect the sperm of other males. Functional Ecology 23, 180–186. https://doi.org/10.1111/j.1365-2435.2008.01509.x
Hopkins, B.R., Sepil, I., Bonham, S., Miller, T., Charles, P.D., Fischer, R., Kessler, B.M., Wilson, C., Wigby, S., 2019a. BMP signaling inhibition in *Drosophila* secondary cells remodels the seminal proteome and self and rival ejaculate functions. Proc. Natl. Acad. Sci. U.S.A. 116, 24719–24728. https://doi.org/10.1073/pnas.1914491116
Hopkins, B.R., Sepil, I., Thézénas, M.-L., Craig, J.F., Miller, T., Charles, P.D., Fischer, R., Kessler, B.M., Bretman, A., Pizzari, T., Wigby, S., 2019b. Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 116, 17925–17933. https://doi.org/10.1073/pnas.1906149116
Jahn, M.T., Lachnit, T., Markert, S.M., Stigloher, C., Pita, L., Ribes, M., Dutilh, B.E., Hentschel, U., 2021. Lifestyle of sponge symbiont phages by host prediction and correlative microscopy. The ISME Journal 15, 2001–2011. https://doi.org/10.1038/s41396-021-00900-6
Johnson, S.L., Dunleavy, J., Gemmell, N.J., Nakagawa, S., 2015. Consistent age-dependent declines in human semen quality: A systematic review and meta-analysis. Ageing Research Reviews 19, 22–33. https://doi.org/10.1016/j.arr.2014.10.007
Jones, O.R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C.G., Schaible, R., Casper, B.B., Dahlgren, J.P., Ehrlén, J., García, M.B., Menges, E.S., Quintana-Ascencio, P.F., Caswell, H., Baudisch, A., Vaupel, J.W., 2014. Diversity of ageing across the tree of life. Nature 505, 169–173. https://doi.org/10.1038/nature12789
Kalb, J.M., DiBenedetto, A.J., Wolfner, M.F., 1993. Probing the function of Drosophila melanogaster accessory glands by directed cell ablation. Proc Natl Acad Sci U S A 90, 8093–8097. https://doi.org/10.1073/pnas.90.17.8093
Kehl, T., Beaulieu, M., Kehl, A., Fischer, K., 2015. Old male sex: large ejaculate, many sperm, but few offspring. Behav Ecol Sociobiol 69, 1543–1552. https://doi.org/10.1007/s00265-015-1966-1
Kehl, T., Karl, I., Fischer, K., 2013. Old-male paternity advantage is a function of accumulating sperm and last-male precedence in a butterfly. Mol Ecol 22, 4289–4297. https://doi.org/10.1111/mec.12386
Koppik, M., Fricke, C., 2017. Gene expression changes in male accessory glands during ageing are accompanied by reproductive decline in *Drosophila melanogaster*. Mol Ecol 26, 6704–6716. https://doi.org/10.1111/mec.14384
Lemaître, J.-F., Gaillard, J.-M., 2017. Reproductive senescence: new perspectives in the wild: Reproductive senescence in the wild. Biol Rev 92, 2182–2199. https://doi.org/10.1111/brv.12328
Lewis, S.M., 2004. Multiple mating and repeated copulations: effects on male reproductive success in red flour beetles. Animal Behaviour 67, 799–804. https://doi.org/10.1016/j.anbehav.2003.05.013
Linklater, J.R., Wertheim, B., Wigby, S., Chapman, T., 2007. EJACULATE DEPLETION PATTERNS EVOLVE IN RESPONSE TO EXPERIMENTAL MANIPULATION OF SEX RATIO IN *DROSOPHILA MELANOGASTER*. Evolution 61, 2027–2034. https://doi.org/10.1111/j.1558-5646.2007.00157.x
Loyau, A., Blanchet, S., Van Laere, P., Clobert, J., Danchin, E., 2012. When not to copy: female fruit flies use sophisticated public information to avoid mated males. Sci Rep 2, 768. https://doi.org/10.1038/srep00768

Lupold, S., Manier, M.K., Ala-Honkola, O., Belote, J.M., Pitnick, S., 2011. Male Drosophila melanogaster adjust ejaculate size based on female mating status, fecundity, and age. Behavioral Ecology 22, 184–191. https://doi.org/10.1093/beheco/arq193
Macartney, E.L., Bonduriansky, R., Crean, A.J., 2020. Frequent mating reduces male mating rate but not offspring quality or quantity in a neriid fly. Evol Ecol 34, 915–927. https://doi.org/10.1007/s10682-020-10076-8
Maklakov, A.A., Immler, S., 2016. The Expensive Germline and the Evolution of Ageing. Current Biology 26, R577–R586. https://doi.org/10.1016/j.cub.2016.04.012
Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving Mechanisms of Competitive Fertilization Success in *Drosophila melanogaster*. Science 328, 354–357. https://doi.org/10.1126/science.1187096
Milholland, B., Dong, X., Zhang, L., Hao, X., Suh, Y., Vijg, J., 2017. Differences between germline and somatic mutation rates in humans and mice. Nat Commun 8, 15183. https://doi.org/10.1038/ncomms15183
Monaghan, P., Charmantier, A., Nussey, D.H., Ricklefs, R.E., 2008. The evolutionary ecology of senescence. Functional Ecology 22, 371–378. https://doi.org/10.1111/j.1365-2435.2008.01418.x
Monaghan, P., Maklakov, A.A., Metcalfe, N.B., 2020. Intergenerational Transfer of Ageing: Parental Age and Offspring Lifespan. Trends in Ecology & Evolution 35, 927–937. https://doi.org/10.1016/j.tree.2020.07.005
Monaghan, P., Metcalfe, N.B., 2019. The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. Proc. R. Soc. B. 286, 20192187. https://doi.org/10.1098/rspb.2019.2187
Nguyen, T.T.X., Moehring, A.J., 2018. A male's seminal fluid increases later competitors' productivity. J. Evol. Biol. 31, 1572–1581. https://doi.org/10.1111/jeb.13352
Noguera, J.C., Dean, R., Isaksson, C., Velando, A., Pizzari, T., 2012. Age-specific oxidative status and the expression of pre- and postcopulatory sexually selected traits in male red junglefowl, Gallus gallus. Ecol Evol 2, 2155–2167. https://doi.org/10.1002/ece3.300
Olsson, M., Madsen, T., Shine, R., 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. Proc. R. Soc. Lond. B 264, 455–459. https://doi.org/10.1098/rspb.1997.0065
Part, T., Gustafsson, L., Moreno, J., 1992. "Terminal investment" and a sexual conflict in the Collared Flycatcher (Ficedula albicollis). Am Nat 140, 868–882. https://doi.org/10.1086/285445
Pitnick, S., Markow, T.A., 1994. Male Gametic Strategies: Sperm Size, Testes Size, and the Allocation of Ejaculate Among Successive Mates by the Sperm-Limited Fly Drosophila pachea and Its Relatives. The American Naturalist 143, 785–819. https://doi.org/10.1086/285633
Pizzari, T., Dean, R., Pacey, A., Moore, H., Bonsall, M.B., 2008. The evolutionary ecology of pre- and post-meiotic sperm senescence. Trends in Ecology & Evolution 23, 131–140. https://doi.org/10.1016/j.tree.2007.12.003
Poiani, A., 2006. Complexity of seminal fluid: a review. Behav Ecol Sociobiol 60, 289–310. https://doi.org/10.1007/s00265-006-0178-0
Preston, B.T., Saint Jalme, M., Hingrat, Y., Lacroix, F., Sorci, G., 2015. The sperm of aging male bustards retards their offspring's development. Nat Commun 6, 6146. https://doi.org/10.1038/ncomms7146
Price, T.A.R., Lizé, A., Marcello, M., Bretman, A., 2012. Experience of mating rivals causes males to modulate sperm transfer in the fly Drosophila pseudoobscura. Journal of Insect Physiology 58, 1669–1675. https://doi.org/10.1016/j.jinsphys.2012.10.008
Radwan, J., 2003. Male age, germline mutations and the benefits of polyandry. Ecology Letters 6, 581–586. https://doi.org/10.1046/j.1461-0248.2003.00484.x
Ramm, S.A., 2020. Seminal fluid and accessory male investment in sperm competition. Phil. Trans. R. Soc. B 375, 20200068. https://doi.org/10.1098/rstb.2020.0068
Ramm, S.A., Edward, D.A., Claydon, A.J., Hammond, D.E., Brownridge, P., Hurst, J.L., Beynon, R.J., Stockley, P., 2015. Sperm competition risk drives plasticity in seminal fluid composition. BMC Biol 13, 87. https://doi.org/10.1186/s12915-015-0197-2
Ravi Ram, K., Wolfner, M.F., 2007. Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. Integrative and Comparative Biology 47, 427–445. https://doi.org/10.1093/icb/icm046
Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2011. Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, Cimex lectularius. PLoS ONE 6, e22082. https://doi.org/10.1371/journal.pone.0022082
Rezaei, A., Krishna, M.S., Santhosh, H.T., 2015. Male Age Affects Female Mate Preference, Quantity of Accessory Gland Proteins, and Sperm Traits and Female Fitness in D. melanogaster. Zoological Science 32, 16. https://doi.org/10.2108/zs140121
Rubolini, D., Galeotti, P., Pupin, F., Sacchi, R., Nardi, P.A., Fasola, M., 2007. Repeated matings and sperm depletion in the freshwater crayfish Austropotamobius italicus. Freshwater Biol 52, 1898–1906. https://doi.org/10.1111/j.1365-2427.2007.01814.x
Ruhmann, H., Koppik, M., Wolfner, M.F., Fricke, C., 2018. The impact of ageing on male reproductive success in Drosophila melanogaster. Experimental Gerontology 103, 1–10. https://doi.org/10.1016/j.exger.2017.12.013
Sanghvi, K., Iglesias-Carrasco, M., Zajitschek, F., Kruuk, L.E.B., Head, M.L., 2022. Effects of developmental and adult environments on ageing. Evolution evo.14567. https://doi.org/10.1111/evo.14567
Sanghvi, K., Pizzari, T., Sepil, I., 2023. What doesn't kill you makes you stronger? Effects of paternal age at conception on fathers and sons (preprint). Evolutionary Biology. https://doi.org/10.1101/2023.12.20.572572
Sanghvi, K., Vega-Trejo, R., Nakagawa, S., Gascoigne, S.J.L., Johnson, S.L., Salguero-Gómez, R., Pizzari, T., Sepil, I., 2024. Meta-analysis shows no consistent evidence for senescence in ejaculate traits across animals. Nat Commun 15, 558. https://doi.org/10.1038/s41467-024-44768-4
Santos, I.B., Wainman, A., Garrido-Maraver, J., Pires, V., Riparbelli, M.G., Kovács, L., Callaini, G., Glover, D.M., Tavares, Á.A., 2023. Mob4 is essential for spermatogenesis in *Drosophila melanogaster* (preprint). Developmental Biology. https://doi.org/10.1101/2023.02.05.527206
Sasson, D.A., Johnson, S.L., Brockmann, H.J., 2012. The role of age on sperm traits in the American horseshoe crab, Limulus polyphemus. Animal Behaviour 84, 975–981. https://doi.org/10.1016/j.anbehav.2012.07.023

Savalli, U.M., Fox, C.W., 1999. The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus maculatus*. Functional Ecology 13, 169–177. https://doi.org/10.1046/j.1365-2435.1999.00287.x
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019
Sepil, I., Hopkins, B.R., Dean, R., Bath, E., Friedman, S., Swanson, B., Ostridge, H.J., Harper, L., Buehner, N.A., Wolfner, M.F., Konietzny, R., Thézénas, M.-L., Sandham, E., Charles, P.D., Fischer, R., Steinhauer, J., Kessler, B.M., Wigby, S., 2020. Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 117, 17094–17103. https://doi.org/10.1073/pnas.2009053117
Sirot, L.K., Buehner, N.A., Fiumera, A.C., Wolfner, M.F., 2009a. Seminal fluid protein depletion and replenishment in the fruit fly, Drosophila melanogaster: an ELISA-based method for tracking individual ejaculates. Behav Ecol Sociobiol 63, 1505–1513. https://doi.org/10.1007/s00265-009-0806-6
Sirot, L.K., LaFlamme, B.A., Sitnik, J.L., Rubinstein, C.D., Avila, F.W., Chow, C.Y., Wolfner, M.F., 2009b. Molecular social interactions: Drosophila melanogaster seminal fluid proteins as a case study. Adv Genet 68, 23–56. https://doi.org/10.1016/S0065-2660(09)68002-0
Snoke, M.S., Promislow, D.E.L., 2003. Quantitative genetic tests of recent senescence theory: age-specific mortality and male fertility in Drosophila melanogaster. Heredity 91, 546–556. https://doi.org/10.1038/sj.hdy.6800353
Snook, R.R., Hosken, D.J., 2004. Sperm death and dumping in Drosophila. Nature 428, 939–941. https://doi.org/10.1038/nature02455
Sturup, M., Baer-Imhoof, B., Nash, D.R., Boomsma, J.J., Baer, B., 2013. When every sperm counts: factors affecting male fertility in the honeybee Apis mellifera. Behavioral Ecology 24, 1192–1198. https://doi.org/10.1093/beheco/art049
Swierk, L., Tennessen, J.B., Langkilde, T., 2015. Sperm depletion may not limit male reproduction in a capital breeder. Biol. J. Linn. Soc. 116, 684–690. https://doi.org/10.1111/bij.12603
Thomas, M.L., Simmons, L.W., 2009. Male-derived cuticular hydrocarbons signal sperm competition intensity and affect ejaculate expenditure in crickets. Proc. R. Soc. B. 276, 383–388. https://doi.org/10.1098/rspb.2008.1206
Turnell, B.R., Reinhardt, K., 2020. Metabolic Rate and Oxygen Radical Levels Increase But Radical Generation Rate Decreases with Male Age in *Drosophila melanogaster* Sperm. The Journals of Gerontology: Series A 75, 2278–2285. https://doi.org/10.1093/gerona/glaa078
Vega-Trejo, R., Fox, R.J., Iglesias-Carrasco, M., Head, M.L., Jennions, M.D., 2019. The effects of male age, sperm age and mating history on ejaculate senescence. Funct Ecol 33, 1267–1279. https://doi.org/10.1111/1365-2435.13305
Vrtílek, M., Žák, J., Reichard, M., 2022. Evidence for reproductive senescence across ray-finned fishes: A review. Front. Ecol. Evol. 10, 982915. https://doi.org/10.3389/fevo.2022.982915
Vuarin, P., Bouchard, A., Lesobre, L., Levêque, G., Chalah, T., Jalme, M.S., Lacroix, F., Hingrat, Y., Sorci, G., 2019. Post-copulatory sexual selection allows females to alleviate the fitness costs incurred when mating with senescing males. Proc. R. Soc. B. 286, 20191675. https://doi.org/10.1098/rspb.2019.1675
Wagner, R.H., Helfenstein, F., Danchin, E., 2004. Female choice of young sperm in a genetically monogamous bird. Proc Biol Sci 271 Suppl 4, S134-137. https://doi.org/10.1098/rsbl.2003.0142
Wedell, N., Cook, P.A., 1999. Butterflies tailor their ejaculate in response to sperm competition risk and intensity. Proc. R. Soc. Lond. B 266, 1033–1039. https://doi.org/10.1098/rspb.1999.0740
Wedell, N., Gage, M.J.G., Parker, G.A., 2002. Sperm competition, male prudence and sperm-limited females. Trends in Ecology & Evolution 17, 313–320. https://doi.org/10.1016/S0169-5347(02)02533-8
White, J., Wagner, R.H., Helfenstein, F., Hatch, S.A., Mulard, H., Naves, L.C., Danchin, E., 2008. Multiple deleterious effects of experimentally aged sperm in a monogamous bird. Proc Natl Acad Sci U S A 105, 13947–13952. https://doi.org/10.1073/pnas.0803067105
Wigby, S., Sirot, L.K., Linklater, J.R., Buehner, N., Calboli, F.C.F., Bretman, A., Wolfner, M.F., Chapman, T., 2009. Seminal Fluid Protein Allocation and Male Reproductive Success. Current Biology 19, 751–757. https://doi.org/10.1016/j.cub.2009.03.036
Win, A., Kojima, W., Ishikawa, Y., 2013. Age-Related Male Reproductive Investment in Courtship Display and Nuptial Gifts in a Moth, *Ostrinia scapulalis*. Ethology 119, 325–334. https://doi.org/10.1111/eth.12069
Xue, L., Noll, M., 2000. *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. Proc. Natl. Acad. Sci. U.S.A. 97, 3272–3275. https://doi.org/10.1073/pnas.97.7.3272

Supplementary information

Appendix 1: Scenarios for 'male age x female number' interactions

Some possible scenarios for how male age could interact with female number in a male's multiple-mating sequence, to affect male reproductive output. We assume that male reproductive success (W) is a negative function of progression through the mating sequence, such that $W(i) < W(i+1)$, where i is the ith female in a mating sequence. For simplicity we assume that the function is linear; i.e. $W = -ax + b$, where x is the order of female in the mating sequence, a is the slope, and b is the intercept. The null hypothesis is that old and young males have the same size and quality of ejaculates, and allocate ejaculates in similar ways to females in their mating sequence, thus produce similar numbers of offspring across a mating sequence:

$$\int_1^{10} W_{Old}(x)dx = \int_1^{10} W_{Young}(x)dx$$

However, under scenarios of reproductive senescence (scenario A), the general prediction is that old males have overall lower total reproductive success than young males (i.e. lower intercepts; $b_{Old}<b_{Young}$)). However, old and young males do not differ in their slopes ($a_{Old} = a_{Young}$) of decline through a mating sequence and allocate similar proportions of their ejaculate to each female. Here:

$$\int_1^{10} W_{Old}(x)dx < \int_1^{10} W_{Young}(x)dx$$

where W_{Old} and W_{Young} are the reproductive output functions of old and young males over a mating sequence of 10 available females, respectively.

Scenario B: old males due to fewer mating opportunities in the future, might terminally invest in reproduction, allocating a higher proportion of ejaculate to early than late females compared to young males (i.e. $|a_{Old}| > |a_{Young}|$). This would lead to relatively higher reproductive output of old males early in a mating sequence, but steeper slopes of decline through the mating sequence, compared to young males. Scenario C: Old males, due to having lower quantities of ejaculates, might more prudently allocate ejaculates to each female (i.e. $|a_{Old}| < |a_{Young}|$). This would lead old males to have lower intercepts but shallower slopes of decline in reproductive output than young males.

Scenario D: Old males have smaller ejaculates than young males, however, they would transfer the same size of ejaculates to females as young males until old males run out of ejaculates. The point where old males run out of ejaculates would be sooner than that of young males, and old males would not have any reproductive success beyond this point.

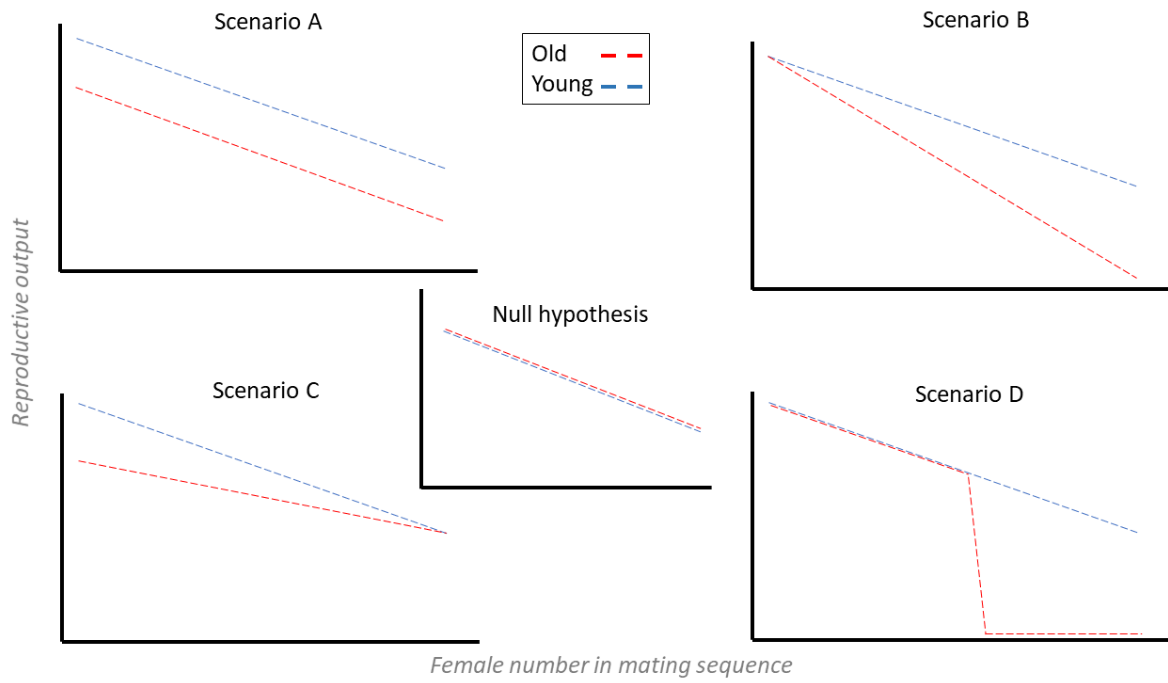


Figure 1: Possible scenarios for how advancing male age can interact with female order in the male's mating sequence, to influence male reproductive output.

Appendix 2: *son-of-tudor* crossing scheme

Sot males were generated by mating brown-eyed straight-winged homozygous *Tudor* females (backcrossed into *dah* background), to *dah* males. To ensure that this crossing scheme produced sterile flies, we used a sub-set of 120 non-experimental virgin *sot* males (3-4 days old), and kept them in bottles containing virgin *dah* females (~20-30 males and females per bottle), for 14 days. None of the bottles had any larvae developing after 14 days, indicating that the males were sterile. We additionally dissected three *sot* males and examined their testes and AGs under a light microscope, revealing the testes and SV to be shrunk and without sperm, but AGs appearing normal, indicating that the males were sterile but producing SF.

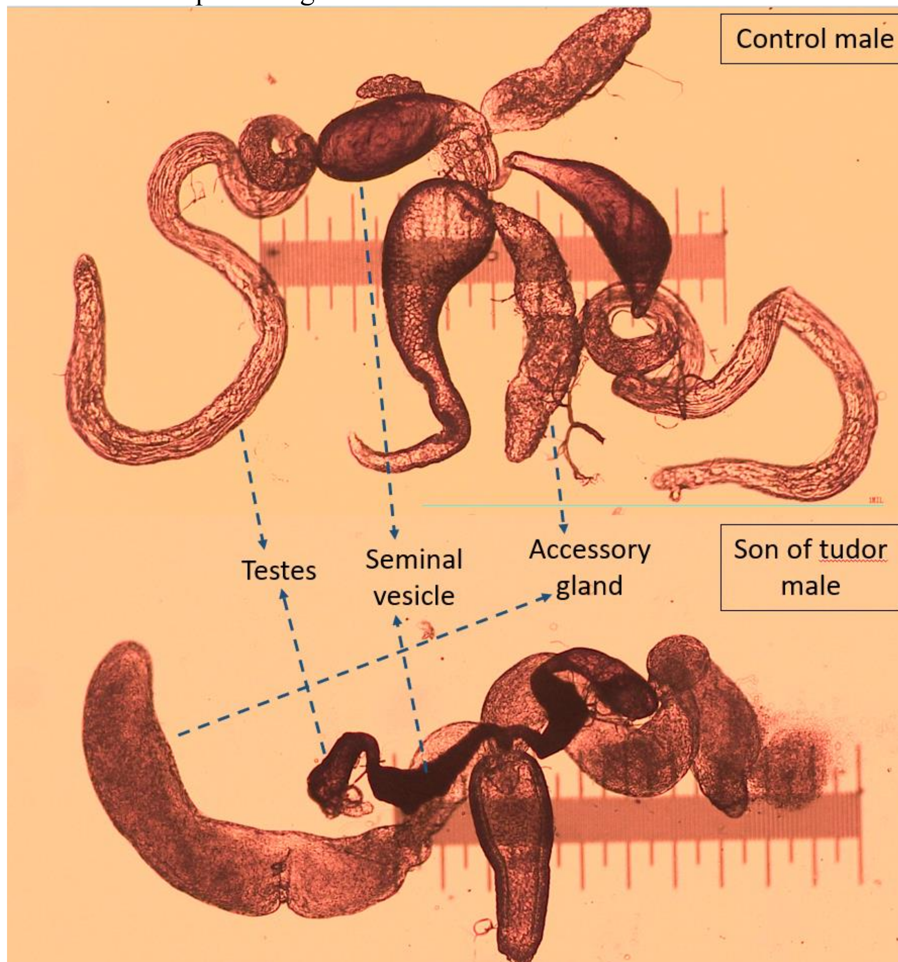


Image1: Disrupted testis and seminal vesicles (SV) with no sperm, but normal accessory glands (AG), in *sot* male, compared to control male. Scale = 1mm. Magnification = 40x (4x objective, 10x eyepiece).

Appendix 3: Female dissections

Odd-numbered females (frozen after 24 hours) in a *gfp* male's mating sequence, were dissected to count the number of sperm stored by a female in her long-term sperm storage organs. Even-numbered females in a *gfp* male's mating sequence (frozen after 30 minutes), were dissected to count the number of sperm transferred by a male to a female.

To dissect frozen females, three droplets of 50uL of Phosphate buffer solution each, were placed on a slide coated with an aqueous solution of gelatine and chromium potassium sulphate. Each female to be dissected was placed on the first droplet, and her reproductive tract was removed gently by separating the last two abdominal segments from the rest of her body, using Inox Biology forceps. Her reproductive organs (bursa, seminal receptacle, and spermathecae) were washed in the second droplet of PBS, and extra surrounding tissue removed. The reproductive organs were then placed on a third droplet. Here, using microneedles (0.1mm thick), the two spermathecae and seminal receptacle were gently spread and the sample covered with a coverslip. Rubber cement (fixogum) was used to glue the coverslip edges to the slide for imaging (Appendix 4) conducted on the following day.

Appendix 4: sperm imaging and counts in females

For odd numbered females (frozen after 24 hours of mating), female reproductive tracts (spermathecae and seminal receptacle) fixed on as slide (described in Appendix 3) were imaged (Image 2) using a Nikon Eclipse50i fluorescence microscope (magnification = 10x objective, 10x eyepiece, wavelength = 480nm) with a chromix HD camera, under UV light from a CoolLED pe300 light source. The number of sperm heads (which appeared as fluorescent green under UV light) were later counted manually from images, using the cell counter plugin on FIJI/ImageJ version win32 (Schindelin et al, 2012). The GFP label in *gfp* flies is expressed at the *Mst35Ba* and *Mst35Bb* loci (Manier et al, 2010). To ensure repeatability of the manual counts, an independent analyst (TLC) counted 30 randomly chosen samples blind to counts made by the first analyst. Repeatability between the counts by the two analysts was high ($R^2 = 0.98$, Figure S2A). For even numbered females (frozen within 30 mins of mating), the bursa, spermathecae, and seminal receptacle, were imaged using a 5x air objective on a Zeiss LSM880 confocal laser scanning unit microscope (laser strength = 15; pinhole size = 34.1; laser wavelength = 488nm; pixel strength = 1196 x 1196, objective magnification = 5x, eyepiece magnification = 10x), and the Zen black v14.0.18.201 software (Image 2). Images were analysed with ImageJ win32, and the find maxima plugin (with prominence = 10000, strict setting, output type = single point) was used to estimate the number of fluorescent green sperm heads in each sample. The prominence was chosen as 10000 because this was the point where the power function relationship between prominence and estimates of sperm number by the find maxima plugin, became linear and flat (Figure S3, based on 8 samples, following Jahn et al (2021) who do this using threshold against signal:noise ratio). To ensure that sperm numbers estimated by the find maxima plugin were robust, we tested for repeatability between sperm number counted manually using the cell counter plugin, and sperm number estimated by the find maxima plugin. This repeatability test was done on a subset of 24 randomly chosen samples of even-numbered females (repeatability: $R^2 = 0.98$, Figure S2B).

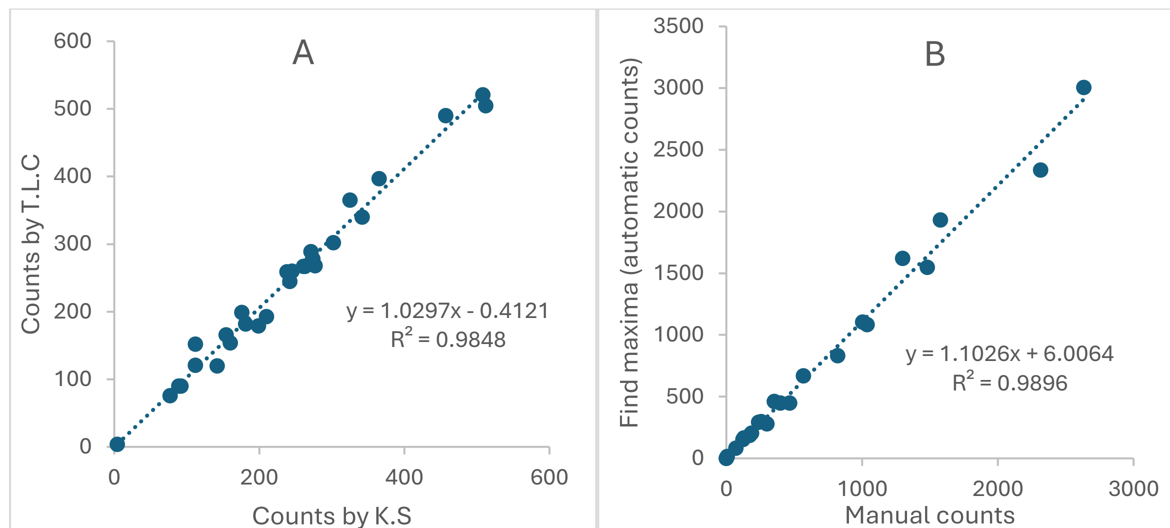


Figure S2: A: high repeatability score of $R^2 = 0.98$ between two analysts, when counting sperm numbers stored in odd-numbered females after 24 hours, using the cell counter plugin. B: high repeatability score of $R^2 = 0.98$, between sperm numbers when sperm are manually counted using the cell counter plugin, versus estimated using the find maxima plugin, for sperm transferred to even-numbered females.

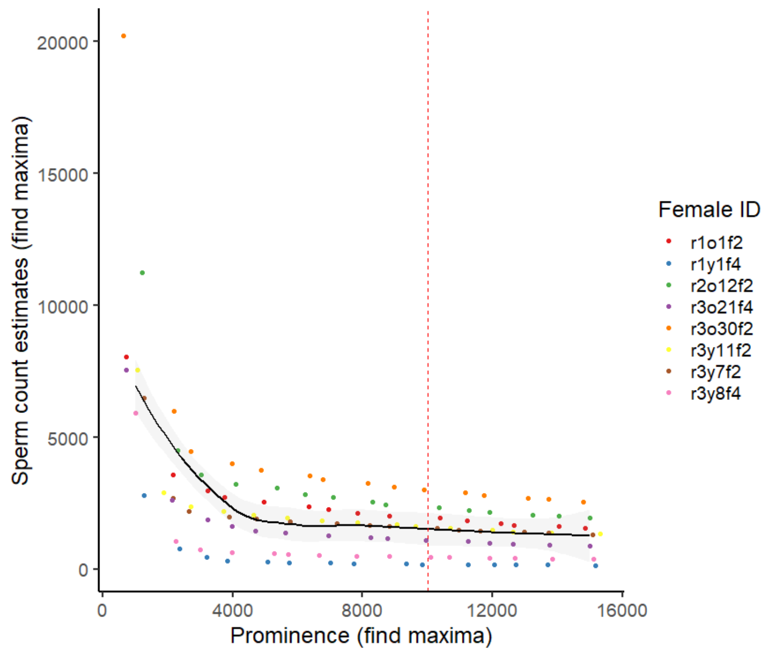


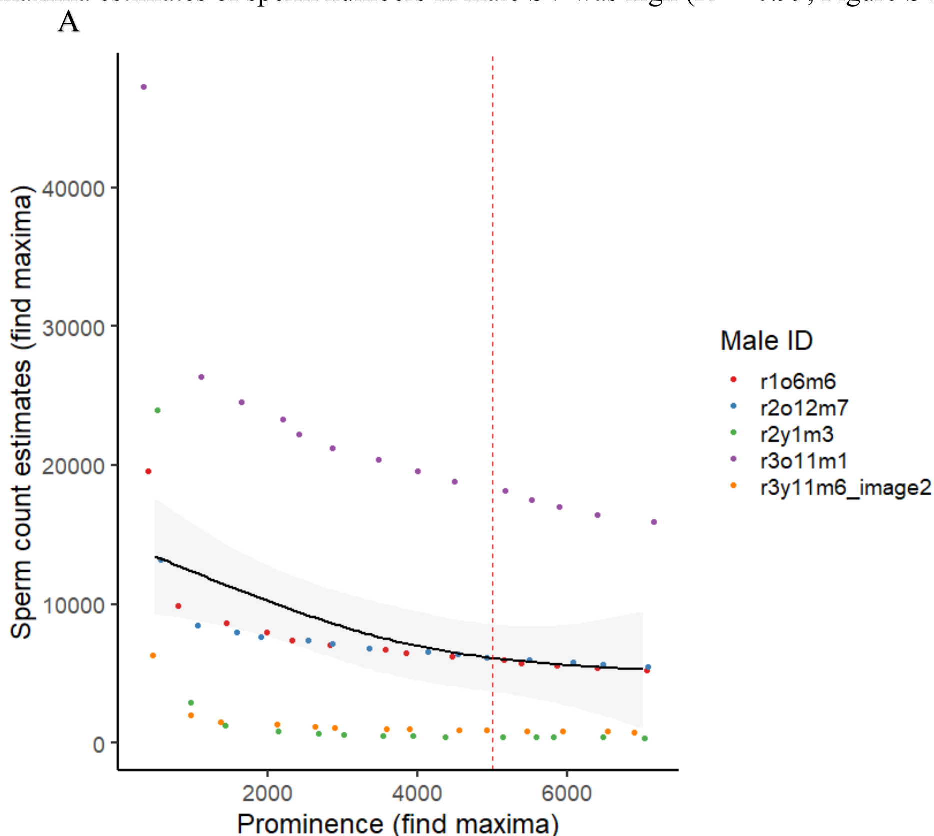
Figure S3: prominence on find maxima chosen as 10000, when estimating sperm stored in females, because this is where the power function relationship between prominence and estimates of sperm number by the find maxima plugin, became linear and flat based on visual inspection.

<u>Appendix 5: male dissections and imaging</u>

Males were dissected, and later imaged (see Image 2) to estimate sperm numbers stored in male seminal vesicles, and the size of male AGs. To dissect each male, three droplets of 50uL of PBS were placed on a slide covered with an aqueous solution of gelatine and chromium potassium sulphate. The reproductive tract of males (testes, accessory glands, seminal vesicles, and ejaculatory bulb) were separated from the rest of the body, by carefully pulling away the upper abdomen of the male using Inox Biology forceps. The male's reproductive tract was washed in a second droplet of PBS and surrounding tissue separated. A clean sample of only reproductive tissue was placed on the third droplet. The accessory glands were then separated from the rest of the reproductive tract using microneedles (0.1mm thick), and placed on a new slide which had a measuring scale, inside a droplet of 5uL of PBS. The two accessory glands of each male were immediately imaged without a coverslip on, using the brightfield setting on a Nikon Eclipse 50i microscope with magnification of 4x (objective) and 10x (eyepiece), with each image calibrated to the scale of 1mm. Then, the male's seminal vesicles were punctured, and sperm present in both seminal vesicles were carefully spread in the droplet of PBS using microneedles. A coverslip was then placed on this sample and then glued to the slide using rubber cement. The sperm were imaged the following day using a 5x air objective on a Zeiss LSM880 confocal laser scanning unit microscope (laser strength = 20; pinhole size = 20.1; Laser wavelength = 488; Pixel strength = 1196 x 1196; objective magnification = 5x, eyepiece magnification = 10x) and Zen black software.

To measure the area of accessory glands on FIJI/ImageJ win32, we used the freehand selection tool to outline the area of each accessory gland separately, and then used the "measure" option under "analyze" to measure the area of each accessory gland. To estimate the number of sperm in the seminal vesicles of males, we used the find maxima plugin (Prominence = 5000, strict setting, output type = single point). The prominence was chosen as 5000 because this point was where the power function relationship between prominence and estimates of sperm number by the find maxima plugin, became linear and flat (Image S4A, based on visual inspection of 5 samples). To ensure robustness of sperm numbers in male SV estimated by find maxima, we manually counted (using cell counter on FIJI) sperm in eight images of dissected male SV. Repeatability between manual counts and find maxima estimates of sperm numbers in male SV was high ($R^2 = 0.99$, Figure S4B).

A



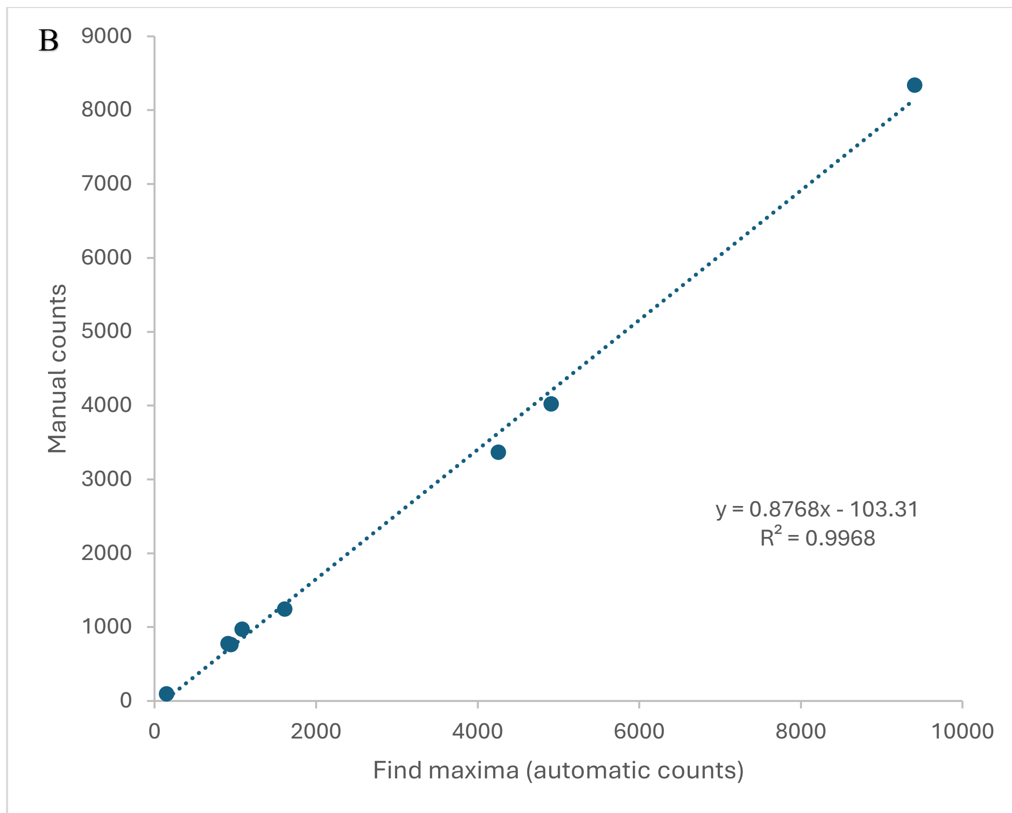


Figure S4A: prominence on find maxima chosen as 5000 when estimating sperm stored in males, because this is where the power function relationship between prominence and estimates of sperm number by the find maxima plugin, became linear and flat based on visual inspection. S4B: High repeatability score of $R^2 = 0.99$ between sperm numbers estimated manually (cell counter plugin) versus using find maxima plugin on FIJI.

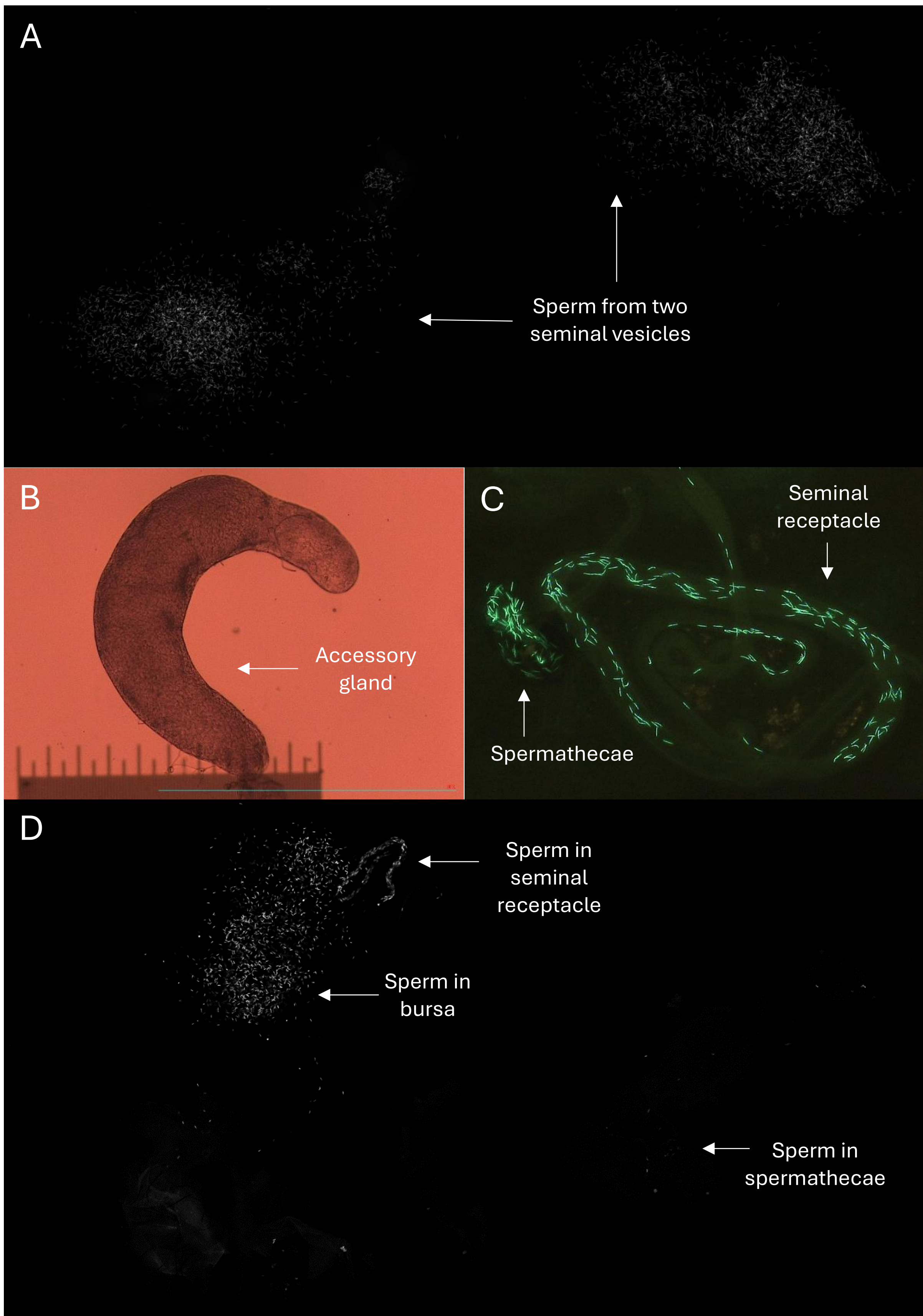


Image 2: Examples of images from dissected individuals in experiment C. **A.** sperm in seminal vesicles of males. **B.** accessory gland in male. **C.** sperm in long-term storage organs of even-numbered female after 24 hours. **D.** sperm transferred to odd-numbered female.

Appendix 6: male mating success and latency

We compared the mating success (i.e. total number of females a male mated with) of young and old males in our three experiments. For this, we created a generalised linear model with Poisson error distribution, and included male mating success as our dependent variable. We modelled male age, experiment (A, B, or C), and their two-way interaction as fixed effects, and observation-level ID as a random effect to account for overdispersion. We also compared male mating latency (time elapsed between male being paired with a female, and the start of copulation). For this, we modelled male mating latency as our dependent variable using an LMM with Gaussian error distribution. Male age, a linear and quadratic term of female number, their two-way interactions with male age, and experiment number, were included as fixed effects, with male ID as a random effect.

Old males consistently mated with fewer females compared to young males ($z = 6.423$, $P < 0.001$, Figure S10A). However, this difference was greater in experiments C (age*experiment C: $z = 3.491$, $P < 0.001$) and B (age*experiment C: $z = 2.027$, $P < 0.043$) than experiment A. Old males had a lower mating latency than young males early in the mating sequence but not later (age*female number: $t = 3.159$, $P = 0.002$, Figure S10B, S10C).

Ejaculate limitation seems to be an unlikely explanation for the lower mating success of old males, because they had more sperm in their SV and larger AGs than young males (Figure 2, 3). Instead, the lower mating success of old males might be a consequence of their longer mating latencies. These longer latencies could be due to old males being less attractive to females or worse at courtship, thus females taking longer to accept mating with an old than a young male (e.g. Amin et al, 2012; Rezaei et al, 2015). Lower mating success of old males could also be due to old males being more frail than young males (Sanghvi et al, 2023). Furthermore, differences between old and young males in their mating latencies were greater in experiment B (when females were first mated to *sot* males) than experiment A (Figure 10C). This result suggests that females show stronger choice for young males than old males, when females have previously been mated compared to when females are virgins.

Appendix 7: Bateman's gradients

Note: These results are only presented as exploratory analyses to generate new hypotheses regarding sexual selection and as such are not a part of our study aims.

We first investigated whether the age of males and seminal fluid availability to females, might influence the strength and slope of the Bateman's gradient, to explore whether the opportunity for sexual selection is influenced by demography and ejaculate limitation. For this, we calculated Bateman's gradients, i.e. the slope of the linear relationship between male mating success and reproductive success (Anthes et al, 2017), for old and young males used in Experiments A and C. We first calculated the total mating success (sum of successful copulations by a male in his mating sequence- one data point per male), and total reproductive success (sum of offspring produced by all females that copulated with a male- one data point per male), for each male that mated in Experiments A and C. We then calculated the slopes of the linear regression between male mating success and reproductive success (Figure S5A). Visual inspection indicated that males in Experiment C had steeper slopes than males in Experiment A, suggesting that the opportunity for sexual selection might be greater when females are not seminal fluid limited. Additionally, young males had steeper gradients, suggesting a greater opportunity for pre-copulatory sexual selection in young compared to old males.

Next, we investigated whether covariances between male reproductive output (i.e. number of offspring produced with each female- multiple data points per male) and total male mating success (i.e. total number of females a male mated with in the mating sequence- on data point per male) exist, as these can lead to a biased estimation of Bateman's gradients. For example, if males that produce more offspring with each female also mate with more females (for instance, due to being more attractive), then females later in a mating sequence will be exposed to a biased group of high reproductive quality males (i.e. males who produce more offspring). These positive covariances between male pre- and post-copulatory traits, and "selective disappearance" of low-quality males through a mating sequence, can inflate the Bateman's gradient (Anthes et al, 2017). We visually tested this hypothesis using data from experiment A. We plotted the number of offspring produced by a male with each female in his mating sequence (Y axis) against the female number in the mating sequence (X axis). Here, we grouped males by their total mating success and visually inspected the plots. Generally, males who produced more offspring initially in the mating sequence, also mating with more females (i.e. had higher total mating success) compared to males who produced fewer offspring initially in the sequence. However, males who produced more offspring initially also experienced steeper declines in their reproductive output through the mating sequence (Figure S5B). Our results reveal positive covariances between initial reproductive output in a mating sequence and male mating success, but negative covariances between initial reproductive output and later reproductive output. These results emphasize the need for studies on Bateman's gradients to consider the exact sequence in which females encounter males, as well as covariances between male reproductive measures which may lead to biased estimates of the strength of sexual selection.

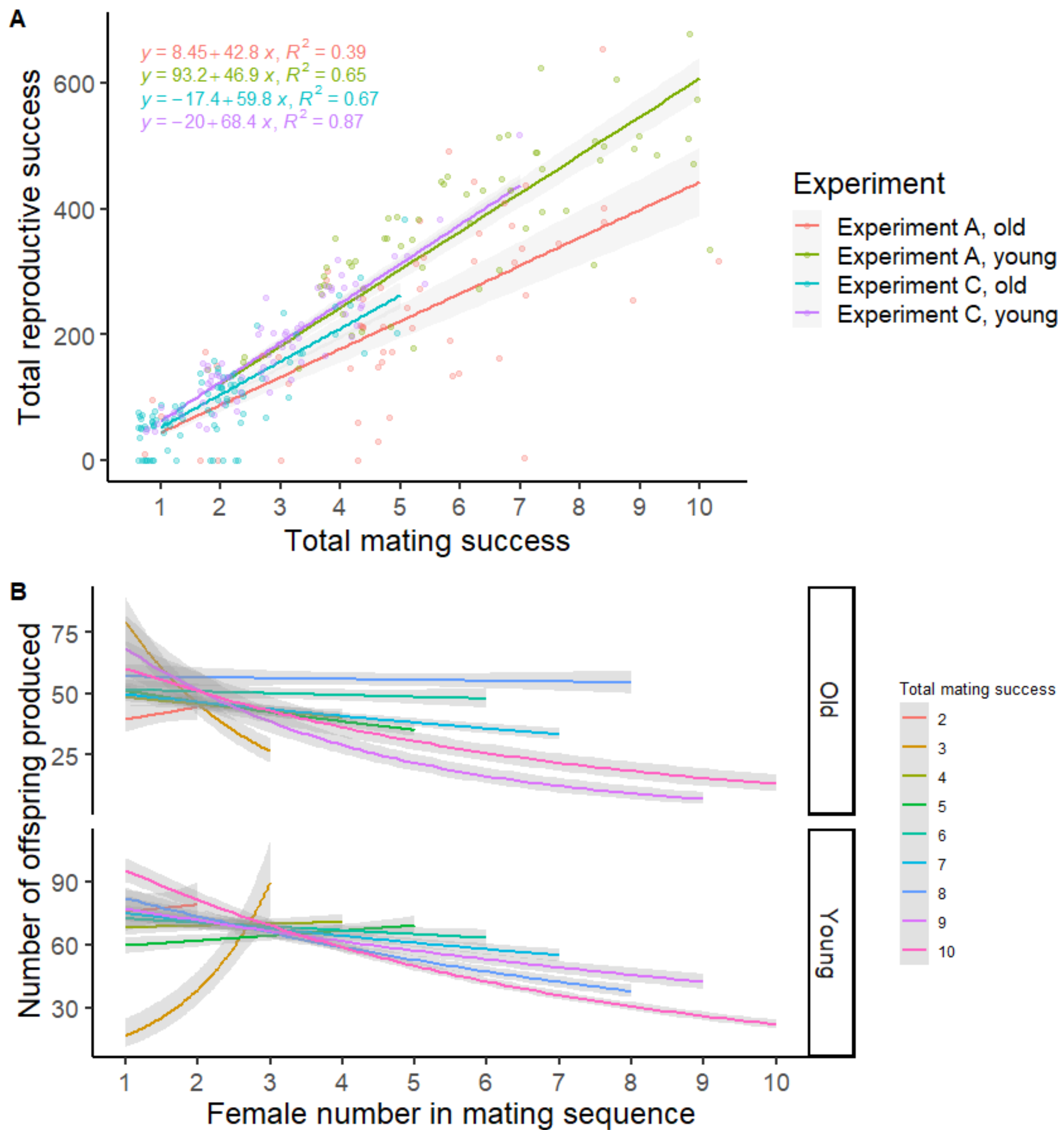


Figure S5: **A.** Bateman's gradients showing the linear relationship between male mating success and reproductive success, for old and young males used in Experiments A and C. Slopes and R^2 values shown. Intercepts set to zero to allow direct comparisons of slopes. Each dot represents one male. **B.** Positive covariances between initial reproductive output in a mating sequence and male's total mating success, but negative covariances between initial reproductive output and later reproductive output in the sequence, using data from experiment A. Dark lines show means, shaded regions show 95% C.I.

Supplementary figures (S6-S12)

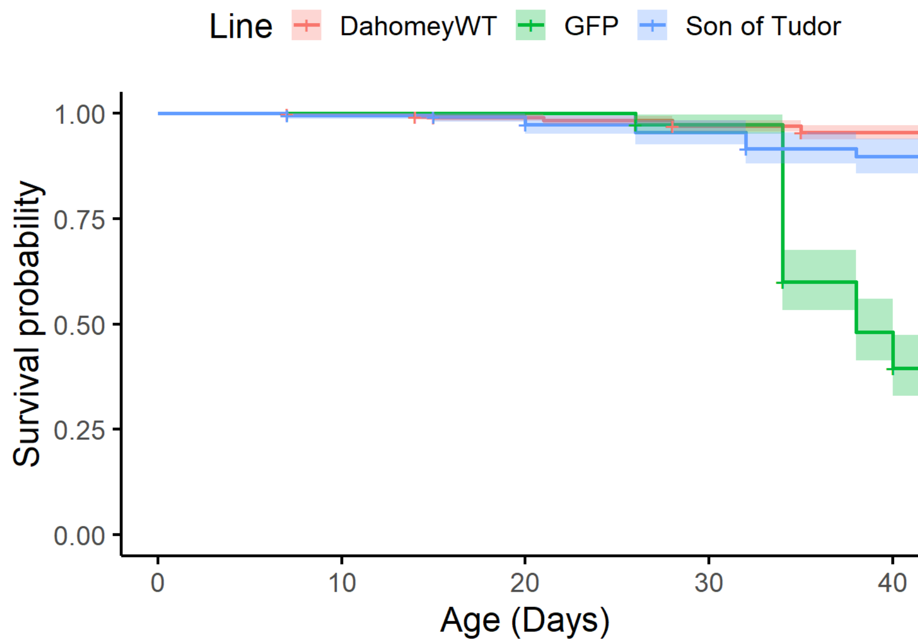


Figure S6: Age-dependent survival probability of males used in our study, from three distinct lines of *Drosophila melanogaster: dah, gfp, and sot.* Gfp males experienced higher mortality than *sot* or *dah* males. Means and 95% C.I. shown.

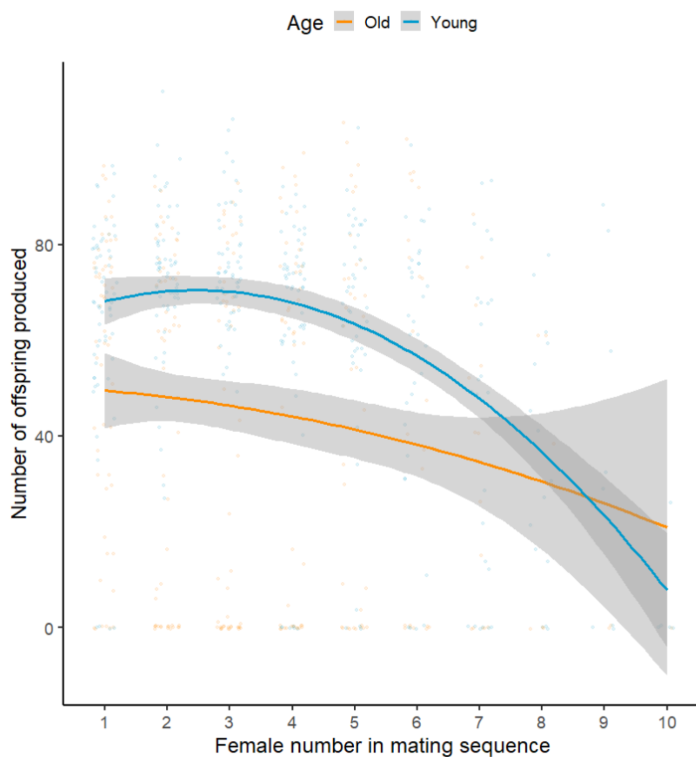


Fig S7: Significant interaction between male age and female number in a male's mating sequence, to affect the number of offspring produced by a female over 24 hours of egg laying, in experiment A. Old males produce fewer offspring than young males only early on in a mating sequence. Means and 95% C.I. shown.

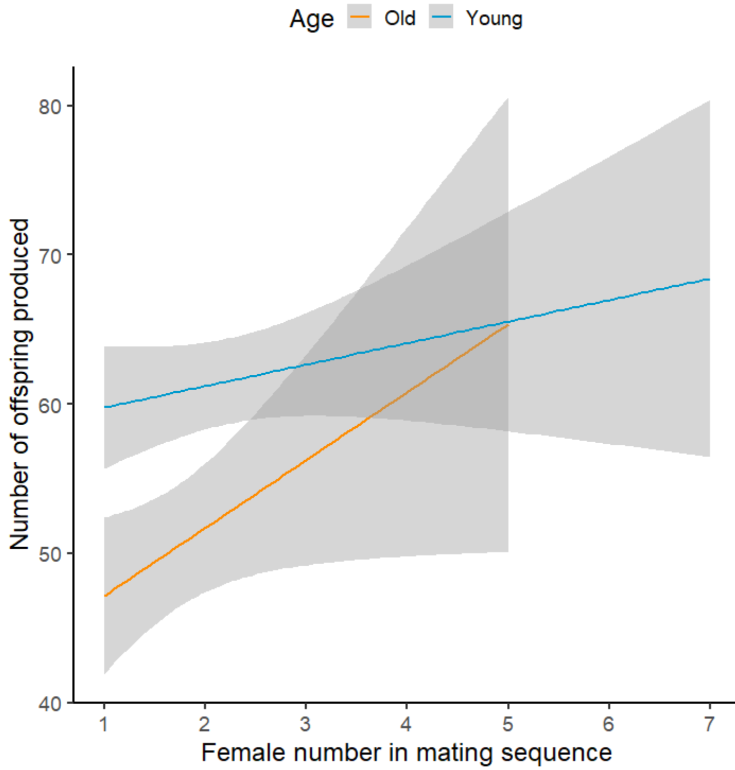


Fig S8: No significant effect of male age or female number in a male's mating sequence, to affect the number of offspring produced by a female over 24 hours of egg laying, in experiment B. Means and 95% C.I. shown.

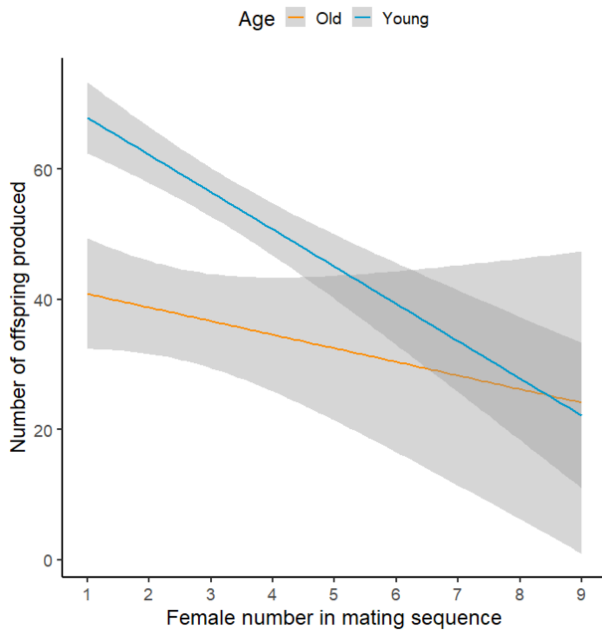


Fig S9: Significant effect of male age and female number in a male's mating sequence, to affect the number of offspring produced by a female over 24 hours of egg laying, in experiment C. Old males produce fewer offspring than young males, and males produce fewer offspring with females later than earlier in a mating sequence. Means and 95% C.I. shown.

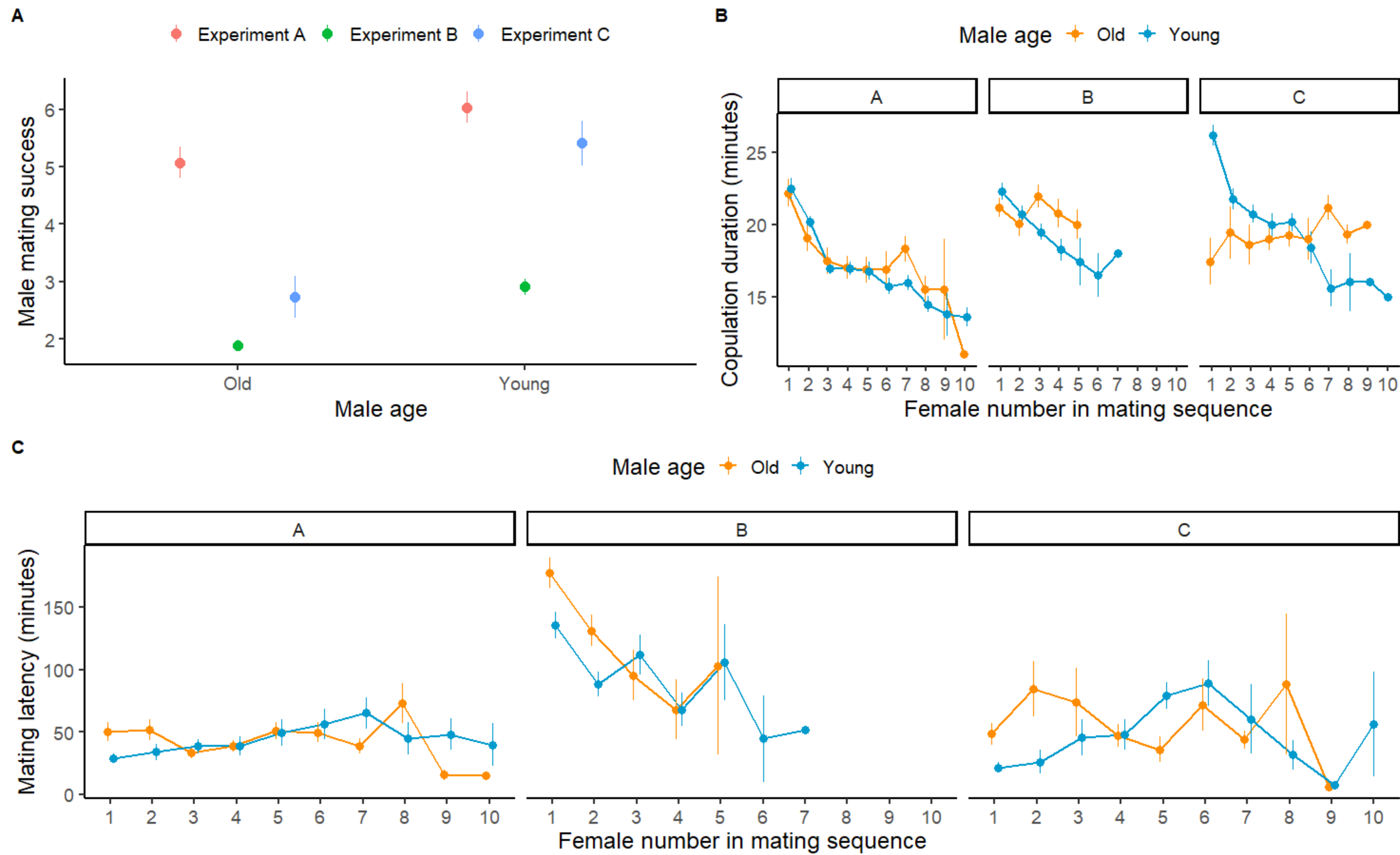


Figure S10: **A**. Mating success of focal old and young males used in our three experiments. **B.** Copulation duration of focal old and young males across experiments A, B, and C. **C.** Mating latency of focal young and old males across experiments A, B, and C. Means and SE shown.

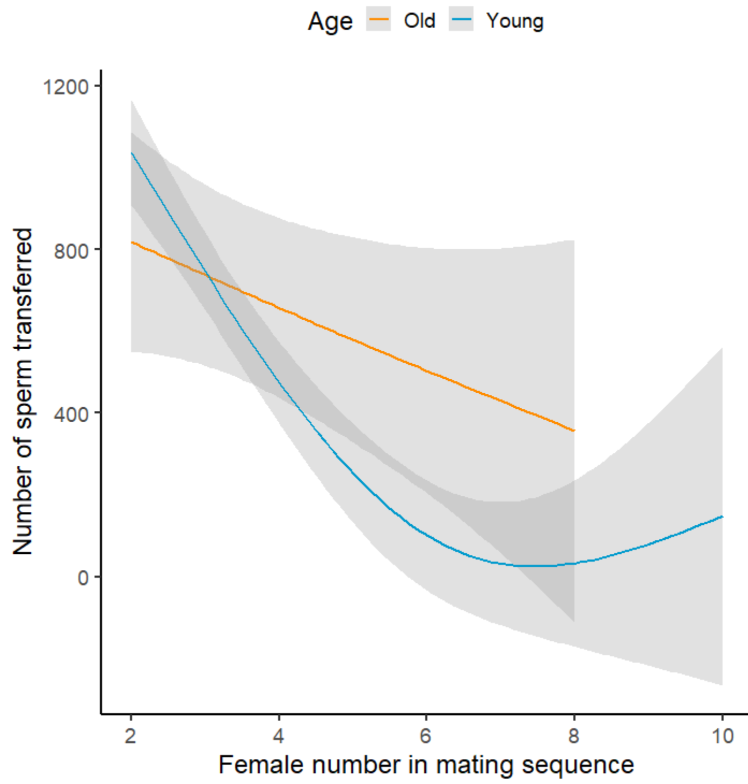


Figure S11: Effect of male age and female number in a male's mating sequence, on the number of sperm transferred by the male to the female. Means and 95% C.I. shown.

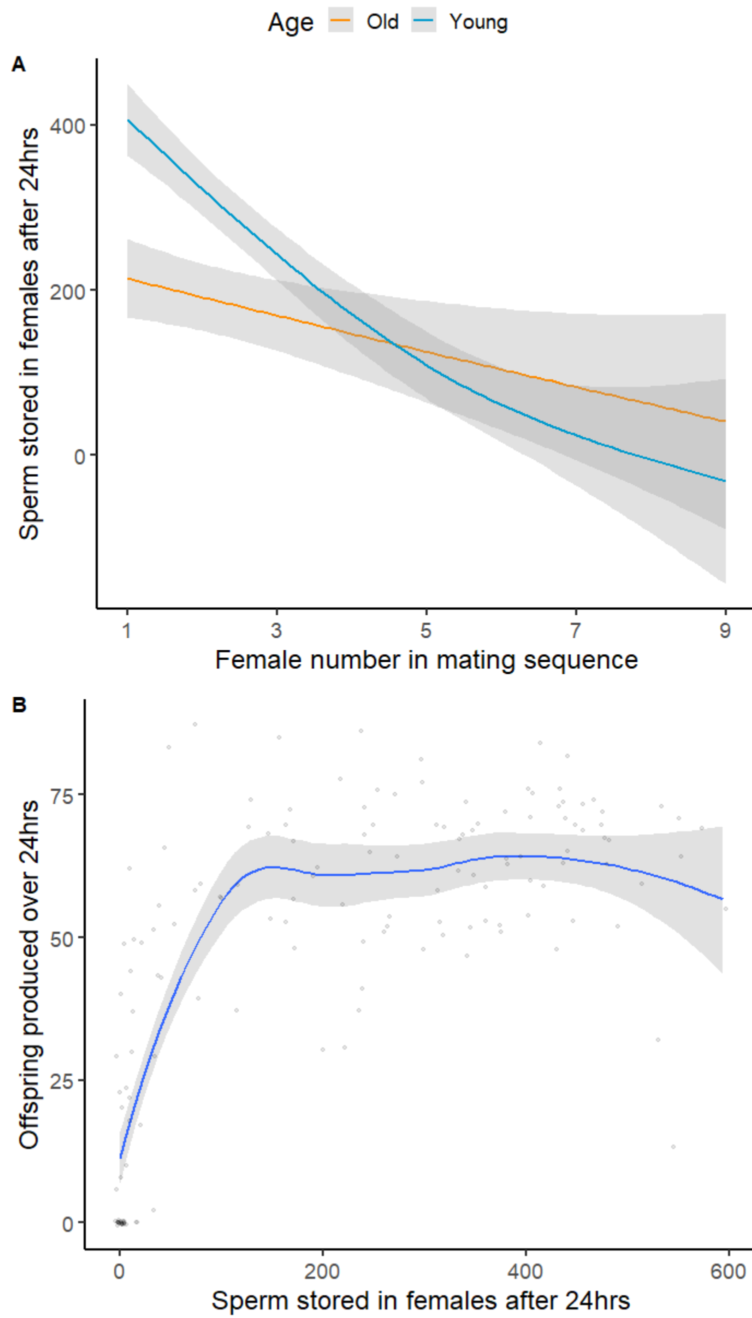


Figure S12: A. Effect of male age and female number in a male's mating sequence, on the number of sperm stored by mated females after 24 hours of egg laying. B. Co-variance between number of sperm stored in odd-numbered females after 24 hours and the number of offspring produced by these females over 24 hours. Plot created using a loess smooth, to illustrate the non-linear (asymptotic) relationship. Means and 95% C.I. shown.

Supplementary tables (S1-S9)

Table S1a: sample sizes for number of successful copulations by old and young males with females in a mating sequence, for experiments A, B, and C. Table 1b: sample sizes for total mating success (i.e. sum of females a male mated with) of old and young males, in experiments A-C

Table S1a				
Male Age	Female number in mating sequence (i.e. female mating order)	Sample sizes of successful copulations		
		Experiment A	Experiment B	Experiment C
Old	Starting male sample size	60	115	50
Old	1st	57	89	45
Old	2nd	52	54	21
Old	3rd	49	19	15
Old	4th	45	8	14
Old	5th	35	2	9
Old	6th	22		9
Old	7th	15		6
Old	8th	6		3
Old	9th	2		1
Old	10th	1		
Young	Starting male sample size	60	85	28
Young	1st	60	74	27
Young	2nd	60	67	26
Young	3rd	57	45	26
Young	4th	54	23	22
Young	5th	42	5	19
Young	6th	33	2	15
Young	7th	25	1	5
Young	8th	15		2
Young	9th	9		2
Young	10th	5		2

Table S1b				
Male Age	Male mating success	Sample sizes of males		
		Experiment A	Experiment B	Experiment C
Old	0	3	26	5
Old	1	5	35	24
Old	2	3	35	6
Old	3	4	11	1
Old	4	10	6	5
Old	5	13	2	0
Old	6	7		3
Old	7	9		3
Old	8	4		2
Old	9	1		1
Old	10	1		
Young	0	0	11	1
Young	1	0	7	1
Young	2	3	22	0
Young	3	3	22	4
Young	4	12	18	3
Young	5	9	3	4
Young	6	8	1	10
Young	7	10	1	3
Young	8	6	0	0
Young	9	4	0	0
Young	10	5	0	2

Table S2: Descriptions of the best-fit models (final) models for each analysis in our study, with details on model aims, dependent and fixed terms, random effects, model error structure, and marginal variance explained. Model outputs of each best-fit model can be found in Tables S3-S9 below.

Experiment	Aim	Dependent term	Fixed effects	Random effects	Error distribution	$R^2_{marginal}$%
A	Compare reproductive output of old and young *dah* males in a mating sequence	Number of offspring produced by female	Male age * female number + I(female number^2) + replicate	1|Male ID + 1|Observation-level	Zero-inflated Poisson	7.7
B	Compare reproductive output of old and young *dah* males in a mating sequence, when females are first mated to *sot* males	Number of offspring produced by female	Male age + female number + replicate	1|Male ID + 1|Observation-level	Zero-inflated Poisson	1
C	Compare reproductive output of old and young *gfp* males in a mating sequence	Number of offspring produced by female	Male age + female number + replicate	1|Male ID + 1|Observation-level	Zero-inflated Poisson	6.4
	Compare sperm transferred by old and young *gfp* males in a mating sequence	Number of sperm in females frozen within 30 minutes of mating	Male age * female number + replicate	1|Male ID + 1|Observation-level	Zero-inflated Poisson	25.7
	Compare sperm stored by females mated to old and young *gfp* males in a mating sequence	Number of sperm in females frozen 24 hours after mating	Male age * female number + I(female number^2) + replicate	1|Male ID + 1|Observation-level	Poisson	21.9
	Compare sperm in SV of old and young *gfp*	Number of sperm in SV of males	Male age * mating success + I(mating	1|Observation-level	Poisson	75.7

	males with varying mating success		success ^2) + replicate			
	Compare accessory gland size of old and young *gfp* males with varying mating success	Accessory gland size	Male age * mating success + replicate		Gaussian	89.7

Table S3: Effects of male age and female number in a male's mating sequence, on the number of offspring produced by mated females in Experiment A. Model constructed with zero inflated Poisson error distribution. Two-way interaction model used to interpret interaction only, main-effects model used to interpret main-effects only when two-way interaction is non-significant. Effects of interest highlighted in grey, significant P values of interest in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.813	0.073	52.250	<0.001
I(Female number^2)	-0.014	0.004	-3.430	0.001
Age (Young)	0.318	0.075	4.240	<0.001
Female number	0.109	0.033	3.300	0.001
RepA2	0.116	0.039	2.990	0.003
Age (Young)*Female number	-0.043	0.019	-2.240	**0.025**

Random effects	Variance	SD
Male ID	0.006	0.078
Observation level	0.146	0.382

Table S4: Effects of male age and female number in a male's mating sequence, on the number of offspring produced by mated females in Experiment B. Model constructed with zero inflated Poisson error distribution. Two-way interaction model used to interpret interaction only, main-effects model used to interpret main-effects only when two-way interaction is non-significant. Effects of interest highlighted in grey, significant P values of interest in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.953	0.059	66.540	<0.001
Age (Young)	0.154	0.074	2.080	0.038
Female number	0.055	0.029	1.940	0.053
RepB2	0.038	0.034	1.120	0.264
Age (Young)*Female number	-0.050	0.034	-1.460	0.145

Random effects	Variance	SD
Male ID	0.000	0.020
Observation level	0.083	0.288

Main-effects model

Fixed effects	Estimate	SE	z	P
(Intercept)	4.015	0.041	96.820	<0.001
Age (Young)	0.059	0.036	1.650	0.099
Female number	0.020	0.016	1.300	0.193
RepB2	0.037	0.034	1.080	0.280

Table S5: Effects of male age and female number in a male's mating sequence, on the number of offspring produced by mated females in Experiment C. Model constructed with zero inflated Poisson error distribution. Two-way interaction model used to interpret interaction only, main-effects model used to interpret main-effects only when two-way interaction is non-significant. Effects of interest highlighted in grey, significant P values of interest in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.923	0.111	35.260	<0.001
Age (Young)	0.266	0.126	2.110	0.035
Female number	-0.037	0.030	-1.240	0.216
RepCR2	0.112	0.108	1.040	0.299
RepCR3	0.082	0.092	0.890	0.376
Age (Young)*Female number	-0.046	0.040	-1.140	0.254

Random effects	Variance	SD
Male ID	0.000	0.000
Observation level	0.141	0.375

Main-effects model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.979	0.101	39.540	<0.001
Age (Young)	0.149	0.074	2.010	**0.044**
Female number	-0.064	0.020	-3.230	**0.001**
RepCR2	0.124	0.108	1.150	0.250
RepCR3	0.083	0.093	0.900	0.370

Table S6: Effects of male age and female number in a male's mating sequence, on the number of sperm transferred by males to mated females in Experiment C. Model constructed with zero inflated Poisson error distribution. Two-way interaction model used to interpret interaction only, main-effects model used to interpret main-effects only when two-way interaction is non-significant. Effects used for interpretation highlighted in grey, significant P values of interest in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	4.866	0.569	8.554	<0.001
Age (Young)	2.433	0.594	4.099	<0.001
Female number	-0.033	0.115	-0.288	0.773
RepCR2	0.182	0.526	0.346	0.730
RepCR3	0.675	0.461	1.464	0.143
Age (Young)*Female number	-0.414	0.142	-2.905	**0.004**

Random effects	Variance	SD
Male ID	0.631	0.795
Observation level	1.421	1.192

Table S7: Effects of male age and female number in a male's mating sequence, on the number of sperm transferred stored by mated females in Experiment C, after 24 hours of egg laying. Model constructed with Poisson error distribution. Two-way interaction model used to interpret interaction only, main-effects model used to interpret main-effects only when two-way interaction is non-significant. Effects used for interpretation highlighted in grey, significant P values of interest in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.488	0.706	4.939	<0.001
I(Female number^2)	-0.032	0.039	-0.821	0.412
Age (Young)	2.700	0.644	4.195	<0.001
Female number	-0.053	0.326	-0.162	0.872
RepCR2	-0.304	0.651	-0.467	0.641
RepCR3	0.680	0.573	1.188	0.235
Age (Young)*Female number	-0.373	0.184	-2.030	**0.042**

Random effects	Variance	SD
Male ID	0.746	0.864
Observation level	4.244	2.060

Table S8: Effects of male age and male mating success, on the number of sperm in seminal vesicles of males. Model constructed with Poisson error distribution. Two-way interaction model used to interpret interaction only, main-effects model used to interpret main-effects only when two-way interaction is non-significant. Effects used for interpretation highlighted in grey, significant P values of interest in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	9.632	0.165	58.350	<0.001
I(Mating success^2)	0.035	0.007	4.990	<0.001
Age (Young)	-1.483	0.157	-9.420	<0.001
Mating success	-0.377	0.062	-6.120	<0.001
RepCR2	0.144	0.158	0.910	0.363
RepCR3	0.514	0.143	3.600	<0.001
Age (Young)*Mating success	-0.166	0.036	-4.630	**<0.001**

Random effects	Variance	SD
Observation level	0.179	0.423

Table S9: Effects of male age and male mating success, on the area of male accessory glands (cm^2). Model constructed with Gaussian error distribution. Two-way interaction model used to interpret interaction only. Effects used for interpretation highlighted in grey, significant P of interest values in bold.

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	0.372	0.015	24.724	<0.001
Age (Young)	-0.161	0.014	-11.677	<0.001
Mating success	-0.040	0.003	-11.831	<0.001
RepCR2	-0.011	0.014	-0.785	0.437
RepCR3	0.027	0.014	1.910	0.064
Age (Young)*Mating success	0.027	0.004	6.577	**<0.001**

Chapter 5

No evidence for paternal age effects on sons or daughters, when accounting for paternal sperm storage duration

Abstract

The age at which fathers reproduce is predicted to affect offspring phenotypes, with offspring born to old fathers often being of a lower quality than those born to young fathers. However, when fathers have low mating rates, paternal age might be confounded with the duration for which mature sperm are stored in fathers prior to ejaculation. Few studies have been able to disentangle the confounding effects of paternal sperm storage from those of paternal age, on offspring. Here, we use *Drosophila melanogaster* to test the separate and interactive effects of paternal age and sperm storage duration, on the age-dependent survival and lifetime reproduction of offspring. Contrary to prediction, we find no effect of paternal age on the survival or lifetime reproduction of either sons or daughters, but find weak evidence for paternal sperm storage duration on the reproductive output of sons. We further discover that daughters of low reproductive quality selectively disappear with age, but sons do not. This result emphasizes that studies should account for demographic processes when testing for paternal age effects. Our study suggests that paternal age effects might not be as pervasive as previously thought, with studies possibly misattributing effects of paternal sperm storage to paternal age. Overall, we highlight the need for a more nuanced understanding of the mechanisms driving paternal age effects, and the importance of measuring sex-specific responses in offspring.

Keywords: trade-offs, parental effects, post-meiotic sperm ageing, senescence, sex-specific

Introduction

Parental environments and phenotypes can influence offspring phenotypes (Badyaev and Uller, 2009; Liu and Chen, 2018) via differential resource allocation by parents (Uller, 2008), and genetic (e.g. mutation accumulation in gametes) or epigenetic mechanisms (Bauch et al, 2019; Chen et al, 2016; Heidinger et al, 2016; Perez and Lehner, 2019; Rando, 2016; Rodgers et al, 2015; Sharma, 2019; Yoshizaki et al, 2021). One parental effect that has received considerable attention is the 'paternal age effect'. Paternal age effects are caused when the age at which a father conceives offspring affects the offspring's phenotype (Monaghan and Metcalfe, 2019). These effects have broad implications for organismal healthspan (Chan and Robaire, 2022), life-history evolution, and population dynamics (Evans, et al, 2019).

Paternal age has been hypothesized to be more potent in influencing offspring phenotypes than maternal age (de Manuel et al, 2022; Gao et al, 2019). The rationale stems from sperm producing more reactive oxygen species, but have poorer DNA repair machinery than eggs, and male germlines accumulating more mutations with advancing age than female germlines (Crow, 2000; Ellegren, 2007; Girard et al, 2016; Reinhardt and Turnell, 2000; Venn et al, 2014). Evidence for deleterious paternal age effects includes old fathers producing offspring with poorer development (e.g. Janecka et al, 2017; Preston et al, 2015), lower juvenile survival (e.g. Fay et al, 2016), reduced adult lifespans (e.g. Crow, 2003; Noguera et al, 2018; Priest et al, 2002; Sharma et al, 2015; Wylde et al, 2019; Xie et al, 2018), and lower reproductive output (e.g. Arslan, 2017; Schroeder et al, 2015; Vuarin et al, 2021), compared to offspring of young fathers.

The effects of paternal age can, in some cases, be confounded by the duration for which sperm are stored post-meiosis and prior to ejaculation in extra-gonadal reserves (Pizzari et al, 2008; Reinhardt, 2007; Siva-Jothy, 2000). These confounding effects typically arise in studies where fathers are kept virgins for long durations (reviewed in Sanghvi et al,

2024), have low rates of sperm loss or resorption, and have life-long spermatogenesis (e.g. some vertebrates and insects: Bjork et al, 2007; Demarco et al, 2014; Reinhardt et al, 2011; Santos et al, 2023; Sepil et al, 2020). In such studies, old, virgin males not only have a more senescent germline, but also have sperm stored for longer durations than young virgin males (Pizzari et al, 2008). Differences in durations of sperm storage between old and young fathers could also arise when fathers of different ages differ in their mating rates (Aich et al, 2022). In natural settings for instance, old males could be mating less often, thus storing sperm for longer durations on average, than young males. When the effects of paternal age and sexual rest cannot be disentangled, it remains unclear how these processes independently and interactively influence paternal reproductive output. Studies that disentangle these effects on paternal reproductive output, report contrasting results to each other (Gasparini et al, 2019; Vega-Trejo et al, 2019). Furthermore, no study has yet tested the interactive effects of these processes on the reproductive output of offspring. Previous studies that measure offspring phenotypes focus mainly on offspring survival (Meunier et al, 2022), which might not be informative of offspring fitness if offspring survival and reproduction co-vary negatively.

Testing the separate and combined effects of paternal age and sperm storage duration on offspring is necessary to ensure that studies are not incorrectly attributing the effects of these factors to each other. Sperm storage after meiosis and before ejaculation, can influence ejaculate traits and the overall fertility of a male, as well as his offspring phenotype and fitness, independent of male age (Pizzari et al, 2008). Prolonged storage of sperm in males or females can deteriorate sperm quality (Brindle et al, 2023; Cattelan and Gasparini, 2021; Comar et al, 2017; Gasparini et al, 2014, 2019; Hettyey et al, 2012; Levitas et al, 2005; Radhakrishnan and Fedorka, 2011), increase the number of mutations in sperm (Agarwal et al, 2016; Rinehart, 1969), and reduce male fertilisation success (Gasparini et al, 2018; Reinhardt and Siva-Jothy, 2005). Sperm storage can also negatively affect the development

(Dharmarajan, 1950; Lodge et al, 1971; Pineaux et al, 2019; White et al, 2008), quality (Tarin, 2000; Wagner et al, 2004; White et al, 2008), and fertility (Gasparini et al, 2017) of resultant offspring. This deterioration of stored sperm mainly occurs because sperm accumulate DNA and oxidative damage over prolonged periods of sexual rest (Barbagallo et al, 2022; Sorensen et al, 2023). Further, paternal age and sperm storage duration might interact (Pizzari et al, 2008), for example, if old fathers are worse at repairing damage in stored sperm, than young fathers (Gorbunova et al, 2007; Selvaratnam et al, 2015; Weirich-Schwaiger et al, 1994). Studies that manipulate both, paternal sperm storage duration and age, and simultaneously measuring offspring and paternal fitness components are lacking, leaving such hypotheses untested.

Here, we experimentally test the independent and interactive effects of paternal age at conception and paternal duration of sexual rest (henceforth, sperm storage duration), on the age-dependent survival and lifetime reproduction of sons and daughters. We use the fruit fly, *Drosophila melanogaster,* a model organism for investigating parental age effects (Aguilar et al, 2023; Hercus and Hoffmann, 2000; Mossman et al, 2019; Nystrand and Dowling, 2014; Price and Hansen, 1998; Sanghvi et al, 2023; Sepil et al, 2020; Tan et al, 2013), owing to their short generation time and absence of parental care. Fruit flies show life-long spermatogenesis (e.g. Bjork et al, 2007; Sepil et al, 2020) and low rates of sperm loss (Demarco et al, 2014; Santos et al, 2023), leading to age-dependent accumulation of mature sperm in unmated males (Pisano et al, 1993; Sepil et al. 2020). Sperm storage reduces up to 50% of sperm viability over 8 days (Radhakrishnan and Fedorka, 2011), leads to sperm ejection by females (Snook and Hosken, 2004), and lowers offspring viability (Tan et al, 2013) in flies.

We test predictions of five hypotheses (H1-H5). First, paternal sperm storage could confound with paternal age, to influence the reproductive output of fathers, and the

phenotypes of their offspring. If previous studies that do not separate these effects are misattributing effects of paternal sperm storage to paternal age, we predict that separating these effects will reveal no significant evidence for paternal age effects on offspring. Second, old males might be worse at repairing cellular damage than young males (Gorbunova et al, 2007; Weirich-Schwaiger et al, 1994; Witt et al, 2023), causing deleterious effects of sperm storage to be exacerbated in old than young fathers (Zubkova and Robaire, 2006). We thus predict offspring of old fathers with long durations of sexual rest to have lower fitness than offspring of young fathers or of fathers with short sperm storage durations (H2). Third, offspring of old fathers might inherit a higher mutation load than offspring of young fathers (Chen et al, 2023; de Manuel et al, 2022; Girard et al, 2016; Jonsson et al, 2017; Kong et al, 2012; Wang et al, 2020). If paternally inherited mutations are exacerbated as offspring grow older (Bregdahl et al, 2020, 2023; Monaghan et al, 2020; Moorad and Promislow, 2008; Shindyapina et al, 2020), we predict that the effects of old paternal age would be more deleterious when offspring are old than young (H3). Fourth, paternal age effects might be sex-specific (e.g. Aich et al, 2022; Angell et al, 2022; Gasparini et al, 2017; Krishna et al, 2012; Sparks et al, 2022). For instance, advancing paternal age might deteriorate the Y chromosome (Byrne et al, 2003; Carothers et al, 1978) or imprinted genes (Denomme et al, 2020; Paczkowski et al, 2015). Similarly, telomeres or epigenetic markers (which are affected by paternal age) from might be inherited sex-specifically (Bouwhuis et al, 2015; Olsson et al, 2011; Schroeder et al, 2015). In line with these hypotheses, we predict that the quality of sons will be more affected by paternal age more than that of daughters (H4). Lastly, fathers might trade-off investment in sperm production against repair/maintenance of sperm quality (e.g. Koppik et al, 2023). This trade-off might manifest as trade-offs between offspring quantity and quality, therefore modulate paternal age effects (Fischer et al, 2011; Ratikainen et al,

2018; Johnson et al, 2018). In line with these hypotheses, we predict that fathers who produce more offspring would produce poorer quality offspring (H5).

Methods

Stock and experimental individuals

We used *Dahomey* wild-type *Drosophila melanogaster* flies maintained at a 12:12hr light cycle, at a constant temperature of 25°C, and fed with Lewis medium (Lewis, 1960) supplemented with *ad libitum* live yeast (following Sepil et al, 2020). The stock from which these flies were taken have been reared in the lab since the 1970s. Under these conditions, flies have an egg-to-adult development of ~10 days, and virgin adult males have median and maximum lifespans of ~45 and 90 days, respectively (Sepil et al, 2020; also see Fig S1). Our experiment consisted of two parts: first, fathers were generated and assigned across four treatments (old or young paternal age, with long or short sperm storage duration) in a fully balanced design (henceforth "F0 assays"). Then, sons and daughters (F1 individuals) from fathers in each paternal treatment were collected, and their survival and lifetime reproduction measured (henceforth "F1 assays). Our experiment (Figure 1) was conducted using a total of 60 fathers per treatment (our independent sample size), spread across four replicates (see Table S1 for sample sizes).

Experimental design

F0 assays

Paternal treatments

We first reared experimental flies using a standard larval density method by placing ~200 eggs obtained from our stock population cage, on 50 mL of food in 250-mL bottles (Clancy and Kennington, 2001). We then collected virgin "F0 males" using ice anaesthesia within 7

hours of eclosion from across ~15 of these bottles. F0 males were kept in groups of 10 and haphazardly assigned to one of four paternal treatments. These four paternal treatments were: old fathers with sperm stored for long durations (OO), old fathers with sperm stored for short durations (OY), young fathers with sperm stored for long durations (YO), and young fathers with sperm stored for short durations (YY). To generate fathers with sperm of known storage durations, we manipulated the duration of sexual rest. For this, we "stripped" (i.e. depleted stored ejaculates) virgin young and old F0 males of their stored ejaculates (see "F0 stripping assay" below), and then allowed F0 males to replenish their ejaculates for a known duration, until they were mated to a single young virgin experimental female to obtain offspring (see F0 mating assay below). Specifically, F0 males assigned to the OO treatment were stripped when 37 days old and mated at 45 days old, OY males were stripped when 43 days old and mated at 45 days old, YO males were stripped when 3 days old and mated at 11 days old, while YY males were stripped when 9 days old and mated at 11 days old (Figure 1). This design gave us F0 males who were young (11 days old) or old (45 days old), and with ejaculates stored for short (2 days) or long (8 days) durations.

F0 stripping assay

To deplete ("strip") experimental F0 males and create the four paternal treatments, we placed single old or young F0 males with 10 virgin young (3-4 days old) females (henceforth, "stripping females"), and allowed males to mate *ad-libitum* with these stripping females for 24 hours. Mating with 10 females over 24 hours is sufficient to deplete *D. melanogaster* males of their ejaculate reserves (Douglas et al, 2020; Hopkins et al, 2019; Linklater et al, 2007; Loyau et al, 2010; Macartney et al, 2021, also see Appendix 1). After 24 hours of being with the 10 stripping females, F0 males were separated, sexually rested for two or eight days depending on their treatment, and used subsequently for the "F0 mating assay". Finally, we

measured whether the F0 males used in the stripping assay produced offspring with the stripping females, to ensure the stripping assay's effectiveness (see Appendix 1 for details).

F0 mating assay

After being with the 10 stripping females for 24 hours, all F0 males were transferred to new vials and kept individually. Only F0 males that produced offspring in the "F0 stripping assay", were used in the "F0 mating assay" (see Appendix 1 for details). Next, old and young F0 males assigned to the short sperm storage treatment (OY and YY respectively) were mated once with a young virgin (3-4 days old) experimental female two days after the F0 stripping assay. Old and young F0 males in the long sperm storage treatment (OO and YO respectively) were mated once with a young virgin (3-4 days old) experimental female 8 days after the stripping assay. All experimental females (mothers) were obtained from standard stock cages using the standard larval density method described above. F0 males from all four treatments (henceforth called "fathers") were presented with an experimental female on the same day, given a maximum of 5 hours to mate only once. Each mating event was observed, and mating latency and copulation duration recorded. Following mating, experimental females were left singly in the mating vial for 24h to enable oviposition. The resultant offspring ("F1") produced by experimental females (mothers) and fathers, over 24 hours of eggs laying in mating vials, were used in the F1 assays (see below).

F1 assays

The vials with eggs laid from experimental parents in the four treatments were checked every day for eclosing offspring (F1). Within seven hours of eclosion, three male (henceforth "sons") and three female (henceforth "daughters") offspring were haphazardly collected from each vial, into individual vials with a unique ID. These six offspring from each parental pair

were then used for subsequent “F1 assays” (see below). The remaining eclosed offspring in each parental vial were frozen four days later and counted, to compare the reproductive output of fathers from the four treatments. Overall, we conducted F1 assays on 3 sons and 3 daughters from ~60 fathers in each treatment.

Once every two weeks, surviving sons and daughters were moved to a new vial with a virgin young mate (3-4 days old) of the opposite sex for 24 hours and the resultant offspring (“F2”) were counted. This gave us data on the reproductive ageing patterns of both sons and daughters, from fathers in all four paternal treatments. We checked offspring survival every one to three days. Using an aspirator, sons were transferred to new food vials once a week, while daughters were transferred to new vials twice a week to reduce female mortality caused by larvae softening the food medium.

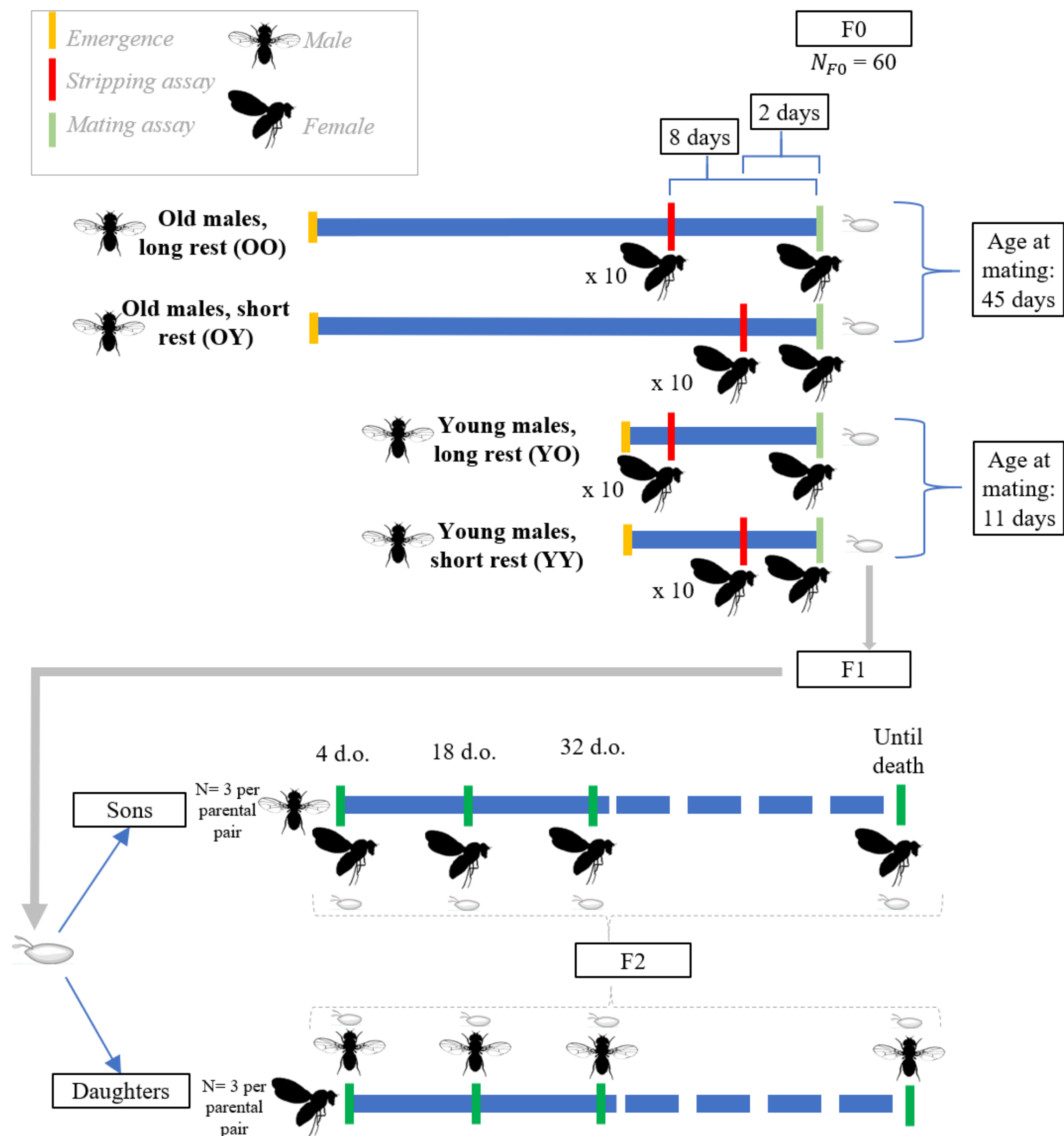


Figure 1: Experimental design to test how paternal age (Y: young, O: old) and paternal sperm storage duration (Y: short, O: long) affect lifetime reproduction and survival of sons and daughters. We first depleted old and young males of their ejaculates by keeping them with 10 females for 24 hours, then allowed them to replenish their ejaculate reserves for either two days (short storage) or eight days (long storage), and subsequently mated them to a single female. Three sons and three daughters produced from this mating (F1) were chosen haphazardly, and used for the F1 assays. Here, each experimental F1 offspring was mated to an individual of the opposite sex from the stock population once every two weeks, until death, and offspring reproductive output (number of eclosed F2 individuals) from eggs laid over 24 hours were counted. In total, each paternal treatment had 60 fathers. Images from PhyloPics by Thomas Hegna and Ramiro Morales Hojas (PD1.0 and CC01.0 licence).

Data analysis

General modelling approach

We used linear mixed-effects models (LMM) and generalised linear mixed-effects models (GLMM) to understand how paternal age and paternal sperm storage duration affected the reproductive output of fathers and their offspring (Table 1). We included an optimizer ("BFGS") whenever GLMM models did not converge. We used Cox mixed-effects proportional-hazards models (coxme) to understand how paternal age and paternal sperm storage duration affected the age-dependent mortality of offspring. All analyses were done in R v3.5.2 (R Core team, 2012), using the packages *stats* (R Core team, 2012), *lme4* (Bates et al, 2015)*, glmmTMB* (Brooks et al, 2017)*, and coxme* (Therneau, 2015). All LMMs were checked for normality and homoscedasticity of residuals using the *stats* package, and GLMMs were checked for overdispersion whenever appropriate, using the *DHARMa* package (Hartig and Hartig, 2017). We analysed data on sons and daughters separately. Marginal variance ($R^2_{marginal}$) explained by fixed effects in our models, was calculated using the *sjPlot* (Ludecke, 2023) and *CoxR2* (You and Xu, 2020) packages. Post-hoc pairwise comparisons whenever conducted, were done using Hedges' g in the *effectsize* (R Core team, 2012) package ($\alpha = 0.05$ for significance tests of effect size). Model comparisons were done using AIC function in the *stats* package.

Unless mentioned otherwise, we started with a "full model" that included two-way interactions between paternal sperm storage duration and paternal age. For models on reproductive ageing in offspring, our full model additionally included offspring age in a three-way interaction with paternal age and sperm storage. These full models were used to interpret the highest order interactions only. To then interpret lower order interactions or main-effects whenever higher order-interactions were non-significant, we fitted models with

the highest level of interaction removed (following Engqvist, 2005). Main effects indicated independent effects of variables when averaged across the effects of other variables.

F0 assays

We analysed how paternal age and sperm storage duration affected the reproductive output of fathers. We modelled the number of offspring produced by fathers as our dependent variable, with zero-inflated negative binomial error distribution, because this distribution fit data better than Poisson ($\Delta AIC = 6624$, $\Delta DF = 2$) or zero-inflated Poisson ($\Delta AIC = 1283$, $\Delta DF = 1$) error distributions. We modelled paternal age, sperm storage duration, their interaction, copulation duration, and replicate as fixed effects. Copulation duration was included to account for males who copulate for longer durations transferring more sperm to females, thus producing more offspring.

F1 assays

Reproductive ageing (Table 1)

To understand the effects of paternal treatment on age-dependent reproductive output of daughters, we built a GLMM with zero-inflated negative binomial error distribution. This model was a better fit to the data than one with Poisson ($\Delta AIC = 20967$, $\Delta DF = 3$) or zero-inflated Poisson ($\Delta AIC = 3151$, $\Delta DF = 1$) error distribution. We modelled the number of offspring produced over 24 hours by daughters, measured once every two weeks from birth to death, as the dependent variable. We included paternal age, paternal sperm storage duration, the age of daughters, their three-way interaction, and replicate as fixed effects. In the same model, we included a quadratic term for daughter's age (which significantly improved model fit compared to only a linear term, $P < 0.001$), because reproductive ageing patterns are often curvilinear (Jones et al, 2014; Sanghvi et al, 2024). Effects of paternal age on offspring

lifetime reproduction might be biased, if demographic processes such as selective disappearance cause non-random death of offspring over time. In our model, we thus additionally included the lifespan of daughters as a fixed effect, to account for selective disappearance (Bouwhuis et al, 2009; Sanghvi et al, 2022). We further included the number of offspring that fathers produced as a fixed effect, to investigate whether fathers compensate for lower quality offspring by producing more offspring. We modelled daughter ID nested within paternal ID as random effects.

To understand the effects of paternal treatment on age-dependent reproductive output of sons, we built a GLMM with a negative binomial error distribution. This model was a better fit to the data than one with Poisson (ΔAIC = 13177, ΔDF = 1) or zero inflated Poisson (ΔAIC = 4211, ΔDF = 0) error distributions. The fixed and random effects in the model for reproductive output of sons were identical to those in our model for reproductive output of daughters (as described above), except for one difference. Specifically, for sons, we modelled only a linear term for the age at reproduction of sons, because a quadratic term did not improve model fit (ΔAIC = 2, ΔDF = 1, P = 0.766 with L.R.T. under a Chi-sq. distribution).

Actuarial ageing (Table 1)

We used Cox- mixed-effects proportional hazards models to investigate the age-dependent mortality risk to sons and daughters separately. However, our models for both sons and daughters were identically structured. We modelled the lifespan of offspring as the dependent variable. Paternal age, paternal sperm storage duration, their two-way interaction, and replicate were included as fixed effects. The number of offspring sired by fathers was modelled as a fixed effect, to test whether fathers compensate for low survival of offspring by producing more offspring. We modelled paternal ID as a random effect.

Table 1: Detailed model structure for each statistical model in our study. Model aim, dependent variables, fixed and random effects, and model type for the best-fitting model (determined using AIC comparisons) are reported. Proportion of variance in data explained by fixed effects reported as marginal R^2.

Generation	Model	Dependent variable	Fixed effects	Random effects	Model type	$R^2_{marginal}$
F0 (fathers)	Reproductive output	Number of offspring produced	Age*sperm storage + copulation duration + replicate		Zero-inflated negative binomial	3.3%
F1 (offspring)	Reproductive ageing (daughters)	Number of offspring produced in a day of egg laying	Paternal age*paternal sperm storage*age + age^2 + lifespan + paternal fecundity + replicate	1|Paternal ID/daughter ID	Zero-inflated negative binomial	38.8%
	Reproductive ageing (sons)	Number of offspring produced in a day of egg laying	Paternal age*paternal sperm storage*age + lifespan + paternal fecundity + replicate	1|Paternal ID/son ID	Negative binomial	2.6%
	Actuarial ageing (daughters)	Lifespan	Paternal age*paternal sperm storage + paternal fecundity + replicate	1|Paternal ID	Cox-proportional hazards	6.5%
	Actuarial ageing (sons)	Lifespan	Paternal age*paternal sperm storage + paternal fecundity + replicate	1|Paternal ID	Cox-proportional hazards	4.8%

Results

F0 assays

We found no interaction between paternal age and sperm storage duration influencing the number of offspring produced by fathers ($z = 0.343$, $P = 0.731$, Figure 2B, Table S2). However, paternal age ($z = 2.130$, $P = 0.033$), but not sperm storage duration ($z = 0.810$, $P = 0.419$, Figure 2B), independently affected the number of offspring fathers produced, with young fathers producing more offspring than old fathers.

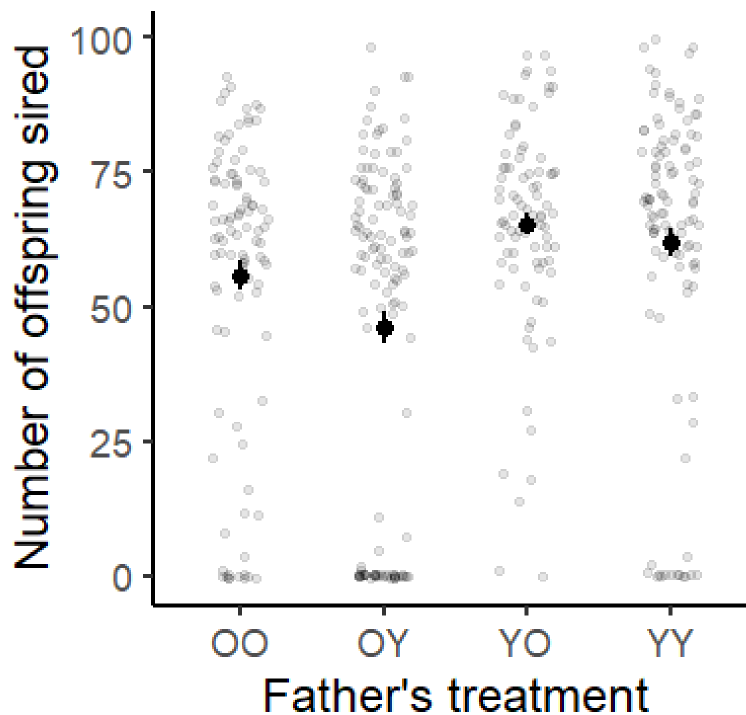


Figure 2: Effects of age and sperm storage treatment, on the number of offspring sired by fathers across the four paternal treatments: old age, long sperm storage (OO); old age, short sperm storage (OY); young age, long sperm storage (YO); young age, short sperm storage (YY). Means and SE shown along with the raw data points.

F1 (offspring) assays

Reproductive ageing

The three- or two- way interaction of paternal age, paternal sperm storage duration, and the age of daughters, had no significant effect on the number of offspring produced by daughters (Figure 3, Table S3). Furthermore, paternal age ($z = -1.14$, $P = 0.254$) or paternal sperm storage duration ($z = 1.43$, $P = 0.153$) did not significantly influence the fecundity of daughters (Table S3). However, daughters produced fewer offspring when old than when young (daughter age as quadratic: $z = -3.410$, $P = 0.001$; as linear: $z = -6.050$, $P < 0.001$). Daughters that produced fewer offspring died earlier than daughters that produced more offspring (effect of daughter's lifespan: $z = 2.710$, $P = 0.007$, Figure 4A), consistent with

selective disappearance. Paternal reproductive output did not influence the reproductive output of daughters (z = -0.020, P = 0.984, Figure S3A).

We found no significant effect of three-way or two-way interactions between paternal age, paternal sperm storage duration, and the age of sons, to affect the number of offspring produced by sons (Figure S4, Table S4). Similarly, paternal age did not have a significant effect (z = 0.160, P = 0.875). However, we found a marginally significant effect of paternal sperm storage duration (z = 2.030, P = 0.043, Figure 5A), on the number of offspring produced by sons. Sons born to fathers who stored sperm for eight days (i.e. long sperm storage) had on average a 4.2% lower reproductive output across their lifespan, than sons born to fathers who stored sperm for two days. Post-hoc tests revealed that this difference was significant only in the early life of sons (Hedge's g at 4 days old: -0.22, 6.5% difference; Figure 5A, 5B). The number of offspring sired by fathers was positively correlated with the number of offspring sired by their sons (z = 2.930, P = 0.003, Figure S5). However, unlike in daughters, we did not find significant evidence for selective disappearance in sons (z = 1.660, P = 0.097; Figure 4B). Overall, sons produced fewer offspring with advancing age (z = -4.43, P < 0.001).

Actuarial ageing

Paternal age, paternal sperm storage duration, or their two-way interaction, did not significantly influence age-dependent mortality risk of daughters (Figure 6A, Table S5) or sons (Table S6). However, the number of offspring sired by fathers was significantly correlated with the mortality risk of their sons (z = -2.260, P = 0.024, Figure S5), but not daughters (z = 0.500, P = 0.610, Figure S5). Specifically, fathers who produced more offspring conceived sons that had higher rates of survival.

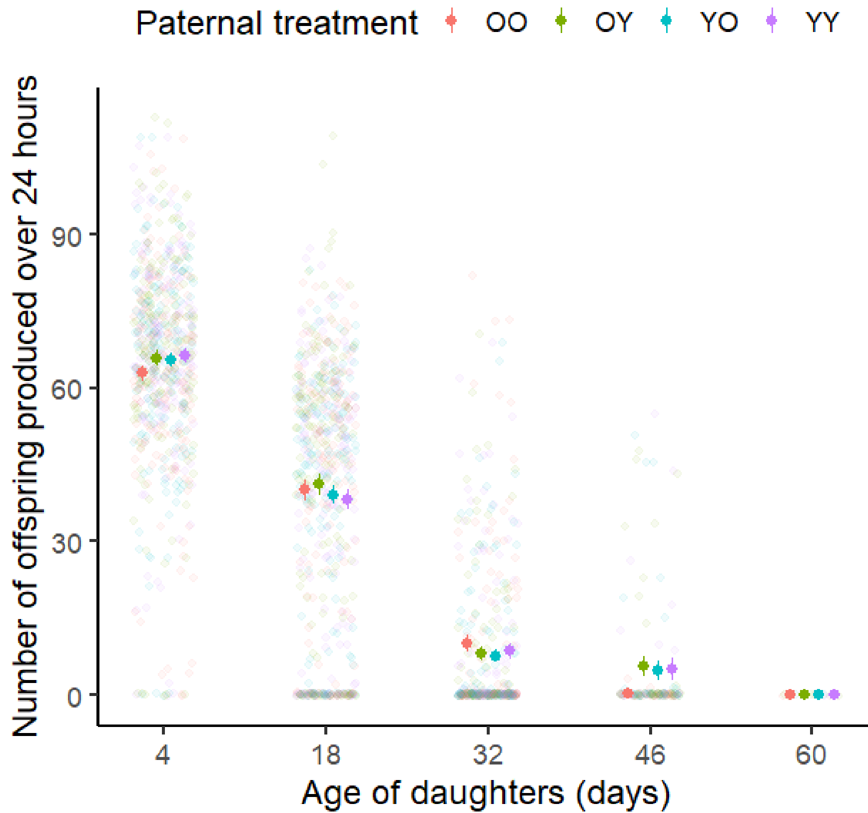


Figure 3: No effect of paternal age or paternal sperm storage duration on the number of offspring produced by daughters. OO: Old paternal age, long paternal sperm storage; OY: Old paternal age, short paternal sperm storage; YO: young paternal age, long paternal sperm storage; YY: young paternal age, short paternal sperm storage. Means and SE shown along with raw data. Each light dot represents measurements on a single daughter for a given age.

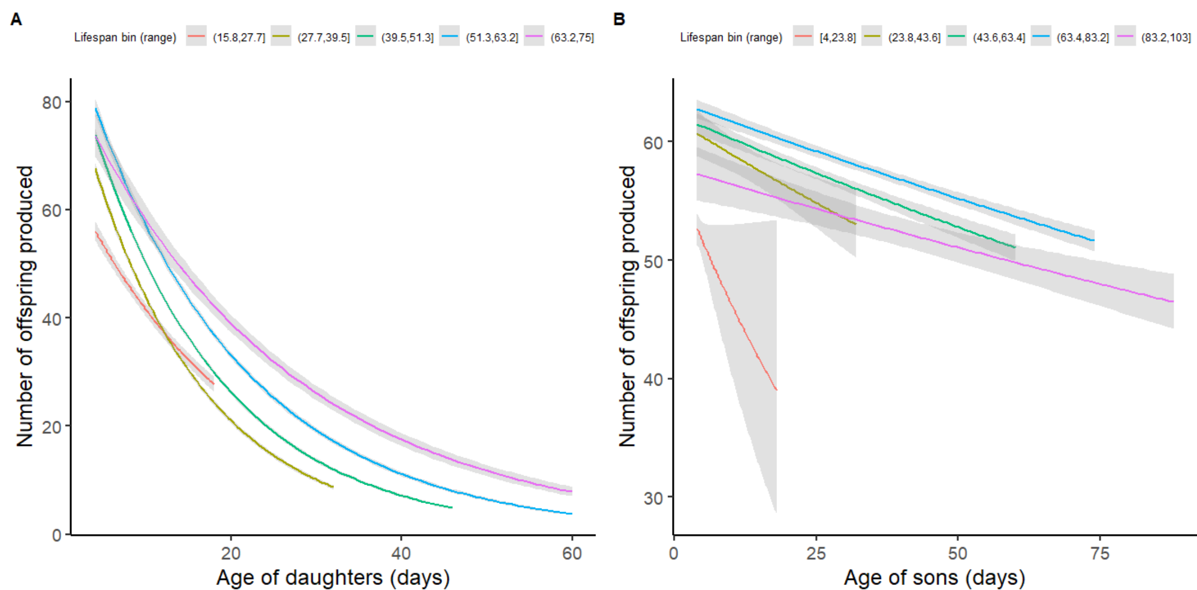


Figure 4: A- Daughters who lived longer consistently produced more offspring throughout life, than daughters who lived shorter lives, suggesting selective disappearance. B- We found no significant evidence for selective disappearance in sons. Data binned within 5 lifespan ranges. Curves plotted as glm-poisson. Shaded areas represent 95% C.I.

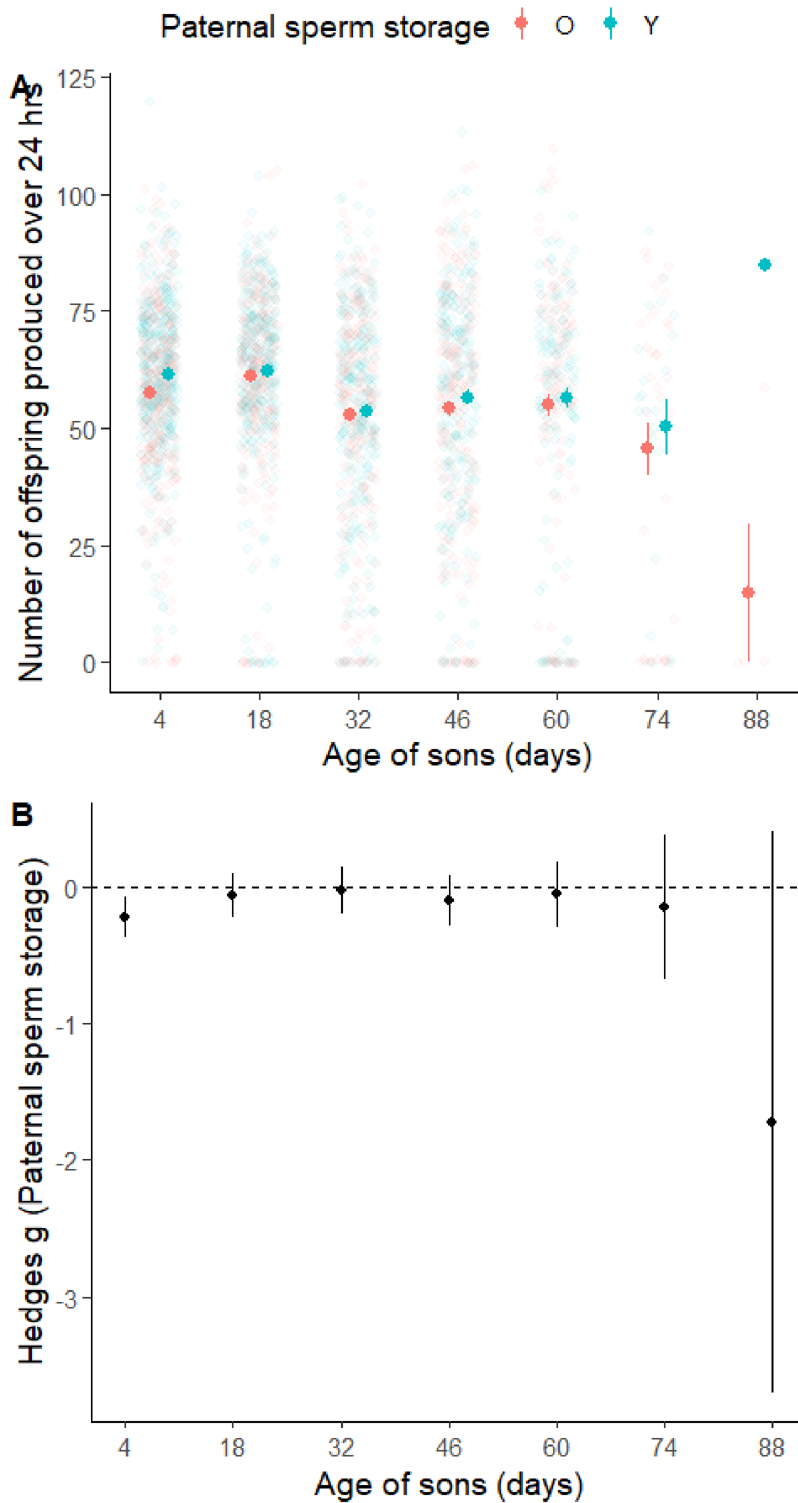


Figure 5: A- Effect of paternal sperm storage duration on the number of offspring produced by sons, when averaged across effects of other variables. Means and SE shown along with raw data. O: long paternal sperm storage, Y: short paternal sperm storage. Each light dot represents measurements on a single son at a given age. B- Effect of paternal sperm storage duration on reproductive success of sons is significant when sons are 4 days old. Effect sizes (Hedges; g) used for comparisons, and significance tests based on whether the 95% C.I. overlaps with zero or not. Negative effect sizes indicate that sons from fathers with long sperm storage duration, have lower reproductive output than sons from fathers with short sperm storage. Means and C.I. shown.

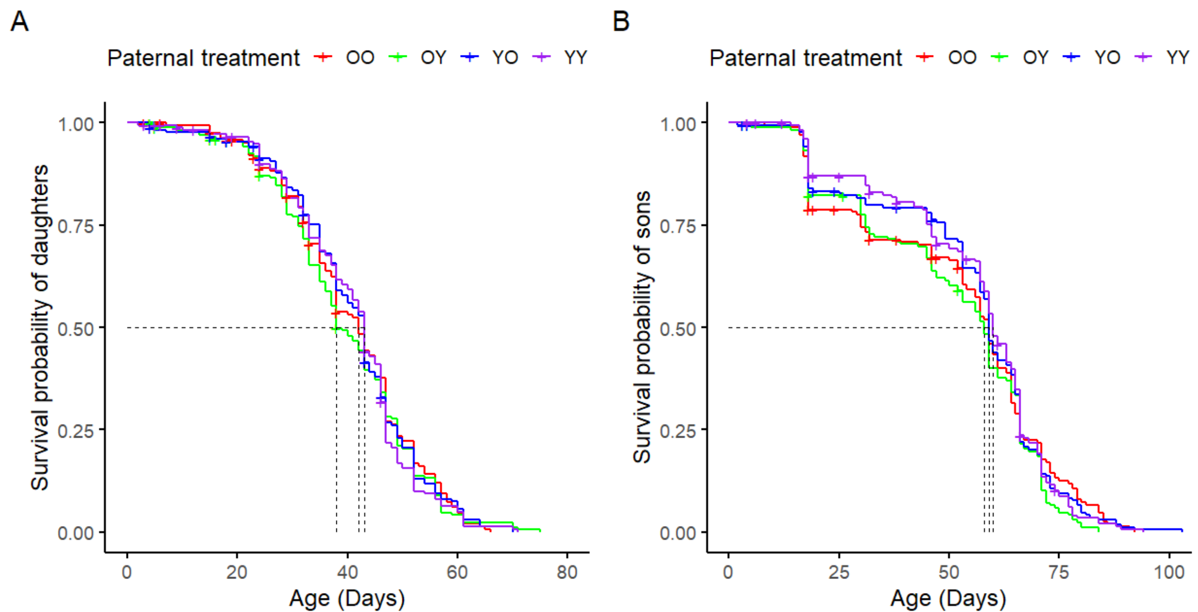


Figure 6: No significant effect of paternal age or paternal sperm storage duration on the age-dependent survival probability of A- daughters, or B- sons. Dotted lines show age at median survival probability. OO: old paternal age, long paternal sperm storage; OY: old paternal age, short paternal sperm storage; YO: young paternal age, long paternal sperm storage; YY: young paternal age, short paternal sperm storage. "+" shows censoring (lost, accidentally killed, or mislabelled).

Discussion

Paternal age effects can have far-reaching ramifications for organismal evolution. However, demonstrating a causal effect of paternal age on offspring phenotype and fitness requires experimental control of potential confounds, which is challenging. We investigated how paternal age and paternal sperm storage duration affect the reproductive output of fathers, and the reproductive and actuarial ageing of their offspring. We found older fathers to have lower reproductive output, suggesting reproductive senescence, but found no significant evidence for paternal age effects on offspring (H1). Additionally, we found no evidence for paternal sperm storage duration interacting with paternal age, to influence offspring phenotypes (H2). Instead, we found some evidence for an independent effect of paternal sperm storage duration on the reproductive output of sons. Furthermore, the lack of paternal age effects was consistently observed irrespective of offspring age, which is inconsistent with the mutation accumulation hypothesis (H3). We also found no evidence for paternal age to affect sons

more than daughters (H4). Furthermore, we did not find trade-offs between offspring quantity versus quality produced by fathers. Instead, fathers that produced more offspring also produced higher quality sons (H5).

We found no significant effect of paternal age on offspring, when separated from effects of paternal sperm storage duration (H1). Paternal age effects have been widely reported across taxa (e.g. Crow, 2003; Monaghan et al, 2020; Schroeder et al, 2015; Vuarin et al, 2019), including in humans (Chang and Robaire, 2022), with offspring from old fathers having lower fitness than offspring from young fathers (reviewed in Monaghan and Metcalfe, 2019). However, most studies on paternal age effects do not control for duration of sperm storage. The lack of an effect in our study might suggest some of the previous reports misattributing deleterious effects of paternal sperm storage to paternal age (reviewed in Pizzari et al, 2008, Sanghvi et al, 2024). In fruit flies for example, experimental males are often unmated until they are used to sire progeny (e.g. Aguilar et al, 2023; Mossman et al, 2019; Nystrand and Dowling, 2014; Price and Hansen, 1998; Priest et al, 2002). Male fruit flies are characterised by spermatogenesis throughout their adult life (Bjork et al, 2007; Sepil et al, 2020) and low rates of sperm loss (Demarco et al, 2014; Santos et al, 2023). These might lead to the accumulation of deteriorating sperm within the male reproductive tract, such that old virgin fathers have more deteriorated sperm than young virgin fathers. Only few studies have attempted to separate paternal age versus sperm storage effects, albeit in other species. For example, Gasparini et al (2019) report independent negative effects of both, paternal age and sperm storage duration, on paternal fertility in guppies; Jones et al (2004) show interactive effects between paternal age and sperm storage duration on paternal fertilisation success, in hide beetles; Vega-Trejo et al (2019) show no deleterious effects of paternal age or sperm storage duration on paternal sperm traits, in mosquitofish; while Meunier et al (2022) show deleterious effects of paternal age but not of sperm storage

duration on sperm traits and offspring survival, in bustards. None of these studies however, measured offspring lifetime reproductive success (a better metric of evolutionary fitness), as we were able to do in the present study.

The lack of a paternal age effect in our study could also reflect deleterious effects being balanced by beneficial effects of having old fathers (Sanghvi et al, 2023). For instance, fathers who have survived to older ages might represent a biased subset of fathers with alleles that confer longer lifespans (due to viability selection: Brooks and Kemp, 2001; Hansen and Price, 1995; Johnson and Gemmell, 2012; Kokko, 1998). Similarly, fathers of poor reproductive value could selectively disappear with age (Bouwhuis et al, 2009; Hamalainen et al, 2014; Sanghvi et al, 2022), leading to old surviving fathers having alleles for higher reproductive output. These processes (selective disappearance and viability selection) might have underestimated paternal age effects in our study, due to fathers being sampled cross-sectionally, rather than the same fathers being sampled longitudinally across life. The lack of a paternal age effect in our study could also occur due to female-driven processes. For example, female fruit flies might bias fertilisation toward good quality sperm via cryptic female choice (e.g Hadlow et al, 2023; reviewed in Firman et al, 2017; Sanghvi et al, 2024; Vuarin et al, 2019). Here, even if the ejaculates of old males contained more deteriorated sperm than ejaculates of young males, females could select for higher quality sperm from the ejaculates of old [and young] males, thus buffering deleterious paternal age effects.

We did not find evidence that old fathers who stored sperm for long durations, produce lower quality offspring than other treatments (H2). Old males are hypothesized to have poorer DNA repair machinery than young males (Chen et al, 2023; Gorbunova et al, 2007; Selvaratnam et al, 2015; Weirich-Schwaiger et al, 1994), which could lead to old fathers being worse at repairing sperm damage than young fathers (Pizzari et al, 2008). The lack of an interaction between paternal age and sperm storage duration in our study could be

due to sperm storage for eight days not being sufficient to increase mutation load in paternal sperm, or old fathers being able to repair DNA damage in mature sperm, well enough to ameliorate deleterious effects of sperm storage.

We found weak evidence for deleterious effects of paternal sperm storage duration on the early-life reproductive output of sons. Several studies have demonstrated that storage of mature sperm can deteriorate sperm (Agarwal et al, 2016; Brindle et al, 2023; Cattelan and Gasparini, 2021; Comar et al, 2017; Gasparini et al, 2014, 2019; Hettyey et al, 2012; Levitas et al, 2005; Radhakrishnan and Fedorka, 2011), and lead to offspring having lower fitness (Gasparini et al, 2017; Wagner et al, 2004; White et al, 2008). Other studies however, have not obtained evidence for such deleterious effects (Firman et al, 2015; Hotzy et al, 2020; Meunier et al, 2022; Vega-Trejo et al, 2019). There are several explanations for only a weak effect of paternal sperm storage duration on offspring observed in our study. First, sperm could to some extent be re-absorbed or lost in fathers, leading to low levels of sperm damage despite long durations of sexual rest (Pizzari et al, 2008; Reinhardt and Siva-Jothy, 2005; Reinhardt, 2007). Second, a weak effect could indicate sperm not being stratified in males (Reinhardt, 2007), causing fresh and stored sperm to be mixed in paternal ejaculates, whereby fathers with 8 days of sexual rest would have ejaculates of sperm varying in ages between zero and eight days. Third, not all stored sperm might have been ejaculated by F0 males in our stripping assay, leading to less effective sperm storage treatments. Fourth, if sperm haploid genomes are expressed in fruit flies, selection on sperm haplotypes could lead to the death of poor-quality sperm in fathers who stored sperm for long durations (Alavioon et al, 2017; Immler, 2019; Otto et al, 2015). This selective death (thus lack of fertilisation capacity) of old sperm, could buffer deleterious effects of paternal sperm storage duration on offspring. Fifth, females could be actively ejecting sperm stored for longer durations in males, thus buffering the effects of sperm storage (Reinhardt and Siva-Jothy, 2005; Snook and Hosken,

2004). In our study, the effect of sperm storage on the reproductive output of sons was significant, despite its magnitude being small. This statistical significance could be an artefact of having a large sample size (of ~180 sons per treatment) rather than a true biological pattern. We only manipulated sperm storage duration in fathers, but future studies can investigate whether sperm storage in mothers (reviewed in Orr and Brennan, 2015) also influence offspring phenotypes.

We predicted (H3) that deleterious effects of old paternal age would be more severe for old than young offspring (Brengdahl et al, 2023; Moorad and Promislow, 2008; reviewed in Monaghan et al, 2020). Such an interaction could occur if senescence in offspring exacerbates effects of deleterious mutations inherited from old parents (Chen et al, 2023; Girard et al, 2016; Kong et al, 2012; Yatsenko and Turek, 2018). Studies on fruit flies show that male germlines accumulate mutations with age (Garcia et al, 2010; Wang et al, 2022), that age-specific mutation accumulation can lead to accelerated senescence (Brengdahl et al, 2020; Yampolsky et al, 2000), and that old fathers have a higher germline mutation load than young fathers (Witt et al, 2023). A lack of support for the predicted interactive effect in our study might indicate that inherited mutations from old fathers in fruit flies are mostly selectively neutral (reviewed in de Jong et al, 2023) in their fitness effects, rather than deleterious, as predicted by the mutation accumulation hypothesis of senescence (Moorad and Promislow, 2008). In daughters, selective disappearance could have further masked such an interactive effect, because daughters of worse reproductive quality selectively died with age. Overall, we encourage future studies to not only quantify age-dependent mutation rates and epigenetic changes in old paternal sperm (e.g. Oakes et a, 2003; Suvorov et al, 2020), but also investigate their inter-generational phenotypic effects.

We did not find paternal age to have sex-specific effects on offspring (H4), likely due to an overall absence of paternal age effects altogether in our study. This lack of evidence

could also be due to mechanisms of inheritance causing sex-specific paternal age effects, being absent in fruit flies. Lastly, we did not find evidence that fathers who produced more offspring also produced offspring of poorer quality (H5). Instead, paternal fecundity co-varied positively with the survival and reproductive output of their sons. These results might be explained by heterogeneity in paternal condition, such that fathers who can acquire more resources are able to invest in both, offspring production (e.g. via higher ejaculate production) and offspring quality (e.g. via sperm repair) without trade-offs (Reznick et al, 2000; Roff and Fairbairn, 2007). Due to these covariances being apparent only in sons but not in daughters, these results might alternatively be explained by sex-specific heritability of fitness-components (Calsbeek et al, 2015; Connallon, 2012; Weiss et al, 2006) in fruit flies.

Conclusions

Our study challenges the commonly held prediction that older fathers produce lower quality offspring, and we find no evidence for deleterious paternal age effects. The absence of a paternal age effect persists irrespective of the age of sons and daughters, or the duration of paternal sperm storage. These results call for a re-evaluation of the causes and consequences of paternal age effects, under a framework that incorporates selective disappearance, condition dependence, life-history trade-offs, and variable paternal mating rates. We emphasize that sperm damage during sperm storage in fathers, might be an under-appreciated modulator of offspring phenotypic variation, especially in species where low male mating rates and long-term female sperm storage are commonplace. The interactive influence of male age and sexual rest on male reproductive output and his offspring's phenotypes, might be crucial in modulating female sperm ejection (Wagner et al, 2004), mate choice (Johnson and Gemmell, 2012), polyandry, and last-male sperm precedence (Snook and Hosken, 2014). Overall, we highlight the importance of simultaneously understanding various mechanisms by which advancing organismal age might directly (e.g. sex-specific inheritance, mutation accumulation) or indirectly (e.g. sperm storage, selective disappearance, trade-offs, condition dependence) influence offspring.

Data availability

All code and data are available at OSF: https://osf.io/gq9j2/ under DOI 10.17605/OSF.IO/GQ9J2.

References

Agarwal, A., Gupta, S., Du Plessis, S., Sharma, R., Esteves, S.C., Cirenza, C., Eliwa, J., Al-Najjar, W., Kumaresan, D., Haroun, N., Philby, S., Sabanegh, E., 2016. Abstinence Time and Its Impact on Basic and Advanced Semen Parameters. Urology 94, 102–110. https://doi.org/10.1016/j.urology.2016.03.059

Aguilar, P., Dag, B., Carazo, P., Sultanova, Z., 2023. Sex-specific paternal age effects on offspring quality in *Drosophila melanogaster*. J of Evolutionary Biology jeb.14167. https://doi.org/10.1111/jeb.14167

Aich, U., Chowdhury, S., Jennions, M.D., 2022. Separating the effects of paternal age and mating history: Evidence for sex-specific paternal effect in eastern mosquitofish. Evolution 76, 1565–1577. https://doi.org/10.1111/evo.14498

Alavioon, G., Hotzy, C., Nakhro, K., Rudolf, S., Scofield, D.G., Zajitschek, S., Maklakov, A.A., Immler, S., 2017. Haploid selection within a single ejaculate increases offspring fitness. Proc. Natl. Acad. Sci. U.S.A. 114, 8053–8058. https://doi.org/10.1073/pnas.1705601114

Angell, C.S., Janacek, R., Rundle, H.D., 2022. Maternal and Paternal Age Effects on Male Antler Flies: A Field Experiment. The American Naturalist 199, 436–442. https://doi.org/10.1086/718236

Arslan, R.C., Willführ, K.P., Frans, E.M., Verweij, K.J.H., Bürkner, P.-C., Myrskylä, M., Voland, E., Almqvist, C., Zietsch, B.P., Penke, L., 2017. Older fathers' children have lower evolutionary fitness across four centuries and in four populations. Proc. R. Soc. B. 284, 20171562. https://doi.org/10.1098/rspb.2017.1562

Badyaev, A.V., Uller, T., 2009. Parental effects in ecology and evolution: mechanisms, processes and implications. Phil. Trans. R. Soc. B 364, 1169–1177. https://doi.org/10.1098/rstb.2008.0302

Bates, D., Kliegl, R., Vasishth, S., Baayen, H., 2015. Parsimonious Mixed Models. https://doi.org/10.48550/ARXIV.1506.04967

Bauch, C., Boonekamp, J.J., Korsten, P., Mulder, E., Verhulst, S., 2019. Epigenetic inheritance of telomere length in wild birds. PLoS Genet 15, e1007827. https://doi.org/10.1371/journal.pgen.1007827

Barbagallo, F., Cannarella, R., Crafa, A., Manna, C., La Vignera, S., Condorelli, R.A., Calogero, A.E., 2022. The Impact of a Very Short Abstinence Period on Conventional Sperm Parameters and Sperm DNA Fragmentation: A Systematic Review and Meta-Analysis. JCM 11, 7303. https://doi.org/10.3390/jcm11247303

Bjork, A., Dallai, R., Pitnick, S., 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca* , a species with giant sperm. Biol. Lett. 3, 517–519. https://doi.org/10.1098/rsbl.2007.0219

Bouwhuis, S., Sheldon, B.C., Verhulst, S., Charmantier, A., 2009. Great tits growing old: selective disappearance and the partitioning of senescence to stages within the breeding cycle. Proc. R. Soc. B. 276, 2769–2777. https://doi.org/10.1098/rspb.2009.0457

Bouwhuis, S., Vedder, O., Becker, P.H., 2015. Sex-specific pathways of parental age effects on offspring lifetime reproductive success in a long-lived seabird: SEX-SPECIFIC PARENTAL EFFECTS ON OFFSPRING FITNESS. Evolution 69, 1760–1771. https://doi.org/10.1111/evo.12692

Brengdahl, M.I., Kimber, C.M., Elias, P., Thompson, J., Friberg, U., 2020. Deleterious mutations show increasing negative effects with age in Drosophila melanogaster. BMC Biol 18, 128. https://doi.org/10.1186/s12915-020-00858-5

Brengdahl, M.I., Kimber, C.M., Shenoi, V.N., Dumea, M., Mital, A., Friberg, U., 2023. Age-specific effects of deletions: implications for aging theories. Evolution 77, 254–263. https://doi.org/10.1093/evolut/qpac027

Brindle, M., Ferguson-Gow, H., Williamson, J., Thomsen, R., Sommer, V., 2023. The evolution of masturbation is associated with postcopulatory selection and pathogen avoidance in primates. Proc. R. Soc. B. 290, 20230061. https://doi.org/10.1098/rspb.2023.0061

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Mächler, M., Bolker, B.M., 2017. Modeling zero-inflated count data with glmmTMB (preprint). Ecology. https://doi.org/10.1101/132753

Brooks, R., Kemp, D.J., 2001. Can older males deliver the good genes? Trends in Ecology & Evolution 16, 308–313. https://doi.org/10.1016/S0169-5347(01)02147-4

Byrne, M., Agerbo, E., Ewald, H., Eaton, W.W., Mortensen, P.B., 2003. Parental Age and Risk of Schizophrenia: A Case-control Study. Arch Gen Psychiatry 60, 673. https://doi.org/10.1001/archpsyc.60.7.673

Calsbeek, R., Duryea, M.C., Goedert, D., Bergeron, P., Cox, R.M., 2015. Intralocus sexual conflict, adaptive sex allocation, and the heritability of fitness. J of Evolutionary Biology 28, 1975–1985. https://doi.org/10.1111/jeb.12713

Carnes, B.A., Riesch, R., Schlupp, I., 2012. The Delayed Impact of Parental Age on Offspring Mortality in Mice. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 67A, 351–357. https://doi.org/10.1093/gerona/glr116

Carothers, A.D., Collyer, S., Mey, R., Frackiewicz, A., 1978. Parental age and birth order in the aetiology of some sex chromosome aneuploidies. Ann Human Genet 41, 277–287. https://doi.org/10.1111/j.1469-1809.1978.tb01895.x

Cattelan, S., Gasparini, C., 2021. Male sperm storage impairs sperm quality in the zebrafish. Sci Rep 11, 16689. https://doi.org/10.1038/s41598-021-94976-x

Chan, P.T.K., Robaire, B., 2022. Advanced Paternal Age and Future Generations. Front. Endocrinol. 13, 897101. https://doi.org/10.3389/fendo.2022.897101

Chen, H., Krieg, T., Mautz, B., Jolly, C., Scofield, D., Maklakov, A.A., Immler, S., 2023. Germline mutation rate is elevated in young and old parents in *Caenorhabditis remanei*. Evolution Letters qrad052. https://doi.org/10.1093/evlett/qrad052

Chen, Q., Yan, W., Duan, E., 2016. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. Nat Rev Genet 17, 733–743. https://doi.org/10.1038/nrg.2016.106

Clancy, Kennington, 2001. A simple method to achieve consistent larval density in bottle cultures. Dros. Inf. Serv. 84.

Comar, V.A., Petersen, C.G., Mauri, A.L., Mattila, M., Vagnini, L.D., Renzi, A., Petersen, B., Nicoletti, A., Dieamant, F., Oliveira, J.B.A., Baruffi, R.L.R., Franco Jr, J.G., 2017. Influence of the abstinence period on human sperm quality: analysis of 2,458 semen samples. JBRA Assisted Reproduction. https://doi.org/10.5935/1518-0557.20170052

Connallon, T., 2010. Genic Capture, Sex Linkage, and the Heritability of Fitness. The American Naturalist 175, 564–576. https://doi.org/10.1086/651590

Criscuolo, F., Zahn, S., Bize, P., 2017. Offspring telomere length in the long lived Alpine swift is negatively related to the age of their biological father and foster mother. Biol. Lett. 13, 20170188. https://doi.org/10.1098/rsbl.2017.0188

Crow, J.F., 2003. There's Something Curious About Paternal-Age Effects. Science 301, 606–607. https://doi.org/10.1126/science.1088552
Crow, J.F., 2000. The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet 1, 40–47. https://doi.org/10.1038/35049558
De Jong, M.J., Van Oosterhout, C., Hoelzel, A.R., Janke, A., 2023. Moderating the neutralist–selectionist debate: exactly which propositions are we debating, and which arguments are valid? Biological Reviews brv.13010. https://doi.org/10.1111/brv.13010
de Manuel, M., Wu, F.L., Przeworski, M., 2022. A paternal bias in germline mutation is widespread in amniotes and can arise independently of cell division numbers. eLife 11, e80008. https://doi.org/10.7554/eLife.80008
Demarco, R.S., Eikenes, Å.H., Haglund, K., Jones, D.L., 2014. Investigating spermatogenesis in Drosophila melanogaster. Methods 68, 218–227. https://doi.org/10.1016/j.ymeth.2014.04.020
Denomme, M.M., Parks, J.C., McCallie, B.R., McCubbin, N.I., Schoolcraft, W.B., Katz-Jaffe, M.G., 2020. Advanced paternal age directly impacts mouse embryonic placental imprinting. PLoS ONE 15, e0229904. https://doi.org/10.1371/journal.pone.0229904
Dharmarajan, M., 1950. Effect on the Embryo of Staleness of the Sperm at the Time of Fertilization in the Domestic Hen. Nature 165, 398–398. https://doi.org/10.1038/165398a0
Douglas, T., Anderson, R., Saltz, J.B., 2020. Limits to male reproductive potential across mating bouts in Drosophila melanogaster. Animal Behaviour 160, 25–33. https://doi.org/10.1016/j.anbehav.2019.11.009
Ellegren, H., 2007. Characteristics, causes and evolutionary consequences of male-biased mutation. Proc. R. Soc. B. 274, 1–10. https://doi.org/10.1098/rspb.2006.3720
Engqvist, L., 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. Animal Behaviour 70, 967–971. https://doi.org/10.1016/j.anbehav.2005.01.016
Evans, J.P., Wilson, A.J., Pilastro, A., Garcia-Gonzalez, F., 2019. Ejaculate-mediated paternal effects: evidence, mechanisms and evolutionary implications. Reproduction 157, R109–R126. https://doi.org/10.1530/REP-18-0524
Fay, R., Barbraud, C., Delord, K., Weimerskirch, H., 2016. Paternal but not maternal age influences early-life performance of offspring in a long-lived seabird. Proc. R. Soc. B. 283, 20152318. https://doi.org/10.1098/rspb.2015.2318
Firman, R.C., Gasparini, C., Manier, M.K., Pizzari, T., 2017. Postmating Female Control: 20 Years of Cryptic Female Choice. Trends Ecol Evol 32, 368–382. https://doi.org/10.1016/j.tree.2017.02.010
Firman, R.C., Young, F.J., Rowe, D.C., Duong, H.T., Gasparini, C., 2015. Sexual rest and post-meiotic sperm ageing in house mice. J. Evol. Biol. 28, 1373–1382. https://doi.org/10.1111/jeb.12661
Fischer, B., Taborsky, B., Kokko, H., 2011. How to balance the offspring quality–quantity tradeoff when environmental cues are unreliable. Oikos 120, 258–270. https://doi.org/10.1111/j.1600-0706.2010.18642.x
Gao, Z., Moorjani, P., Sasani, T.A., Pedersen, B.S., Quinlan, A.R., Jorde, L.B., Amster, G., Przeworski, M., 2019. Overlooked roles of DNA damage and maternal age in generating human germline mutations. Proc. Natl. Acad. Sci. U.S.A. 116, 9491–9500. https://doi.org/10.1073/pnas.1901259116
Garcia, A.M., Calder, R.B., Dollé, M.E.T., Lundell, M., Kapahi, P., Vijg, J., 2010. Age- and Temperature-Dependent Somatic Mutation Accumulation in Drosophila melanogaster. PLoS Genet 6, e1000950. https://doi.org/10.1371/journal.pgen.1000950
Gasparini, C., Daymond, E., Evans, J.P., 2018. Extreme fertilization bias towards freshly inseminated sperm in a species exhibiting prolonged female sperm storage. R. Soc. open sci. 5, 172195. https://doi.org/10.1098/rsos.172195
Gasparini, C., Devigili, A., Pilastro, A., 2019. Sexual selection and ageing: interplay between pre- and post-copulatory traits senescence in the guppy. Proc. R. Soc. B. 286, 20182873. https://doi.org/10.1098/rspb.2018.2873
Gasparini, C., Dosselli, R., Evans, J.P., 2017. Sperm storage by males causes changes in sperm phenotype and influences the reproductive fitness of males and their sons. Evol Lett 1, 16–25. https://doi.org/10.1002/evl3.2
Gasparini, C., Kelley, J.L., Evans, J.P., 2014. Male sperm storage compromises sperm motility in guppies. Biol Lett 10, 20140681. https://doi.org/10.1098/rsbl.2014.0681
Girard, S.L., Bourassa, C.V., Lemieux Perreault, L.-P., Legault, M.-A., Barhdadi, A., Ambalavanan, A., Brendgen, M., Vitaro, F., Noreau, A., Dionne, G., Tremblay, R.E., Dion, P.A., Boivin, M., Dubé, M.-P., Rouleau, G.A., 2016. Paternal Age Explains a Major Portion of De Novo Germline Mutation Rate Variability in Healthy Individuals. PLoS ONE 11, e0164212. https://doi.org/10.1371/journal.pone.0164212
Gorbunova, V., Seluanov, A., Mao, Z., Hine, C., 2007. Changes in DNA repair during aging. Nucleic Acids Res 35, 7466–7474. https://doi.org/10.1093/nar/gkm756
Hadlow, J.H., Lymbery, R.A., Evans, J.P., 2023. Paternal environment effects are driven by female reproductive fluid but not sperm age in an external fertilizer. Biol. Lett. 19, 20230368. https://doi.org/10.1098/rsbl.2023.0368
Hämäläinen, A., Dammhahn, M., Aujard, F., Eberle, M., Hardy, I., Kappeler, P.M., Perret, M., Schliehe-Diecks, S., Kraus, C., 2014. Senescence or selective disappearance? Age trajectories of body mass in wild and captive populations of a small-bodied primate. Proc. R. Soc. B. 281, 20140830. https://doi.org/10.1098/rspb.2014.0830
Hansen, T.F., Price, D.K., 1995. Good genes and old age: Do old mates provide superior genes? J Evolution Biol 8, 759–778. https://doi.org/10.1046/j.1420-9101.1995.8060759.x
Hartig, F., Hartig, M.F., 2017. Package 'DHARMa'. R package. https://cran.r-hub.io/web/packages/DHARMa/DHARMa.pdf
Heidinger, B.J., Herborn, K.A., Granroth-Wilding, H.M.V., Boner, W., Burthe, S., Newell, M., Wanless, S., Daunt, F., Monaghan, P., 2016. Parental age influences offspring telomere loss. Funct Ecol 30, 1531–1538. https://doi.org/10.1111/1365-2435.12630
Hercus, M.J., Hoffmann, A.A., 2000. Maternal and grandmaternal age influence offspring fitness in *Drosophila*. Proc. R. Soc. Lond. B 267, 2105–2110. https://doi.org/10.1098/rspb.2000.1256
Hettyey, A., Vági, B., Penn, D.J., Hoi, H., Wagner, R.H., 2012. Post-Meiotic Intra-Testicular Sperm Senescence in a Wild Vertebrate. PLoS ONE 7, e50820. https://doi.org/10.1371/journal.pone.0050820

Hopkins, B.R., Sepil, I., Thézénas, M.-L., Craig, J.F., Miller, T., Charles, P.D., Fischer, R., Kessler, B.M., Bretman, A., Pizzari, T., Wigby, S., 2019. Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 116, 17925–17933. https://doi.org/10.1073/pnas.1906149116

Hotzy, C., Xuhui, B., Larva, T., Immler, S., 2020. Intrinsic post-ejaculation sperm ageing does not affect offspring fitness in Atlantic salmon. J Evol Biol 33, 576–583. https://doi.org/10.1111/jeb.13590

Immler, S., 2019. Haploid Selection in "Diploid" Organisms. Annu. Rev. Ecol. Evol. Syst. 50, 219–236. https://doi.org/10.1146/annurev-ecolsys-110218-024709

Janecka, M., Mill, J., Basson, M.A., Goriely, A., Spiers, H., Reichenberg, A., Schalkwyk, L., Fernandes, C., 2017. Advanced paternal age effects in neurodevelopmental disorders—review of potential underlying mechanisms. Transl Psychiatry 7, e1019–e1019. https://doi.org/10.1038/tp.2016.294

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 off-set costs related to fertility, or are we missing something else? Bioessays 34, 609–619. https://doi.org/10.1002/bies.201100157

Johnson, S.L., Zellhuber-McMillan, S., Gillum, J., Dunleavy, J., Evans, J.P., Nakagawa, S., Gemmell, N.J., 2018. Evidence that fertility trades off with early offspring fitness as males age. Proc. R. Soc. B. 285, 20172174. https://doi.org/10.1098/rspb.2017.2174

Jones, O.R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C.G., Schaible, R., Casper, B.B., Dahlgren, J.P., Ehrlén, J., García, M.B., Menges, E.S., Quintana-Ascencio, P.F., Caswell, H., Baudisch, A., Vaupel, J.W., 2014. Diversity of ageing across the tree of life. Nature 505, 169–173. https://doi.org/10.1038/nature12789

Jones, T.M., Elgar, M.A., 2004. The role of male age, sperm age and mating history on fecundity and fertilization success in the hide beetle. Proc. R. Soc. Lond. B 271, 1311–1318. https://doi.org/10.1098/rspb.2004.2723

Jónsson, H., Sulem, P., Kehr, B., Kristmundsdottir, S., Zink, F., Hjartarson, E., Hardarson, M.T., Hjorleifsson, K.E., Eggertsson, H.P., Gudjonsson, S.A., Ward, L.D., Arnadottir, G.A., Helgason, E.A., Helgason, H., Gylfason, A., Jonasdottir, Adalbjorg, Jonasdottir, Aslaug, Rafnar, T., Frigge, M., Stacey, S.N., Th. Magnusson, O., Thorsteinsdottir, U., Masson, G., Kong, A., Halldorsson, B.V., Helgason, A., Gudbjartsson, D.F., Stefansson, K., 2017. Parental influence on human germline de novo mutations in 1,548 trios from Iceland. Nature 549, 519–522. https://doi.org/10.1038/nature24018

Kokko, H., 1998. Good genes, old age and life-history trade-offs. Evolutionary Ecology 12, 739–750. https://doi.org/10.1023/A:1006541701002

Koppik, M., Baur, J., Berger, D., 2023. Increased male investment in sperm competition results in reduced maintenance of gametes. PLoS Biol 21, e3002049. https://doi.org/10.1371/journal.pbio.3002049

Kong, A., Frigge, M.L., Masson, G., Besenbacher, S., Sulem, P., Magnusson, G., Gudjonsson, S.A., Sigurdsson, A., Jonasdottir, Aslaug, Jonasdottir, Adalbjorg, Wong, W.S.W., Sigurdsson, G., Walters, G.B., Steinberg, S., Helgason, H., Thorleifsson, G., Gudbjartsson, D.F., Helgason, A., Magnusson, O.Th., Thorsteinsdottir, U., Stefansson, K., 2012. Rate of de novo mutations and the importance of father's age to disease risk. Nature 488, 471–475. https://doi.org/10.1038/nature11396

Krishna, M.S., Santhosh, H.T., Hegde, S., 2012. Offspring of Older Males Are Superior in Drosophila Bipectinata. Zoological Studies 51.

Levitas, E., Lunenfeld, E., Weiss, N., Friger, M., Har-Vardi, I., Koifman, A., Potashnik, G., 2005. Relationship between the duration of sexual abstinence and semen quality: analysis of 9,489 semen samples. Fertility and Sterility 83, 1680–1686. https://doi.org/10.1016/j.fertnstert.2004.12.045

Linklater, J.R., Wertheim, B., Wigby, S., Chapman, T., 2007. EJACULATE DEPLETION PATTERNS EVOLVE IN RESPONSE TO EXPERIMENTAL MANIPULATION OF SEX RATIO IN *DROSOPHILA MELANOGASTER*. Evolution 61, 2027–2034. https://doi.org/10.1111/j.1558-5646.2007.00157.x

Liu, Y., Chen, Q., 2018. 150 years of Darwin's theory of intercellular flow of hereditary information. Nat Rev Mol Cell Biol 19, 749–750. https://doi.org/10.1038/s41580-018-0072-4

Lodge, J.R., Fechheimer, N.S., Jaap, R.G., 1971. The Relationship of in Vivo Sperm Storage Interval to Fertility and Embryonic Survival in the Chicken1. Biology of Reproduction 5, 252–257. https://doi.org/10.1093/biolreprod/5.3.252

Loyau, A., Blanchet, S., Van Laere, P., Clobert, J., Danchin, E., 2012. When not to copy: female fruit flies use sophisticated public information to avoid mated males. Sci Rep 2, 768. https://doi.org/10.1038/srep00768

Ludecke, D., 2023. sjPlot: Data Visualization for Statistics in Social Science.

Macartney, E.L., Zeender, V., Meena, A., De Nardo, A.N., Bonduriansky, R., Lüpold, S., 2021. Sperm depletion in relation to developmental nutrition and genotype in Drosophila melanogaster. Evolution 75, 2830–2841. https://doi.org/10.1111/evo.14373

Meunier, L., Sorci, G., Abi Hussein, H., Hingrat, Y., Rehspringer, N., Saint-Jalme, M., Lesobre, L., Torres Carreira, J., 2022. Pre- but not post-meiotic senescence affects sperm quality and reproductive success in the North African houbara bustard. Front. Ecol. Evol. 10, 977184. https://doi.org/10.3389/fevo.2022.977184

Monaghan, P., Maklakov, A.A., Metcalfe, N.B., 2020. Intergenerational Transfer of Ageing: Parental Age and Offspring Lifespan. Trends in Ecology & Evolution 35, 927–937. https://doi.org/10.1016/j.tree.2020.07.005

Monaghan, P., Metcalfe, N.B., 2019. The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. Proc. R. Soc. B. 286, 20192187. https://doi.org/10.1098/rspb.2019.2187

Moorad, J.A., Promislow, D.E.L., 2008. A Theory of Age-Dependent Mutation and Senescence. Genetics 179, 2061–2073. https://doi.org/10.1534/genetics.108.088526

Mossman, Jim A, Mabeza, R.M.S., Blake, E., Mehta, N., Rand, D.M., 2019a. Age of Both Parents Influences Reproduction and Egg Dumping Behavior in Drosophila melanogaster. Journal of Heredity 110, 300–309. https://doi.org/10.1093/jhered/esz009

Noguera, J.C., Metcalfe, N.B., Monaghan, P., 2018. Experimental demonstration that offspring fathered by old males have shorter telomeres and reduced lifespans. Proc. R. Soc. B. 285, 20180268. https://doi.org/10.1098/rspb.2018.0268

Nystrand, M., Dowling, D.K., 2014. Transgenerational interactions involving parental age and immune status affect female reproductive success in *Drosophila melanogaster*. Proc. R. Soc. B. 281, 20141242. https://doi.org/10.1098/rspb.2014.1242
Oakes, C.C., Smiraglia, D.J., Plass, C., Trasler, J.M., Robaire, B., 2003. Aging results in hypermethylation of ribosomal DNA in sperm and liver of male rats. Proc. Natl. Acad. Sci. U.S.A. 100, 1775–1780. https://doi.org/10.1073/pnas.0437971100
Olsson, M., Pauliny, A., Wapstra, E., Uller, T., Schwartz, T., Blomqvist, D., 2011. Sex Differences in Sand Lizard Telomere Inheritance: Paternal Epigenetic Effects Increases Telomere Heritability and Offspring Survival. PLoS ONE 6, e17473. https://doi.org/10.1371/journal.pone.0017473
Orr, T.J., Brennan, P.L.R., 2015. Sperm storage: distinguishing selective processes and evaluating criteria. Trends in Ecology & Evolution 30, 261–272. https://doi.org/10.1016/j.tree.2015.03.006
Otto, S.P., Scott, M.F., Immler, S., 2015. Evolution of haploid selection in predominantly diploid organisms. Proc. Natl. Acad. Sci. U.S.A. 112, 15952–15957. https://doi.org/10.1073/pnas.1512004112
Paczkowski, M., Schoolcraft, W.B., Krisher, R.L., 2015. Dysregulation of methylation and expression of imprinted genes in oocytes and reproductive tissues in mice of advanced maternal age. J Assist Reprod Genet 32, 713–723. https://doi.org/10.1007/s10815-015-0463-9
Perez, M.F., Lehner, B., 2019. Intergenerational and transgenerational epigenetic inheritance in animals. Nat Cell Biol 21, 143–151. https://doi.org/10.1038/s41556-018-0242-9
Pineaux, M., Blanchard, P., Danchin, É., Hatch, S.A., Helfenstein, F., Mulard, H., White, J., Leclaire, S., Wagner, R.H., 2019. Behavioural avoidance of sperm ageing depends on genetic similarity of mates in a monogamous seabird. Biological Journal of the Linnean Society 128, 170–180. https://doi.org/10.1093/biolinnean/blz079
Pisano, C., Bonaccorsi, S., Gatti, M., 1993. The kl-3 loop of the Y chromosome of Drosophila melanogaster binds a tektin-like protein. Genetics 133, 569–579. https://doi.org/10.1093/genetics/133.3.569
Pizzari, T., Dean, R., Pacey, A., Moore, H., Bonsall, M.B., 2008. The evolutionary ecology of pre- and post-meiotic sperm senescence. Trends in Ecology & Evolution 23, 131–140. https://doi.org/10.1016/j.tree.2007.12.003
Preston, B.T., Saint Jalme, M., Hingrat, Y., Lacroix, F., Sorci, G., 2015. The sperm of aging male bustards retards their offspring's development. Nat Commun 6, 6146. https://doi.org/10.1038/ncomms7146
Price, D.K., Hansen, T.F., 1998. How Does Offspring Quality Change with Age in Male Drosophila Melanogaster? Behavior Genetics 28, 395–402. https://doi.org/10.1023/A:1021677804038
Priest, N.K., Mackowiak, B., Promislow, D.E.L., 2002. sch. Evolution 56, 927–935. https://doi.org/10.1111/j.0014-3820.2002.tb01405.x
Radhakrishnan, P., Fedorka, K.M., 2011. Influence of female age, sperm senescence and multiple mating on sperm viability in female Drosophila melanogaster. Journal of Insect Physiology 57, 778–783. https://doi.org/10.1016/j.jinsphys.2011.02.017
Rando, O.J., 2016. Intergenerational Transfer of Epigenetic Information in Sperm. Cold Spring Harb Perspect Med 6, a022988. https://doi.org/10.1101/cshperspect.a022988
Ratikainen, I.I., Haaland, T.R., Wright, J., 2018. Differential allocation of parental investment and the trade-off between size and number of offspring. Proc. R. Soc. B. 285, 20181074. https://doi.org/10.1098/rspb.2018.1074
Reinhardt, K., 2007. Evolutionary Consequences of Sperm Cell Aging. The Quarterly Review of Biology 82, 375–393. https://doi.org/10.1086/522811
Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2011. Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, Cimex lectularius. PLoS ONE 6, e22082. https://doi.org/10.1371/journal.pone.0022082
Reinhardt, K., Siva-Jothy, M.T., 2005. An Advantage for Young Sperm in the House Cricket *Acheta domesticus*. The American Naturalist 165, 718–723. https://doi.org/10.1086/430010
Reinhardt, K., Turnell, B., 2019. Sperm ageing: A complex business. Funct Ecol 33, 1188–1189. https://doi.org/10.1111/1365-2435.13350
Reznick, D., Nunney, L., Tessier, A., 2000. Big houses, big cars, superfleas and the costs of reproduction. Trends in Ecology & Evolution 15, 421–425. https://doi.org/10.1016/S0169-5347(00)01941-8
Rinehart, R.R., 1969. Spontaneous sex-linked recessive lethal frequencies from aged and non-aged spermatozoa of Drosophila melanogaster. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 7, 417–423. https://doi.org/10.1016/0027-5107(69)90112-2
Rodgers, A.B., Morgan, C.P., Leu, N.A., Bale, T.L., 2015. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. Proc. Natl. Acad. Sci. U.S.A. 112, 13699–13704. https://doi.org/10.1073/pnas.1508347112
Roff, D.A., Fairbairn, D.J., 2007. The evolution of trade-offs: where are we? J of Evolutionary Biology 20, 433–447. https://doi.org/10.1111/j.1420-9101.2006.01255.x
R Core Team, 2012. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austra. https://www.r-project.org/
Sanghvi, K., Iglesias-Carrasco, M., Zajitschek, F., Kruuk, L.E.B., Head, M.L., 2022. Effects of developmental and adult environments on ageing. Evolution 76, 1868–1882. https://doi.org/10.1111/evo.14567
Sanghvi, K., Vega-Trejo, R., Nakagawa, S., Gascoigne, S.J.L., Johnson, S.L., Salguero-Gómez, R., Pizzari, T., Sepil, I., 2024. Meta-analysis shows no consistent evidence for senescence in ejaculate traits across animals. Nat Commun 15, 558. https://doi.org/10.1038/s41467-024-44768-4
Sanghvi, K., Pizzari, T., Sepil, I., 2023. What doesn't kill you makes you stronger? Effects of paternal age at conception on fathers and sons. bioRxiv Evolutionary Biology. https://doi.org/10.1101/2023.12.20.572572
Santos, I.B., Wainman, A., Garrido-Maraver, J., Pires, V., Riparbelli, M.G., Kovács, L., Callaini, G., Glover, D.M., Tavares, Á.A., 2023. Mob4 is essential for spermatogenesis in *Drosophila melanogaster* (preprint). Developmental Biology. https://doi.org/10.1101/2023.02.05.527206
Schroeder, J., Nakagawa, S., Rees, M., Mannarelli, M.-E., Burke, T., 2015. Reduced fitness in progeny from old parents in a natural population. Proc. Natl. Acad. Sci. U.S.A. 112, 4021–4025. https://doi.org/10.1073/pnas.1422715112

Selvaratnam, J., Paul, C., Robaire, B., 2015. Male Rat Germ Cells Display Age-Dependent and Cell-Specific Susceptibility in Response to Oxidative Stress Challenges1. Biology of Reproduction 93. https://doi.org/10.1095/biolreprod.115.131318
Sepil, I., Hopkins, B.R., Dean, R., Bath, E., Friedman, S., Swanson, B., Ostridge, H.J., Harper, L., Buehner, N.A., Wolfner, M.F., Konietzny, R., Thézénas, M.-L., Sandham, E., Charles, P.D., Fischer, R., Steinhauer, J., Kessler, B.M., Wigby, S., 2020. Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 117, 17094–17103. https://doi.org/10.1073/pnas.2009053117
Sharma, R., Agarwal, A., Rohra, V.K., Assidi, M., Abu-Elmagd, M., Turki, R.F., 2015. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13, 35. https://doi.org/10.1186/s12958-015-0028-x
Sharma, U., 2019. Paternal Contributions to Offspring Health: Role of Sperm Small RNAs in Intergenerational Transmission of Epigenetic Information. Front. Cell Dev. Biol. 7, 215. https://doi.org/10.3389/fcell.2019.00215
Shindyapina, A.V., Zenin, A.A., Tarkhov, A.E., Santesmasses, D., Fedichev, P.O., Gladyshev, V.N., 2020. Germline burden of rare damaging variants negatively affects human healthspan and lifespan. eLife 9, e53449. https://doi.org/10.7554/eLife.53449
Sirot, L.K., Buehner, N.A., Fiumera, A.C., Wolfner, M.F., 2009. Seminal fluid protein depletion and replenishment in the fruit fly, Drosophila melanogaster: an ELISA-based method for tracking individual ejaculates. Behav Ecol Sociobiol 63, 1505–1513. https://doi.org/10.1007/s00265-009-0806-6
Siva-Jothy, 2000. The young sperm gambit. Ecol Letters 3, 172–174. https://doi.org/10.1046/j.1461-0248.2000.00146.x
Snook, R.R., Hosken, D.J., 2004. Sperm death and dumping in Drosophila. Nature 428, 939–941. https://doi.org/10.1038/nature02455
Sørensen, F., Melsen, L.M., Fedder, J., Soltanizadeh, S., 2023. The Influence of Male Ejaculatory Abstinence Time on Pregnancy Rate, Live Birth Rate and DNA Fragmentation: A Systematic Review. JCM 12, 2219. https://doi.org/10.3390/jcm12062219
Sparks, A.M., Hammers, M., Komdeur, J., Burke, T., Richardson, D.S., Dugdale, H.L., 2022. Sex-dependent effects of parental age on offspring fitness in a cooperatively breeding bird. Evolution Letters evl3.300. https://doi.org/10.1002/evl3.300
Suvorov, A., Pilsner, J.R., Naumov, V., Shtratnikova, V., Zheludkevich, A., Gerasimov, E., Logacheva, M., Sergeyev, O., 2020. Aging Induces Profound Changes in sncRNA in Rat Sperm and These Changes Are Modified by Perinatal Exposure to Environmental Flame Retardant. Int J Mol Sci 21, 8252. https://doi.org/10.3390/ijms21218252
Tan, C.K.W., Pizzari, T., Wigby, S., 2013. PARENTAL AGE, GAMETIC AGE, AND INBREEDING INTERACT TO MODULATE OFFSPRING VIABILITY IN *DROSOPHILA MELANOGASTER*: BRIEF COMMUNICATION. Evolution n/a-n/a. https://doi.org/10.1111/evo.12131
Tarin, J.J., 2000. Consequences on offspring of abnormal function in ageing gametes. Human Reproduction Update 6, 532–549. https://doi.org/10.1093/humupd/6.6.532
Therneau, T., 2105. Mixed effects Cox models. CRAN Repository.
Uller, T., 2008. Developmental plasticity and the evolution of parental effects. Trends in Ecology & Evolution 23, 432–438. https://doi.org/10.1016/j.tree.2008.04.005
Vega-Trejo, R., Fox, R.J., Iglesias-Carrasco, M., Head, M.L., Jennions, M.D., 2019. The effects of male age, sperm age and mating history on ejaculate senescence. Funct Ecol 33, 1267–1279. https://doi.org/10.1111/1365-2435.13305
Vega-Trejo, R., Kruuk, L.E.B., Jennions, M.D., Head, M.L., 2018. What happens to offspring when parents are inbred, old or had a poor start in life? Evidence for sex-specific parental effects. J of Evolutionary Biology 31, 1138–1151. https://doi.org/10.1111/jeb.13292
Venn, O., Turner, I., Mathieson, I., De Groot, N., Bontrop, R., McVean, G., 2014. Strong male bias drives germline mutation in chimpanzees. Science 344, 1272–1275. https://doi.org/10.1126/science.344.6189.1272
Vuarin, P., Bouchard, A., Lesobre, L., Levêque, G., Chalah, T., Jalme, M.S., Lacroix, F., Hingrat, Y., Sorci, G., 2019. Post-copulatory sexual selection allows females to alleviate the fitness costs incurred when mating with senescing males. Proc. R. Soc. B. 286, 20191675. https://doi.org/10.1098/rspb.2019.1675
Vuarin, P., Lesobre, L., Levêque, G., Saint Jalme, M., Lacroix, F., Hingrat, Y., Sorci, G., 2021. Paternal age negatively affects sperm production of the progeny. Ecology Letters 24, 719–727. https://doi.org/10.1111/ele.13696
Wagner, R.H., Helfenstein, F., Danchin, E., 2004. Female choice of young sperm in a genetically monogamous bird. Proc Biol Sci 271 Suppl 4, S134-137. https://doi.org/10.1098/rsbl.2003.0142
Wang, R.J., Thomas, G.W.C., Raveendran, M., Harris, R.A., Doddapaneni, H., Muzny, D.M., Capitanio, J.P., Radivojac, P., Rogers, J., Hahn, M.W., 2020. Paternal age in rhesus macaques is positively associated with germline mutation accumulation but not with measures of offspring sociability. Genome Res. 30, 826–834. https://doi.org/10.1101/gr.255174.119
Wang, Y., McNeil, P., Abdulazeez, R., Pascual, M., Johnston, S.E., Keightley, P.D., Obbard, D.J., 2022. Variation in mutation, recombination, and transposition rates in *Drosophila melanogaster* and *Drosophila simulans* (preprint). Evolutionary Biology. https://doi.org/10.1101/2022.09.12.507595
Weirich-Schwaiger, H., Weirich, H.G., Gruber, B., Schweiger, M., Hirsch-Kauffman, M., 1994. Correlation between senescence and DNA repair in cells from young and old individuals and in premature aging syndromes. Mutation Research/DNAging 316, 37–48. https://doi.org/10.1016/0921-8734(94)90006-X
Weiss, L.A., Pan, L., Abney, M., Ober, C., 2006. The sex-specific genetic architecture of quantitative traits in humans. Nat Genet 38, 218–222. https://doi.org/10.1038/ng1726
White, J., Wagner, R.H., Helfenstein, F., Hatch, S.A., Mulard, H., Naves, L.C., Danchin, E., 2008. Multiple deleterious effects of experimentally aged sperm in a monogamous bird. Proc Natl Acad Sci U S A 105, 13947–13952. https://doi.org/10.1073/pnas.0803067105
Witt, E., Langer, C.B., Svetec, N., Zhao, L., 2023. Transcriptional and mutational signatures of the Drosophila ageing germline. Nat Ecol Evol. https://doi.org/10.1038/s41559-022-01958-x
Wylde, Z., Spagopoulou, F., Hooper, A.K., Maklakov, A.A., Bonduriansky, R., 2019. Parental breeding age effects on descendants' longevity interact over 2 generations in matrilines and patrilines. PLoS Biol 17, e3000556. https://doi.org/10.1371/journal.pbio.3000556

Xie, K., Ryan, D.P., Pearson, B.L., Henzel, K.S., Neff, F., Vidal, R.O., Hennion, M., Lehmann, I., Schleif, M., Schröder, S., Adler, T., Rathkolb, B., Rozman, J., Schütz, A.-L., Prehn, C., Mickael, M.E., Weiergräber, M., Adamski, J., Busch, D.H., Ehninger, G., Matynia, A., Jackson, W.S., Wolf, E., Fuchs, H., Gailus-Durner, V., Bonn, S., Hrabě de Angelis, M., Ehninger, D., 2018. Epigenetic alterations in longevity regulators, reduced life span, and exacerbated aging-related pathology in old father offspring mice. Proc. Natl. Acad. Sci. U.S.A. 115. https://doi.org/10.1073/pnas.1707337115

Yampolsky, L.Yu., Pearse, L.E., Promislow, D.E.L., 2000. Age-specific Effects of Novel Mutations in Drosophila Melanogaster I. Mortality. Genetica 110, 11–29. https://doi.org/10.1023/A:1017582625191

Yoshizaki, K., Kimura, R., Kobayashi, H., Oki, S., Kikkawa, T., Mai, L., Koike, K., Mochizuki, K., Inada, H., Matsui, Y., Kono, T., Osumi, N., 2021. Paternal age affects offspring via an epigenetic mechanism involving REST/NRSF. EMBO Rep 22. https://doi.org/10.15252/embr.202051524

You, H., Xu, R., 2020. R-Squared Measure Based on Partial LR Statistic, for the Cox PH Regression Model. CRAN Repository.

Zubkova, E.V., Robaire, B., 2006. Effects of ageing on spermatozoal chromatin and its sensitivity to in vivo and in vitro oxidative challenge in the Brown Norway rat. Human Reproduction 21, 2901–2910. https://doi.org/10.1093/humrep/del193

Appendix 1: F0 stripping assay

To create our four paternal treatments, we used old and young experimental F0 males, and kept each male with 10 females in a vial for 24 hours, to deplete males of their ejaculate. This was done so that after depletion of ejaculates, males would produce sperm of a known maximum post-meitoic storage duration. After 24 hours of being with a female, we kept males in individual vials allowing either two or eight days of sexual rest. For all F0 males used in the stripping assay (and eventually in the paternal treatments), we ensured that the 10 females associated with him, collectively, produced at least one offspring. This was done to ensure that all the F0 males that were to be used in subsequent parts of the experiment in the four paternal treatments, were fertile.

In addition to this stripping assay, we tested whether F0 males had mated with the females they were kept with, in the stripping assay. For this, we chose a random subset of F0 males (see Table S1 for sample sizes), and collected each of the 10 females he was with, on individual vials. For each male, we recorded the number of associated stripping females he was with, which produced an offspring (Figure S2A). On average, males from all four paternal treatments produced offspring with >9 out of 10 females (Figure S2A). This "stripping fertility" assay, was done using eggs laid by females within 24 hours of mating. Next, we chose another subset of F0 males (see Table S1 for sample sizes) and moved each of the ten females he was with into individual vials for 24 hours for egg laying. This "stripping counts" assay was done three days after the F0 "stripping fertility" assay. We then counted all the offspring that emerged from laid eggs. This "stripping counts" assay was done to ensure that males did not maintain their level of offspring production across all 10 females, and instead showed a large variation in how many offspring they produced with each female (Figure S2B, S2C), with many females producing very few offspring. Such a pattern in female fecundity would be indicative of males having lower numbers of remaining sperm when mating with each successive female through a mating sequence (Douglas et al, 2020; Hopkins et al, 2019; Linklater et al, 2007; Loyau et al, 2010; Macartney et al, 2021; Sirot et al, 2009; Figure S2B, S2C). On the other hand, maintenance of offspring production across females would indicate that males still had a lot of sperm stored, making our stripping assay ineffective in creating the two sperm storage duration treatments.

To further ensure that ten matings were sufficient to deplete males of their sperm reserves, we used a transgenic line of *Drosophila melanogaster* males that express green fluorescent protein in their sperm heads at the *Mst35Ba* and *Mst35Bb* loci (Manier et al, 2010). We dissected two virgin males who were 6-days old and compared the numbers of sperm in their seminal vesicles to two, 6-day old males who had been with 10 females in a vial for 24 hours. Visual comparison indicated many sperm in the virgin males' seminal vesicles but almost no sperm in the mated males' seminal vesicles (Images 1a and 1b respectively).

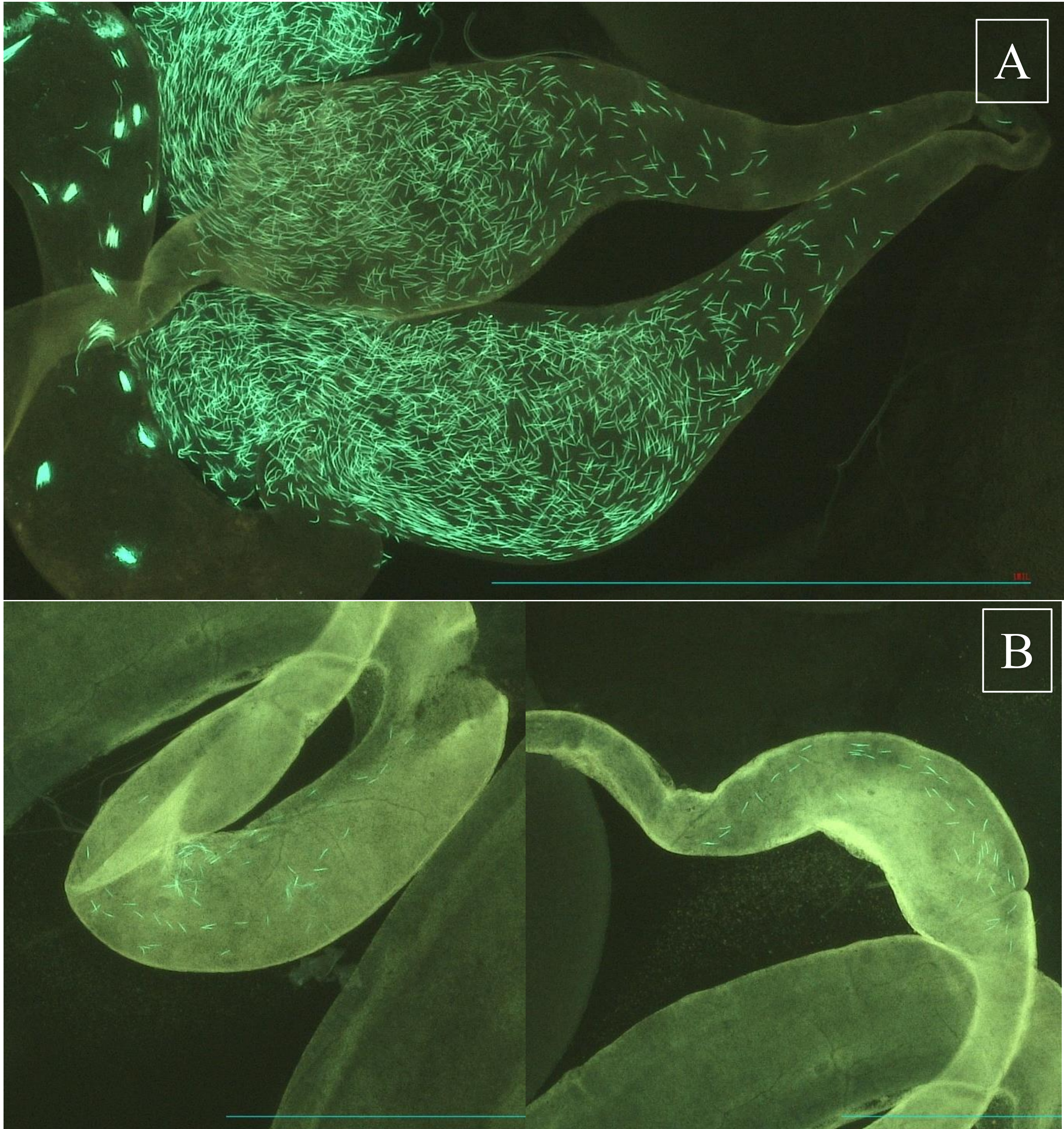


Image 1: A. GFP tagged sperm heads (bright green lines) in the seminal vesicles of virgin males that were six days old. B. GFP tagged sperm heads (bright green lines) in the seminal vesicles of six-day old males, who were kept in a vial with 10 females for 24 hours. Images taken using a Nikon Eclipse50i fluorescence microscope (magnification = 10x objective, 10x eyepiece, wavelength = 480nm) with a chromix HD camera, under UV light from a CoolLED pe300 light source

Appendix 2: Supplementary figures

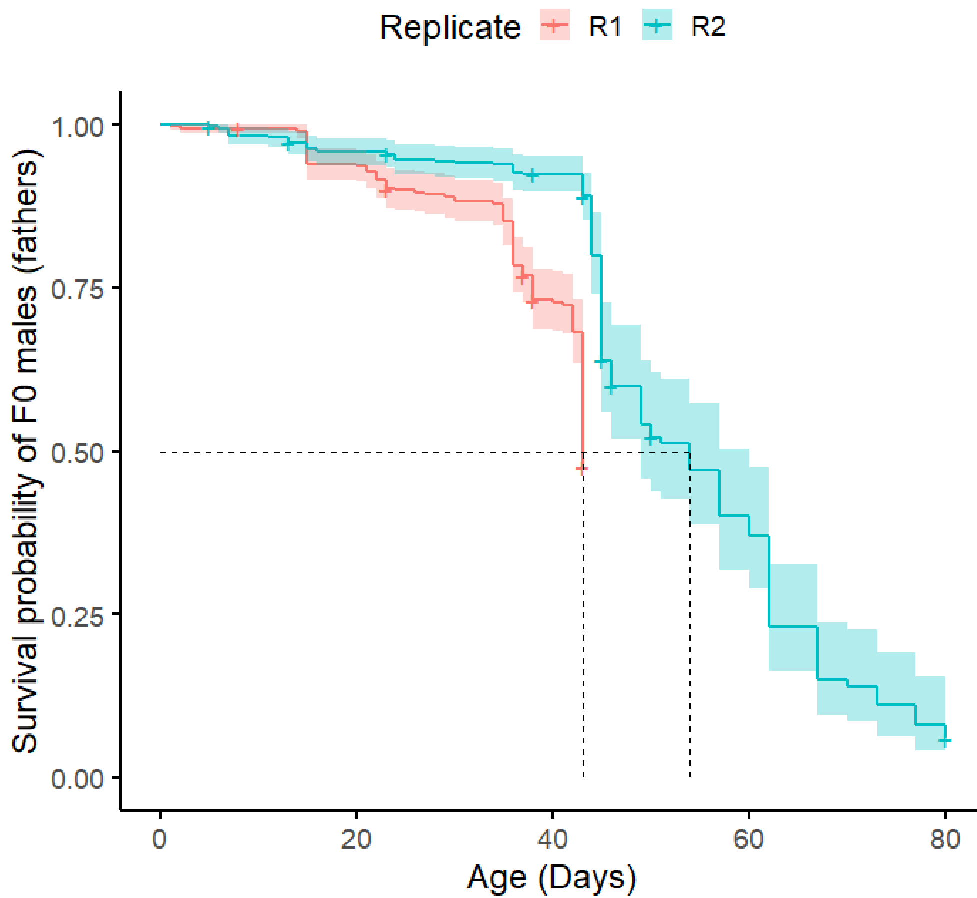


Figure S1: Survival probabilities of F0 males (kept in single-sex groups of 10) from replicates R1 and R2 used in our experiment. Initial sample size = ~370 for both replicates. Median lifespan is ~ 45 days. "+" shows censoring, shaded areas show 95% C.I.

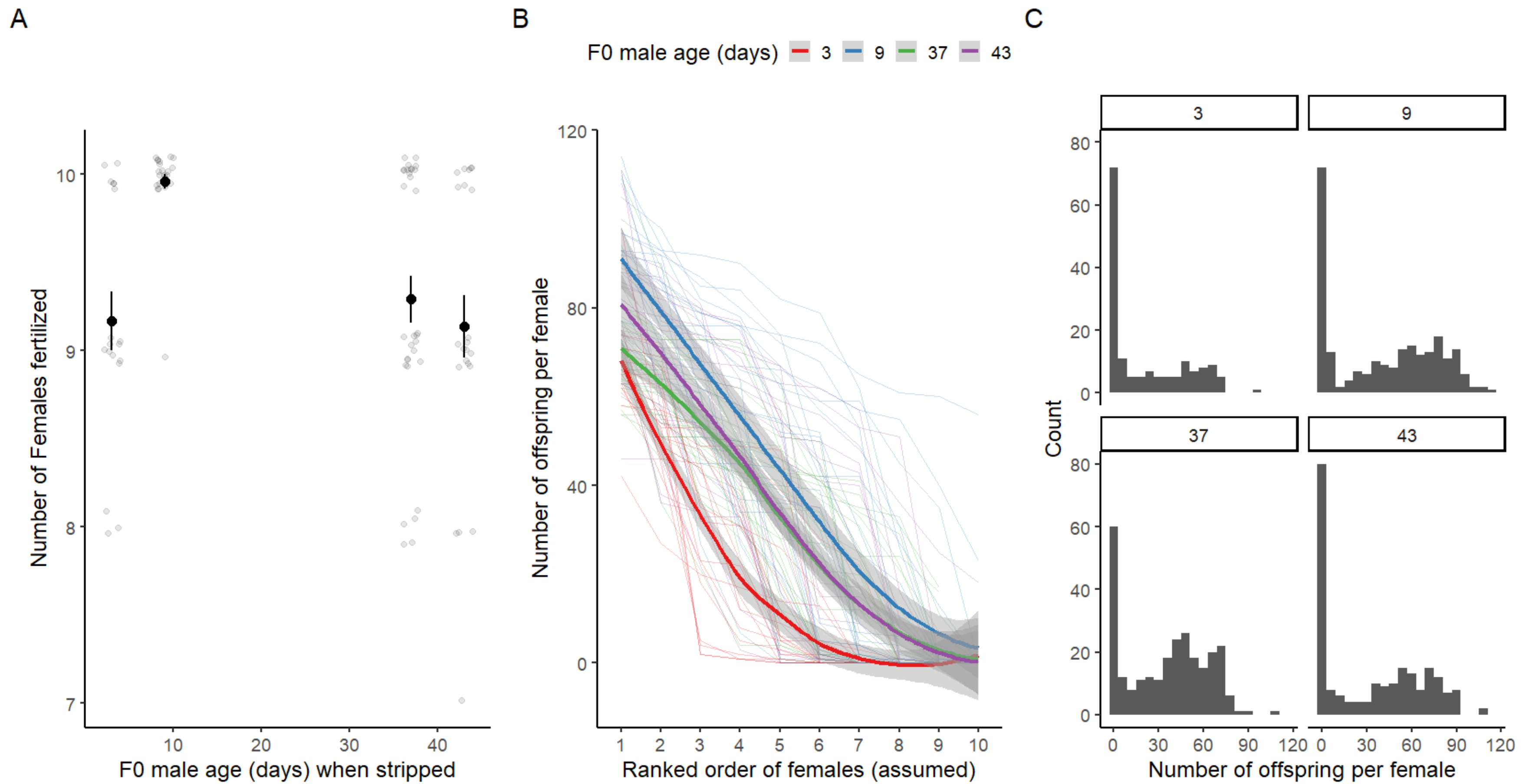


Figure S2: **A-** number of females fertilized by F0 males (fathers) in the stripping assay (>9 out of 10 females on average; means and SE along with raw data presented). **B-** When all 10 stripping females from a subset of males were put on individual vials, and the number of offspring they produced counted, there was a large amount of variation in the number of offspring across females. For each F0 male assayed, we ranked females he was kept with, ascendingly from one to ten, based on the number of offspring they produced. This ranking was assumed to be the order in which females mated to a male, such that males produced fewer offspring with each successive female in a mating sequence. Dark lines show means of each F0 age group, light lines show individual males. Shaded areas show 95% C.I. **C-** Distribution of the number of offspring produced by each of the 10 stripping females a male was kept with, in the stripping-counts assay. F0 males of 3, 9, 37, and 43 days of age were assigned to YO, YY, OO, OY paternal treatments, respectively.

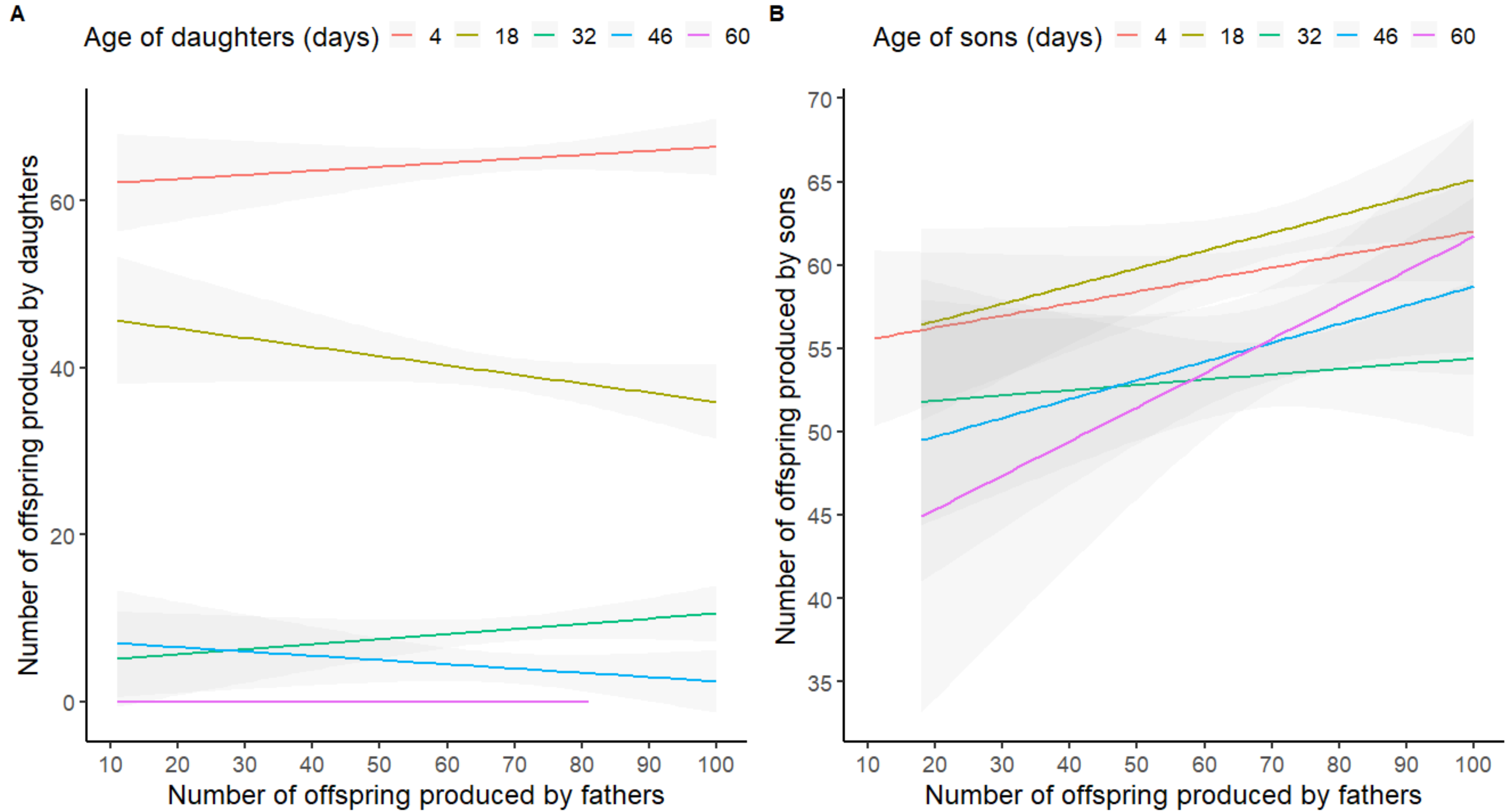


Figure S3. Number of offspring produced by fathers did not affect the number of offspring produced by A) daughters, but had a positive effect on the number of offspring produced by B) sons. Last two age groups (i.e. 74 and 88 days old) were excluded in the figure (but included in the model) for sons, due to very low sample sizes, and to allow better comparison between sons and daughters, given daughters did not live until 74 days.

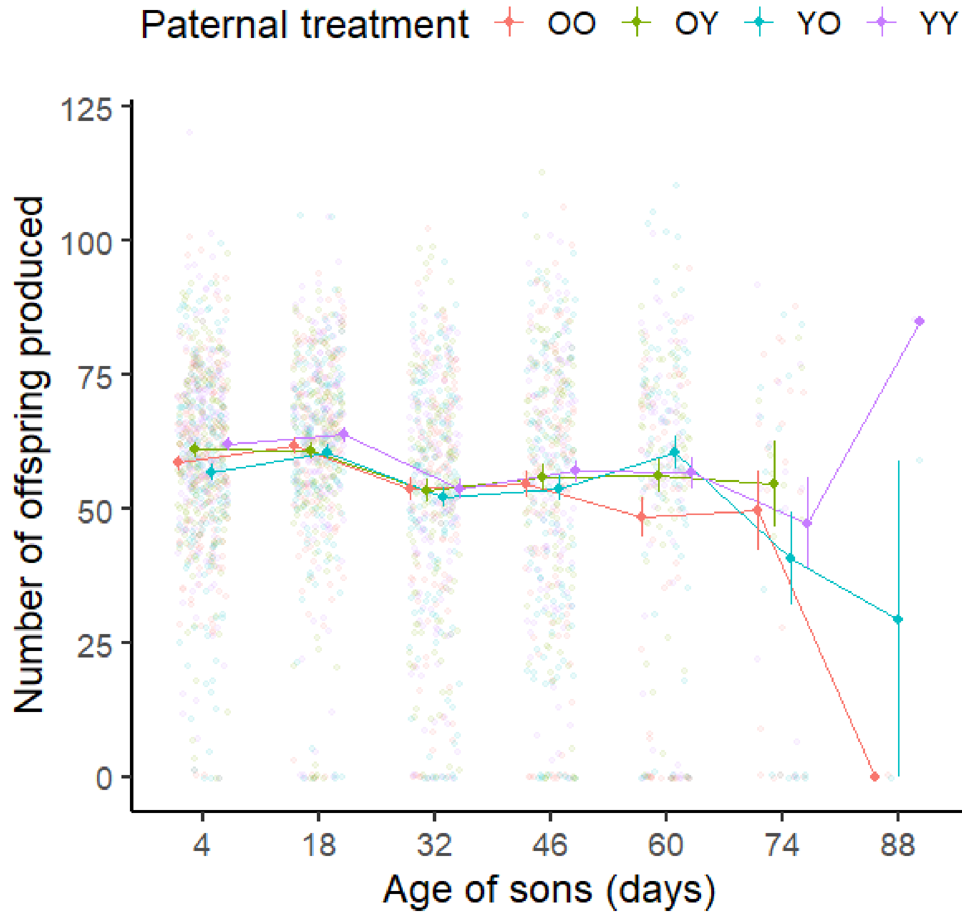


Figure S4: Effect of paternal age and paternal sperm storage duration on the number of offspring produced by sons at different ages. OO: Old paternal age, long paternal sperm storage; OY: Old paternal age, short paternal sperm storage; YO: young paternal age, long paternal sperm storage; YY: young paternal age, short paternal sperm storage. Means and SE along with raw data presented. Each light dot represents measurements on a single son for a given age.

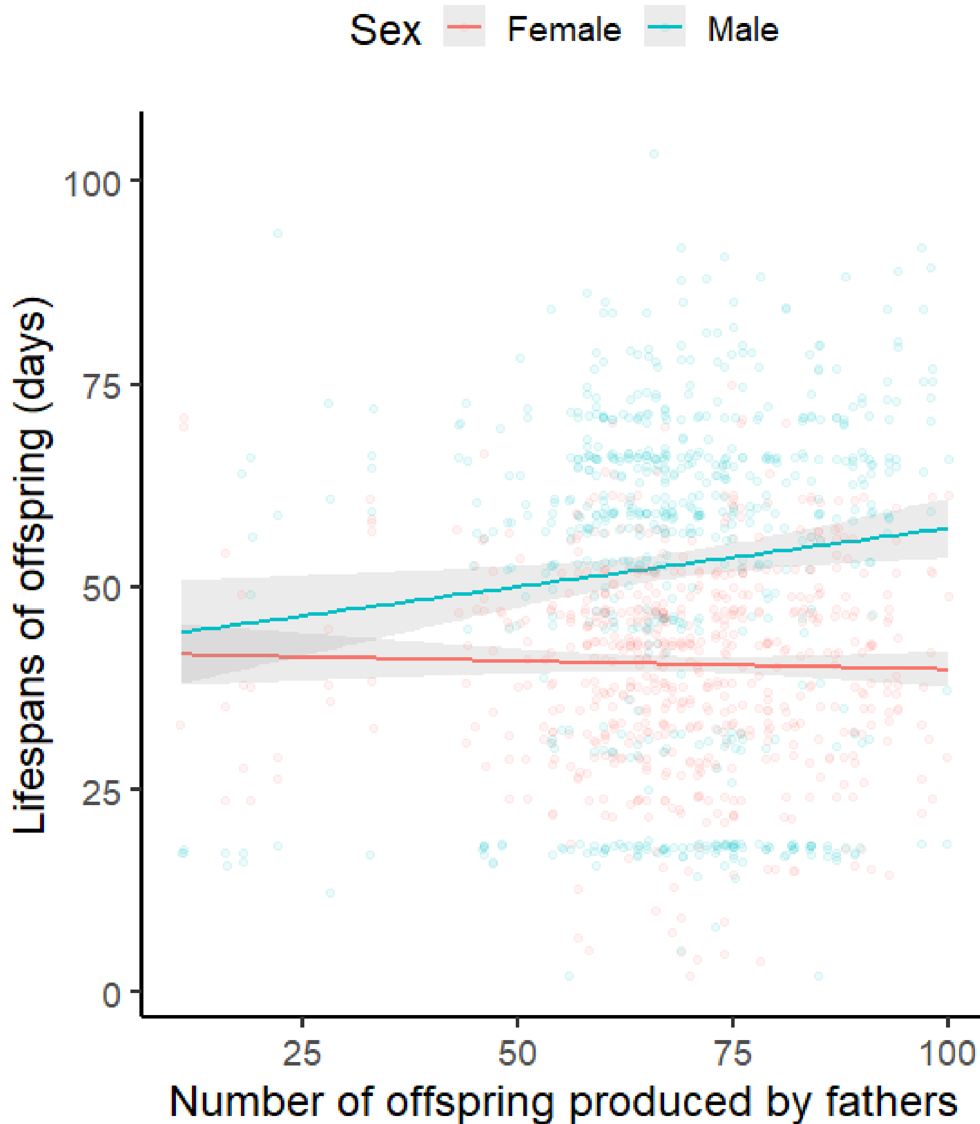


Figure S5: Number of offspring produced by fathers had a significant positive effect on the survival of their sons (male), but not their daughters (female). Lifespans (Y axis), rather than survival probability shown, for simplicity of interpretation, and difficulty of plotting mortality risk hazard function against a continuous variable (paternal reproductive output). Shaded areas show 95% C.I. Each light dot represents measurements on a single offspring.

Appendix 3: Supplementary tables

Table S1: Sample sizes used at each step of our experiment, for each of the four paternal treatments (OO: Old males, long sperm storage; OY: old males, short sperm storage; YO: young males, long sperm storage; YY: young males, short sperm storage). Each row refers to (top to bottom): Number of F0 males measured in the F0 stripping-fertility assay, where each of the 10 females were put on separate vials to assess the proportion of females that produced an offspring; the number of F0 males for whom 10 females from the stripping-counts assay were later kept on individual vials to count the number of offspring produced by each female; the number of fathers mated in the F0 mating assay whose reproductive output was measured; the number of fathers for whom 3 sons and 3 daughters were collected, and later used in the F1 assays.

	OO	OY	YO	YY
N_F0 males stripping-fertility assay	40	34	20	25
N_F0 males stripping-counts assay	31	22	17	24
N_F0 fathers mated	99	126	101	110
N_F1 families	59	60	61	61

Table S2: Effects of age (Y: young, O: old) and sperm storage duration (Y: short, O: long), on number of offspring produced by fathers. Model has zero inflated negative binomial error distribution. Two-way interaction model used to interpret only two-way interaction effect, main effect model used to interpret only main effects. Shaded grey terms were used to interpret associated model effects. Significant terms of interest in bold.

Two-way interaction model				
Dispersion parameter	7.440			
Conditional model:	Estimate	SE	z	P
(Intercept)	4.096	0.131	31.256	<0.001
Mating duration	0.007	0.005	1.270	0.204
RepR2	-0.112	0.056	-2.004	0.045
RepR3	-0.126	0.060	-2.081	0.037
RepR4	-0.212	0.056	-3.812	<0.001
Male ageY	0.081	0.062	1.297	0.195
Sperm storageY	0.022	0.061	0.356	0.722
Male ageY:Sperm storageY	0.028	0.082	0.343	0.732
Zero -inflation model	Estimate	SE	z	P
(Intercept)	-1.852	0.144	-12.890	<0.001

Main-effects model				
Conditional model:	Estimate	SE	z	P
(Intercept)	4.088	0.129	31.670	<0.001
RepR2	-0.112	0.056	-2.000	0.046
RepR3	-0.125	0.060	-2.070	0.039
RepR4	-0.212	0.056	-3.810	<0.001
Mating duration	0.007	0.005	1.270	0.204
Male ageY	0.096	0.045	2.130	**0.033**
Sperm storageY	0.036	0.045	0.810	0.419

Table S3: Effects of paternal age (Y: young, O: old) and paternal sperm storage duration (Y: short, O: long), on age-dependent reproductive output of daughters. Shaded grey terms used to interpret associated model effects. Significant terms of interest in bold. F0_ID = paternal ID, F1_ID = offspring ID.

Three-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	4.182	0.087	47.860	<0.001
RepR2	0.013	0.031	0.410	0.679
RepR3	-0.123	0.039	-3.150	0.002
RepR4	-0.004	0.040	-0.100	0.920
Daughter’s lifespan	0.003	0.001	2.680	0.007
Paternal fecundity	0.000	0.001	0.040	0.969
Paternal ageY	0.045	0.056	0.800	0.421
Paternal sperm storageY	0.028	0.056	0.500	0.615
Daughter's age	-0.020	0.004	-5.160	<0.001
I(Daughter's age^2)	0.000	0.000	-3.380	0.001
Paternal ageY* Paternal sperm storageY	-0.053	0.078	-0.680	0.495
Paternal ageY*Daughter's age	-0.004	0.003	-1.260	0.209
Paternal sperm storageY*Daughter's age	0.002	0.003	0.530	0.597
Paternal ageY* Paternal sperm storageY*Daughter's age	0.001	0.005	0.300	0.761

Random effects	Variance	SD
F0_ID/F1_ID	0.000	0.000
F0_ID	0.002	0.040

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	4.186	0.087	48.320	<0.001
RepR2	0.013	0.031	0.410	0.683
RepR3	-0.123	0.039	-3.150	0.002
RepR4	-0.004	0.040	-0.100	0.922
Daughter’s lifespan	0.003	0.001	2.690	0.007
Paternal fecundity	0.000	0.001	0.050	0.957
Paternal ageY	0.035	0.046	0.770	0.442
Paternal sperm storageY	0.018	0.046	0.400	0.689
Daughter's age	-0.020	0.004	-5.420	0.000
I(Daughter's age^2)	0.000	0.000	-3.450	0.001
Paternal ageY*Paternal sperm storageY	-0.035	0.049	-0.700	0.481
Paternal ageY*Daughter's age	-0.004	0.002	-1.510	0.130
Paternal sperm storageY*Daughter's age	0.003	0.002	1.110	0.268

Random effects	Variance	SD
F0_ID/F1_ID	0.000	0.001
F0_ID	0.002	0.040

Main-effects model

Fixed effects	Estimate	SE	z	P
(Intercept)	4.205	0.081	52.070	<0.001
RepR2	0.012	0.031	0.400	0.689
RepR3	-0.123	0.039	-3.150	0.002
RepR4	-0.005	0.039	-0.140	0.891
Daughter’s lifespan	0.003	0.001	2.710	**0.007**
Paternal fecundity	0.000	0.001	-0.020	0.984
Paternal ageY	-0.028	0.025	-1.140	0.254
Paternal sperm storageY	0.035	0.024	1.430	0.153
Daughter's age	-0.021	0.003	-6.050	**<0.001**

I(Daughter's age^2)	0.000	0.000	-3.410	**0.001**

Random effects	Variance	SD
F0_ID/F1_ID	0.000	0.000
F0_ID	0.001	0.035

Table S4: Effects of paternal age (Y: young, O: old) and paternal sperm storage duration (Y: short, O: long), on number of offspring produced by sons. Shaded grey terms used to interpret associated model effects. Significant terms of interest in bold. F0_ID = paternal ID, F1_ID = offspring ID.

Three-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.850	0.085	45.570	<0.001
RepR2	-0.021	0.029	-0.720	0.473
RepR3	0.102	0.035	2.950	0.003
RepR4	0.123	0.035	3.570	<0.001
Son's lifespan	0.001	0.001	1.770	0.077
Paternal fecundity	0.002	0.001	2.900	0.004
Paternal ageY	-0.065	0.055	-1.180	0.238
Paternal Sperm storageY	0.009	0.055	0.160	0.875
Son's age	-0.004	0.001	-3.420	0.001
Paternal ageY* Paternal Sperm storageY	0.066	0.077	0.850	0.394
Paternal ageY* Son's age	0.002	0.002	1.450	0.148
Paternal Sperm storageY * Son's age	0.001	0.002	0.740	0.458
Paternal ageY* Paternal Sperm storageY * Son's age	-0.002	0.002	-0.910	0.361

Random effects	Variance	SD
F0_ID/F1_ID	0.000	0.000
F0_ID	0.000	0.000

Two-way interaction model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.835	0.083	46.340	<0.001
RepR2	-0.021	0.029	-0.720	0.469
RepR3	0.102	0.035	2.960	0.003
RepR4	0.123	0.035	3.570	<0.001
Son's lifespan	0.001	0.001	1.740	0.083
Paternal fecundity	0.002	0.001	2.940	0.003
Paternal ageY	-0.036	0.045	-0.800	0.422
Paternal Sperm storageY	0.038	0.045	0.850	0.398
Son's age	-0.004	0.001	-3.410	0.001
Paternal ageY* Paternal Sperm storageY	0.009	0.045	0.200	0.845
Paternal ageY*Son's age	0.001	0.001	1.130	0.257
Paternal Sperm storageY *Son's age	0.000	0.001	0.120	0.906

Random effects	Variance	SD
F0_ID/F1_ID	0.000	0.000
F0_ID	0.000	0.000

Main-effects model

Fixed effects	Estimate	SE	z	P
(Intercept)	3.817	0.079	48.270	<0.001
RepR2	-0.021	0.029	-0.730	0.468
RepR3	0.101	0.035	2.940	0.003
RepR4	0.123	0.034	3.550	<0.001
Son's lifespan	0.001	0.001	1.660	0.097
Paternal fecundity	0.002	0.001	2.930	**0.003**
Paternal ageY	0.004	0.023	0.160	0.875
Paternal Sperm storageY	0.046	0.023	2.030	**0.043**
Son's age	-0.003	0.001	-4.430	**<0.001**

Random effects	Variance	SD
F0_ID/F1_ID	0.000	0.000
F0_ID	0.000	0.001

Table S5: Effects of paternal age (Y: young, O: old) and paternal sperm storage duration (Y: short, O: long), on the age-dependent mortality risk for daughters. Shaded terms used to interpret associated model effects. Significant terms of interest in bold. F0_ID = paternal ID.

Two-way interaction model	coef	exp(coef)	se(coef)	z	P
RepR2	-0.503	0.605	0.120	-4.190	0.000
RepR3	0.103	1.108	0.145	0.710	0.480
RepR4	0.151	1.163	0.147	1.030	0.310
Paternal fecundity	0.002	1.002	0.003	0.500	0.610
Paternal ageY	-0.059	0.943	0.135	-0.430	0.660
Paternal sperm storageY	0.039	1.040	0.136	0.290	0.770
Paternal_ageY*Paternal sperm storageY	-0.004	0.996	0.190	-0.020	0.980

Random effects	SD	Variance
F0_ID	0.354	0.125

Main-effects model	coef	exp(coef)	se(coef)	z	P
RepR2	-0.503	0.605	0.120	-4.200	0.000
RepR3	0.102	1.108	0.145	0.710	0.480
RepR4	0.151	1.162	0.147	1.030	0.310
Paternal fecundity	0.002	1.002	0.003	0.500	0.610
Paternal ageY	-0.061	0.941	0.096	-0.630	0.530
Paternal sperm storageY	0.037	1.037	0.094	0.390	0.700

Table S6: Effects of paternal age (Y: young, O: old) and paternal sperm storage duration (Y: short, O: long), on the age-dependent mortality risk for sons. Shaded grey terms used to interpret associated model effects. Significant terms of interest in bold.

Two-way interaction model	coef	exp(coef)	se(coef)	z	P
RepR2	0.402	1.496	0.140	2.880	0.004
RepR3	0.464	1.591	0.167	2.790	0.005
RepR4	0.168	1.183	0.170	0.990	0.320
Paternal fecundity	-0.009	0.991	0.004	-2.200	0.028
Paternal ageY	0.066	1.068	0.156	0.430	0.670
Paternal sperm storageY	0.220	1.245	0.157	1.400	0.160
Paternal_ageY*Paternal sperm storageY	-0.298	0.742	0.219	-1.360	0.170

Random effects	SD	Variance
F0_ID	0.539	0.290

Main-effects model	coef	exp(coef)	se(coef)	z	P
RepR2	0.388	1.473	0.140	2.760	0.006
RepR3	0.454	1.574	0.168	2.700	0.007
RepR4	0.150	1.162	0.171	0.880	0.380
Paternal fecundity	-0.009	0.991	0.004	-2.260	**0.024**
Paternal ageY	-0.086	0.918	0.110	-0.780	0.440
Paternal sperm storageY	0.065	1.067	0.110	0.590	0.550

Chapter 6

What doesn't kill you makes you stronger? Effects of paternal age at conception on fathers and sons*

*published in Evolution

Abstract

Advancing male age is often hypothesised to reduce both, male fertility and offspring quality due to reproductive senescence. However, the effects of advancing male age on reproductive output and offspring quality are not always deleterious. For example, older fathers might buffer effects of reproductive senescence by terminally investing in reproduction. Similarly, males that survive to reproduce at an old age, might carry alleles that confer high viability (viability selection) which are then inherited by offspring, or might have high reproductive potential (selective disappearance). Differentiating these mechanisms requires an integrated experimental study of paternal survival and reproductive performance, as well as offspring quality, which is currently lacking. Using a cross-sectional study in *Drosophila melanogaster*, we test the effects of paternal age at conception (PAC) on paternal survival and reproductive success, and on the lifespans of sons. We discover that mating at an old age is linked with decreased future male survival, suggesting that mating-induced mortality is possibly due to old fathers being frail. We find no evidence for terminal investment, and show that reproductive senescence in fathers does not onset until their late-adult life. Additionally, we find that as a father's lifespan increases, his probability of siring offspring increases, for older PAC treatments only. Lastly, we show that sons born to older fathers live longer than those born to younger fathers, due to viability selection. Collectively, our results suggest that advancing paternal age is not necessarily associated with deleterious effects for offspring, and may even lead to older fathers producing longer-lived offspring.

Keywords: senescence, viability selection, Lansing effect, terminal investment, life-history trade-offs, pleiotropy

Introduction

Reproductive senescence is the age-dependent decline in the reproductive output of organisms (Monaghan et al, 2008; Rose and Charlesworth, 1980). This leads old individuals to have lower gamete quality and quantity (Dean et al, 2010; Gasparini et al, 2010), thus lower fecundity and fertility (Naciri et al, 2022; Sepil et al, 2020), and produce fewer offspring, than young individuals. For instance, old males across different species often have smaller ejaculate sizes (Cornwallis et al, 2014; Sanghvi et al, 2024), poorer quality sperm (Gasparini et al, 2019; Johnson et al, 2015), and lower abundance of seminal fluid proteins (Fricke et al, 2023), than young males. A growing body of literature however, challenges these patterns by documenting that advancing male age does not necessarily result in male reproductive senescence (Aich et al, 2022; Baudisch and Stott, 2019; Brooks and Kemp, 2001; Cooper et al, 2020, 2021; Finch, 2009; Forslund and Part, 1995; Heinze et al, 2018; Johnson and Gemmell, 2012; Jones et al, 2014; Jones and Vaupel, 2017; Lee and Chu, 2023; Moullec et al, 2023; Sandfoss et al, 2023; Segami et al, 2021; Vega-Trejo et al, 2019). In some cases, advancing age may even be associated with increased reproductive output (e.g. Avent et al, 2008; Girndt et al, 2019; Lifjeld et al, 2022; Prathibha et al, 2011; Sanghvi et al, 2024; Santhosh and Krishna, 2013; Verspoor et al, 2015).

Several mechanisms explain why old males might have a reproductive output similar to-, or higher than, young males. At a population level, between individual heterogeneity in quality might lead to selective disappearance of poor-quality individuals. Here, longer living males could represent a non-random cohort with higher reproductive quality (Bouwhuis et al, 2009; Hamalainen et al, 2014; Sanghvi et al, 2022; Sultanova et al, 2023), thus masking reproductive senescence. Alternatively, according to the terminal investment hypothesis, old males, males who perceive themselves as close to dying, or old males close to dying, might allocate proportionally more resources such as ejaculates, to a single current reproductive

event than young males (Duffield et al, 2017; Farchmin et al, 2020; Moullec et al, 2023). This is because as future survival prospects of organisms decline, individuals are hypothesized to invest more in current than future reproductive opportunities (Creighton et al, 2009; Duffield et al, 2018; Froy et al, 2013; Part et al, 1992; Velando et al, 2006).

In addition to impacting a male's own reproductive output, reproductive senescence in males can also influence the phenotypes of the offspring via paternal age effects (Priest et al, 2007; Schroeder et al, 2015). For instance, offspring sired by old fathers are reported to have poorer development (Janecka et al, 2017; Preston et al, 2015), early-life performance (Fay et al, 2016), and reproductive output (Arslan, 2017; Nystrand and Dowling, 2014; Vuarin et al, 2021), than offspring of young fathers. Notably, offspring born to old fathers often have shorter lifespans than those born to young fathers (Crow, 2003; Monaghan et al, 2020; Sharma et al, 2015), a phenomenon known as the 'Lansing effect' (Ivimey-Cook et al, 2023; Lansing, 1947). While these paternal age effects might be caused by age-dependent changes in paternal care (Benowitz et al, 2013; Cope et al, 2022), in species without care, these effects likely occur via age-dependent deterioration in ejaculates (Monaghan and Metcalfe, 2019) or differential resource allocation by females (Harris and Uller, 2009).

In contrast to negative effects of old fathers, few studies report no effects of paternal age on offspring quality (Heinze et al, 2018; Sparks et al, 2022; also see chapter 5 of my thesis). Others yet show that old fathers can produce larger (Aguilar et al, 2023; Mirrhosseini et al, 2014; Pappert et al, 2023), longer lived (Angell et al, 2022; Johnson et al, 2018; Krishna et al, 2012; Lee et al, 2019; Priest et al, 2002), and more fecund (Krishna et al, 2012; Sparks et al, 2022) offspring than young fathers (also see Kroeger et al, 2020 for beneficial maternal age effects). Thus, some mechanisms have been proposed to explain why old fathers produce higher quality offspring compared to young fathers. Males who mate at an old age are predicted to have, on average, longer lifespans than males who mate at younger ages

(Mueller, 2004). If differences in survival between individuals are due to intrinsic reasons, older fathers would carry alleles that confer higher viability (Bowen et al, 2006; Chen and Maklakov, 2012; Kokko and Lindstrom, 1996; Reznick et al, 2004). Consequently, offspring born to older fathers would inherit these alleles, leading to old fathers producing longer-lived offspring, as predicted by the viability selection hypothesis (Beck et al, 2002; Brooks and Kemp, 2001; Hansen and Price, 1995; Kokko, 1998; Johnson and Gemmell, 2012). However, this positive relationship between paternal age, and paternal or offspring lifespan, might be buffered when old males are frail and die soon after mating, or under high extrinsic mortality (Kokko and Lindstrom, 1996).

The effects of paternal age at conception and paternal lifespan on paternal reproductive output and offspring lifespan are unlikely to be independent. Non-mutually exclusive processes (effects on male reproductive output: *reproductive senescence, selective disappearance, terminal investment*; effects on offspring quality: *Lansing effect, viability selection*), might co-occur and interact with each other to shape the life-history of fathers and their offspring. For example, higher paternal allocation toward producing offspring of high quality, could come at the cost of lower allocation toward producing many offspring, or fathers living longer, due to life-history trade-offs (e.g. Johnson et al, 2018; Lemaitre et al, 2015; Travers et al, 2021). Different processes could further interact to shape patterns of ageing in animals. For instance, selective disappearance might lead to beneficial effects of advancing age on reproductive output until mid-life. However, from mid- to late-life, these age-dependent improvements could be outweighed or balanced by deleterious effects of senescence, leading to curvilinear shapes of ageing (e.g. Cooper et al, 2020; Jones et al, 2014; McCleery et al, 2008; Reid et al, 2010; Sanghvi et al, 2024; Torres et al, 2011), or no overall effects of age (e.g. Cooper et al, 2021). Only few studies (on wild populations) have attempted to disentangle the confounding effects of paternal age at conception and paternal

lifespan (e.g. Reid et al, 2010; Torres et al, 2011). While valuable for their ecological relevance, these studies on wild populations have limited control over confounding factors such as maternal ageing and male mating history (a crucial modulator of male survival and reproductive output: Aich et al, 2022; Jones and Elgar, 2004; Partridge and Andrews, 1985; Paukku and Kotiaho, 2005).

Using cross-sectional sampling of fathers at different ages in the fruit fly, *Drosophila melanogaster*, we test the effects of paternal age at conception (PAC) on paternal survival (aim 1), paternal reproductive output (aim 2), and the lifespans of sons (aim 3). A cross-sectional design allows us to test whether between-individual variation in lifespan and reproduction, therefore population-level processes such as viability selection and selective disappearance, explain the influence of PAC on offspring phenotypes. We first investigate the influence of PAC on paternal lifespan and future survival (aim 1). The relationship between PAC and paternal lifespan is often assumed to be positive. However, this relationship might be non-linear, due to processes not usually accounted for (Appendix 1). For instance, males who mate when old might be frail, thus incur more severe costs of mating and die soon after mating, than males who mate at a young age. Such age-dependent frailty could lead to a lower ceiling for maximum lifespan in males who mate when old than in males who mate when young, therefore buffering the expected positive relationship between PAC and paternal lifespan. Next, we investigate whether a male's age at conception affects his reproductive output (aim 2, Appendix 2). Consistent with reproductive senescence, old males might have a lower reproductive output than young males. However, between-individual heterogeneity arising when males are sampled cross-sectionally, might mask reproductive senescence at a population-level. For example, when male mortality is non-random and lifespan and reproductive output co-vary positively, males with low reproductive output might die earlier than males with high reproductive output. Such selective disappearance would lead to an

increase in reproductive output with advancing age. Further, terminal investment could lead old males, males who are close to dying, or old males close to dying, to invest more in a current reproductive event, than young males or males far away from dying. Finally, we investigate how PAC affects the lifespans of offspring, focussing on sons (aim 3, Appendix 2). Here, in line with Lansing effects, old fathers might produce shorter-lived sons than young fathers. However, if old fathers have alleles that confer longer lifespans, then a cross-sectional sampling of paternal ages could lead to sons born to old fathers living for longer, than those born to young fathers, due to viability selection.

Methods

Stock population

To investigate how PAC affects paternal survival and reproductive output, as well as the lifespan of sons, we first set up a population of experimental males. To do this, we collected 300 virgin males (henceforth "unmated experimental males") within 6 hours of eclosion on ice, and placed them in individual vials. These 300 males were collected from a stock population cage of lab-adapted, outbred, wildtype Dahomey *D. melanogaster* flies maintained in our lab since the 1970s. Unmated experimental flies were reared using a standard larval density method at 25°C and 45% r.h. (Clancy and Kennington, 2001). All flies in our experiment were maintained on a 12:12 hour light cycle, and fed with Lewis medium supplemented with ad libitum live yeast (Lewis, 1960). Under these conditions, male flies in our lab have median and maximum lifespans of ~45 days and ~90 days, respectively.

Experimental design

We first generated different PAC treatments of experimental fathers, by sampling fathers using a cross-sectional design. A cross-sectional sampling approach was employed for two

reasons. First, we were interested in testing whether between-individual heterogeneity in paternal lifespan might buffer the deleterious effects of advancing paternal age. Here, only a cross-sectional design could create heterogeneity in paternal lifespan for different PAC treatments. Our second reason for cross-sectional sampling was to prevent paternal age from confounding with paternal mating history, which inevitably occurs when fathers are sampled longitudinally (Aich et al, 2022).

Every two weeks (starting at 4 days of age), we chose between 25 to 37 surviving males from the 300 unmated experimental males (sample sizes in Table S1). In total, we generated a total of six PAC treatments, with males of the following ages (in days): 4, 18, 32, 46, 60, 74 (see Table S1 for sample sizes). We generated random numbers on MS Excel ('randbetween' function) to randomise our choice of males. The chosen experimental males were placed in a vial with a young (4 days old) virgin female of Dahomey background. Chosen experimental males were mated only once with the female, and males that did not mate within four hours (5 in total across all PAC treatments) were censored. Each mating was observed and mating latency and copulation duration recorded. After mating, the males were transferred into a new vial and kept individually until they died, and monitored daily to record their lifespans within 1 day of error. All fathers in our experiment, mated only once across their lifespan. The mated females were left in the mating vial for another 24 hours following exposure to the male, to lay eggs, after which the females were discarded. These vials were left at 25°C and 45% r.h for 9 to 10 days, until offspring emerged (pupae in all vials emerged).

Within six hours of emergence, three sons were selected haphazardly from each parental pair and moved into a separate vial. All three sons from a parental pair were kept together until death. The remaining offspring from each parental pair were frozen at -20°C on the fourth day after eclosion began, and their numbers counted. We additionally recorded the

survival of the unmated experimental males each day, to compare the survival of mated versus unmated males. All experimental flies, including unmated males and sons, were put in new food vials once every four days, using an aspirator.

Data analysis

We used R v3.5.2 for all analyses (See HTML supplement for code and full model outputs), and packages *lme4* (Bates et al, 2014), *glmmTMB* (Brooks et al, 2014), *lavaan* (Rosseel, 2012), and *nlme* (Pinheiro et al, 2017) for building models. P values for all linear models were calculated using Satterthwaite's method. All linear models were checked for model assumptions of normality and homoscedasticity of residuals using the *stats* package (R core team, 2012). All generalised linear models were checked for overdispersion using *DHARMa* (Hartig and Hartig, 2017). We interpreted main effects only when two-way interactions were non-significant (following Engqvist 2005), by fitting a model with the interaction removed. PAC was modelled as a continuous, not categorical variable, in all our models.

Aim 1: Effects of PAC on paternal survival

First, we investigated the effects of increasing PAC on paternal lifespan (see Appendix 1 and 2 for hypotheses and predictions). For this, we created a linear model with PAC as a fixed effect, and paternal lifespan as the dependent variable. Our data violated linear model assumptions of homogenous variance, due to lower variance in paternal lifespans for older PAC treatments. Thus, we specified a heterogeneous variance structure using the function *gls* (Pinheiro et al, 2017). A model with heterogenous variance structure was a better fit to the data than one without (ΔAIC = 7.817; ΔDF = 5).

Second, we tested whether mortality risk differed between mated (i.e. fathers) and unmated experimental males. For this, we compared survival probability between unmated

experimental males and fathers who mated when young (4 days old). Young, rather than older fathers were chosen for this comparison because we wished to compare actuarial senescence patterns across the entire lifetime of mated versus unmated males. Using old fathers would not allow this comparison, because older fathers by definition, survived to older ages. We modelled data using Cox proportional hazards in the *coxme* (Therneau and Therneau, 2015) and *survival* (Therneau and Lumley, 2013) packages, with male lifespan as the dependent variable, and treatment (4 day old mated versus unmated) as a fixed effect.

Third, we compared whether mortality risk due to mating differed between young and old males, using a Cox proportional hazards model. For this, we used two approaches. In our first approach, we used a generalised linear model with binomial error distribution. Here, we compared the proportion of males that were alive (1) or dead (0) as our dependent variable, in each PAC treatment (fixed effect), three days after mating. Here, we additionally included number of offspring each male produced as a fixed effect to test for potential trade-offs between paternal mortality risk and offspring production (Lemaitre et al, 2015). However, differences in survival soon after mating, between different PAC treatments could, in principle, be explained by actuarial senescence alone. We thus used a second approach to specifically test whether mortality risk differed between old and young males due to mating *per se*, by removing effects of actuarial senescence. To do this, we compared survival probabilities between males who mated when 60 days (i.e. old PAC) old and males mated when 4 days old (i.e. young PAC), while only using data of males from both PAC treatments that survived beyond 60 days. Similarly, we also compared survival probabilities between males who mated when 74 days old versus 4 days old, but only using data on males from both PAC treatments that survived beyond 74 days. Out of the 25 fathers initially assigned to the 4 day old treatment, 14 (56%) and 6 (24%) survived beyond 60 and 74 days, respectively.

Aim 2: Effects of PAC on paternal reproductive output

We tested the effect of PAC on paternal probability of siring an offspring (P_s), and on the number of offspring sired (N_s) when only considering fathers who sired offspring. To do this, we used a hurdle generalised linear model with truncated negative binomial error distribution (Brooks et al, 2017), and number of offspring as our dependent variable. A hurdle model allowed us to first, compare the P_s of fathers (i.e. zero inflation model). Then, using only data on fathers that sired offspring (conditional model), compare the N_s of fathers. PAC, paternal lifespan, their interaction, a quadratic effect of PAC, paternal copulation duration, and mating latency, were included as fixed effects. PAC was included to test for senescence, while paternal lifespan was included to test for selective disappearance (van de Pol and Verhulst, 2006). Copulation duration was included to account for males potentially producing more offspring due to transferring more sperm by copulating for longer (Dore et al, 2021). Latency was included to account for differences in female pre-mating preference for males, where females who take longer to mate (i.e. less prefer a male), might produce fewer offspring than females who take shorter to mate. In the same model, we also included a quadratic term for PAC, to test for curvilinear patterns of ageing. This model would allow us to test for the effects of reproductive senescence, as well as selective disappearance, by testing whether within each PAC treatment, fathers with longer lifespans sired more offspring than fathers with shorter lifespans. We conducted a sensitivity analysis to ensure that the patterns observed for reproductive ageing were not driven by the oldest age group (i.e. 74-day old males), by re-analysing the data but excluding the 74-day old males, as this age group had few data points (Table S1).

We then investigated whether observed patterns in paternal reproduction could be due to terminal investment. Terminal investment could occur solely as a response to advancing male age, proximity to death, or due to an interaction between male age and proximity to

death (reviewed in Duffield et al, 2017; Foo et al, 2023). We thus first tested whether old fathers who were close to dying produced more offspring than old fathers not close to dying, or young fathers. For this, we calculated the number of days elapsed between a male's death and the day he mated, henceforth called "days to death". Then, using a generalised linear model with zero-inflated negative binomial error distribution, we tested how the interaction between days to death and PAC (fixed effects), affected the number of offspring produced (using all data, including fathers who produced zero offspring) by fathers (dependent variable). Here, we additionally included paternal copulation duration as a fixed effect. We then tested whether fathers who were close to dying or fathers who were old, produced more offspring than fathers who were not close to dying or than young fathers respectively, by removing the interaction term in the model specified above.

Aim 3: Effects of PAC on lifespans of sons

We investigated the effects of PAC on the lifespans of sons, and whether the observed effects were due to fathers in older PAC treatments living longer, as predicted by the viability selection hypothesis. For this, we used three approaches. In our first approach, we tested how PAC affected the lifespans of sons (dependent variable) using a linear model. Here, we included PAC as fixed effect and paternal ID as a random effect. We also included the number of offspring produced as fixed effect to test for trade-offs between paternal investment in offspring lifespan versus number (Johnson et al, 2018). Our models had heterogenous variance structure specified, as this structure yielded a better fit to the data than a model without heterogenous variance (ΔAIC= 25.66; ΔDF= 1).

In our second approach, we tested whether PAC had a significant effect on the lifespans of sons, after isolating the variance explained by paternal lifespan. This was done to test whether the effect of PAC on lifespans of sons, observed in our first approach, was

mediated by paternal lifespan (i.e. indirectly) as predicted by the viability selection hypothesis. We first built a model with paternal lifespan as a fixed effect, paternal ID as a random effect, and the lifespan of each son as our dependent variable. Then, we used residuals from this model as the dependent variable, and included PAC and the number of offspring produced by fathers as fixed effects, and paternal ID as a random effect. Such a method of residualizing allowed us to test the effects of a PAC while isolating the effects of paternal lifespan which was a collinear predictor (Dormann et al, 2013). This model had a heterogeneous variance structure specified as described above.

In our third approach, we conducted a path analysis using a structural equation model in the package *lavaan* (Rosseel, 2012). This approach was used to obtain a better understanding of the path of causality for how PAC affects lifespans of sons. We modelled the direct effect of PAC on son lifespan and its indirect effect via the influence of paternal lifespan. In this model, we also included covariances between number of offspring produced by fathers with paternal lifespan and with lifespans of sons, to account for trade-offs between offspring quality and number, and between investment in reproduction versus survival by fathers. For all three approaches used for analysing the effects of PAC on son' lifespans, we additionally conducted sensitivity analyses. In these, we excluded data on sons from the 74-day PAC treatment, owing to the small sample size of this treatment.

Results

Aim 1: Effects of PAC on paternal survival

Increased paternal age at conception (PAC) was associated with an increase in the average lifespan of fathers (t = 3.072; P = 0.003, R^2 = 4.3%, Figure 1). Paternal lifespan increased by 0.125 days with an increase of one day in PAC.

There was no significant difference in survival probabilities between the unmated experimental males, and those who mated at 4 days old ($z = 1.574$, $P = 0.115$, Figure 2 and 3A). When comparing only males who mated, males in older PAC treatments had a lower probability of surviving beyond three days after mating, than males in younger PAC treatments ($z = -3.544$, $P < 0.001$, Table S2, Figure S1). For instance, within 3 days after mating, males who mated when 'old' (i.e. at 60 or 74 days old) experienced >50% mortality, compared to no deaths in 'young' PAC treatments (i.e. 4, 18, and 32 days old). While this result could be due to age-specific effects of mating stress, it could also be a consequence of actuarial senescence, whereby older PAC have an overall lower age-dependent survival probability than young PAC treatments ($z = -2.067$, $P = 0.038$, Figure S2). We thus further tested whether the difference in mortality rates between old and young PAC treatments was specifically due to mating. Males who mated at 60 days of age had a significantly lower probability of surviving past 60 days of age than males who mated at 4 days of age, when only data on males that survived past 60 days of age in both PAC treatments were used ($P = 0.045$, Figure 3A). Similarly, males who mated at 74 days of age were less likely to survive past 74 days of age than males who mated at 4 days of age ($P = 0.066$, Figure 3B). Collectively, these results indicate that while advancing PAC is associated with fathers having longer lifespans, the strength of this effect is buffered by old fathers experiencing higher mortality associated with mating stress, compared to fathers who mated when young.

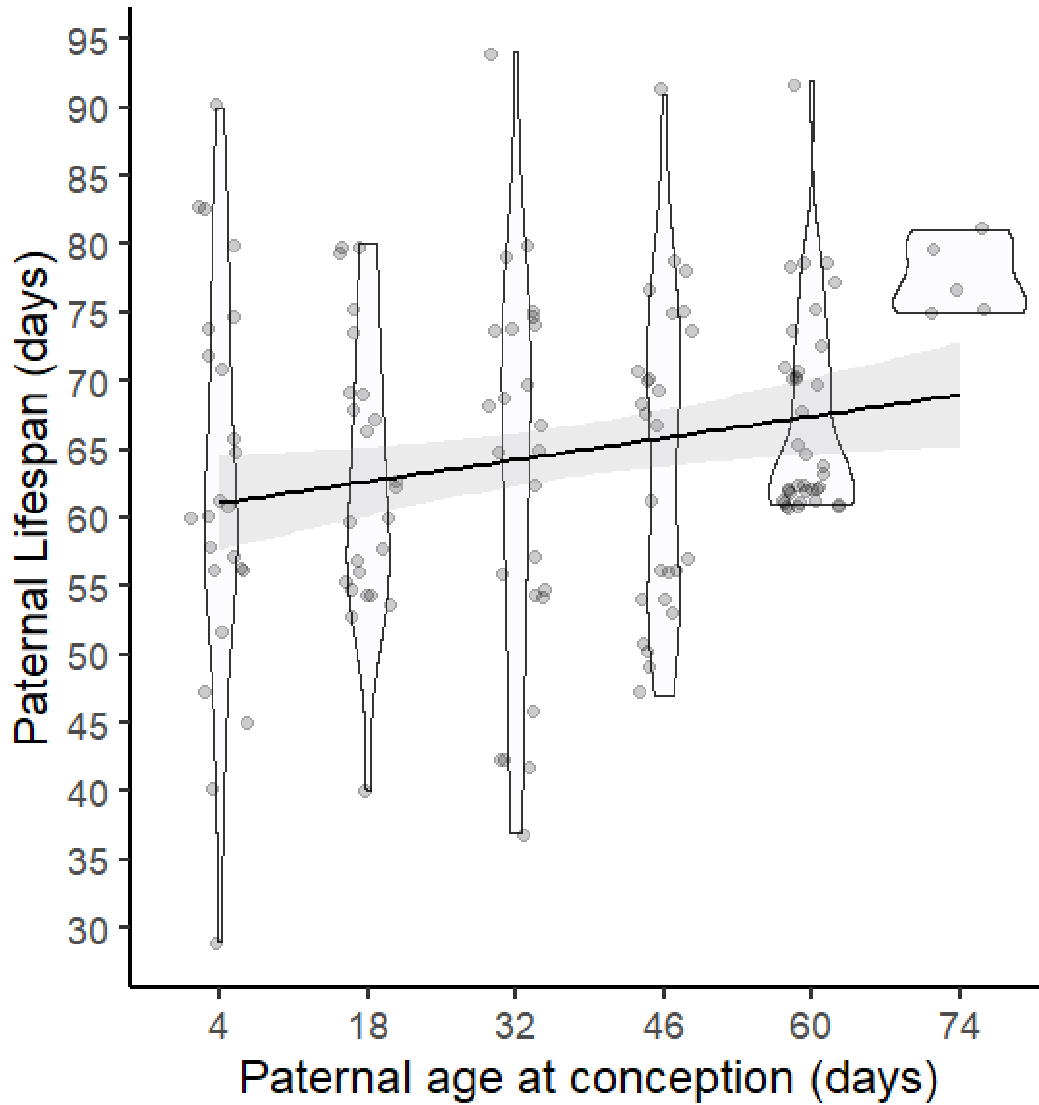


Figure 1: Males who conceive at older ages have longer lifespans on average. Violin plots show smoothed distribution of data, while dark line and shaded areas show regression line and 95% C.I. Lower Y axis limit set to 25 because no deaths occurred before 25 days of age. Sample size of fathers across the six PAC treatments (from 4 to 74 days) is: 25, 25, 26, 26, 37, 5, respectively.

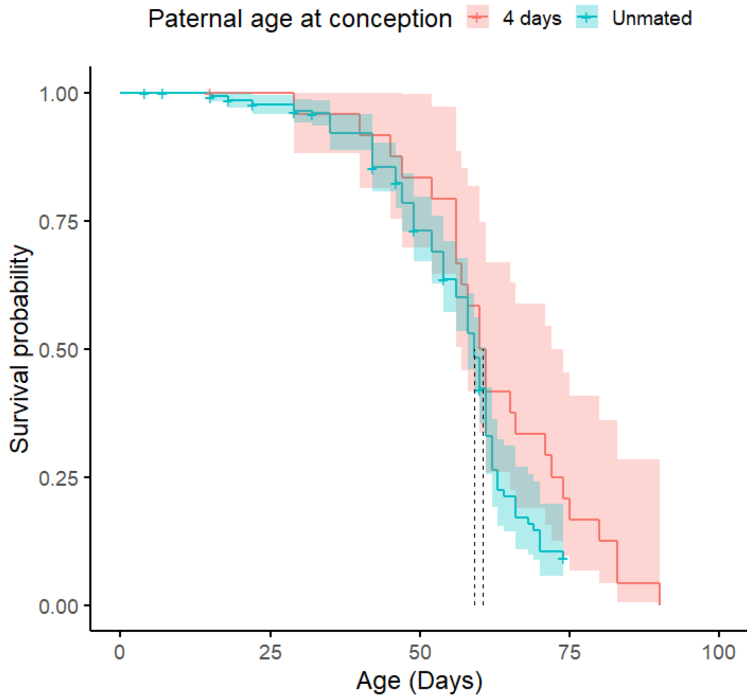


Figure 2: Males who mated at 4 days of age (N=25) did not have a significantly different survival probability compared to the unmated experimental males (N=300). "+" indicates censored individuals. Shaded area shows 95% C.I. Dotted lines show age at median survival probability.

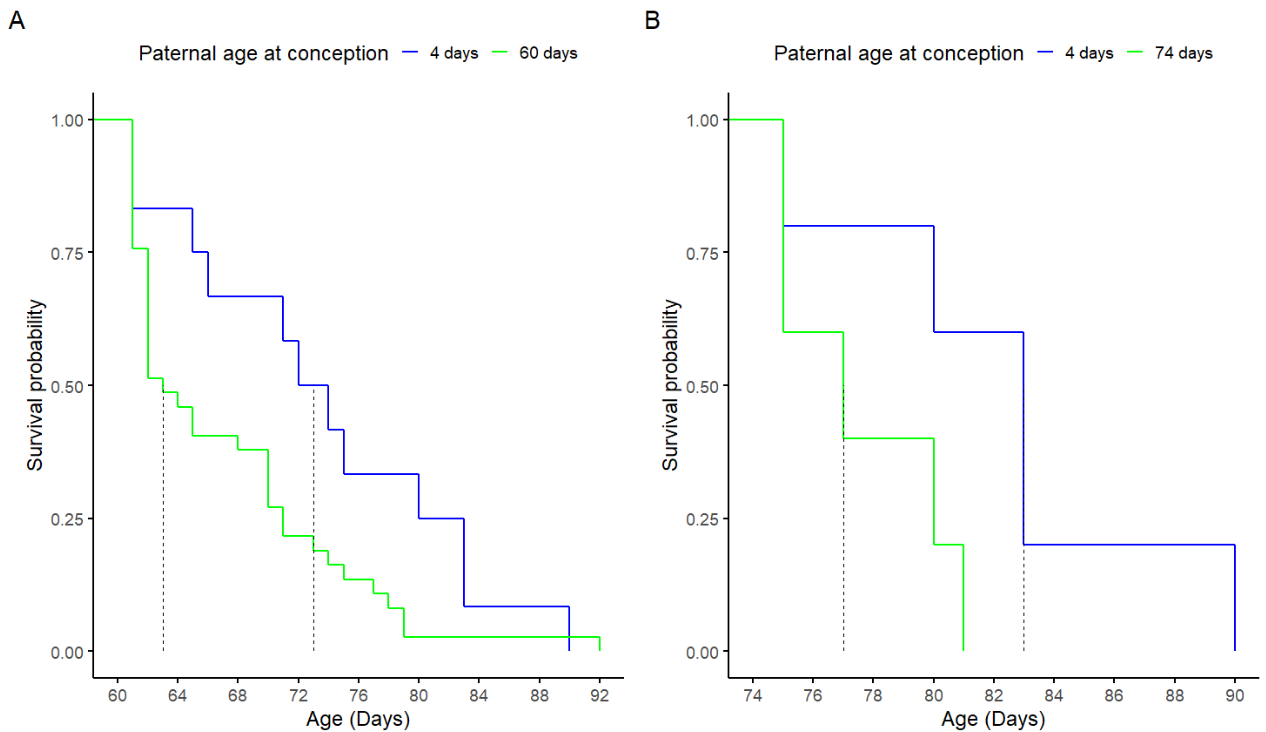


Figure 3: A- Males who mated at 60 days of age (N=37) had a lower survival probability than males who mated at 4 days of age but survived beyond 60 days of age (N=14). B- Males who mated at 74 days of age (N=5) had a lower survival probability than males who mated at 4

days of age but survived beyond 74 days of age (N=6). Dotted lines show age at median survival probability.

Aim 2: Effects of PAC on paternal reproductive output

We found a significant interaction between PAC and paternal lifespan, on the probability of siring offspring (i.e. P_s: z = -2.336, P = 0.020, Table S3). Specifically, P_s increased with paternal lifespan, but only for fathers who conceived at older ages (Figure 4A). When the interaction term between PAC and paternal lifespan was removed, there was an overall positive effect of increasing paternal lifespan leading to a higher P_s (z = -2.526, P = 0.012). However, a model with the interaction term provided a marginally better fit to the data than a model without this term (ΔAIC= 1.4; ΔDF= 2; P = 0.065). When investigating variation in N_s (i.e. number of offspring sired by fathers, who produced at least one offspring), we found no significant effects of the interaction between PAC and paternal lifespan (z = 0.562, P= 0.574, Table S3), and no significant effect of paternal lifespan (z = 0.963; P = 0.336) on paternal N_s. However, we found a significant quadratic effect of PAC on N_s (z = -3.902, P < 0.001, Figure 4B).

Our sensitivity analysis (i.e. a model without data from 74-day old PAC) showed a significant quadratic effect of PAC on N_s (z = -2.084, P = 0.037). However, the shape of PAC on N_s from the sensitivity analysis was shallower (Figure S3, S4) than the original model that included data from 74-day old males. Furthermore, a model with a quadratic and linear term for PAC was no better fit to the data than a model without the quadratic term (ΔAIC= 0.6; ΔDF= 2; P = 0.1). This suggests that a decline in N_s late in life and an overall quadratic effect of reproductive ageing, are most likely driven by low N_s in the 74-day PAC treatment.

We found a significant interaction between days to death and PAC to affect number of offspring sired by fathers. A model with an interaction term between PAC and days to death provided a better fit to the data than a model with the interaction removed (ΔAIC = 2.8, ΔDF

= 1, $P = 0.027$). Specifically, old males who died soon after mating sired fewer offspring than old males who died later after mating, or than young males ($z = 2.201$, $P = 0.027$; Table S4). When averaged across other variables, males who were closer to dying did not produce more offspring than males who were not close to dying ($z = 0.167$, $P = 0.867$). Collectively, these results on reproductive output indicate that reproductive senescence in fathers becomes apparent only in late-adult life; that life-history traits of survival and reproductive output show pleiotropic interactions that depend on the age of the father; and that there is no evidence for terminal investment or selective disappearance.

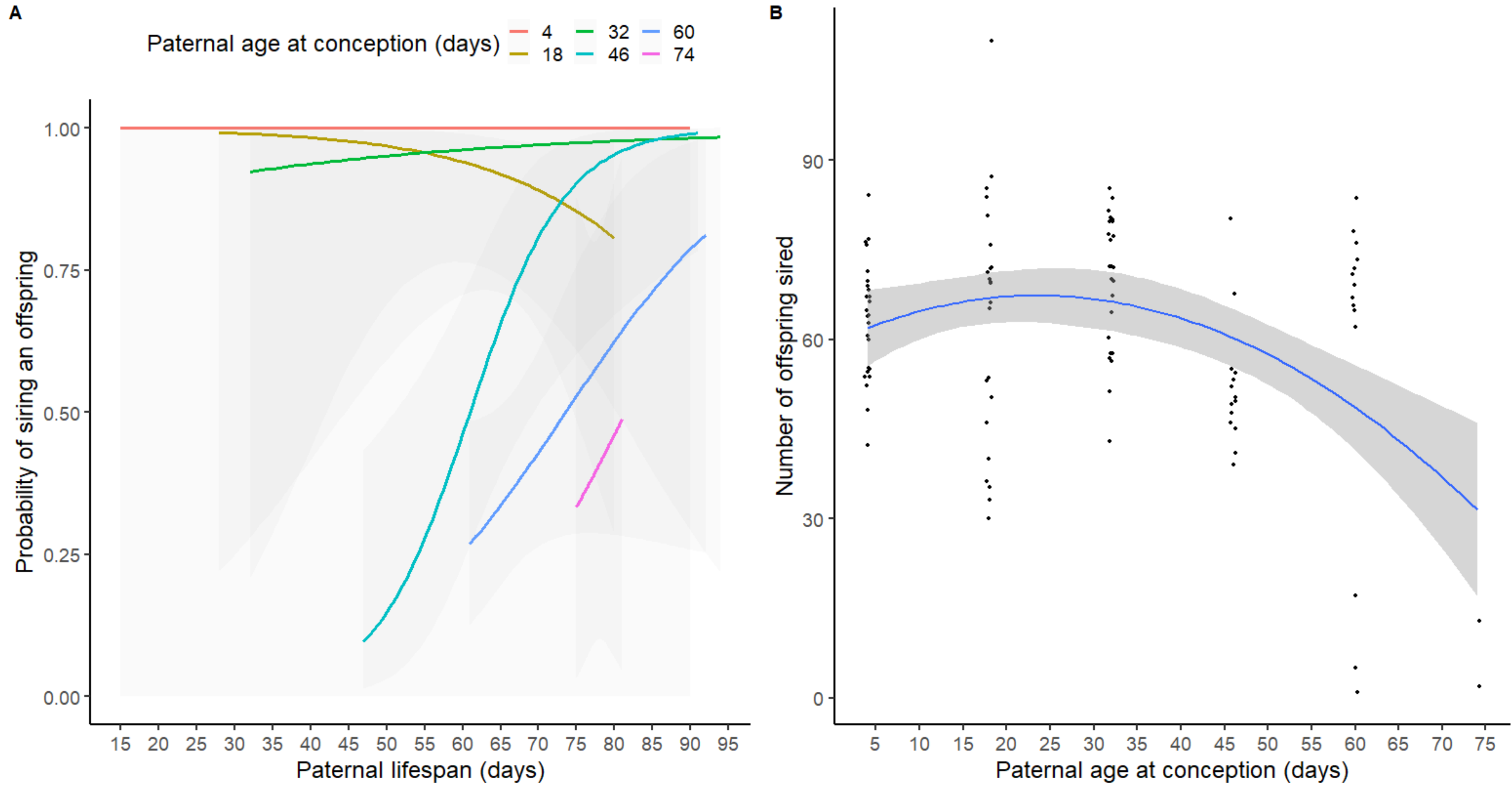


Figure 4: A: Paternal age at conception interacted with paternal lifespan to affect the probability of siring an offspring (P_S). B: Paternal age at conception affected the number of offspring sired (N_S: after excluding fathers who did not produce offspring) in a quadratic way. Shaded areas represent 95% C.I. Sample size of fathers across the six PAC treatments (from 4 to 74 days) is: 25, 25, 26, 26, 37, 5, respectively.

Aim 3: Effects of PAC on lifespans of sons

In our first approach, we tested for the effects of PAC on lifespans of sons without accounting for effects of paternal lifespan. We found a significant positive effect of PAC on the lifespans of sons ($t = 2.532$; $P = 0.013$; Table S5, Figure 5, Figure S5), even when the 74- days old PAC treatment was excluded ($t = 2.983$; $P = 0.004$). Lifespans of sons increased by ~0.1 days with each day of increase in PAC. The number of offspring produced by fathers had no effect on the lifespans of sons ($t = 0.054$, $P = 0.957$). In our second approach, we tested for the effects of PAC on residuals from a model that first tested for effects of paternal lifespans on lifespans of sons. Here, we found no significant effects of PAC on these residuals ($t = 1.532$; $P = 0.129$), including in our sensitivity analysis where data of sons from the 74-day old PAC treatment were excluded ($t = 1.923$; $P = 0.057$). In our third approach (Figure S6; Table S6), our path analysis suggested significant direct effects of PAC on sons' lifespan ($z = 2.443$, $P = 0.015$) and on paternal lifespan ($z = 4.662$, $P < 0.001$). This path analysis also revealed marginally non-significant effects of paternal lifespan on sons' lifespan ($z = 1.920$, $P = 0.055$), as well as marginally non-significant indirect effects of PAC on sons' lifespans via the effect of paternal lifespan ($z = 1.776$, $P = 0.076$). Results from our sensitivity analysis, where sons belonging to the 74-day old PAC treatment were excluded, showed qualitatively similar results. Specifically, increasing PAC significantly increased paternal ($z = 4.245$, $P < 0.001$) and sons' lifespans ($z = 2.723$, $P = 0.006$); paternal lifespan positively correlated with lifespan of sons ($z = 1.994$, $P = 0.046$); and there was a trend for PAC to influence sons' lifespans via the indirect effect of PAC on paternal lifespans ($z = 1.808$, $P = 0.071$). Collectively, our results for aim 3 indicate that the positive effects of advancing PAC on the lifespans of sons is partly due to older fathers living longer. This result is

in line with the viability selection hypothesis rather than Lansing effects, and is not due to trade-offs between paternal reproductive output and sons' lifespans.

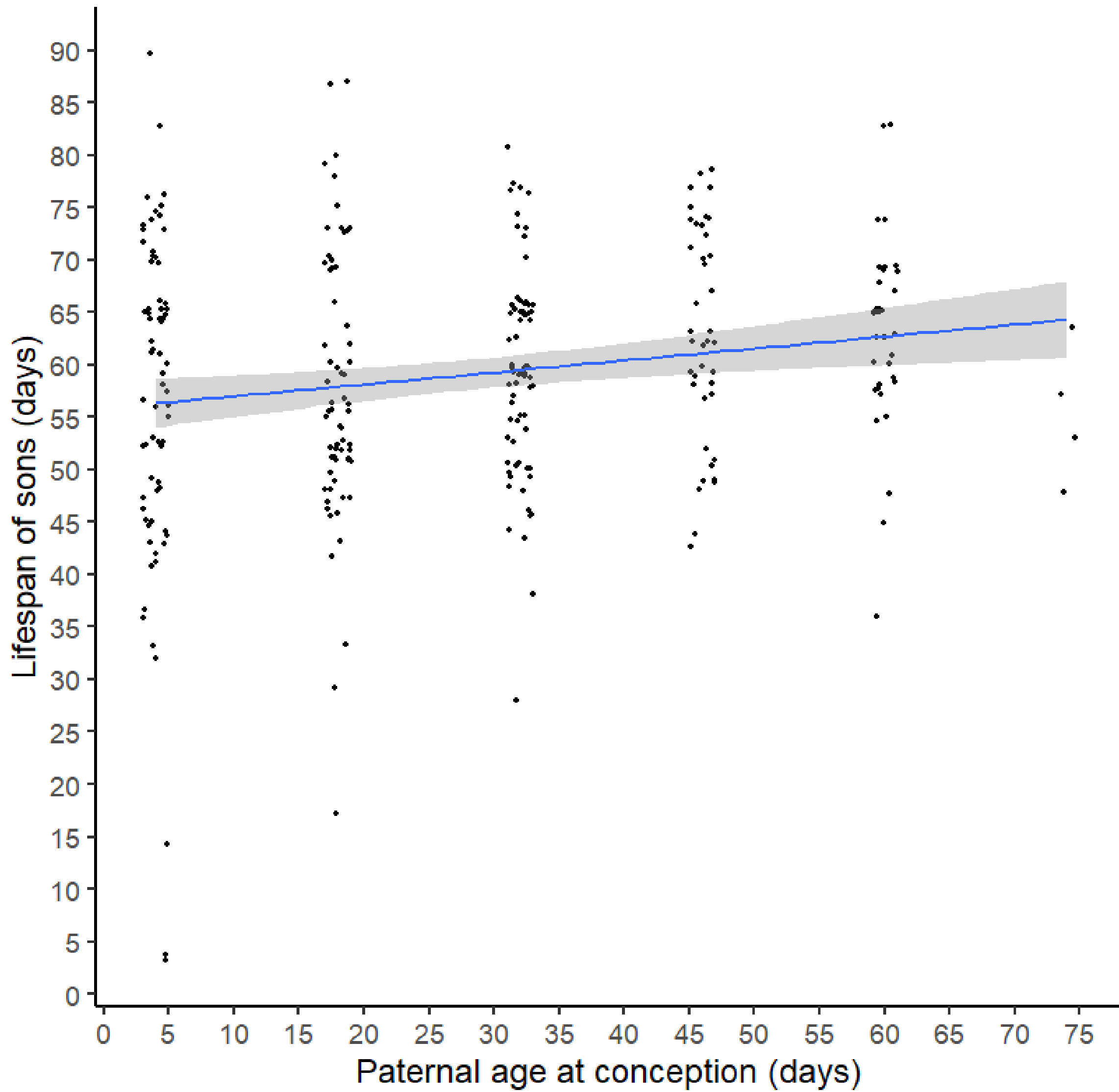


Figure 5: Increase in paternal age at conception led to an increase in the lifespans of sons. Each dot represents the lifespan of a single son, shaded areas show 95% C.I. See figure S5 for means and SE. Sample size of fathers across the six PAC treatments (from 4 to 74 days) is: 25, 25, 26, 26, 37, 5, respectively.

Discussion

Paternal age at conception can affect paternal survival and reproductive success, and the fitness of offspring in complex ways, with several hypothesized outcomes. Studying the effects of paternal age at a population-level therefore requires simultaneously testing for multiple, non-mutually exclusive processes (e.g. reproductive senescence, selective disappearance, terminal investment, Lansing effect, viability selection, trade-offs). Currently, there is a scarcity of studies adopting such an approach. We used experiments in fruit flies to investigate how paternal age at conception (PAC) affects the survival (aim 1) and reproductive output (aim 2) of fathers, and the lifespans of their sons (aim 3), and show that the effects of PAC are more complex than predicted by any single hypothesis (See Appendix 1 and 2 for predictions for each hypothesis).

Aim 1: Effects of PAC on paternal survival

We found that while increased PAC was associated with higher paternal lifespans, mating itself was related to increased mortality in older fathers. Mating is known to be energetically and physiologically costly to individuals, and can often reduce overall lifespan (Heinze, 2016; Kemp and Rutowski, 2004; Koppik et al, 2018; Partridge and Andrews, 1985; Paukku and Kotiaho, 2005; Perry and Tse, 2013; Sanghvi et al, 2021; Scharf et al, 2013). We found no differences in the overall survival of unmated males and that of the fathers who mated when young. However, we found that fathers who mated at older ages had higher mortality than fathers who mated when younger (see Clinton and Le Boeuf, 1993, who show the opposite in seals), even after removing the effects of actuarial senescence, which has not been previously reported. This result is likely due to old males being frailer and physiologically more vulnerable than young males (e.g. Bissett

and Howlett, 2019; Minois and LeBourg, 1999), such that the cost of mating affects the survival of old males more than young males.

Aim 2: Effects of PAC on paternal reproductive output

We found significant interactive effects of paternal lifespan and PAC on paternal P_S (the probability of siring an offspring). Specifically, paternal lifespan covaried positively with paternal P_S, but only for fathers who conceived at older ages. This result could be because most (>92%) males in the three youngest PAC treatments sired offspring, and there was little variance in P_S for younger PAC treatments. However, infertility became more apparent in older PAC treatments, leading to more variance in P_S for older PAC treatments. Therefore, positive covariances (that are less apparent when there is lower variance in dependent variables) between paternal lifespans and P_S in old but not young PAC treatments, might be due to statistical effects rather than true biological effects. While the covariance between lifespans and P_S could have also been negative, a positive covariance would be expected if reproductive output is condition-dependent (Bonduriansky and Brassil, 2005; Chen et al, 2016; Sultanova et al, 2020). Thus, for an older male, ceasing of offspring production could be a reliable indication of the individual's proximity to death. However, for young males this would not be so, because of low variance in P_S. Similarly, this result might indicate that the lifespans of older, but not younger males, might be a reliable cue of their reproductive quality (Brooks and Kemp, 2001; Johnson and Gemmell, 2012). Future studies could investigate whether these patterns hold true, in systems where variance in reproductive success is the same for old and young males.

We found no evidence for the terminal investment hypothesis. Specifically, older males who died soon after mating produced fewer offspring than older males who died later, or than

young males. This result is inconsistent with some studies in birds and insects, that have found older males (Duffield et al, 2018; Farchmin et al, 2020; Velando et al, 2006) and females (Creighton et al, 2009; Froy et al, 2013; Part et al, 1992) to invest terminally in reproduction. However, males in many of these studies were also immune-challenged (reviewed in Duffield et al, 2017), which can cause individuals to perceive themselves as being diseased, which was not the case in our study. Moreover, all fathers in our study only mated once. If males perceive their remaining reproductive opportunities based on the number of mates previously encountered, then the lack of variance in the number of matings for each father imposed by our study, could have eliminated variation in the perception of residual reproductive opportunities for fathers, thus preventing terminal investment. When averaged across the effects of male age, we found no evidence for paternal lifespan co-varying positively with the number of offspring sired by a male (N_s). While we found evidence for longer lived fathers having a higher probability of producing offspring, this result was significant only in older PAC treatments. Evidence for selective disappearance would require detecting positive covariances between paternal lifespan and N_s or P_s across all PAC treatments. Our results therefore do not support selective disappearance occurring across all PAC treatments in our study.

Our results showed some evidence for reproductive senescence (also seen in other fruit fly studies: Grotewiel et al, 2005; Ruhmann et al, 2018; Sepil et al, 2020). Reproductive senescence in our study was driven by old males (46 days and older) being less likely to sire an offspring, and the oldest PAC treatments (i.e. 74 days old) siring the fewest numbers of offspring. Overall, our results suggest that male reproductive output increases from mid- to adult-life, and reproductive senescence onsets in only much later in adult life (Baudisch and Stott, 2019; Jones et al, 2014; Sanghvi et al, 2024). Future studies should investigate the extent to

which the patterns of reproductive senescence observed in our study are due to declines in sperm number (Sepil et al, 2020; Gasparini et al, 2010, 2019), sperm performance (Dean et al, 2010; Vuarin et al, 2019), seminal fluid quantity (Reinhardt et al, 2019), or changes in seminal fluid composition (Fricke et al, 2023). In our study, PAC correlated with the period for which fathers remained sexually and socially isolated, therefore we cannot disentangle the role of period of isolation on paternal reproductive output.

Aim 3: Effects of PAC on lifespans of sons

Older fathers produced longer-lived sons, a result that is not in line with the Lansing effect (Crow, 2003; Lansing, 1947; Monaghan et al, 2020; Sharma et al, 2015; Wylde et al, 2019). Our results contrast with other studies in fruit flies that show a Lansing effect (e.g. Mossman et al, 2019; Price and Hanson, 1998), whereby offspring born to older fathers have lower survival. There could be several reasons for this discrepancy.

First, fathers in our experiment were kept individually isolated, without exposure to rival males, whereas Mossman et al (2019) and Price and Hanson (1998) kept males in single-sexed groups. Exposure to rivals has been shown to cause *Drosophila* males to invest more in sperm production (Bjork et al, 2007; Hopkins et al, 2019) at the cost of reduced investment in maintaining sperm quality (Koppik et al, 2023; Silva et al, 2019) compared to males kept individually. Thus, it is possible that fathers in our experiment would have been able to prevent deleterious paternal age effects mediated via deterioration in sperm quality, due to being kept individually. Second, lack of exposure to rival males in our study could have reduced the rate of sperm production, thus germline cell division and mutation rates in fathers (de Manuel et al, 2022; Crow, 2000; Girard et al, 2016; Monaghan and Metcalfe, 2019), compared to studies

showing Lansing effects that kept males in groups. This possible reduction in germline mutation rate could further buffer the effects of paternal age. Third, sons in our experiment were not mated. Life-history theory predicts trade-offs between allocating energy to somatic maintenance versus reproduction (Lemaitre et al, 2015; Stearns, 1989). Being kept unmated could have allowed sons to invest in somatic maintenance, possibly masking deleterious paternal age effects, and future studies could test this by repeating our experiments but introducing another treatment where sons mate. Fourth, we sampled fathers at extremely old lifespans (i.e. up to 74 days, representing >75% of maximum lifespan). However, studies that have shown deleterious paternal age effects in *D. melanogaster* sampled 'old' fathers between 14 and 45 days of age (e.g. Mossman et al, 2019; Nystrand and Dowling, 2014; Price and Hanson, 1998; Sepil et al, 2020). It is possible that at ages as extreme as in our study, deleterious effects of PAC are outweighed by positive effects of selection on paternal viability. Fifth, we sampled fathers cross-sectionally to create heterogeneity in paternal lifespans between PAC treatments, because we were explicitly interested in testing the viability selection hypothesis (Beck and Powell, 2000; Kokko, 1998). However, this sampling could have masked within-individual deterioration of sperm, thus the Lansing effect, which might be better revealed when fathers are sampled longitudinally.

Our results provided some support for the viability selection hypothesis (Brooks and Kemp, 2001; Hansen and Price, 1995; Kokko, 1998; Johnson and Gemmell, 2012). Specifically, fathers that mated when older had longer lifespans, and produced sons with longer lifespans. When effects of paternal lifespans on sons' lifespans were removed (i.e. second statistical approach), PAC no longer had a significant effect on the lifespans of sons. These results suggest that significant positive effects of PAC on lifespans of sons were driven by paternal lifespans. There could however, be other mechanisms which could explain the direct effect of PAC on the

lifespans of sons. For instance, poor quality offspring of old fathers, could have experienced death at the larval or pupal stage, thus eclosed sons representing a biased sample of high-quality offspring (e.g. Sanghvi et al, 2022). Additionally, a sample size of three sons per father might not have been representative of the average lifespans of sons, which could have increased noise in our results. Unlike Johnson et al (2018), we found no evidence that increased lifespans of sons from old males was due to trade-offs between paternal investment in reproductive output versus investment in offspring quality (i.e. increased lifespan of sons). Our results overall suggest that increasing PAC selects for fathers that have alleles which confer higher viability, which are then inherited by the sons of older fathers. Some previous studies have found positive effects of PAC on offspring lifespans (Angell et al, 2022; Johnson et al, 2018; Krishna et al, 2012; Lee et al, 2019; Priest et al, 2002). However, to our knowledge, ours is the first to formally test the viability selection hypothesis, by investigating separately, the direct as well as indirect effects (via paternal lifespans) of PAC, on offspring lifespans. It is possible that sons from older fathers have longer lifespans but lower reproductive output (e.g. Long and Pischedda, 2005), or that paternal age effects are sex-specific (Angell et al, 2022; Bouwhuis et al, 2015; Priest et al, 2022; Schroeder et al, 2015; Sparks et al, 2022), something future studies ought to investigate. Collectively, our results indicate that being born to old fathers need not be deleterious to offspring lifespan.

Conclusions

Our study simultaneously tests various mechanisms that may link PAC to paternal survival (age-dependent frailty) and reproductive output (reproductive senescence, selective disappearance, terminal investment), and offspring lifespans (Lansing effect, viability selection, trade-offs

between paternal investment in offspring quality versus quantity). We show that mating at older ages can lead to an increased risk of mortality for fathers, thus buffering the magnitude of positive covariances between PAC and paternal lifespans. We further reveal that positive pleiotropy between survival and reproductive output depends on the age of the father. We also find that senescence in reproductive output occurs late in life, and its onset differs for the likelihood of producing offspring versus the number of offspring produced. Lastly, we show that advancing PAC has a positive effect on the lifespans of sons, and that this effect is likely due to older fathers having longer lifespans. Our study highlights that demographic processes such as differential mortality risk and viability selection can mask possible within-individual deterioration in reproduction. We thus recommend that future studies employ a broader theoretical framework that weighs costs of reproducing at an old age against its direct and indirect benefits, to better understand organismal health and life-history. Fitness benefits provided by old fathers producing longer lived sons, might offset the fitness costs of reduced fertility in old fathers. If so, this offset could lead to the evolution of female preference for old males (Beck and Powell, 2000; Beck and Promislow, 2007; Beck et al, 2002; Johnson and Gemmell, 2012; Kokko and Lindstrom, 1996), and future studies can investigate this exciting but relatively unexplored avenue of research.

Data archiving

Data and all associated code are available on OSF with DOI: 10.17605/OSF.IO/38CXN at https://osf.io/38cxn/. Supplementary information is provided as a separate word file, along with this manuscript.

References

Aguilar, P., Dag, B., Carazo, P., Sultanova, Z., 2023. Sex-specific paternal age effects on offspring quality in *Drosophila melanogaster*. J of Evolutionary Biology jeb.14167. https://doi.org/10.1111/jeb.14167

Aich, U., Chowdhury, S., Jennions, M.D., 2022. Separating the effects of paternal age and mating history: Evidence for sex-specific paternal effect in eastern mosquitofish. Evolution 76, 1565–1577. https://doi.org/10.1111/evo.14498

Angell, C.S., Janacek, R., Rundle, H.D., 2022. Maternal and Paternal Age Effects on Male Antler Flies: A Field Experiment. The American Naturalist 199, 436–442. https://doi.org/10.1086/718236

Arslan, R.C., Willführ, K.P., Frans, E.M., Verweij, K.J.H., Bürkner, P.-C., Myrskylä, M., Voland, E., Almqvist, C., Zietsch, B.P., Penke, L., 2017. Older fathers' children have lower evolutionary fitness across four centuries and in four populations. Proc. R. Soc. B. 284, 20171562. https://doi.org/10.1098/rspb.2017.1562

Avent, T.D., Price, T.A.R., Wedell, N., 2008. Age-based female preference in the fruit fly Drosophila pseudoobscura. Animal Behaviour 75, 1413–1421. https://doi.org/10.1016/j.anbehav.2007.09.015

Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting Linear Mixed-Effects Models using lme4. https://doi.org/10.48550/ARXIV.1406.5823

Baudisch, A., Stott, I., 2019. A pace and shape perspective on fertility. Methods Ecol Evol 10, 1941–1951. https://doi.org/10.1111/2041-210X.13289

Beck, C., Powell, 2000. Evolution of female mate choice based on male age: Are older males better mates? Evolutionary Ecology Research 2, 107–118.

Beck, C.W., Promislow, D.E.L., 2007. Evolution of Female Preference for Younger Males. PLoS ONE 2, e939. https://doi.org/10.1371/journal.pone.0000939

Beck, C., Shapiro, B., Choksi, S., Promislow, D., 2002. A genetic algorithm approach to study the evolution of female preference based on male age. Evolutionary Ecology research 4, 275–292.

Benowitz, K.M., Head, M.L., Williams, C.A., Moore, A.J., Royle, N.J., 2013. Male age mediates reproductive investment and response to paternity assurance. Proc. R. Soc. B. 280, 20131124. https://doi.org/10.1098/rspb.2013.1124

Bisset, E.S., Howlett, S.E., 2019. The biology of frailty in humans and animals: Understanding frailty and promoting translation. Aging Med 2, 27–34. https://doi.org/10.1002/agm2.12058

Bjork, A., Dallai, R., Pitnick, S., 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca* , a species with giant sperm. Biol. Lett. 3, 517–519. https://doi.org/10.1098/rsbl.2007.0219

Bonduriansky, R., Brassil, C.E., 2005. Reproductive ageing and sexual selection on male body size in a wild population of antler flies (Protopiophila litigata): Ageing and sexual selection. Journal of Evolutionary Biology 18, 1332–1340. https://doi.org/10.1111/j.1420-9101.2005.00957.x

Bouwhuis, S., Sheldon, B.C., Verhulst, S., Charmantier, A., 2009. Great tits growing old: selective disappearance and the partitioning of senescence to stages within the breeding cycle. Proc. R. Soc. B. 276, 2769–2777. https://doi.org/10.1098/rspb.2009.0457

Bouwhuis, S., Vedder, O., Becker, P.H., 2015. Sex-specific pathways of parental age effects on offspring lifetime reproductive success in a long-lived seabird: SEX-SPECIFIC PARENTAL EFFECTS ON OFFSPRING FITNESS. Evolution 69, 1760–1771. https://doi.org/10.1111/evo.12692

Bowen, W.D., Iverson, S.J., Mcmillan, J.I., Boness, D.J., 2006. Reproductive performance in grey seals: age-related improvement and senescence in a capital breeder. J Anim Ecology 75, 1340–1351. https://doi.org/10.1111/j.1365-2656.2006.01157.x

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Mächler, M., Bolker, B.M., 2017. Modeling zero-inflated count data with glmmTMB (preprint). Ecology. https://doi.org/10.1101/132753

Brooks, R., Kemp, D.J., 2001. Can older males deliver the good genes? Trends in Ecology & Evolution 16, 308–313. https://doi.org/10.1016/S0169-5347(01)02147-4

Chen, H., Maklakov, A.A., 2012. Longer Life Span Evolves under High Rates of Condition-Dependent Mortality. Current Biology 22, 2140–2143. https://doi.org/10.1016/j.cub.2012.09.021

Chen, H. -y., Spagopoulou, F., Maklakov, A.A., 2016. Evolution of male age-specific reproduction under differential risks and causes of death: males pay the cost of high female fitness. J. Evol. Biol. 29, 848–856. https://doi.org/10.1111/jeb.12833

Clancy, David J., and W. Jason Kennington. 2001. A simple method to achieve consistent larval density in bottle cultures. Dros. Inf. Serv. 84: 168-169.

Clinton, W.L., Le Boeuf, B.J., 1993. Sexual Selection's Effects on Male Life History and the Pattern of Male Mortality. Ecology 74, 1884–1892. https://doi.org/10.2307/1939945

Cope, H., Ivimey-Cook, E.R., Moorad, J., 2022. Triparental ageing in a laboratory population of an insect with maternal care. Behavioral Ecology 33, 1123–1132. https://doi.org/10.1093/beheco/arac078

Cooper, E.B., Bonnet, T., Osmond, H., Cockburn, A., Kruuk, L.E.B., 2020. Do the ages of parents or helpers affect offspring fitness in a cooperatively breeding bird? J of Evolutionary Biology 33, 1735–1748. https://doi.org/10.1111/jeb.13712

Cooper, E.B., Bonnet, T., Osmond, H.L., Cockburn, A., Kruuk, L.E.B., 2021. Aging and Senescence across Reproductive Traits and Survival in Superb Fairy-Wrens (*Malurus cyaneus*). The American Naturalist 197, 111–127. https://doi.org/10.1086/711755

Cornwallis, C.K., Dean, R., Pizzari, T., 2014. Sex-specific patterns of aging in sexual ornaments and gametes. The American Naturalist 184, E66–E78. https://doi.org/10.1086/677385

Creighton, J.C., Heflin, N.D., Belk, M.C., 2009. Cost of Reproduction, Resource Quality, and Terminal Investment in a Burying Beetle. The American Naturalist 174, 673–684. https://doi.org/10.1086/605963
Crow, J.F., 2003. There's Something Curious About Paternal-Age Effects. Science 301, 606–607. https://doi.org/10.1126/science.1088552
Dean, R., Cornwallis, C.K., Løvlie, H., Worley, K., Richardson, D.S., Pizzari, T., 2010. Male Reproductive Senescence Causes Potential for Sexual Conflict over Mating. Current Biology 20, 1192–1196. https://doi.org/10.1016/j.cub.2010.04.059
de Manuel, M., Wu, F.L., Przeworski, M., 2022. A paternal bias in germline mutation is widespread in amniotes and can arise independently of cell division numbers. eLife 11, e80008. https://doi.org/10.7554/eLife.80008
Dore, A.A., Rostant, W.G., Bretman, A., Chapman, T., 2021. Plastic male mating behavior evolves in response to the competitive environment. Evolution 75, 101–115. https://doi.org/10.1111/evo.14089
Dormann, C.F., Elith, J., Bacher, S., Buchmann, C., Carl, G., Carré, G., Marquéz, J.R.G., Gruber, B., Lafourcade, B., Leitão, P.J., Münkemüller, T., McClean, C., Osborne, P.E., Reineking, B., Schröder, B., Skidmore, A.K., Zurell, D., Lautenbach, S., 2013. Collinearity: a review of methods to deal with it and a simulation study evaluating their performance. Ecography 36, 27–46. https://doi.org/10.1111/j.1600-0587.2012.07348.x
Duffield, K.R., Bowers, E.K., Sakaluk, S.K., Sadd, B.M., 2017. A dynamic threshold model for terminal investment. Behav Ecol Sociobiol 71, 185. https://doi.org/10.1007/s00265-017-2416-z
Duffield, K.R., Hampton, K.J., Houslay, T.M., Hunt, J., Rapkin, J., Sakaluk, S.K., Sadd, B.M., 2018. Age-dependent variation in the terminal investment threshold in male crickets. Evolution 72, 578–589. https://doi.org/10.1111/evo.13443
Engqvist, L., 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. Animal Behaviour 70, 967–971. https://doi.org/10.1016/j.anbehav.2005.01.016
Farchmin, P.A., Eggert, A.-K., Duffield, K.R., Sakaluk, S.K., 2020. Dynamic terminal investment in male burying beetles. Animal Behaviour 163, 1–7. https://doi.org/10.1016/j.anbehav.2020.02.015
Fay, R., Barbraud, C., Delord, K., Weimerskirch, H., 2016. Paternal but not maternal age influences early-life performance of offspring in a long-lived seabird. Proc. R. Soc. B. 283, 20152318. https://doi.org/10.1098/rspb.2015.2318
Finch, C.E., 2009. Update on Slow Aging and Negligible Senescence – A Mini-Review. Gerontology 55, 307–313. https://doi.org/10.1159/000215589
Foo, Y.Z., Lagisz, M., O'Dea, R.E., Nakagawa, S., 2023. The influence of immune challenges on the mean and variance in reproductive investment: a meta-analysis of the terminal investment hypothesis. BMC Biol 21, 107. https://doi.org/10.1186/s12915-023-01603-4
Forslund, P., Pärt, T., 1995. Age and reproduction in birds — hypotheses and tests. Trends in Ecology & Evolution 10, 374–378. https://doi.org/10.1016/S0169-5347(00)89141-7
Fricke, C., Sanghvi, K., Emery, M., Lindenbaum, I., Wigby, S., Ramm, S.A., Sepil, I., 2023. Timeless or tainted? The effects of male ageing on seminal fluid. Front. Ecol. Evol. 11, 1066022. https://doi.org/10.3389/fevo.2023.1066022
Froy, H., Phillips, R.A., Wood, A.G., Nussey, D.H., Lewis, S., 2013. Age-related variation in reproductive traits in the wandering albatross: evidence for terminal improvement following senescence. Ecol Lett 16, 642–649. https://doi.org/10.1111/ele.12092
Gasparini, C., Devigili, A., Pilastro, A., 2019. Sexual selection and ageing: interplay between pre- and post-copulatory traits senescence in the guppy. Proc. R. Soc. B. 286, 20182873. https://doi.org/10.1098/rspb.2018.2873
Gasparini, C., Marino, I.A.M., Boschetto, C., Pilastro, A., 2010. Effect of male age on sperm traits and sperm competition success in the guppy (*Poecilia reticulata*). Journal of Evolutionary Biology 23, 124–135. https://doi.org/10.1111/j.1420-9101.2009.01889.x
Girard, S.L., Bourassa, C.V., Lemieux Perreault, L.-P., Legault, M.-A., Barhdadi, A., Ambalavanan, A., Brendgen, M., Vitaro, F., Noreau, A., Dionne, G., Tremblay, R.E., Dion, P.A., Boivin, M., Dubé, M.-P., Rouleau, G.A., 2016. Paternal Age Explains a Major Portion of De Novo Germline Mutation Rate Variability in Healthy Individuals. PLoS ONE 11, e0164212. https://doi.org/10.1371/journal.pone.0164212
Girndt, A., Cockburn, G., Sánchez-Tójar, A., Hertel, M., Burke, T., Schroeder, J., 2019. Male age and its association with reproductive traits in captive and wild house sparrows. J Evol Biol 32, 1432–1443. https://doi.org/10.1111/jeb.13542
Grotewiel, M.S., Martin, I., Bhandari, P., Cook-Wiens, E., 2005. Functional senescence in Drosophila melanogaster. Ageing Research Reviews 4, 372–397. https://doi.org/10.1016/j.arr.2005.04.001
Hartig, F. and Hartig, M.F., 2017. Package 'DHARMa'. *R package*. https://cran.r-hub.io/web/packages/DHARMa/DHARMa.pdf
Hämäläinen, A., Dammhahn, M., Aujard, F., Eberle, M., Hardy, I., Kappeler, P.M., Perret, M., Schliehe-Diecks, S., Kraus, C., 2014. Senescence or selective disappearance? Age trajectories of body mass in wild and captive populations of a small-bodied primate. Proc. R. Soc. B. 281, 20140830. https://doi.org/10.1098/rspb.2014.0830
Hansen, T.F., Price, D.K., 1995. Good genes and old age: Do old mates provide superior genes? J Evolution Biol 8, 759–778. https://doi.org/10.1046/j.1420-9101.1995.8060759.x
Harris, W.E., Uller, T., 2009. Reproductive investment when mate quality varies: differential allocation versus reproductive compensation. Phil. Trans. R. Soc. B 364, 1039–1048. https://doi.org/10.1098/rstb.2008.0299
Heinze, J., 2016. The male has done his work — the male may go. Current Opinion in Insect Science 16, 22–27. https://doi.org/10.1016/j.cois.2016.05.005
Heinze, J., Hanoeffner, M., Delabie, J.H.C., Schrempf, A., 2018. Methuselah's daughters: Paternal age has little effect on offspring number and quality in *Cardiocondyla* ants. Ecol Evol 8, 12066–12072. https://doi.org/10.1002/ece3.4666

Hopkins, B.R., Sepil, I., Thézénas, M.-L., Craig, J.F., Miller, T., Charles, P.D., Fischer, R., Kessler, B.M., Bretman, A., Pizzari, T., Wigby, S., 2019. Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 116, 17925–17933. https://doi.org/10.1073/pnas.1906149116

Ivimey-Cook, E.R., Shorr, S., Moorad, J.A., 2023. The distribution of the Lansing Effect across animal species. Evolution 77, 608–615. https://doi.org/10.1093/evolut/qpac045

Janecka, M., Mill, J., Basson, M.A., Goriely, A., Spiers, H., Reichenberg, A., Schalkwyk, L., Fernandes, C., 2017. Advanced paternal age effects in neurodevelopmental disorders—review of potential underlying mechanisms. Transl Psychiatry 7, e1019–e1019. https://doi.org/10.1038/tp.2016.294

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 off-set costs related to fertility, or are we missing something else? Bioessays 34, 609–619. https://doi.org/10.1002/bies.201100157

Johnson, S.L., Dunleavy, J., Gemmell, N.J., Nakagawa, S., 2015. Consistent age-dependent declines in human semen quality: A systematic review and meta-analysis. Ageing Research Reviews 19, 22–33. https://doi.org/10.1016/j.arr.2014.10.007

Johnson, S.L., Zellhuber-McMillan, S., Gillum, J., Dunleavy, J., Evans, J.P., Nakagawa, S., Gemmell, N.J., 2018. Evidence that fertility trades off with early offspring fitness as males age. Proc. R. Soc. B. 285, 20172174. https://doi.org/10.1098/rspb.2017.2174

Jones, O.R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C.G., Schaible, R., Casper, B.B., Dahlgren, J.P., Ehrlén, J., García, M.B., Menges, E.S., Quintana-Ascencio, P.F., Caswell, H., Baudisch, A., Vaupel, J.W., 2014. Diversity of ageing across the tree of life. Nature 505, 169–173. https://doi.org/10.1038/nature12789

Jones, O.R., Vaupel, J.W., 2017. Senescence is not inevitable. Biogerontology 18, 965–971. https://doi.org/10.1007/s10522-017-9727-3

Jones, T.M., Elgar, M.A., 2004. The role of male age, sperm age and mating history on fecundity and fertilization success in the hide beetle. Proc. R. Soc. Lond. B 271, 1311–1318. https://doi.org/10.1098/rspb.2004.2723

Kemp, D.J., Rutowski, R.L., 2004. A survival cost to mating in a polyandrous butterfly, *Colias eurytheme*. Oikos 105, 65–70. https://doi.org/10.1111/j.0030-1299.2004.12874.x

Kokko, H., 1998. Good genes, old age and life-history trade-offs. Evolutionary Ecology 12, 739–750. https://doi.org/10.1023/A:1006541701002

Kokko, H., Lindström, J., 1996. Evolution of female preference for old mates. Proc. R. Soc. Lond. B 263, 1533–1538. https://doi.org/10.1098/rspb.1996.0224

Koppik, M., Baur, J., Berger, D., 2023. Increased male investment in sperm competition results in reduced maintenance of gametes. PLoS Biol 21, e3002049. https://doi.org/10.1371/journal.pbio.3002049

Koppik, M., Ruhmann, H., Fricke, C., 2018. The effect of mating history on male reproductive ageing in Drosophila melanogaster. Journal of Insect Physiology 111, 16–24. https://doi.org/10.1016/j.jinsphys.2018.10.003

Krishna, M.S., Santhosh, H.T., Hegde, S., 2012. Offspring of Older Males Are Superior in Drosophila Bipectinata. Zoological Studies 51.

Kroeger, S.B., Blumstein, D.T., Armitage, K.B., Reid, J.M., Martin, J.G.A., 2020. Older mothers produce more successful daughters. Proc. Natl. Acad. Sci. U.S.A. 117, 4809–4814. https://doi.org/10.1073/pnas.1908551117

Lansing, A.I., 1947. A Transmissible, Cumulative, and Reversible Factor in Aging. Journal of Gerontology 2, 228–239. https://doi.org/10.1093/geronj/2.3.228

Lee, J., Seo, W., Lee, S., Lee, H., Min, K., 2019. Strain-specific effects of parental age on offspring in *DROSOPHILA MELANOGASTER* . Entomological Research 49, 187–202. https://doi.org/10.1111/1748-5967.12344

Lee, R., Chu, C.Y.C., 2023. Theoretical perspectives on reproductive aging. Front. Ecol. Evol. 10, 934732. https://doi.org/10.3389/fevo.2022.934732

Lemaître, J.-F., Berger, V., Bonenfant, C., Douhard, M., Gamelon, M., Plard, F., Gaillard, J.-M., 2015. Early-late life trade-offs and the evolution of ageing in the wild. Proc. R. Soc. B. 282, 20150209. https://doi.org/10.1098/rspb.2015.0209

Lewis EB. 1960. A new standard food medium. Drosophila Information Service 34: 117–118.

Lifjeld, J.T., Kleven, O., Fossøy, F., Jacobsen, F., Laskemoen, T., Rudolfsen, G., Robertson, R.J., 2022. When Older Males Sire More Offspring—Increased Attractiveness or Higher Fertility? Behav Ecol Sociobiol 76, 61. https://doi.org/10.1007/s00265-022-03170-0

Long, T.A.F., Pischedda, A., 2005. Do female *Drosophila melanogaster* adaptively bias offspring sex ratios in relation to the age of their mate? Proc. R. Soc. B. 272, 1781–1787. https://doi.org/10.1098/rspb.2005.3165

McCleery, R.H., Perrins, C.M., Sheldon, B.C., Charmantier, A., 2008. Age-specific reproduction in a long-lived species: the combined effects of senescence and individual quality. Proc. R. Soc. B. 275, 963–970. https://doi.org/10.1098/rspb.2007.1418

Minois, N., Le Bourg, É., 1999. Resistance to stress as a function of age in Drosophila melanogaster living in hypergravity. Mechanisms of Ageing and Development 109, 53–64. https://doi.org/10.1016/S0047-6374(99)00025-1

Mirhosseini, M.A., Michaud, J.P., Jalali, M.A., Ziaaddini, M., 2014. Paternal effects correlate with female reproductive stimulation in the polyandrous ladybird *Cheilomenes sexmaculata*. Bull. Entomol. Res. 104, 480–485. https://doi.org/10.1017/S0007485314000194

Monaghan, P., Charmantier, A., Nussey, D.H., Ricklefs, R.E., 2008. The evolutionary ecology of senescence. Functional Ecology 22, 371–378. https://doi.org/10.1111/j.1365-2435.2008.01418.x

Monaghan, P., Maklakov, A.A., Metcalfe, N.B., 2020. Intergenerational Transfer of Ageing: Parental Age and Offspring Lifespan. Trends in Ecology & Evolution 35, 927–937. https://doi.org/10.1016/j.tree.2020.07.005

Monaghan, P., Metcalfe, N.B., 2019. The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. Proc. R. Soc. B. 286, 20192187. https://doi.org/10.1098/rspb.2019.2187
Mossman, J.A., Mabeza, R.M.S., Blake, E., Mehta, N., Rand, D.M., 2019. Age of Both Parents Influences Reproduction and Egg Dumping Behavior in Drosophila melanogaster. Journal of Heredity 110, 300–309. https://doi.org/10.1093/jhered/esz009
Moullec, H., Reichert, S., Bize, P., 2023. Aging trajectories are trait- and sex-specific in the long-lived Alpine swift. Front. Ecol. Evol. 11, 983266. https://doi.org/10.3389/fevo.2023.983266
Mueller, U., 2004. Does Late Reproduction Extend the Life Span? Findings from European Royalty. Population & Development Review 30, 449–466. https://doi.org/10.1111/j.1728-4457.2004.00023.x
Naciri, M., Aars, J., Blanchet, M.-A., Gimenez, O., Cubaynes, S., 2022. Reproductive senescence in polar bears in a variable environment. Front. Ecol. Evol. 10, 920481. https://doi.org/10.3389/fevo.2022.920481
Nystrand, M., Dowling, D.K., 2014. Transgenerational interactions involving parental age and immune status affect female reproductive success in *Drosophila melanogaster*. Proc. R. Soc. B. 281, 20141242. https://doi.org/10.1098/rspb.2014.1242
Pappert, F.A., Kolbe, D., Dubin, A., Roth, O., 2023. The effect of parental age on the quantity and quality of offspring in *Syngnathus typhle* , a species with male pregnancy (preprint). Evolutionary Biology. https://doi.org/10.1101/2023.06.12.544574
Part, T., Gustafsson, L., Moreno, J., 1992. “Terminal Investment” and a Sexual Conflict in the Collared Flycatcher (Ficedula albicollis). The American Naturalist 140, 868–882. https://doi.org/10.1086/285445
Partridge, L., Andrews, R., 1985. The effect of reproductive activity on the longevity of male Drosophila melanogaster is not caused by an acceleration of ageing. Journal of Insect Physiology 31, 393–395. https://doi.org/10.1016/0022-1910(85)90084-8
Paukku, S., Kotiaho, J.S., 2005. Cost of reproduction in Callosobruchus maculatus: effects of mating on male longevity and the effect of male mating status on female longevity. Journal of Insect Physiology 51, 1220–1226. https://doi.org/10.1016/j.jinsphys.2005.06.012
Perry, J.C., Tse, C.T., 2013. Extreme Costs of Mating for Male Two-Spot Ladybird Beetles. PLoS ONE 8, e81934. https://doi.org/10.1371/journal.pone.0081934
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B. and Maintainer, R., 2017. Package ‘nlme’. *Linear and nonlinear mixed effects models, version*, *3*, 274. https://cran.r-project.org/web/packages/nlme/nlme.pdf
Prathibha, M., Krishna, M.S., Jayaramu, S.C., 2011. Male age influence on male reproductive success in *Drosophila ananassae* (Diptera: Drosophilidae). Italian Journal of Zoology 78, 168–173. https://doi.org/10.1080/11250003.2011.564214
Preston, B.T., Saint Jalme, M., Hingrat, Y., Lacroix, F., Sorci, G., 2015. The sperm of aging male bustards retards their offspring’s development. Nat Commun 6, 6146. https://doi.org/10.1038/ncomms7146
Price, D.K., Hansen, T.F., 1998. How Does Offspring Quality Change with Age in Male Drosophila Melanogaster? Behavior Genetics 28, 395–402. https://doi.org/10.1023/A:1021677804038
Priest, N.K., Mackowiak, B., Promislow, D.E.L., 2002. THE ROLE OF PARENTAL AGE EFFECTS ON THE EVOLUTION OF AGING. Evolution 56, 927–935. https://doi.org/10.1111/j.0014-3820.2002.tb01405.x
Reid, J.M., Bignal, E.M., Bignal, S., McCracken, D.I., Bogdanova, M.I., Monaghan, P., 2010. Parent age, lifespan and offspring survival: structured variation in life history in a wild population. Journal of Animal Ecology. https://doi.org/10.1111/j.1365-2656.2010.01669.x
Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2011. Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, Cimex lectularius. PLoS ONE 6, e22082. https://doi.org/10.1371/journal.pone.0022082
Reznick, D.N., Bryant, M.J., Roff, D., Ghalambor, C.K., Ghalambor, D.E., 2004. Effect of extrinsic mortality on the evolution of senescence in guppies. Nature 431, 1095–1099. https://doi.org/10.1038/nature02936
Rose, M., Charlesworth, B., 1980. A test of evolutionary theories of senescence. Nature 287, 141–142. https://doi.org/10.1038/287141a0
Rosseel, Y., 2012. **lavaan** : An *R* Package for Structural Equation Modeling. J. Stat. Soft. 48. https://doi.org/10.18637/jss.v048.i02
Ruhmann, H., Koppik, M., Wolfner, M.F., Fricke, C., 2018. The impact of ageing on male reproductive success in Drosophila melanogaster. Experimental Gerontology 103, 1–10. https://doi.org/10.1016/j.exger.2017.12.013
Sandfoss, M.R., Reichling, S., Roberts, B.M., 2023. Sperm morphology and forward motility are indicators of reproductive success and are not age- or condition-dependent in a captive breeding population of endangered snake. PLoS ONE 18, e0282845. https://doi.org/10.1371/journal.pone.0282845
Sanghvi, K., Iglesias-Carrasco, M., Zajitschek, F., Kruuk, L.E.B., Head, M.L., 2022. Effects of developmental and adult environments on ageing. Evolution 76, 1868–1882. https://doi.org/10.1111/evo.14567
Sanghvi, K., Zajitschek, F., Iglesias-Carrasco, M., Head, M.L., 2021. Sex- and trait-specific silver-spoon effects of developmental environments, on ageing. Evol Ecol 35, 367–385. https://doi.org/10.1007/s10682-021-10115-y
Sanghvi, K., Vega-Trejo, R., Nakagawa, S., Gascoigne, S.J.L., Johnson, S.L., Salguero-Gómez, R., Pizzari, T., Sepil, I., 2024. Meta-analysis shows no consistent evidence for senescence in ejaculate traits across animals. Nat Commun 15, 558. https://doi.org/10.1038/s41467-024-44768-4
Santhosh, H.T., Krishna, M.S., 2013. Relationship between Male Age, Accessory Gland, Sperm Transferred, and Fitness Traits in *Drosophila bipectinata*. Journal of Insect Science 13, 1–14. https://doi.org/10.1673/031.013.15901
Scharf, I., Peter, F., Martin, O.Y., 2013. Reproductive Trade-Offs and Direct Costs for Males in Arthropods. Evol Biol 40, 169–184. https://doi.org/10.1007/s11692-012-9213-4
Schroeder, J., Nakagawa, S., Rees, M., Mannarelli, M.-E., Burke, T., 2015. Reduced fitness in progeny from old parents in a natural population. Proc. Natl. Acad. Sci. U.S.A. 112, 4021–4025. https://doi.org/10.1073/pnas.1422715112

Segami, J.C., Lind, M.I., Qvarnström, A., 2021. Should females prefer old males? Evolution Letters 5, 507–520. https://doi.org/10.1002/evl3.250
Sepil, I., Hopkins, B.R., Dean, R., Bath, E., Friedman, S., Swanson, B., Ostridge, H.J., Harper, L., Buehner, N.A., Wolfner, M.F., Konietzny, R., Thézénas, M.-L., Sandham, E., Charles, P.D., Fischer, R., Steinhauer, J., Kessler, B.M., Wigby, S., 2020. Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 117, 17094–17103. https://doi.org/10.1073/pnas.2009053117
Sharma, R., Agarwal, A., Rohra, V.K., Assidi, M., Abu-Elmagd, M., Turki, R.F., 2015. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13, 35. https://doi.org/10.1186/s12958-015-0028-x
Silva, W.T.A.F., Sáez-Espinosa, P., Torijo-Boix, S., Romero, A., Devaux, C., Durieux, M., Gómez-Torres, M.J., Immler, S., 2019. The effects of male social environment on sperm phenotype and genome integrity. J Evol Biol 32, 535–544. https://doi.org/10.1111/jeb.13435
Sparks, A.M., Hammers, M., Komdeur, J., Burke, T., Richardson, D.S., Dugdale, H.L., 2022. Sex-dependent effects of parental age on offspring fitness in a cooperatively breeding bird. Evolution Letters evl3.300. https://doi.org/10.1002/evl3.300
Stearns, S.C., 1989. Trade-Offs in Life-History Evolution. Functional Ecology 3, 259. https://doi.org/10.2307/2389364
Sultanova, Z., García-Roa, R., Carazo, P., 2020. Condition-dependent mortality exacerbates male (but not female) reproductive senescence and the potential for sexual conflict. J Evol Biol 33, 1086–1096. https://doi.org/10.1111/jeb.13636
Therneau, T., Lumley, T., 2013. R survival package. *R Core Team*.
Therneau, T.M., Therneau, M.T.M., 2015. Package 'coxme'. R package version, 2, 5.
Torres, R., Drummond, H., Velando, A., 2011. Parental Age and Lifespan Influence Offspring Recruitment: A Long-Term Study in a Seabird. PLoS ONE 6, e27245. https://doi.org/10.1371/journal.pone.0027245
Travers, L.M., Carlsson, H., Lind, M.I., Maklakov, A.A., 2021. Beneficial cumulative effects of old parental age on offspring fitness. Proc. R. Soc. B. 288, 20211843. https://doi.org/10.1098/rspb.2021.1843
van de Pol, M., Verhulst, S., 2006. Age-Dependent Traits: A New Statistical Model to Separate Within- and Between-Individual Effects. The American Naturalist 167, 766–773. https://doi.org/10.1086/503331
Vega-Trejo, R., Fox, R.J., Iglesias-Carrasco, M., Head, M.L., Jennions, M.D., 2019. The effects of male age, sperm age and mating history on ejaculate senescence. Funct Ecol 33, 1267–1279. https://doi.org/10.1111/1365-2435.13305
Velando, A., Drummond, H., Torres, R., 2006. Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. Proc. R. Soc. B. 273, 1443–1448. https://doi.org/10.1098/rspb.2006.3480
Verspoor, R.L., Cuss, M., Price, T.A.R., 2015. Age-based mate choice in the monandrous fruit fly Drosophila subobscura. Animal Behaviour 102, 199–207. https://doi.org/10.1016/j.anbehav.2015.01.024
Vuarin, P., Lesobre, L., Levêque, G., Saint Jalme, M., Lacroix, F., Hingrat, Y., Sorci, G., 2021. Paternal age negatively affects sperm production of the progeny. Ecology Letters 24, 719–727. https://doi.org/10.1111/ele.13696
Wylde, Z., Spagopoulou, F., Hooper, A.K., Maklakov, A.A., Bonduriansky, R., 2019. Parental breeding age effects on descendants' longevity interact over 2 generations in matrilines and patrilines. PLoS Biol 17, e3000556. https://doi.org/10.1371/journal.pbio.3000556

Supplementary information

Appendix 1: Effects of paternal age on paternal survival (hypotheses and predictions)

It is commonly assumed that as age at conception increases, lifespan should increase, i.e., fathers who mate at an older age should have higher lifespans (H1A). A corollary prediction is that at older age groups, there should be lower variances in lifespan. These predictions are based on the fact that individuals who mate at an older age have a lower limit for what lifespans they can have, because they have by definition survived to the age at which they conceive. For instance, if males mate at age 60 days, all males have survived until at least 60 days of age. If all individuals have a similar upper limit to lifespans, irrespective of the age at which they mate, then older ages of conception would have lower variance but higher means in lifespans. This leads to the effects of age at conception on lifespan to look as follows (Figure A):

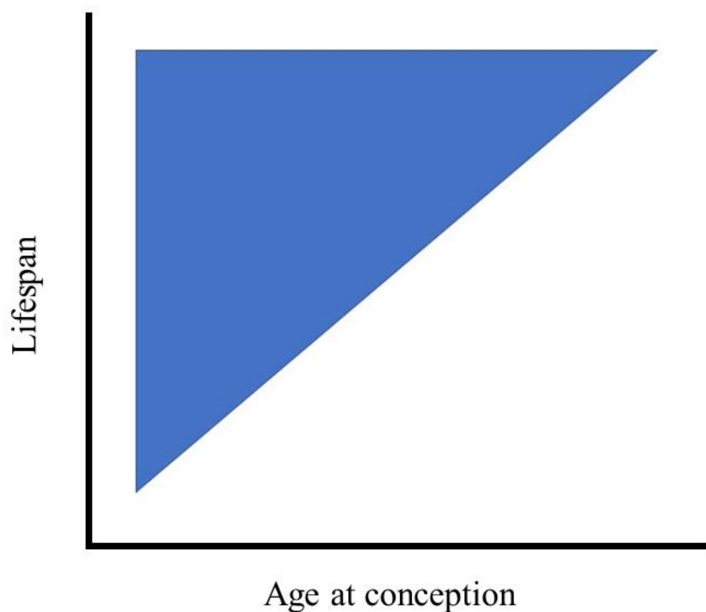


Figure A: Advancing age at conception leads to higher means but lower variance in lifespan of older individuals

However, this prediction has two assumptions which are not usually explicitly tested. The first assumption is that individuals in a population start dying from birth onwards. Thus, all age classes at which individuals conceive offspring, would have experienced some mortality, even if the rate of mortality would only increase after maturity. However, it is possible that death in a population does not occur until a certain age (e.g. if survival curves are sigmoid shaped, and risk of mortality only increases after a certain age). Here, the lower limit for lifespans for various ages at conception might be the same. For instance, if in a population, males mate at ages 10, 20, 30, 40 …. to 90 days of age, but no males die until 40 days of age, then for males who mate at ages 10, 20, 30, and 40 days, the bottom limit of lifespan will be 40 days of age. This can lead to effects of age at conception on lifespan to look as follows (Figure B):

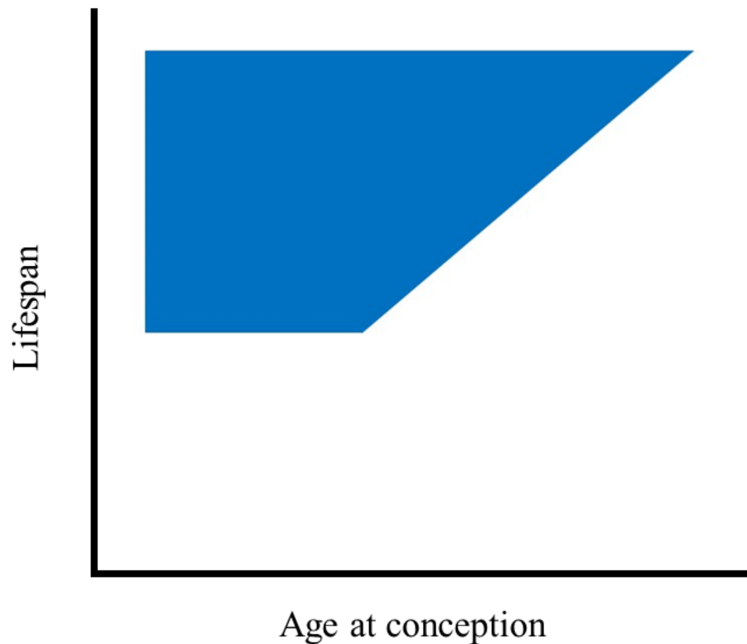


Figure B: Advancing age at conception leads to higher means but lower variance in lifespan of older individuals, however, mortality does not occur until a certain age, thus age at conception groups until that age have the same average lifespans.

The second assumption is that the upper limit for lifespans across all ages of conception is the same. That is, irrespective of at what age individuals reproduce, they have the same probability of surviving until the upper limit of lifespan for that population. This however might not always be true. For instance, if older males are frailer than younger males, males mated when old might have a lower ceiling for longevity. An example of this is if mating related stress increases mortality in males who are more vulnerable (e.g. old mated males), thus reduces lifespans of males who mate when old but not when they mate young. This can lead to effects of age at conception on lifespan to look like Figure C.

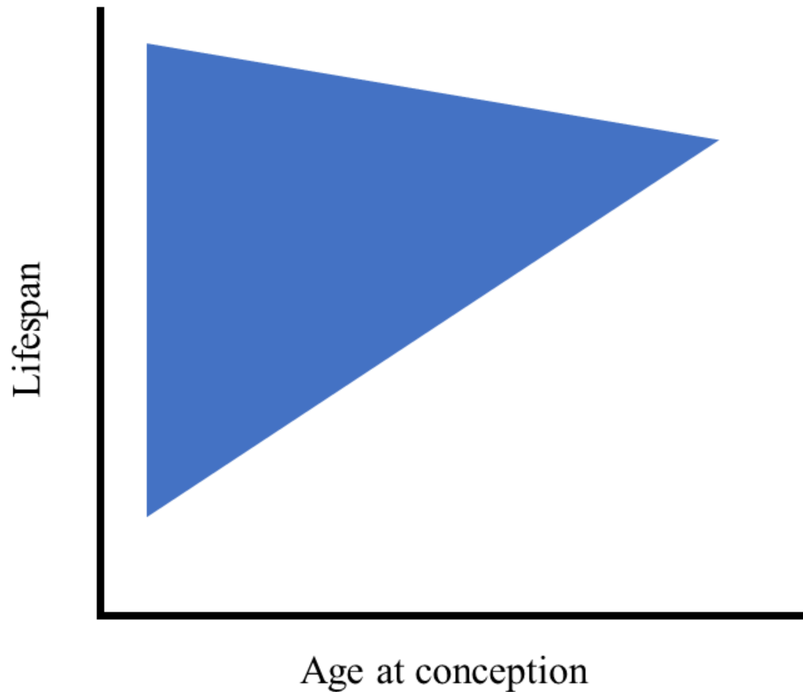


Figure C: Advancing age at conception leads to higher means but lower variance in lifespan of older individuals. However, males who mate when older experience higher mating-related stress due to being frailer.

Combining the effects of assumption 1 and 2, we get a complex relationship between age at conception and lifespan (Figure D), which is quite different from the commonly held prediction in Figure A. What this complex relationship would look like in our data is presented in Figure E.

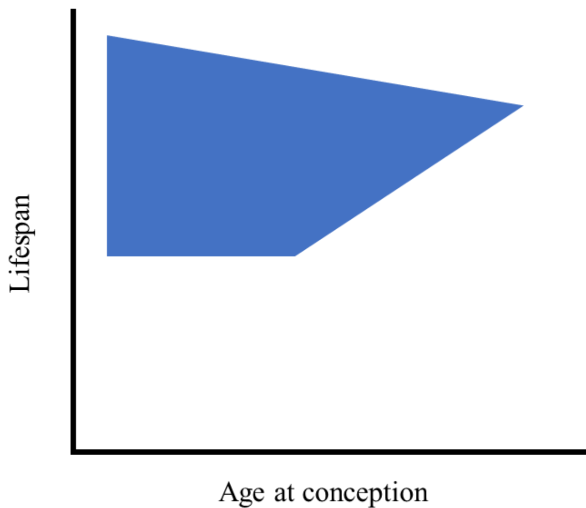


Figure D: Advancing age at conception leads to higher means but lower variance in lifespan of older individuals. However, males who mate when older, experience higher mating-related stress due to being frailer, and mortality does not occur until a certain age, thus age at conception groups until that age have the same average lifespans. This leads to a complex relationship between age at conception and lifespan.

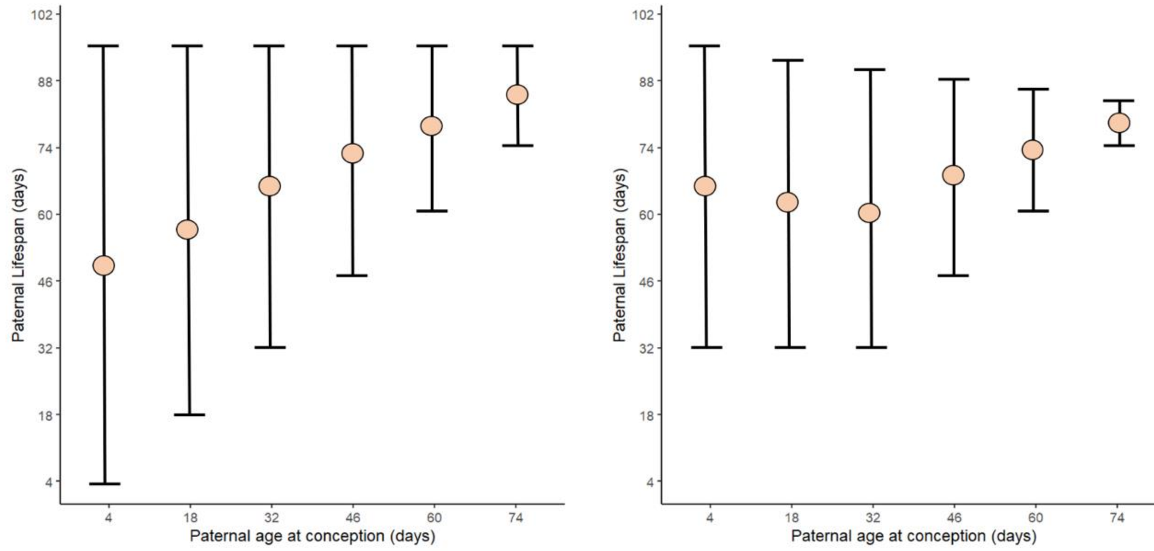


Figure E: (Left) Based on our experimental design, if there is no age-dependent frailty or late onset of death, then advancing paternal age at conception leads to a linear increase in lifespans of fathers. (Right) However, if males who mate when older experience higher mating-induced mortality due to being frailer (creating a lower ceiling for lifespan with increasing PAC), and if death does not occur until a certain age (say 32 days), then a non-linear relationship between PAC and paternal lifespan ensues. Range of (max-min), and mean lifespans for, each PAC group shown, assuming that the mean is the middle value of the range.

Appendix 2: Graphical predictions for different hypotheses in each of the three aims, for how paternal lifespan, PAC, paternal reproductive output, and offspring lifespans, might be linked.
Note that presented graphs are hypothetical

Aim 1: Effects of PAC on paternal survival
Age-dependent frailty hypothesis
Prediction: If mating does not affect survival of males, then unmated males, and males who mate when young or when old, should all have same lifespans.

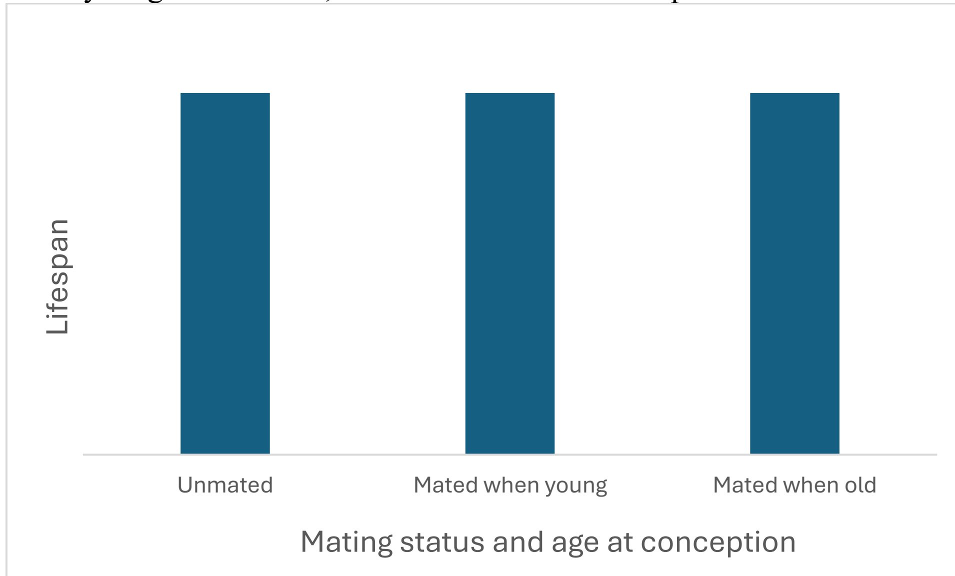


Figure F: No effects of age and/or mating status to affect lifespan.

If mating-stress reduces the survival of males irrespective of the age at which males mate, then unmated males should have higher lifespans than mated males.

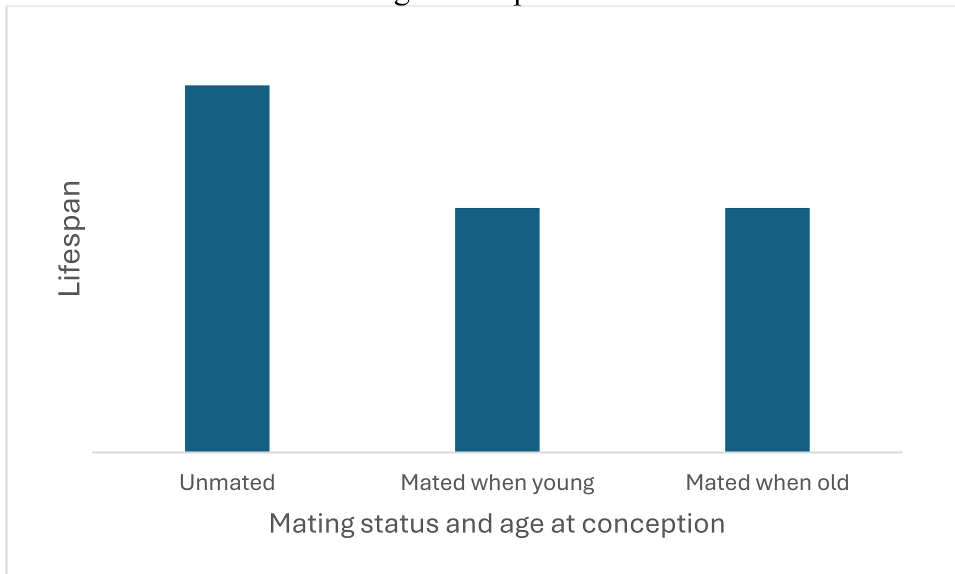


Figure G: Mating status, but not age, affects lifespan, with unmated males living longer.

However, if old are vulnerable to mating stress due to frailty, then mating-related stress should reduce the survival of males who mate when old, but not when young.

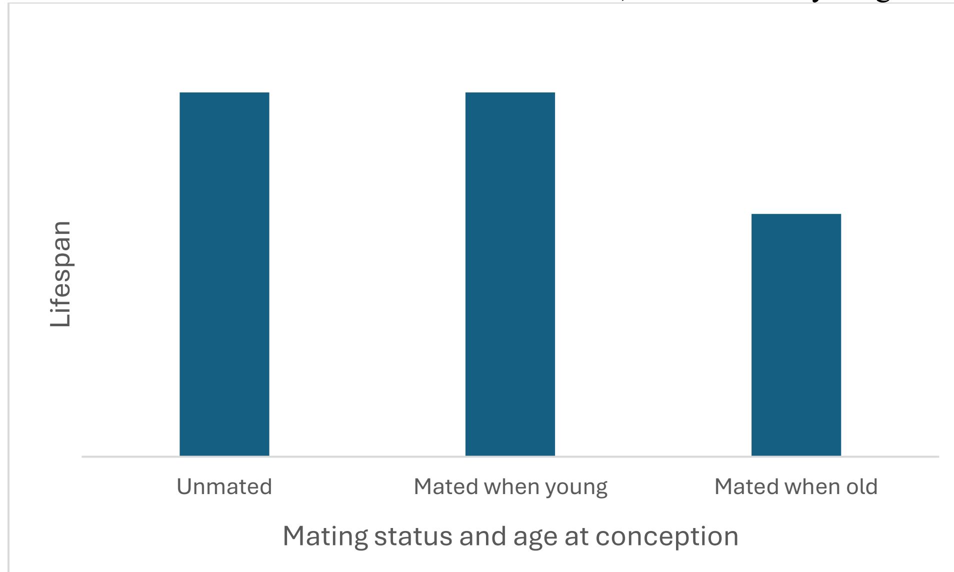


Figure H: Mating status interacts with age to affect lifespan, with mated, older males living the shortest.

Aim 2: Effects of PAC on paternal lifespan

Reproductive senescence hypothesis

Prediction: If reproductive senescence occurs, old males should have lower reproductive output than young males.

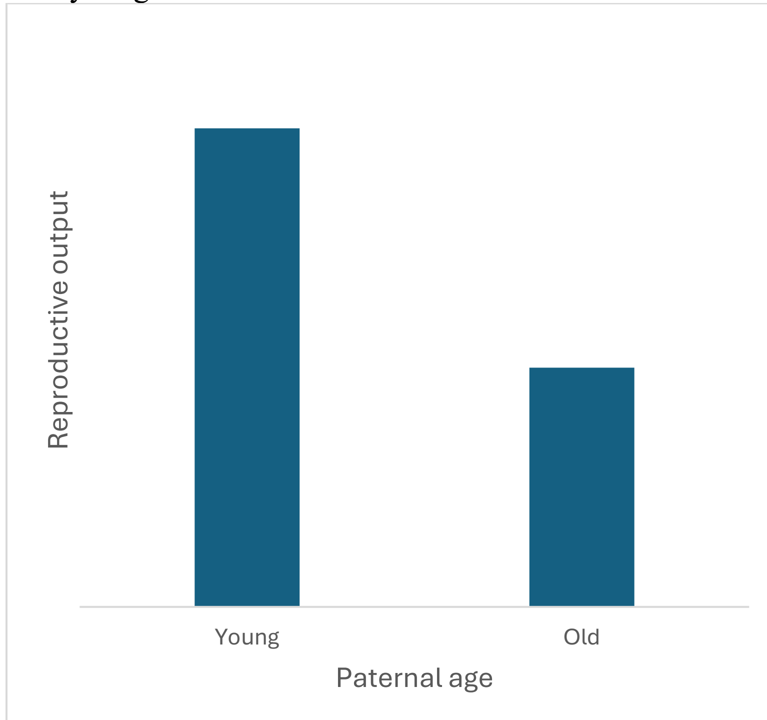


Figure I: Old males have lower reproductive output than young males due to reproductive senescence

Selective disappearance hypothesis
Prediction: Males who produce fewer offspring will selectively disappear (i.e. die) with age, leading to population level increases in reproductive output with advancing male age. Younger age groups will contain males of poor and high reproductive output while older age groups will contain males of only higher reproductive output, thus have lower variance in reproductive output.

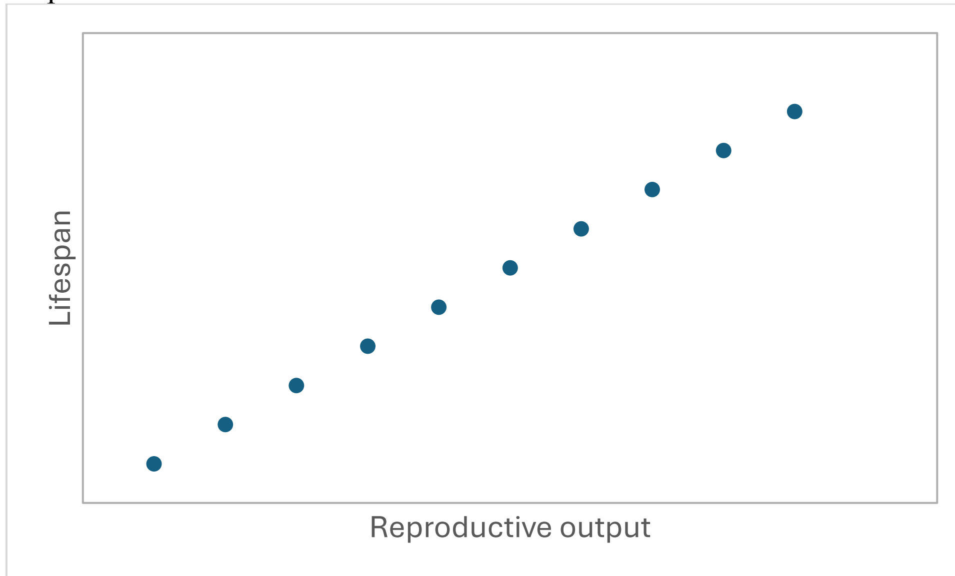


Figure J: Reproductive output and lifespan co-vary positively due to positive pleiotropy between life-history traits

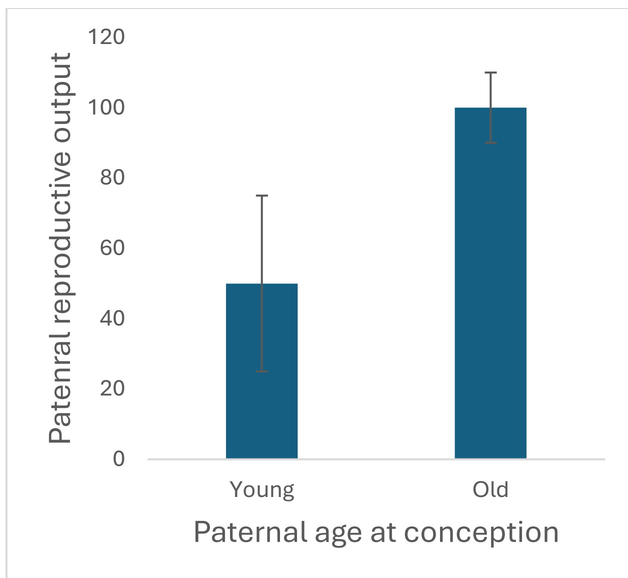


Figure K: Positive covariances between reproductive output and survival leads to selective disappearance of males and old males having higher reproductive output but lower variance in reproductive output, than young males

Dynamic terminal investment hypothesis
Prediction: Older males who are close to dying should invest more in reproduction than young males, or than old males who are not close to dying.

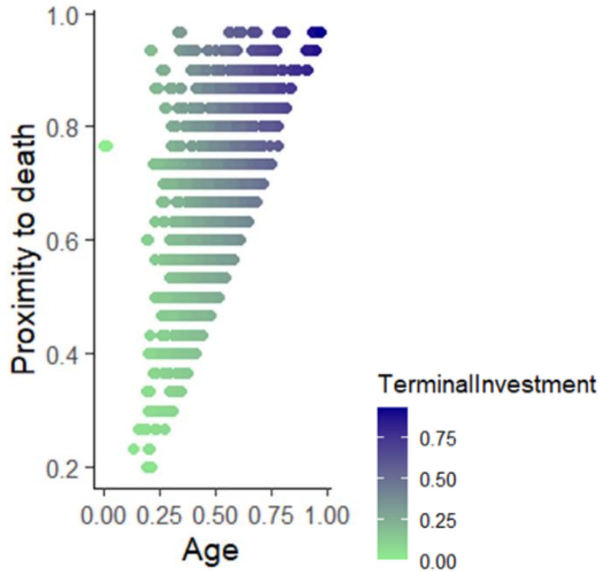


Figure L: Age at conception and proximity to death would interact to influence likelihood of terminal investment. Here, old males who are about to die soon would terminally invest in reproductive output, and have higher reproductive output, than old males who are not about to die soon, or than young males.

Aim 3: Effects of PAC on sons' lifespans
Lansing effect hypothesis
Prediction: Old fathers produce sons with shorter lifespans

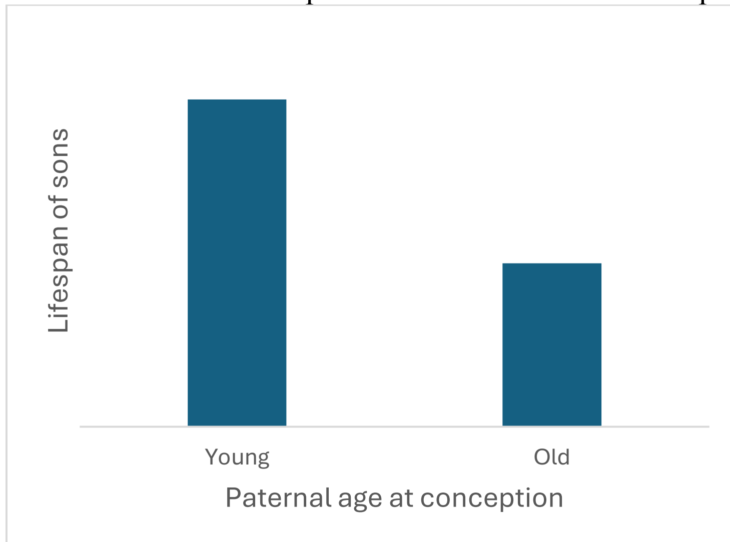


Figure M: Old father produce sons with lower lifespans due to Lansing effect

<u>Viability selection hypothesis</u>
Prediction: Fathers who mate at older ages are on average longer lived (however have lower variances in lifespan), and produce sons with longer lifespans (but lower variances in lifespan).

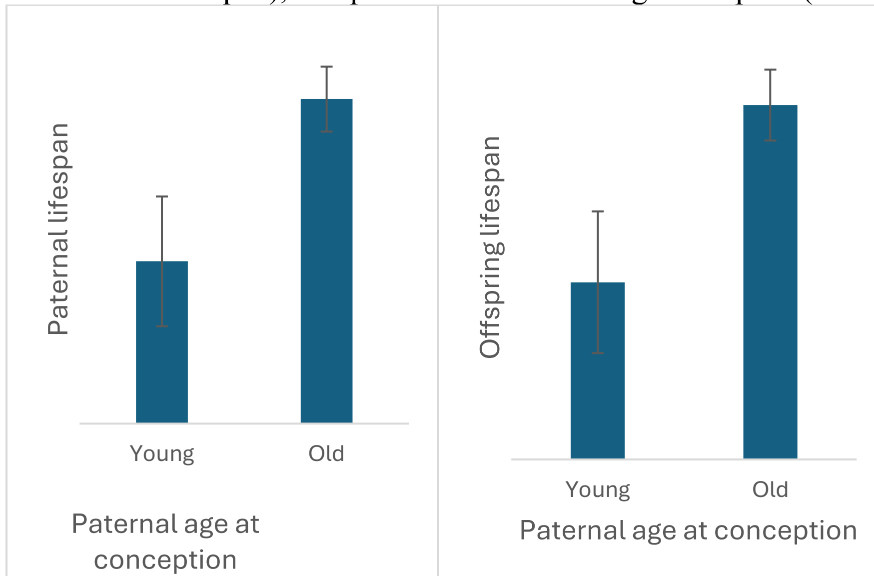


Figure N: Old fathers have longer lifespans but lower variance in lifespans (also corresponding to H1A), and produce sons with longer lifespans but lower variance in lifespans, than young fathers.

Appendix 3: Tables and model outputs

Table S1: Sample sizes for number of males who were mated at a particular age, and from these, the number of males who produced offspring. Note that at age 60 days, a higher number of males were selected to mate because of the high rate of infertility of old males. Additionally, at 74 days of age, very few males were surviving in the unmated stock population, thus the low sample size.

PAC (days)	N mated	N produced offspring
4	25	25
18	25	23
32	26	25
46	26	15
60	37	14
74	5	2

Table S2: Model output for effects of paternal age at conception (PAC) on probability of surviving three days after mating, with paternal offspring production (F1_count) included as a covariate. Terms highlighted in grey used for interpretation of interaction and main effects.

	Estimate	SE	z	P
(Intercept)	7.726	2.176	3.551	<0.001
PAC	-0.131	0.037	-3.544	<0.001
F1_count	0.018	0.010	1.764	0.078

Table S3: Hurdle model to test interactive effects of paternal age at conception and paternal lifespan, on reproductive output of fathers. Zero inflation model tests effects on the probability of not producing an offspring. Conditional model tests effects on the number of offspring produced, when only data on fathers that produced an offspring were analysed. PAC = paternal age at conception, Paternal_LS = paternal lifespan. Terms highlighted in grey used for interpretation of interaction and main effects.

Two-way interaction model:

Dispersion parameter	6.19

Conditional model (>0)	Estimate	SE	z	P
(Intercept)	3.928	0.284	13.819	<0.001
PAC	0.011	0.011	1.021	0.307
Paternal_LS	0.001	0.004	0.126	0.899
I(PAC^2)	0.000	0.000	-3.870	<0.001
Latency	0.000	0.001	-0.092	0.927
Copulation duration	0.006	0.007	0.951	0.342
PAC:Paternal_LS	0.000	0.000	0.562	0.574

Zero-inflation model (1 vs 0)	Estimate	SE	z	P
(Intercept)	-9.046	5.040	-1.795	0.073
PAC	0.349	0.129	2.703	0.007
Paternal_LS	0.094	0.071	1.312	0.190
I(PAC^2)	0.000	0.001	0.270	0.787
Latency	-0.029	0.012	-2.438	0.015
Copulation duration	-0.089	0.046	-1.937	0.053
PAC:Paternal_LS	-0.004	0.002	-2.336	**0.020**

Main-effects model:

Conditional model (>0)	Estimate	SE	z	P
(Intercept)	3.829	0.226	16.921	<0.001
Latency	0.000	0.001	-0.189	0.850
Copulation duration	0.006	0.007	0.882	0.378
Paternal_LS	0.002	0.002	0.963	0.336
PAC	0.016	0.006	2.598	**0.009**
I(PAC^2)	0.000	0.000	-3.902	**<0.001**

Zero-inflation model (1 vs 0)	Estimate	SE	z	P
(Intercept)	-0.621	2.335	-0.266	0.790
Latency	-0.023	0.010	-2.229	0.026
Copulation duration	-0.101	0.043	-2.340	0.019
Paternal_LS	-0.065	0.026	-2.526	0.012
PAC	0.192	0.092	2.081	0.037
I(PAC^2)	-0.001	0.001	-0.993	0.321

Table S4: Effects of paternal age at conception and days to death (i.e. time elapsed between death and mating) on the number of offspring produced by fathers. PAC = paternal age at conception. Terms highlighted in grey used for interpretation of interaction and main effects.

Two-way interaction model	Estimate	SE	z	P
(Intercept)	4.204	0.096	43.830	<0.001
Days_to_death	-0.004	0.001	-2.700	0.007
PAC	-0.009	0.002	-6.050	<0.001
Copulation duration	0.008	0.003	2.970	0.003
Days_to_death:PAC	0.000	0.000	6.850	**<0.001**

Main-effects model	Estimate	SE	z	P
(Intercept)	4.008	0.253	15.823	<0.001
Days_to_death	0.001	0.003	0.167	0.867
PAC	-0.004	0.003	-1.329	0.184
Copulation duration	0.011	0.008	1.381	0.167

Table S5: Effects of paternal age at conception on lifespans of sons, without accounting for effects of paternal lifespan. Heterogeneous variance function specified as a power function, with parameter showing change in variance in lifespans of sons, with the increasing PAC. PAC = paternal age at conception, F1_count= number of offspring produced by fathers. Terms highlighted in grey used for interpretation of main effects.

Random effects	SD
Paternal ID	4.659
Residual	22.002
Heterogenous variance parameter (power)	-0.24169

Fixed effects	Estimate	SE	DF	t	P
(Intercept)	55.904	3.409	188.000	16.399	<0.001
F1_count	0.002	0.043	98.000	0.054	0.957
PAC	0.108	0.042	98.000	2.532	**0.013**

Table S6: Model output from path analysis (structural equation model), showing the direct and indirect effects (via paternal lifespan) of paternal age at conception, on lifespans of sons. F1_LS = lifespans of sons, PAC = paternal age at conception, Paternal_LS = paternal lifespan, F1_count = number of offspring produced by fathers. Terms highlighted in grey used for interpretation of effects.

	Estimate	SE	z	P
F1_LS ~ PAC (c)	0.094	0.039	2.443	**0.015**
Paternal_LS ~ PAC (a)	0.171	0.037	4.662	**<0.001**
F1_LS ~ Paternal_LS (b)	0.115	0.060	1.920	*0.055*

Covariances:	Estimate	SE	z	P
Paternal_LS ~~ F1_count	3.907	12.102	0.323	0.747
F1_LS ~~ F1_count	-0.718	12.272	-0.059	0.953

Variances:	Estimate	SE	z	P
F1_LS	149.855	12.466	12.021	<0.001
Paternal_LS	145.645	12.116	12.021	<0.001
F1_count	290.526	24.169	12.021	<0.001

Defined Parameters	Estimate	SE	z	P
a*b (indirect effect)	0.020	0.011	1.776	*0.076*
c + a*b (total effect)	0.114	0.037	3.040	**0.002**

Appendix 4: Supplementary figures

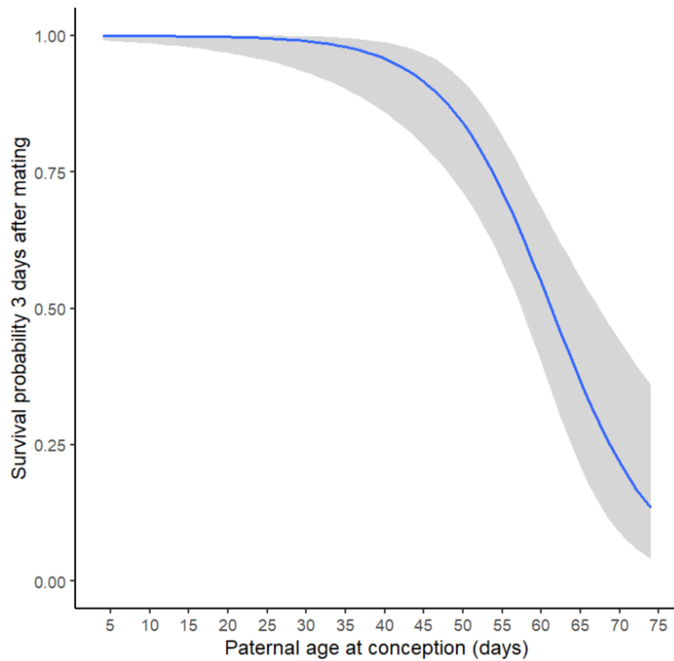


Figure S1: Lower proportion of males are alive 3 days after mating, in older PAC treatments than younger PAC treatments. Shaded areas represent 95% C.I.

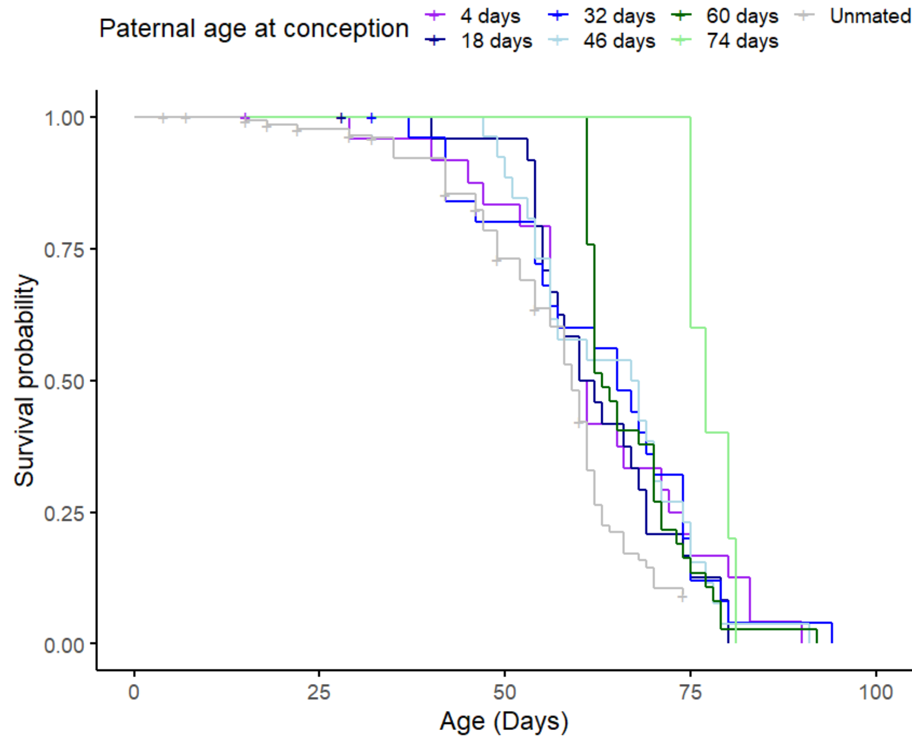
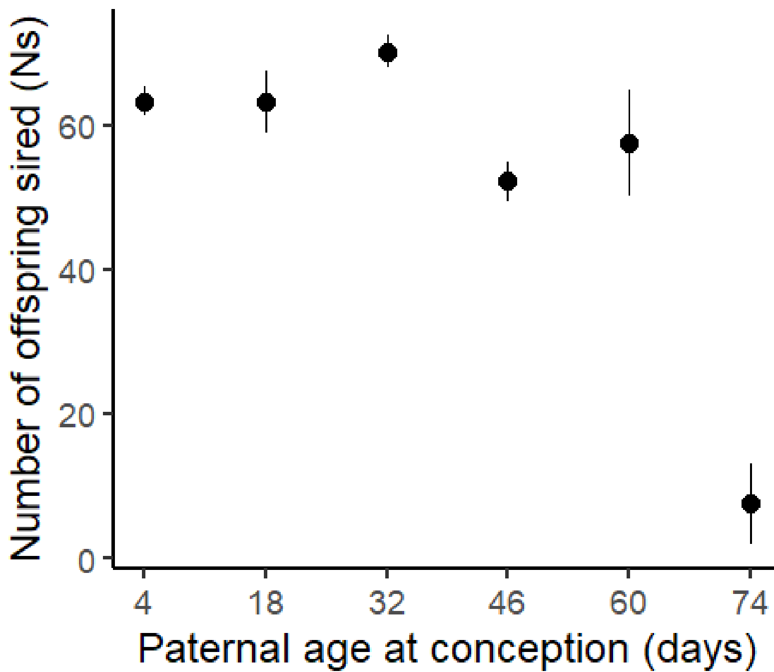


Figure S2: Survival probability of fathers mated at different ages, and males from the unmated experimental population. Fathers who mate at an older age have a lower survival probability at a given age than father who mate at a younger age. “+” signs show censored males.



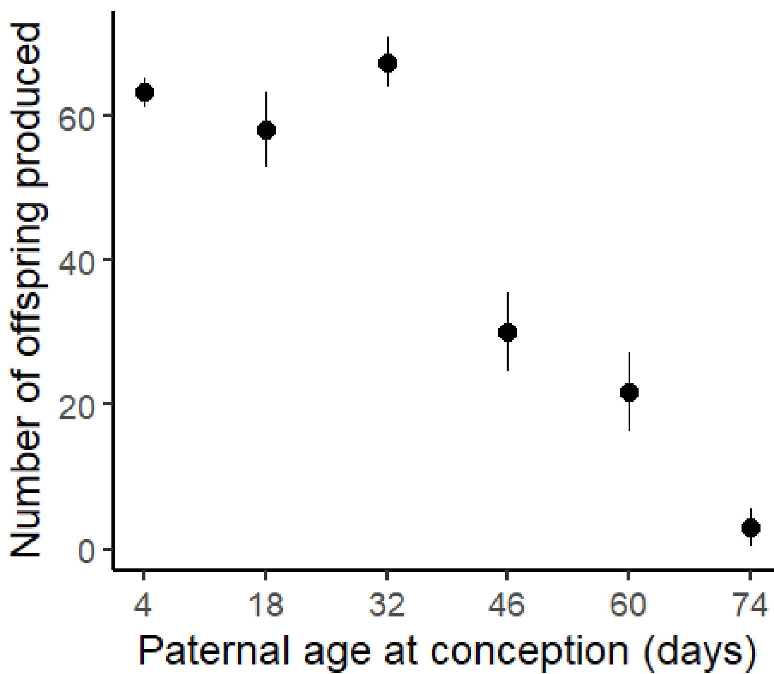


Figure S3: (Top) Paternal age at conception affected the number of offspring sired (excluding fathers who did not produce offspring) in a quadratic way. However, this effect was likely driven by data from PAC of 74 days being lower than other PAC data (also see Figure S4). (Bottom): Effects of paternal at conception on number of offspring produced (including fathers who produced zero offspring). Means and SE shown.

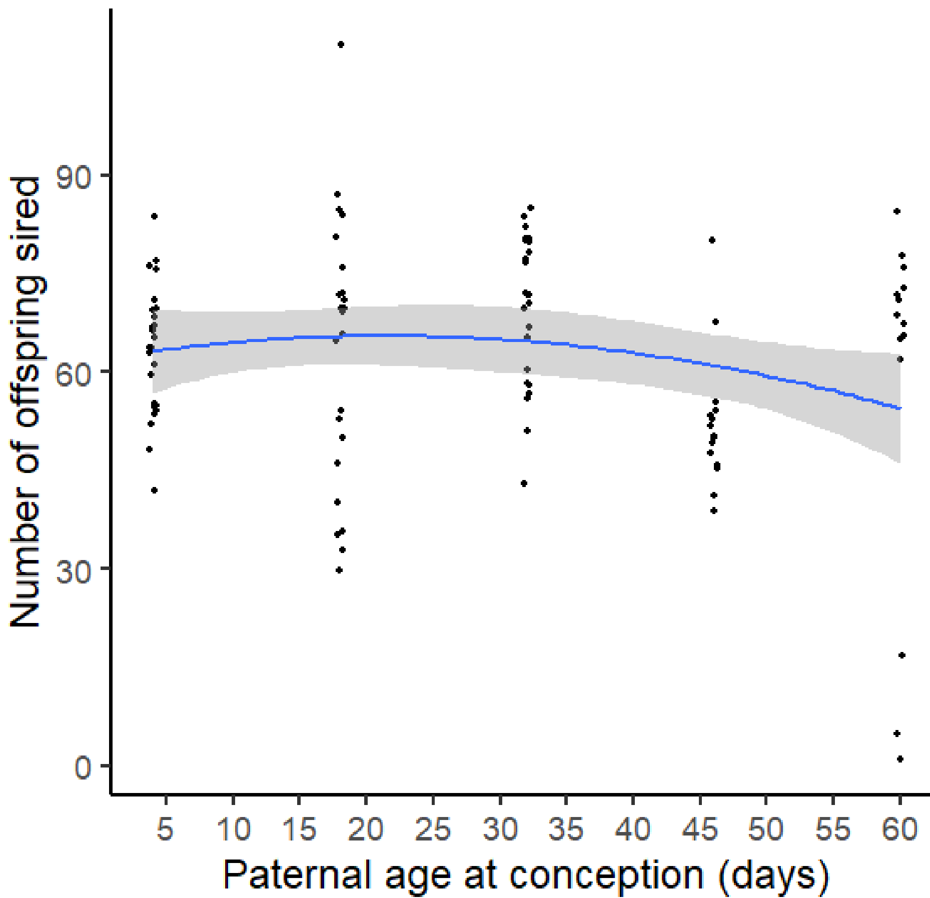


Figure S4: Paternal age at conception affected the number of offspring sired (after excluding fathers who did not produce offspring) in a quadratic way (even when PAC group of 74 days was excluded). However, excluding data from PAC of age 74 days led to a shallow shape of the quadratic curve compared to when this data was included (i.e. Figure 4B). Shaded areas represent 95% C.I.

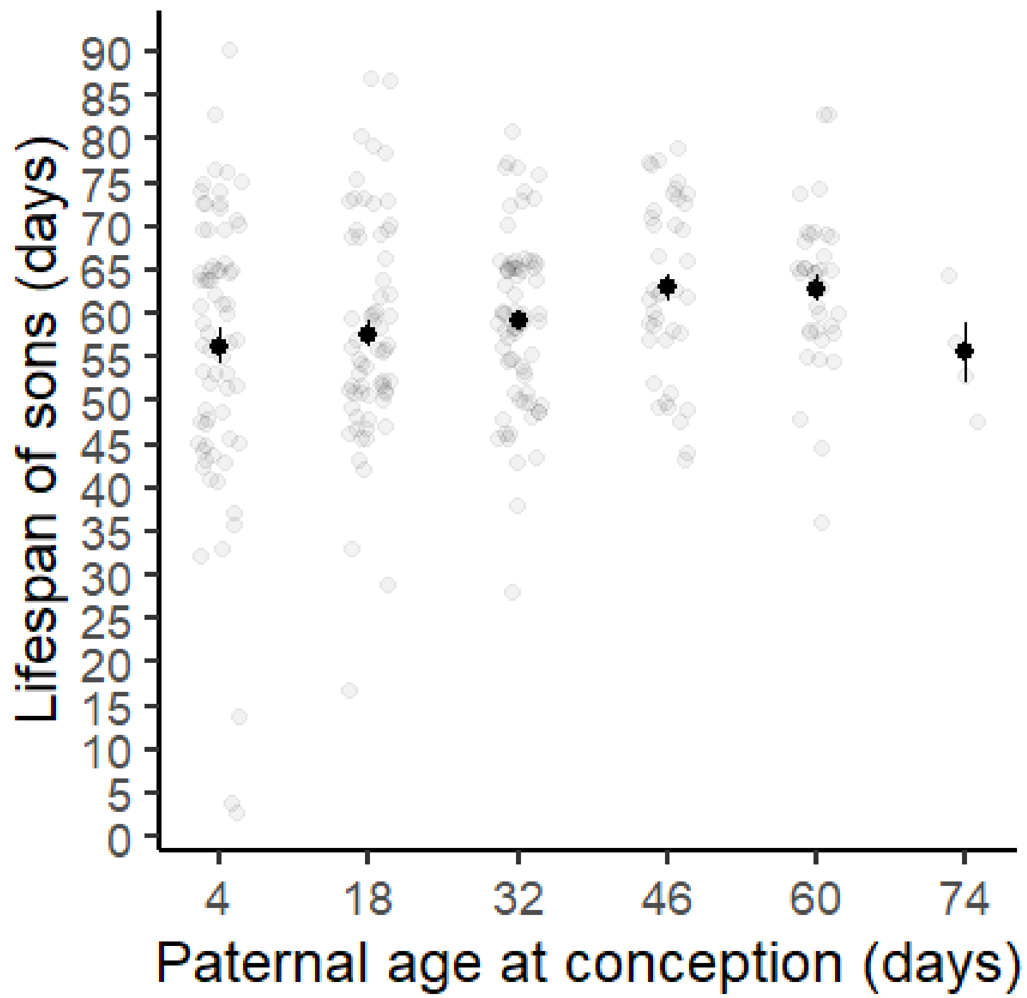


Figure S5: Advancing paternal age at conception increased lifespan of offspring. Means and SE presented

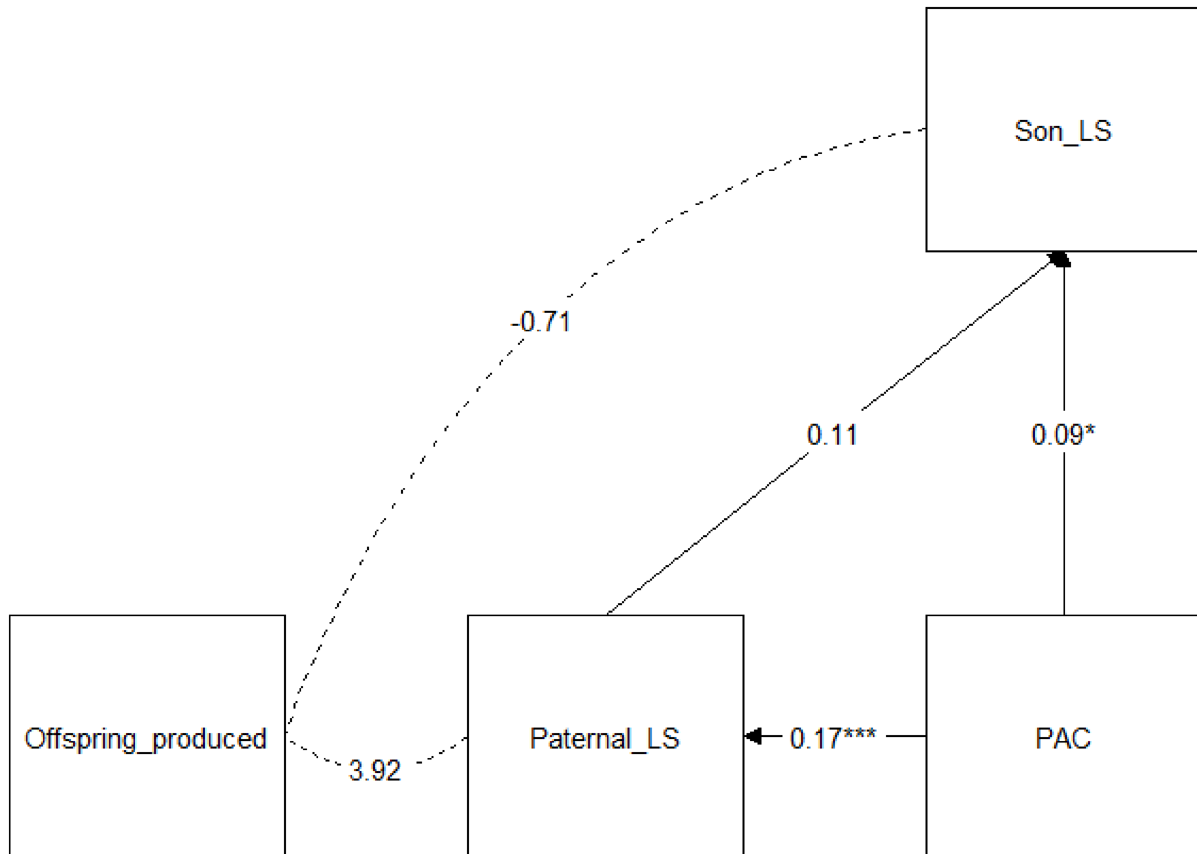


Figure S6: Path analysis showing direct effects of paternal age at conception, and indirect effects via paternal lifespan, on sons' lifespans, along with covariances (dotted) of paternal lifespan, lifespans of sons, and number of offspring produced. Values show estimates of models, and asterisks represent level of significance.

Chapter 7

Thesis discussion

Summary of thesis goals

My thesis used a combination of comparative approaches (chapters 2 and 3) and experimental studies of the fruit fly, *Drosophila melanogaster* (chapters 4, 5 and 6), to investigate the causes and consequences of male reproductive senescence (Figure 1). In addition to chapter-specific aims, my thesis had five broad overarching goals: 1. document patterns of ageing at the level of the ejaculate using comparative and experimental approaches; 2. interpret patterns observed across chapters using predictions from evolutionary hypotheses of senescence; 3. investigate how between-individual processes buffer reproductive senescence; 4. investigate the inter-generational effects of paternal age on offspring; 5. infer the contribution of age-dependent changes within different traits and via different pathways in driving male reproductive senescence. A synopsis of the main findings of my thesis is outlined in Figure 1. Below I discuss findings of my thesis with respect to the five overarching goals.

Pertaining to goal 1, I found that male reproductive senescence at the level of the ejaculate is not universal across taxa and is ejaculate-trait specific. Using comparative approaches (chapter 2), I showed that advancing age did not consistently lead to senescence in sperm traits. Generally, sperm and ejaculate quantity traits senesced at a slower rate than sperm quality traits (chapter 2). Surprisingly, ejaculate/sperm quantity traits even improved with advancing age in certain taxa, which was likely related to indeterminate growth (e.g. crustacea and fish) or sexual rest (e.g. insects). In contrast to sperm traits, seminal fluid quality and composition showed more consistent deterioration with age. This deterioration in seminal fluid was starker in seminal fluid oxidative stress than in individual seminal fluid proteins (chapter 3). My experiments on fruit flies were consistent with the results from the two comparative chapters. Specifically, my results

from chapter 4 showed that the decline in reproductive output with age was associated with seminal fluid senescence or a decline in sperm quality, rather than a decline in sperm numbers with age, in flies. Chapters 2, 3, and 4 combined suggest that senescence might be occurring more intensely in seminal fluid than in sperm number. These patterns could be explained by the disposable soma hypothesis (Kirkwood and Rose, 1991; Lemaitre et al, 2015). This hypothesis predicts that age-dependent maintenance of the germline across all ages is prioritised, over the maintenance of somatic tissue (Maklakov and Immler, 2016). This differential investment would cause faster declines in seminal fluid (produced by somatic tissue) than in sperm, with advancing age. The lack of consistent evidence for senescence in sperm but more consistent senescence in seminal fluid traits, could also be due data collected in Chapter 3 having lesser heterogeneity and being more taxonomically biased, compared to data in Chapter 2. Results pertaining to goal 1 generally emphasize that only measuring sperm quantity traits might not be informative about male reproductive senescence.

Relating to goal 2, I tested some predictions derived from evolutionary theories of senescence (disposable soma, trade-offs, mutations accumulation over generations). Some of my results were in line with the disposable-soma hypothesis (chapters 2, 3, 4; see previous paragraph), whereby somatic components of the ejaculate (seminal fluid) likely senesced at faster rates than gametic components (sperm). However, my results did not support predictions from the life-history trade-offs hypothesis (Nussey et al, 2006). Specifically, my results showed that fruit fly fathers who produced more offspring also survived for longer durations than fathers who produced fewer offspring (i.e. no trade-off between survival and reproduction; chapter 5). Similarly, daughters who were more fecund lived to longer ages than less fecund daughters

(chapter 5). Here, daughters who were more fecund in early life were also more fecund in later life, compared to daughters who were less fecund in early life, indicating a lack of early- versus late-life reproduction trade-off (chapter 5). I additionally found that fathers who produced more offspring also produced sons with longer lifespans and higher fecundity (i.e. no trade-offs between offspring quantity and quality; chapter 5). These positive covariances between various life-history traits might be explained by heterogeneity in individual quality and condition (Cohen et al, 2019; Vedder and Bouwhuis, 2018). Here, individuals of better genetic quality, and/or in higher condition (i.e. better environments), would be able to invest more energy in all life-history traits, than individuals of low condition/genetic quality who would invest fewer resources across life-history traits (Reznick et al, 2000). Additionally, my results are inconsistent with predictions derived from the mutation accumulation hypothesis (Lee and Chu, 2023; Rose and Charlesworth, 2002), because the effects of advancing paternal age on offspring reproduction, did not become more apparent in the late-life of offspring (chapter 5).

To address goal 3, I investigated how between-individual processes (selective disappearance and viability selection) might influence reproductive senescence. I generally found support for between-individual processes buffering the magnitude of reproductive senescence (chapter 5, 6). For instance, fruit fly daughters with longer lifespans also produced more offspring than shorter lived daughters (chapter 6). Similarly, fathers who survived for longer were on average, more fertile than shorter lived fathers (chapter 5). Such selective disappearance has been primarily demonstrated in wild animals (e.g. Hayward et al, 2013; Zhang et al, 2015), with evidence from lab populations being limited. My results highlight that between-individual processes can drive

the patterns observed in lab populations as well, hence the contribution of these processes ought to be incorporated in experimental ageing studies.

For goal 4, I investigated paternal age effects on offspring, and compared these inter-generational effects (i.e. of paternal age on offspring) to intra-generational effects (i.e. of paternal age on a father's own reproduction). Contrary to expectations, my results did not find evidence for advancing paternal age being deleterious to offspring survival or lifetime reproduction (chapters 5, 6). However, older fathers produced fewer offspring than young fathers, an effect consistently observed across my experiments (chapters 4, 5, 6), suggesting that intra-generational effects might be primarily driving male reproductive senescence (see goal 5 on how this might happen).

Lastly with regards to goal 5, drawing on evidence emerging from across my thesis, I suggest that reproductive senescence in males is likely a result intra-generational processes, i.e. age-related declines in fertility (chapter 4, 5, 6) and mating success (chapter 4), rather than inter-generational processes, i.e. older males producing lower quality offspring (chapters 5, 6). My results further suggest that declining male fertility is likely caused by senescence in seminal fluid quality (i.e. changes in seminal fluid protein composition or quality, or increase in seminal fluid oxidative stress) rather than sperm numbers (chapters 2, 3, 4). My thesis highlights the importance of partitioning male reproductive output into the influence of different traits, and partitioning reproductive senescence into age-dependent changes in fertility versus offspring quality. Such an approach can allow a better understanding of the pathways that mediate reproductive senescence, and of how senescence influences individual fitness overall.

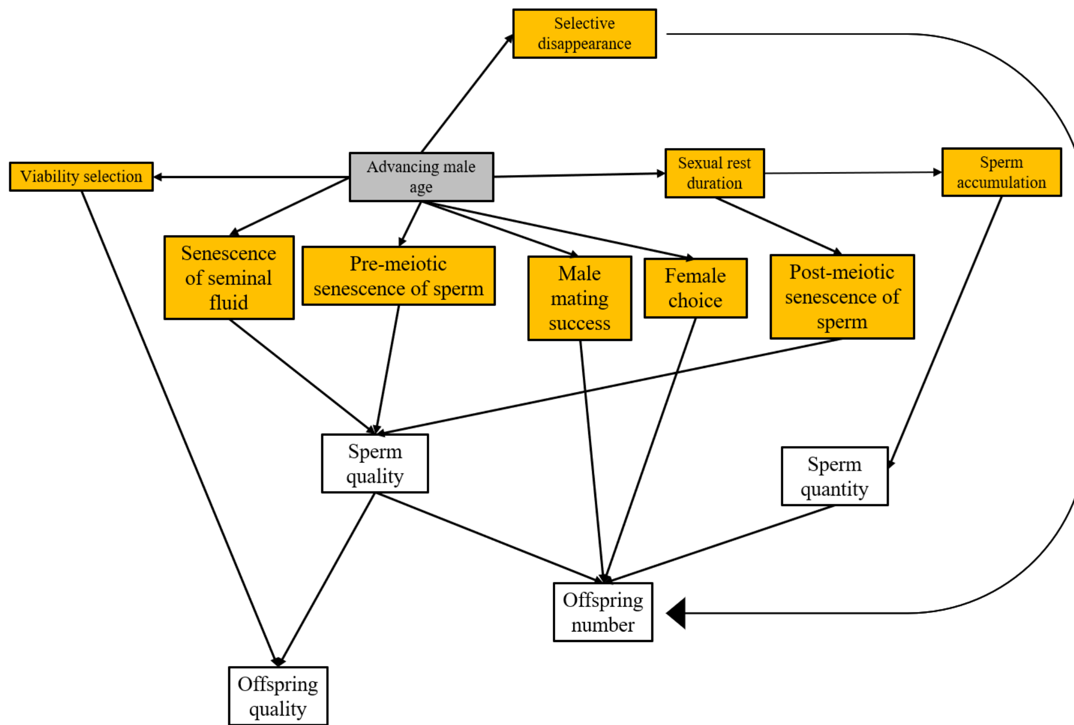


Figure 1: A schematic diagram outlining how various processes and pathways might mediate the influence of male age on intra- and inter-generational components of male fitness, as suggested by the results of my thesis. Orange boxes represent processes that mediate the effects/outcomes of advancing male age, on male fitness components (white boxes). Arrows represent associations for which I have found some evidence (direct or indirect) in my thesis. Direction of arrow indicates likely direction of causality.

Thesis limitations

The general limitations of my thesis might provide opportunities for future research. First, my experiments were limited by their sampling method, and mainly focussed on sampling males cross-sectionally. While cross-sectional sampling is informative about how demographic processes influence reproductive ageing, they cannot elucidate within-individual changes with age (Nussey et al, 2008; van de Pol and Verhulst, 2006). Thus, my thesis could have underestimated the magnitude of senescence in paternal reproduction as well as paternal age effects.

Second, my thesis focussed on male-specific reproductive ageing only, and I did not manipulate the ages of females. However, female age can interact with male age to affect male reproductive ageing, by exacerbating or buffering the deleterious effects of advancing male age (Lemaitre and Gaillard, 2017; Tidiere et al, 2018; Wylde et al, 2019). Investigating the influence of both, male and female age, simultaneously, is especially relevant in scenarios where males and females mate assortatively by age (Bowers et al, 2017; Lenart et al, 2018), and the effects of male and female age act concurrently.

Third, I did not directly measure age-related changes in sperm or seminal fluid *quality* in my experiments. Age-related changes in sperm and seminal fluid *quality* can influence male fertility (Meunier et al, 2022; Sepil et al, 2020; Sharma et al, 2015), independent of changes in *quantity*. Measuring sperm quality is especially crucial to elucidate the mechanisms, such as mutation and reactive oxygen species accumulation in ejaculates, that contribute towards reproductive senescence and deleterious paternal age effects (Monaghan and Metcalfe, 2019).

Fourth, I did not measure age-related changes in male behavioural, cognitive, or secondary sexual traits. Male mate finding ability, courtship ability, and courtship song, are affected by male age in fruit flies, and can ultimately influence male mating success, thus, reproductive success (Hoikkala et al, 2008; Iliadi and Boulianne, 2010; Long et al, 1980; Rezaei et al, 2015). For instance, lower rates of senescence in male pre- copulatory traits compared to ejaculate traits, could allow males to compensate for reproductive senescence by having increased mating success. Therefore, my thesis does not provide a complete picture of the various pathways through which advancing male age could influence the fitness of males.

Fifth, my experiments were conducted on the fruit fly system, which are lab-adapted. Therefore, results from flies might not be applicable to natural systems or other taxa, and might be influenced by the ecology of the fly system. For instance, I controlled for mating experience of flies in my experiments, by maintaining old and young males as virgins. However, in the wild, old males are unlikely to be virgins, and would have had more cumulative mating opportunities than young males. Additionally, fruit flies are polygynandrous, with females mating with multiple mates (Gowaty, 2012) and sperm competition between males being commonplace (Price et al, 1999). However, I did not allow females to remate when measuring reproductive success of males in my experiments. Thus, measures of reproductive output (measured as absolute numbers of offspring produced without sperm competition), may not be representative of true male reproductive ageing patterns. Furthermore, the lack of trade-offs between life-history traits, such as survival versus reproduction or offspring quality versus quantity, might be an artefact of the fly system. Theory predicts trade-offs between life-history traits to be more apparent under resource limitation (Fischer et al, 2011; Roff and Fairbairn, 2007) and less apparent under resources abundance (Reznick et al, 2000; Zera and Harshman, 2001). Therefore, my experiments being conducted on flies with *ad libitum* access to food, might have prevented trade-offs between traits from occurring. Moreover, the lifespans of fruit flies in the wild are considerably shorter than those seen in my experiments (Promislow et al, 2022). Therefore, sampling males to old ages, such as 40+ days old in my experiments (chapters 4, 5, 6), in the absence of predation and extrinsic mortality, may not be realistic (Cordero and Eberhard, 2003).

Sixth, my meta-analysis and review chapters (2, 3) were comparative studies, and did not involve experimental manipulation of male age or other moderators. While I found some evidence for

advancing male age influencing sperm and seminal fluid traits, whether the links between age and ejaculate traits, and between various moderators and ejaculate traits, are causal, cannot be inferred from comparative studies. Additionally, my review on seminal fluid ageing was qualitative and the results were descriptive, therefore we cannot ascertain the magnitude of effects of age on seminal fluid in chapter 3, making the results less useful.

Mathematical representation of thesis outcomes

The aims, outcomes, and limitations of my thesis, highlight three conceptual gaps in male reproductive ageing research. First, studies rarely simultaneously compare the intra- and inter-generational effects of advancing age, i.e. on one's own reproduction and on offspring quality, respectively. Second, studies on ageing seldom simultaneously investigate within-individual changes as well as demographic processes. Third, studies do not partition reproductive success into the combined influence of different traits. These traits could be related to mating success, ejaculate (sperm or seminal fluid) quantity or quality, and even include female-related traits, such as female choice. Such a partitioning might be crucial if traits senesce at varying rates or differentially contribute towards overall fitness.

Here, I formalise a single theoretical framework that incorporates these three knowledge gaps, which future studies can use to conceptualise reproductive senescence more holistically. While I use males as an example, this framework can be extended to female reproductive ageing too.

First, to incorporate intra- and inter- generational effects of advancing paternal age (first gap), a father's age-specific contribution to his overall fitness (F_{age}) could be calculated as the numbers

of grand-offspring he produces at a given age (*x*). Here, the numbers of grand offspring produced at the given age *x* can be partitioned into the reproductive success (i.e. numbers of offspring produced) of the father at age *x*, multiplied by the lifetime reproductive success (cumulative from birth to death) of each offspring he produced at age *x*. This can be written as follows:

Age-specific fitness contribution

Sum of LRS of all offspring

LRS of each offspring

$$F_{age} = f\left(x_f\right) \cdot \sum_{off=0}^{off=y_f{}^{th}} \left[\int_{birth}^{death} \left[f\left(x_{off}\right) \right] \right]$$

RS of fathers at age x

RS of offspring at age x

Number of grand-offspring

The male's reproductive success (i.e. number of offspring he produces at age *x*) is denoted by '*y'*. Here, the fitness contribution of a male at age *x* is given by the number of offspring he produces at age *x* multiplied by the sum of the lifetime reproductive success of each of his offspring (from 0th offspring to the yth offspring). This formulation incorporates processes studied in chapters 5 and 6 of my thesis, where male reproductive ageing affects a male's own reproductive success, but also the lifespan (i.e. *death*) and reproduction of his offspring. In the above formula, male reproductive success at age *x* is derived from a function that describes age-specific changes in reproductive success of a male. The reproduce ageing function can be modelled as linear or non-linear. For realism, I assume that y_f, the reproductive success of a male at age *x* is obtained from a quadratic function, where:

$f(x_f): y_f = -nx^2 + mx + c_f$

Similarly, y_{off}, the reproductive success of each offspring at age x, comes from a quadratic function:

$f(x_{off}): y_{off} = -px^2 + qx + c_{off}$

Where n, p, are coefficients of the quadratic term, m, q, are coefficients of the linear term, c_f, and c_{off} are constants determining the intercept.

To now incorporate selective disappearance at the level of the fathers (second gap), we can co-vary the intercept of the ageing function (c_f) positively with paternal lifespan (*PLS*), such that: $Cov(PLS, c_f)$. This covariance will lead to longer lived fathers producing more offspring than shorter lived fathers, at a given age. Similarly, to incorporate selective disappearance in offspring, we can co-vary offspring lifespans with the intercept of the offspring ageing function, such that $Cov(LS_{off}, c_{off})$. We can further add the process of viability selection, such that older fathers on average represent longer-lived fathers, and produce longer lived offspring. Here, paternal age at reproduction (x) would co-vary with paternal lifespan, such that: $Cov(PLS, x)$. Additionally, paternal lifespan would co-vary with offspring lifespan, such that: $Cov(PLS, LS_{off})$. These formulations represent processes studied in chapters 5 and 6 in my thesis, where between-individual heterogeneity in lifespan and reproduction can buffer reproductive senescence.

To address the third conceptual gap, we can compartmentalize male reproductive success at a given age x, into the combined influence of male mating success and male paternity share. This can be written as:

$$RS_x = M_x * N * P_x$$

Where the age-specific reproductive success of a male (y_f) at age x, is mating success of a male (M) at a given age x, multiplied by the average number of eggs the females he mates to have (N), multiplied by the proportion of those eggs a male fertilizes on average at age x (P). Here, the proportion of a female's eggs that a male fertilizes (i.e. paternity share) would be a function of sperm quality (e.g. sperm viability, velocity), seminal fluid quality and quantity, female sperm ejection rates, and sperm number. Instead of deriving age-specific changes in male reproductive output (y_f) from a general ageing function, y_f can be now calculated by deriving M_x and P_x from independent ageing functions. This formulation represents some of the results from chapter 4, where male reproductive success at a given age is likely influenced by age-related changes in male mating success, ejaculate quantity, ejaculate quality, and female choice (e.g. sperm ejection) in varying degrees.

The mathematical formalisation of the three conceptual gaps highlighted by my thesis, could greatly aid future experimental and theoretical ageing research, by providing a general framework for male reproductive ageing.

The life-history of sperm

My thesis investigated male reproductive senescence, however much of the focus of my thesis was on sperm and ejaculates. My thesis therefore has led me to draw parallels between individual (whole organism) life-history, and sperm life-history, and contemplate about whether such a parallel might give us new insights. Below, I discuss some of these parallels, in the hope that they provide a new perspective for the study of male reproductive ageing, and generate novel hypotheses for studying the biology of sperm. Generally, these parallels emphasize that similar

demographic processes might occur at different levels of biological organisation (i.e. the haploid gamete *and* the diploid individual), highlighting the importance of studying life-history at different levels of selection.

The first parallel compares within-individual (whole organismal) deterioration with age, and within-sperm deterioration with increased sperm storage duration. Sperm are known to deteriorate post-meiosis, when stored either in male extragonadal reserves or in female reproductive tracts (Pizzari et al, 2008; Reinhardt, 2007; see chapter 5). The molecular mechanisms that lead to within-sperm deterioration with time are to some degree similar to the mechanisms that cause within-individual deterioration, namely: accumulation of DNA and oxidative damage (Barbagallo et al, 2023; Pizzari et al, 2008). Therefore, within-sperm deterioration with increased storage duration can be seen as a process akin to within-individual deterioration with age.

The second parallel can be made between viability selection and haploid selection. Viability selection is when individuals reproducing at an older age are on average longer lived and conceive similarly longer-lived offspring, than individuals reproducing at a younger age. This can lead to females choosing older males in some species, because these males could have alleles conferring higher viability (Johnson and Gemmell, 2012; Kokko, 1998; see chapter 6). This process can be akin to haploid selection in sperm. Specifically, when the sperm haploid genome is expressed, sperm haplotypes can influence sperm longevity, and fertilisation from longer lived sperm might lead to the production of more viable offspring (Alavioon et al, 2017; Immler, 2019; Immler and Otto, 2018). In species where the sperm haplotype is expressed post-meiosis or when

the sperm phenotype is associated with the sperm haplotype (e.g. Bhutani et al, 2021; Joseph and Kirkpatrick, 2004; Wykes et al, 1997), the demographic influence of sperm longevity could be conceptually alike that of individual longevity via viability selection (e.g. Crean et al, 2012). Hence, females could prolong sperm storage duration to select longer lived sperm and conceive longer lived offspring.

The third parallel can be made between selective disappearance and covariances between different sperm traits. Selective disappearance is the consequence of positive correlations between reproductive output and lifespan, such that longer lived individuals represent a biased subset of individuals with a high reproductive value (Nussey et al, 2008; see chapters 5, 6). Similarly, if the longevity of individual sperm correlates positively with the fertilisation ability of sperm, then ejaculates with older mature-sperm (e.g. due to long durations of post-meiotic sperm storage) could contain viable sperm with higher fertilisation ability than ejaculates containing younger sperm (Yasui, 1997). Such a process of "selective disappearance" in sperm, might bias estimates of sperm quality, when ejaculates are stored for long durations.

The fourth parallel is between sperm accumulation in male extragonadal sperm reserves and population growth. Population growth rates are determined by birth rates, death rates, and migration (Pearl, 1927; Iannelli and Milner, 2017). In ejaculates, the rate of sperm accumulation in extra gonadal male reserves is determined by sperm production rate (akin to birth rate), rate of passive sperm loss or loss of sperm viability (akin to death rate), and sperm ejaculation (akin to emigration). Therefore, the rate of sperm accumulation with increased duration of sperm storage (for instance, when males are kept virgins, as seen in chapters 2 and 4), can be calculated using

these three processes. In demographic research, age structure is often incorporated to understand age-specific contributions to population growth rates (Iannelli and Milner, 2017). Similarly, at the level of ejaculates, the age of the germline can be incorporated to assess germline age-specific changes in sperm production rates, sperm viability, and sperm loss, and how these influence sperm accumulation in extragonadal reserves of males as the germline itself ages. Additionally, akin to a population carrying capacity, the maximum numbers of sperm that can accumulate in male reserves could be further incorporated. This maximum number would likely be a function of the volume of the sperm storage organ (e.g. seminal vesicles in fruit flies) and of each mature sperm.

The fifth parallel can be made between organismal life-history strategies, and possible sperm strategies. Organismal life-history strategies are usually defined using pace-of-life syndromes. These range from organisms developing quickly, reproducing early, and dying young, to organisms developing slowly, reproducing late, and dying old (Dammhahn et al, 2019). Similarly, variation in the duration of spermatogenesis (akin to development rate), average post-meitoic age of sperm during fertilisation (akin to age of reproduction), and duration of sperm longevity (akin to lifespan), can be used as metrics for classifying possible sperm life-history strategies. Such a pace-of-life framework to describe sperm strategies, might explain heterogeneity observed in sperm traits (Levitan et al, 2000; Taborsky et al, 2018) and can be used to generate novel hypotheses. For instance, a develop-quick, live-fast, die-young strategy in sperm might be expected when fertilisation is immediate (akin to early reproduction). However, a develop-slow, die-later strategy could occur when fertilisation is delayed, for instance, when mature sperm are stored for long durations in vivo.

Drawing parallels between the demography of individual whole-organisms and of sperm could provide a useful conceptual approach for investigating the diversity in sperm phenotypes and fertilisation patterns observed in nature.

Future directions

Within each chapter, I have provided suggestions for future research directions. Additionally, in my 'thesis limitations' section, I highlighted shortcomings and knowledge gaps that my thesis was unable to address, which future studies could address. Furthermore, in the sections 'mathematical representation of thesis outcomes' and the 'life-history of sperm', I suggested new perspectives that could provide a guiding framework for future research on male reproductive ageing. Below I suggest four additional avenues to guide broader research programs:

Cryptic female choice and male age: In chapter 2, I showed that senescence in male post-fertilisation reproductive outcomes was lower than senescence in male ejaculate traits. In chapter 4, I showed that although old and young males transferred similar numbers of sperm to females, females mated to old males stored fewer sperm than females mated to young males. Both chapters suggest that cryptic female choice (CFC) might alleviate the costs of mating with old males (Jones, 2002). CFC has been demonstrated in several contexts (Firman et al, 2017), such as females ejecting sperm from less preferred males (Pizzari and Birkhead, 2000) or ejecting deteriorated sperm (Reinhardt and Siva-Jothy, 2005; Snook and Hosken, 2004; Wagner et al, 2004). However, there is surprisingly little evidence for CFC as a causal mechanism that females might use to bias fertilisation towards young males. The difficulty partly arises from separating sperm competition from CFC in driving observed patterns, whereby fertilisation bias for young

males could be a result of young males having more competitive sperm than old males (e.g. Vuarin et al, 2019). Future studies could investigate whether CFC via sperm ejection might be a mechanism for females to prefer young males. Here, a demonstration of CFC could be possible using the fruit fly system, due to the availability of transgenic lines in which females do not eject sperm at all (Lee et al, 2015) and lines with green and red fluorescent sperm labels (Manier et al, 2010). Specifically, wild-type (females can eject sperm) and transgenic females (females do not eject sperm) could be mated with young or old males in a 2x2 factorial design, and the proportion of sperm stored versus ejected, and the fertilisation success of males, could be compared for each of the four treatments. Here, sperm viability assays could first be used to investigate the contribution of differential viability of sperm in driving results. Additionally, using old and young males with green or red fluorescent protein in sperm, a competitive fertilization scenario could be designed to test whether females eject more sperm from old (i.e. less preferred) compared to young males.

Sperm versus seminal fluid senescence: I showed that the deleterious effects of advancing age on male reproduction can be alleviated by females having young seminal fluid (chapter 4). Future studies in fruit flies could manipulate the age of the seminal fluid donor (i.e. son of tudor) in addition to the age of the focal (whole ejaculate producing) male. Such an experiment will allow us to specifically ask whether it is just the presence of extra seminal fluid that alleviates senescence in reproduction, or whether it is both, the presence and age (thus quality, quantity, and composition) of the seminal fluid. In other animals, a similar experiment could be conducted by first separating sperm and seminal fluid in ejaculates of males (e.g. via centrifugation), and then transplanting/mixing the seminal fluid of young or old males with the sperm of young or old

males in a 2x2 factorial design. These ejaculates then could be assessed for their quality and fertilising ability to assess the relative contribution of sperm and seminal fluid in driving male reproductive senescence. If such experiments consistently show improvement in sperm of old males when mixed with seminal fluid from young males, then seminal fluid transplantation could have groundbreaking biomedical applications (e.g. Eini et al, 2021).

Resource allocation and disposable soma: My thesis (chapters 2, 3 and 4) suggests that sperm senesce at slower rates than seminal fluid, indicating that males might have evolved to allocate more resources into maintaining the germline than somatic tissue (Maklakov and Immler, 2016). Some studies indirectly show that such resource allocation trade-offs might be occurring. For instance, Ivimey-Cook et al (2023) show in zebrafish that fasted males and females, upon refeeding, have rapid fin growth but also suppress their fertility (due to possible germline-soma trade-offs). Chen et al (2020) also show in zebrafish that ablation of the germline leads to faster rates of fin (somatic) repair, compared to when the germline is intact. However, in these experiments, comparing changes in somatic tissues that do not directly influence fertility (fins), versus the germline that directly influences fertility, might not provide a fair comparison. Male *Drosophila melanogaster* offer a promising system to address this shortcoming, where a fair comparison can be made by investigating allocation towards gametic and somatic components *of the ejaculate*- sperm and seminal fluid respectively. Here, techniques developed in insects (Levin et al, 2017) including fruit flies (O'Brien et al, 2008; Min et al, 2006) can be employed, where carbon isotopes in food are used to assess the amount of carbon allocated to different tissues. Using male fruit flies, future studies could thus test whether accessory glands receive relatively less carbon compared to testes, and whether male age modulates this differential allocation.

Sex-specific sperm storage: My thesis showed that prolonged paternal sperm storage duration led to reduced reproductive output of sons (chapter 5). Males in certain species store sperm for longer durations than in fruit flies (e.g. many months in bats- Sato et al, 2023). Additionally, females in some animal species store sperm for many years (e.g. in reptiles and Hymenoptera (Gotoh et al, 2023; Orr and Brenan, 2015)). It is yet a mystery how females in these taxa store sperm for prolonged durations without sperm becoming inviable. Some studies show experimentally that female reproductive fluid alleviates declines in stored sperm (Gasparini et al, 2013; Hadlow et al, 2023), which might facilitate long-term sperm storage in females. Reinhardt and Ribou (2013) show that sperm oxidative damage is higher in sperm stored within males compared to females, indicating that female-specific antioxidants might buffer declines in stored-sperm quality. Additionally, seminal fluid transferred along with sperm by males to females, can play a crucial role in protecting the functionality of sperm stored inside females (Holman, 2009; King et al, 2011). To effectively investigate adaptations that facilitate sperm storage, studies could compare the combined and separate influence of sperm storage in females and males, on sperm and offspring quality. Here, species with long-term sperm storage where transgenic lines can be developed, such that males do not produce seminal fluid (e.g. fruit flies- Tram and Wolfner, 1999) or females do not produce ovarian fluid, could be particularly useful. Changes in sperm and offspring quality due to sperm storage in males and/or females, could be compared between the transgenic and wildtype lines, to demonstrate the separate and combined roles of male and female reproductive fluids in facilitating long-term sperm storage inside females.

Conclusions

Various intricate mechanisms and pathways lead to, modulate, and mediate male reproductive senescence. Through comparative and experimental approaches with a special focus on ejaculates, I show that male reproductive senescence manifests differently across taxa. I also highlight that male reproductive senescence is a consequence of age-related changes in a multitude of traits, such as ejaculate traits (sperm and seminal fluid quantity and quality), and pre-copulatory traits (such as male mating success). My research emphasizes the importance of assaying both inter- and intra-generational effects of advancing male age, the role of demographic processes in buffering senescence, and the need to disentangle effects of factors confounding with age. The mathematical formalisation of my thesis, and the parallels drawn between whole-organism and sperm life-history, provide a novel conceptual foundation and theoretical framework for future research on this topic. While my experiments focus on fruit flies, the results herein can be relevant across taxa, including humans. Overall, my thesis highlights key gaps in ageing research, improves our understanding of the complexities of male reproductive ageing, and generates new avenues for future studies.

References

Alavioon, G., Hotzy, C., Nakhro, K., Rudolf, S., Scofield, D.G., Zajitschek, S., Maklakov, A.A., Immler, S., 2017. Haploid selection within a single ejaculate increases offspring fitness. Proc. Natl. Acad. Sci. U.S.A. 114, 8053–8058. https://doi.org/10.1073/pnas.1705601114

Barbagallo, F., Cannarella, R., Crafa, A., La Vignera, S., Condorelli, R.A., Manna, C., Calogero, A.E., 2023. The Impact of a Very Short Abstinence Period on Assisted Reproductive Technique Outcomes: A Systematic Review and Meta-Analysis. Antioxidants 12, 752. https://doi.org/10.3390/antiox12030752

Bhutani, K., Stansifer, K., Ticau, S., Bojic, L., Villani, A.-C., Slisz, J., Cremers, C.M., Roy, C., Donovan, J., Fiske, B., Friedman, R.C., 2021. Widespread haploid-biased gene expression enables sperm-level natural selection. Science 371, eabb1723. https://doi.org/10.1126/science.abb1723

Bonduriansky, R., Maklakov, A., Zajitschek, F., Brooks, R., 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. Functional Ecology 22, 443–453. https://doi.org/10.1111/j.1365-2435.2008.01417.x

Bowers, E.K., Sakaluk, S.K., Thompson, C.F., 2017. Interactive effects of parental age on offspring fitness and age-assortative mating in a wild bird. J Exp Zool Pt A 327, 302–310. https://doi.org/10.1002/jez.2090

Chen, H., Jolly, C., Bublys, K., Marcu, D., Immler, S., 2020. Trade-off between somatic and germline repair in a vertebrate supports the expensive germ line hypothesis. Proc. Natl. Acad. Sci. U.S.A. 117, 8973–8979. https://doi.org/10.1073/pnas.1918205117

Cohen, A.A., Coste, C.F.D., Li, X., Bourg, S., Pavard, S., 2020. Are trade-offs really the key drivers of ageing and life span? Functional Ecology 34, 153–166. https://doi.org/10.1111/1365-2435.13444

Cordero, C., Eberhard, W.G., 2003. Female choice of sexually antagonistic male adaptations: a critical review of some current research. Journal of Evolutionary Biology 16, 1–6. https://doi.org/10.1046/j.1420-9101.2003.00506.x

Crean, A.J., Dwyer, J.M., Marshall, D.J., 2012. Fertilization Is Not a New Beginning: The Relationship between Sperm Longevity and Offspring Performance. PLoS ONE 7, e49167. https://doi.org/10.1371/journal.pone.0049167

Dammhahn, M., Dingemanse, N.J., Niemelä, P.T., Réale, D., 2018. Pace-of-life syndromes: a framework for the adaptive integration of behaviour, physiology and life history. Behav Ecol Sociobiol 72, 62, s00265-018-2473-y. https://doi.org/10.1007/s00265-018-2473-y

Eini, F., Kutenaei, M.A., Shirzeyli, M.H., Dastjerdi, Z.S., Omidi, M., Novin, M.G., 2021. Normal seminal plasma could preserve human spermatozoa against cryopreservation damages in Oligozoospermic patients. BMC Mol and Cell Biol 22, 50. https://doi.org/10.1186/s12860-021-00390-6

Firman, R.C., Gasparini, C., Manier, M.K., Pizzari, T., 2017. Postmating Female Control: 20 Years of Cryptic Female Choice. Trends in Ecology & Evolution 32, 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Gasparini, C., Evans, J.P., 2013. Ovarian Fluid Mediates the Temporal Decline in Sperm Viability in a Fish with Sperm Storage. PLoS ONE 8, e64431. https://doi.org/10.1371/journal.pone.0064431

Gotoh, A., Takeshima, M., Mizutani, K., 2023. Near-anoxia induces immobilization and sustains viability of sperm stored in ant queens. Sci Rep 13, 3029. https://doi.org/10.1038/s41598-023-29705-7

Gowaty, P.A., 2012. The evolution of multiple mating: Costs and benefits of polyandry to females and of polygyny to males. Fly 6, 3–11. https://doi.org/10.4161/fly.18330

Hadlow, J.H., Evans, J.P., Lymbery, R.A., 2023. Female reproductive fluids 'rescue' sperm from phenotypic ageing in an external fertilizer. Proc. R. Soc. B. 290, 20230574. https://doi.org/10.1098/rspb.2023.0574

Hayward, A.D., Wilson, A.J., Pilkington, J.G., Clutton-Brock, T.H., Pemberton, J.M., Kruuk, L.E.B., 2013. Reproductive senescence in female S oay sheep: variation across traits and contributions of individual ageing and selective disappearance. Functional Ecology 27, 184–195. https://doi.org/10.1111/1365-2435.12029

Hoikkala, A., Saarikettu, M., Kotiaho, J.S., Liimatainen, J.O., 2008. Age-related decrease in male reproductive success and song quality in Drosophila montana. Behavioral Ecology 19, 94–99. https://doi.org/10.1093/beheco/arm101

Holman, L., 2009. *Drosophila melanogaster* seminal fluid can protect the sperm of other males. Functional Ecology 23, 180–186. https://doi.org/10.1111/j.1365-2435.2008.01509.x

Iannelli, M., Milner, F., 2017. The Basic Approach to Age-Structured Population Dynamics: Models, Methods and Numerics, Lecture Notes on Mathematical Modelling in the Life Sciences. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-024-1146-1

Iliadi, K.G., Boulianne, G.L., 2010. Age-related behavioral changes in *Drosophila*. Annals of the New York Academy of Sciences 1197, 9–18. https://doi.org/10.1111/j.1749-6632.2009.05372.x

Immler, S., 2019. Haploid Selection in "Diploid" Organisms. Annu. Rev. Ecol. Evol. Syst. 50, 219–236. https://doi.org/10.1146/annurev-ecolsys-110218-024709

Immler, S., Otto, S.P., 2018. The Evolutionary Consequences of Selection at the Haploid Gametic Stage. The American Naturalist 192, 241–249. https://doi.org/10.1086/698483

Ivimey-Cook, E.R., Murray, D.S., De Coriolis, J.-C., Edden, N., Immler, S., Maklakov, A.A., 2023. Fasting increases investment in soma upon refeeding at the cost of gamete quality in zebrafish. Proc. R. Soc. B. 290, 20221556. https://doi.org/10.1098/rspb.2022.1556

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 off-set costs related to fertility, or are we missing something else? BioEssays 34, 609–619. https://doi.org/10.1002/bies.201100157
Johnson, S.L., Zellhuber-McMillan, S., Gillum, J., Dunleavy, J., Evans, J.P., Nakagawa, S., Gemmell, N.J., 2018. Evidence that fertility trades off with early offspring fitness as males age. Proc. R. Soc. B. 285, 20172174. https://doi.org/10.1098/rspb.2017.2174
Jones, A.G., 2002. THE EVOLUTION OF ALTERNATIVE CRYPTIC FEMALE CHOICE STRATEGIES IN AGE-STRUCTURED POPULATIONS. Evolution 56, 2530–2536. https://doi.org/10.1111/j.0014-3820.2002.tb00177.x
Joseph, S., Kirkpatrick, M., 2004. Haploid selection in animals. Trends in Ecology & Evolution 19, 592–597. https://doi.org/10.1016/j.tree.2004.08.004
King, M., Eubel, H., Millar, A.H., Baer, B., 2011. Proteins within the seminal fluid are crucial to keep sperm viable in the honeybee Apis mellifera. Journal of Insect Physiology 57, 409–414. https://doi.org/10.1016/j.jinsphys.2010.12.011
Kirkwood, T.B.L., Rose, M., 1991. Evolution of senescence: late survival sacrificed for reproduction. Phil. Trans. R. Soc. Lond. B 332, 15–24. https://doi.org/10.1098/rstb.1991.0028
Kokko, H., 1998. Good genes, old age and life-history trade-offs. Evolutionary Ecology 12, 739–750. https://doi.org/10.1023/A:1006541701002
Lee, K.-M., Daubnerová, I., Isaac, R.E., Zhang, C., Choi, S., Chung, J., Kim, Y.-J., 2015. A Neuronal Pathway that Controls Sperm Ejection and Storage in Female Drosophila. Current Biology 25, 790–797. https://doi.org/10.1016/j.cub.2015.01.050
Lee, R., Chu, C.Y.C., 2023. Theoretical perspectives on reproductive aging. Front. Ecol. Evol. 10, 934732. https://doi.org/10.3389/fevo.2022.934732
Lemaître, J., Gaillard, J., 2017. Reproductive senescence: new perspectives in the wild. Biological Reviews 92, 2182–2199. https://doi.org/10.1111/brv.12328
Lemaître, J.-F., Berger, V., Bonenfant, C., Douhard, M., Gamelon, M., Plard, F., Gaillard, J.-M., 2015. Early-late life trade-offs and the evolution of ageing in the wild. Proc. R. Soc. B. 282, 20150209. https://doi.org/10.1098/rspb.2015.0209
Lenart, P., Bienertová-Vašků, J., Berec, L., 2018. Evolution favours aging in populations with assortative mating and in sexually dimorphic populations. Sci Rep 8, 16072. https://doi.org/10.1038/s41598-018-34391-x
Levin, E., McCue, M.D., Davidowitz, G., 2017. More than just sugar: allocation of nectar amino acids and fatty acids in a Lepidopteran. Proc. R. Soc. B. 284, 20162126. https://doi.org/10.1098/rspb.2016.2126
Levitan, D.R., 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. Proc. R. Soc. Lond. B 267, 531–534. https://doi.org/10.1098/rspb.2000.1032
Long, C.E., Markow, T.A., Yaeger, P., 1980. Relative male age, fertility, and competitive mating success inDrosophila melanogaster. Behav Genet 10, 163–170. https://doi.org/10.1007/BF01066266
Maklakov, A.A., Immler, S., 2016. The Expensive Germline and the Evolution of Ageing. Current Biology 26, R577–R586. https://doi.org/10.1016/j.cub.2016.04.012
Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving Mechanisms of Competitive Fertilization Success in *Drosophila melanogaster*. Science 328, 354–357. https://doi.org/10.1126/science.1187096
Meunier, L., Sorci, G., Abi Hussein, H., Hingrat, Y., Rehspringer, N., Saint-Jalme, M., Lesobre, L., Torres Carreira, J., 2022. Pre-but not post-meiotic senescence affects sperm quality and reproductive success in the North African houbara bustard. Front. Ecol. Evol. 10, 977184. https://doi.org/10.3389/fevo.2022.977184
Min, K.-J., Hogan, M.F., Tatar, M., O'Brien, D.M., 2006. Resource allocation to reproduction and soma in Drosophila: A stable isotope analysis of carbon from dietary sugar. Journal of Insect Physiology 52, 763–770. https://doi.org/10.1016/j.jinsphys.2006.04.004
Monaghan, P., Metcalfe, N.B., 2019. The deteriorating soma and the indispensable germline: gamete senescence and offspring fitness. Proc. R. Soc. B. 286, 20192187. https://doi.org/10.1098/rspb.2019.2187
Nussey, D.H., Coulson, T., Festa-Bianchet, M., Gaillard, J. -M., 2008. Measuring senescence in wild animal populations: towards a longitudinal approach. Functional Ecology 22, 393–406. https://doi.org/10.1111/j.1365-2435.2008.01408.x
Nussey, D.H., Kruuk, L.E.B., Donald, A., Fowlie, M., Clutton-Brock, T.H., 2006. The rate of senescence in maternal performance increases with early-life fecundity in red deer. Ecology Letters 9, 1342–1350. https://doi.org/10.1111/j.1461-0248.2006.00989.x
O'Brien, D.M., Min, K.-J., Larsen, T., Tatar, M., 2008. Use of stable isotopes to examine how dietary restriction extends Drosophila lifespan. Current Biology 18, R155–R156. https://doi.org/10.1016/j.cub.2008.01.021
Orr, T.J., Brennan, P.L.R., 2015. Sperm storage: distinguishing selective processes and evaluating criteria. Trends in Ecology & Evolution 30, 261–272. https://doi.org/10.1016/j.tree.2015.03.006
Pearl, R., 1927. The Growth of Populations. The Quarterly Review of Biology 2, 532–548. https://doi.org/10.1086/394288
Pizzari, T., Birkhead, T.R., 2000. Female feral fowl eject sperm of subdominant males. Nature 405, 787–789. https://doi.org/10.1038/35015558
Pizzari, T., Dean, R., Pacey, A., Moore, H., Bonsall, M.B., 2008. The evolutionary ecology of pre- and post-meiotic sperm senescence. Trends in Ecology & Evolution 23, 131–140. https://doi.org/10.1016/j.tree.2007.12.003
Price, C.S.C., Dyer, K.A., Coyne, J.A., 1999. Sperm competition between Drosophila males involves both displacement and incapacitation. Nature 400, 449–452. https://doi.org/10.1038/22755
Promislow, D.E.L., Flatt, T., Bonduriansky, R., 2022. The Biology of Aging in Insects: From *Drosophila* to Other Insects and Back. Annu. Rev. Entomol. 67, 83–103. https://doi.org/10.1146/annurev-ento-061621-064341
Reinhardt, K., 2007. Evolutionary Consequences of Sperm Cell Aging. The Quarterly Review of Biology 82, 375–393. https://doi.org/10.1086/522811

Reinhardt, K., Ribou, A.-C., 2013. Females become infertile as the stored sperm's oxygen radicals increase. Sci Rep 3, 2888. https://doi.org/10.1038/srep02888
Reinhardt, K., Siva-Jothy, M.T., 2005. An Advantage for Young Sperm in the House Cricket *Acheta domesticus*. The American Naturalist 165, 718–723. https://doi.org/10.1086/430010
Rezaei, A., Krishna, M.S., Santhosh, H.T., 2015. Male Age Affects Female Mate Preference, Quantity of Accessory Gland Proteins, and Sperm Traits and Female Fitness in D. melanogaster. Zoological Science 32, 16. https://doi.org/10.2108/zs140121
Reznick, D., Nunney, L., Tessier, A., 2000. Big houses, big cars, superfleas and the costs of reproduction. Trends in Ecology & Evolution 15, 421–425. https://doi.org/10.1016/S0169-5347(00)01941-8
Roff, D.A., Fairbairn, D.J., 2007. The evolution of trade-offs: where are we? J of Evolutionary Biology 20, 433–447. https://doi.org/10.1111/j.1420-9101.2006.01255.x
Rose, M., Charlesworth, B., 2002. A Test of Evolutionary Theories of Senescence. Sci. Aging Knowl. Environ. 2002. https://doi.org/10.1126/sageke.2002.37.cp17
Sato, T., Sugiyama, T., Sekijima, T., 2023. Mating in the cold. Prolonged sperm storage provides opportunities for forced copulation by male bats during winter. Front. Physiol. 14, 1241470. https://doi.org/10.3389/fphys.2023.1241470
Sepil, I., Hopkins, B.R., Dean, R., Bath, E., Friedman, S., Swanson, B., Ostridge, H.J., Harper, L., Buehner, N.A., Wolfner, M.F., Konietzny, R., Thézénas, M.-L., Sandham, E., Charles, P.D., Fischer, R., Steinhauer, J., Kessler, B.M., Wigby, S., 2020. Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 117, 17094–17103. https://doi.org/10.1073/pnas.2009053117
Sharma, R., Agarwal, A., Rohra, V.K., Assidi, M., Abu-Elmagd, M., Turki, R.F., 2015. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13, 35. https://doi.org/10.1186/s12958-015-0028-x
Snook, R.R., Hosken, D.J., 2004. Sperm death and dumping in Drosophila. Nature 428, 939–941. https://doi.org/10.1038/nature02455
Taborsky, M., Schütz, D., Goffinet, O., Van Doorn, G.S., 2018. Alternative male morphs solve sperm performance/longevity trade-off in opposite directions. Sci. Adv. 4, eaap8563. https://doi.org/10.1126/sciadv.aap8563
Tidière, M., Thevenot, X., Deligiannopoulou, A., Douay, G., Whipple, M., Siberchicot, A., Gaillard, J.-M., Lemaître, J.-F., 2018. Maternal reproductive senescence shapes the fitness consequences of the parental age difference in ruffed lemurs. Proc. R. Soc. B. 285, 20181479. https://doi.org/10.1098/rspb.2018.1479
Tram, U., Wolfner, M.F., 1999. Male Seminal Fluid Proteins Are Essential for Sperm Storage in Drosophila melanogaster. Genetics 153, 837–844. https://doi.org/10.1093/genetics/153.2.837
Van De Pol, M., Verhulst, S., 2006. Age-Dependent Traits: A New Statistical Model to Separate Within- and Between-Individual Effects. The American Naturalist 167, 766–773. https://doi.org/10.1086/503331
Vedder, O., Bouwhuis, S., 2018. Heterogeneity in individual quality in birds: overall patterns and insights from a study on common terns. Oikos 127, 719–727. https://doi.org/10.1111/oik.04273
Vuarin, P., Bouchard, A., Lesobre, L., Levêque, G., Chalah, T., Jalme, M.S., Lacroix, F., Hingrat, Y., Sorci, G., 2019. Post-copulatory sexual selection allows females to alleviate the fitness costs incurred when mating with senescing males. Proc. R. Soc. B. 286, 20191675. https://doi.org/10.1098/rspb.2019.1675
Vuarin, P., Lesobre, L., Levêque, G., Saint Jalme, M., Lacroix, F., Hingrat, Y., Sorci, G., 2021. Paternal age negatively affects sperm production of the progeny. Ecology Letters 24, 719–727. https://doi.org/10.1111/ele.13696
Wagner, R.H., Helfenstein, F., Danchin, E., 2004. Female choice of young sperm in a genetically monogamous bird. Proc. R. Soc. Lond. B 271. https://doi.org/10.1098/rsbl.2003.0142
Wykes, S., Visscher, D., Krawetz, S., 1997. Haploid transcripts persist in mature human spermatozoa. Molecular Human Reproduction 3, 15–19. https://doi.org/10.1093/molehr/3.1.15
Wylde, Z., Spagopoulou, F., Hooper, A.K., Maklakov, A.A., Bonduriansky, R., 2019. Parental breeding age effects on descendants' longevity interact over 2 generations in matrilines and patrilines. PLoS Biol 17, e3000556. https://doi.org/10.1371/journal.pbio.3000556
Yasui, Y., 1997. A "Good-Sperm" Model Can Explain the Evolution of Costly Multiple Mating by Females. The American Naturalist 149, 573–584. https://doi.org/10.1086/286006
Zera, A.J., Harshman, L.G., 2001. The Physiology of Life History Trade-Offs in Animals. Annu. Rev. Ecol. Syst. 32, 95–126. https://doi.org/10.1146/annurev.ecolsys.32.081501.114006
Zhang, H., Vedder, O., Becker, P.H., Bouwhuis, S., 2015. Age-dependent trait variation: the relative contribution of within-individual change, selective appearance and disappearance in a long-lived seabird. Journal of Animal Ecology 84, 797–807. https://doi.org/10.1111/1365-2656.12321

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