# An exposition of ejaculate senescence and its inhibition in *Drosophila*

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**Short title:** Ejaculate senescence in *Drosophila*

**One sentence summary:** Ejaculate senescence arises from mating-dependent sperm and seminal protein quality and quantity loss, but it can be delayed

**Abstract**

Declining male ejaculate performance with age (ejaculate senescence) is thought to be a widespread phenomenon, and a major contributor to the current 'male fertility crisis' in humans. However, the mechanistic basis of ejaculate senescence is poorly understood. Here, we show that, in *Drosophila*, ejaculate senescence arises via distinct changes to both sperm and seminal fluid proteins (Sfps). Sperm production declines with age, while repeated mating depletes sperm reserves and reduces sperm viability. In contrast, Sfps accumulate over time in sexually abstinent males, resulting in compositional change in the seminal proteome, which is associated with reduced Sfp transfer. We also find evidence of age-related qualitative changes in several functionally important Sfps. Finally, we reveal that ablation of insulin-like peptide-producing cells in males can ameliorate declines in fertility and other aspects of ejaculate senescence, suggesting that anti-ageing interventions could be co-opted to promote healthy male reproductive ageing.

**Main Text**

There is increasing evidence that male age reduces ejaculate performance in humans and other animals, which contributes to low fertility (*1*, *2*). However, the mechanisms underlying ejaculate senescence are poorly understood (*3*). This is a pressing concern given the growing trend for delayed parenthood and declines in male sperm counts in high-income countries (*4*), resulting in the current "male fertility crisis" (*5*). Consequently, there is urgent need for experimental models to investigate the fundamental mechanisms underlying ejaculate senescence. Past experimental research on ejaculate senescence has almost exclusively focused on sperm or testicular ageing and documented an age-related decline in the quantities of sperm that males ejaculate, and traits such as sperm swimming speed and morphology (*1*, *6–9*). However, the ejaculate is composed of both sperm and non-sperm seminal fluid. The seminal fluid is a complex cocktail of functionally diverse molecules that have crucial contributions to fertility (*10*, *11*). The seminal fluid proteins (Sfps) in particular are central in supporting sperm function, and in many species also modulate post-mating female physiology and behaviour (*12–15*). Presently, we do not know to what extent age-related

declines in ejaculate performance are driven by sperm versus non-sperm components of the ejaculate, nor any underlying mechanisms for this. We also do not know whether it will be possible to delay ejaculate senescence, perhaps by co-opting interventions known to promote healthy somatic ageing. *Drosophila melanogaster* is a well-established model for both ageing (*16*) and ejaculate research (*17*), but these fields have largely operated independently (but see *7–10*). Here, we use *Drosophila* to identify mechanisms that both drive and slow ejaculate senescence. We dissect the contributions of age-related changes to sperm and Sfps, and investigate the impact of a somatic lifespan-extending intervention on reproductive ageing.

**Reproductive consequences of male ageing and mating history**

We measured reproductive traits in experimental males that were one-week-old (1w), three-weeks-old (3w) or five-weeks-old (5w) and had been maintained in either single-sex groups of twelve (unmated, "U"), or in groups of three males and nine females (frequently mated, "F") (Figure 1A). These time points span male peak reproductive performance (1w, young) and reproductively senesced states (5w, old) while ensuring that most males survive the experiment (*19*): 79% of old, frequently mated (Old-F) males and more than 94% of males in all the other treatments survived the experiment (Figure S1). We therefore avoid heavily biasing samples in favour of long-lived individuals (*6*). As expected based on previous work (*18*, *20–22*), we found clear evidence that male reproductive function declines with age. Old-F males father fewer offspring, are more likely to be infertile, are poorer at suppressing female remating and their sperm perform poorly when competing with the ejaculates of rival males (Figure 1B-E). Old, unmated males (Old-U) are also poor sperm competitors, but their reproductive output, fertility and ability to suppress female remating are not significantly reduced compared to young, unmated (Young-U) males (Figure 1B-E). Old males also show a significant reduction in copulation probability – an effect again exacerbated by frequent mating (Figure S2). Taken together, our results highlight how frequent mating activity is an important contributor to age-dependent reproductive decline in males. Reduced copulation probability likely results from the reduced courtship ability of old males and female discrimination of Old-F mated males (*23*), while the decline in post-mating traits signifies ejaculate senescence.

Next, we determined whether the seminal fluid alone, in the absence of sperm, contributes to age-related reproductive decline. Long-term elevated egg production and sexual refractoriness in females requires the receipt of sperm as well as Sfps, but these responses are elevated in the short term even without sperm (*24*). Using spermless (*son-of-tudor*) males we found that old males and young, frequently mated (Young-F) males are poorer at stimulating female fecundity (Figure 1F). Likewise, Old-F males and, to a smaller extent, Young-F males are poorer at suppressing female remating (Figure 1G). As the ejaculates of *son-of-tudor* males do not contain sperm, the age-related decline in reproductive function in these experiments is due to Sfps.

**Age and mating effects on the seminal proteome**

Having identified loss of ejaculate performance associated with increased age and mating activity, and the direct contribution of the seminal fluid, through experiments with spermless males, we next investigated changes to the seminal fluid proteome to explain these effects. We first applied label-free quantitative proteome analysis to the Sfp-producing tissues of experimental males (*25*).

We focused our analyses on established Sfps (*25*, *26*) and first examined their production by males before transfer to females. We found that the abundance of many Sfps increases with age in unmated males, mirrored by an increase in size of the accessory gland, the tissue that makes most Sfps, whereas Sfp abundance and accessory gland size do not change with age in frequently mated males (Figure 2A & 2B & S3). A principal component analysis supported these findings, showing that the composition of the seminal fluid proteome changes significantly with age in unmated males, but not in frequently mated males (Figure 2C). All of the upregulated Sfps are specific to the accessory glands and include Sfps that function in sperm storage and female post-mating behavior modification (*12*, *15*) (Table S1). In contrast, the abundance of most of the ejaculatory duct derived Sfps (*25*, *27*) do not exhibit a differential response to age and mating; they cluster separately from the rest of the proteins (Figure 2A, Table S2). Notably, this disparity indicates that the two male reproductive tissues respond differentially to age and mating.

By comparing the quantity of Sfps present in males before and after mating we can infer Sfp transfer to females during copulation (*25*). Overall, we again found that the abundance of Sfps transferred changes significantly with age and mating, leading to a significant change in Sfp proteome composition. In contrast to our data of Sfp levels in males, transferred Sfps show an age-related decline in unmated males (Figure 2D-F), in spite of their higher accumulation in these males (Figure 2A & 2B). We again observed separate clustering for several ejaculatory duct specific Sfps, where the trend is an increase in Sfp transfer with age in frequently mated males. However, the differential response of the two tissues are more pronounced for Sfp levels in males compared to Sfp transfer. Together these results suggest that a distinct set of Sfps accumulates with age in the absence of mating, resulting in compositional change of the seminal proteome. In contrast, the abundance and transfer of Sfps is maintained with age in the presence of frequent mating, which, given that their striking decline in ejaculate performance, indicates that quantitative Sfp effects do not cause ejaculate senescence in these males.

Next, we performed Western blot analyses for six Sfps of known functional importance to look for evidence of qualitative changes related to age and mating history. For each Sfp tested, our proteomic data, which are based on trypsin-cleaved peptides, indicate either an age-related increase in unmated males or no change in frequently mated males in Sfp abundances. However, Acp62F, an Sfp which has been implicated in sperm competition (*28*), is largely undetectable by Western blot in Old-F males (Figure 2G and Figure S4). This suggests that ageing degrades Acp62F in such a way that no band is detectable on Western blot but that the peptides are present in mass spectrometry. The double band of Acp26Aa (ovulin), one of the most rapidly evolving proteins in *Drosophila* (*29*), becomes either more condensed due to a new form of protein in old age (through alternative splicing) or loses the top band in old males through an age-specific post-translational modification. Likewise, Semp1, a seminal metalloprotease that cleaves ovulin within females (*30*), shows an additional upper band in old males from both mating groups, indicating a potential age-specific post-translational modification (Figure 2G and Figure S4). Full-length ovulin and two of its cleavage products stimulate ovulation, hence any deterioration with age and mating would negatively impact female ovulation rate following mating (*31*). We saw no qualitative changes in the three other Sfps

tested (Acp70A [sex peptide], Acp36DE and CG9997; Figure S4). Together, these results indicate that a subset of seminal proteins display qualitative alterations in response to age and, in some cases the combination of age and frequent mating. These qualitative changes are consistent with a loss of seminal protein homeostasis and are associated with the compromised post-mating phenotypes in females mating with old males (*32*).

Finally, we investigated whether the previously-identified sperm-protecting function of seminal fluid (*33*) declines with age and frequent mating. Using SYBR-14 and propidium iodide fluorescent staining, we measured the effects of seminal fluid on the survival of sperm from a different one-week-old male. However, we found no evidence that age or mating history compromises the ability of seminal fluid to keep sperm alive (Figure S5).

**Age and mating effects on sperm**

Consistent with previous evidence of declining rates of spermatogenesis with age in flies (*34*), we found that the number of germline cysts in the final individualization stage of spermatogenesis (*35*) declines substantially as males age. Strikingly, this decline occurs at indistinguishable rates in unmated and frequently mated males. This suggests that males undergo a chronological decline in sperm production which is unaffected by mating activity (Figure 3A). This finding is surprising given that sperm production rate is known to be malleable; for example, males elevate sperm production in response to the presence of rivals (*36*), and testis germline stem cell maintenance responds plastically to nutrition (*37*).

In the absence of mating, the size of the seminal vesicles (where mature sperm are stored) increases in unmated males, but decreases in frequently mated males (Figure 3B). This suggests that, like Sfps in the accessory glands, sperm stores accumulate in unmated males, despite the declining rate of sperm production. However, in contrast to Sfps, males are unable to sufficiently replenish sperm when they mate frequently throughout life, leading to depletion of sperm stores and high incidences of no sperm being found in the seminal vesicles (Figure 3C). Frequently mated males also have lower sperm viability, independent of age class suggesting that regular copulation leads to reduced

sperm quality (Figure 3D). As might then be expected, we found a significant reduction in the number of sperm present in sperm storage organs (seminal receptacle and spermatheca) of females mated to Old-F males, relative to all other treatments, although there were also nonsignificant downward trends for both Old-U and 3 week-F males (Figure 3E).

The fact that Old-F males are sperm-depleted, but show no evidence of decline in Sfp quantity, indicates a mismatch develops in the relative capacity to produce sperm and Sfps. In the short term, when males mate several times in rapid succession, seminal fluid rather than sperm is thought to limit fertility in male *Drosophila* and other insects (*38*, *39*), but our data show that over the long term, Sfp replenishment capacity remains strong and is little affected by age. However, our data clearly show that non-sperm components of the ejaculate also contribute to the loss of ejaculate performance in ageing males, as evidenced by a reduced ability to stimulate female post-mating responses (Figure 4), known to be controlled by Sfps (*12*). Moreover, Old-U males, despite accumulating Sfps with age, transferred reduced Sfp amounts to females, which could contribute to their decline in ejaculate performance. However, Old-F males did not show a reduction in Sfp transfer, yet they had poorer reproductive performance. It is therefore likely that quantitative changes in Sfps play a minor role in declining ejaculate performance. Instead, our data are consistent with age-related declines in ejaculate performance resulting from qualitative seminal fluid defects, such as a loss of seminal protein homeostasis, and compositional changes in unmated males. These seminal fluid defects are compounded by declines in sperm numbers and viability in Old-F males (Figure 4).

**Changes in reproductive ageing with lifespan extension**

Manipulations of the insulin-signaling pathway can extend lifespan in a broad range of taxa (*40*, *41*), but it is unclear if lifespan extension results in a trade-off with male reproductive function, or whether it could provide co-benefits to late-life ejaculate health. We used males in which the insulin-like peptide (dilp)-producing median neurosecretory cells (mNSCs) were ablated late in development (*42*) (hereon ‘ablated males’) and confirmed that these males display increased survival (Figure S6). We found clear evidence that the ablated males have reduced reproductive senescence. Old-F

ablated males are significantly less likely to be infertile and significantly better at suppressing female remating than Old-F control males (Figure 5). We did not detect any significant differences between ablated and control males in offspring production and paternity share (Figure S7). We also found that old ablated males are more likely to successfully copulate than controls, although the effect was more striking in the frequently mated treatment (Figure S8).

Our data show that inhibition of the insulin signaling pathway, in addition to extending lifespan, can ameliorate at least some aspects of ejaculate senescence. This result supports the idea that it is possible, with certain manipulations and under certain conditions, to simultaneously reduce both somatic and reproductive ageing in males. This result apparently contrasts with the effects of rapamycin in mice, which extends lifespan, but causes testicular degeneration (*43*). There are a number of possibilities that could explain the discrepancy, including differential action of insulin and rapamycin pathways in male reproductive organs as well as taxon-specific responses to nutrient-sensing pathway interventions.

**Conclusions**

In humans, a number of studies have shown declines in sperm ejaculate volume, sperm count, motility and viability with age, patterns which are often – but not always – seen in other animals (reviewed in *44*). *Drosophila* show age-related changes in gene expression in some Sfps (*6*), while Red junglefowl show associations between distinct seminal proteome profiles and sperm speed in aging males (*9*). However, in general non-sperm components of the ejaculate have received little attention in the context of ejaculate senescence. Furthermore, to the best of our knowledge, no previous studies have identified interventions capable of elevating ejaculate performance in aging males. Our *Drosophila* study provides a uniquely comprehensive exposition of sperm and seminal proteome changes with age, and links these with a suite of fitness-related ejaculate performance phenotypes. Our data show that both the quantity and quality of sperm and seminal fluid proteins can contribute to the age-related decline in male ejaculate performance, but that the role of these different factors is highly-dependent on the mating environment. Moreover, our data indicate that organism-level insulin signaling is a mediator of both organism survival and male ejaculate quality

retention, and represents a viable target for developing interventions to ameliorate the impact of ageing on the ejaculate.

Perhaps surprisingly, quantitative declines in seminal protein production seem to play a minimal role in *Drosophila* ejaculate senescence, although seminal proteome imbalance and sub-optimal transfer may contribute in sexually abstinent males, perhaps due to harmful accumulations within the accessory glands that prevent normal ejaculation. We instead suggest that future studies in this system should focus on the role of age-related changes in seminal protein homeostasis as a potentially key but largely unexplored mediator of non-sperm ejaculate senescence. However, the most striking fertility declines were strongly associated with a numerical loss of sperm resulting from a declining sperm production rate, which leads to a failure to replenish lost sperm stores. Sperm production declines are striking in humans: the daily rate approximately halves between the ages of 20 and 60 (*45*). A prime candidate for interventions to delay ejaculate senescence must therefore be to target the root of declining sperm production. The activity of the male germline stem cells is sensitive to nutrition and nutrient-sensing pathways (*37*, *46*, *47*), so these interventions represent obvious targets for future research. Whether the improvements to late life fertility we observed in ablated males were a result of direct influences of reduced insulin activity in male reproductive tissues, or as part of overall organismal health (or both), remain to be elucidated. However, given that nutrient-sensing pathways are highly evolutionary conserved in their effects on lifespan and reproduction and that there is considerable overlap in the process of spermatogenesis, sperm proteomes, Sfp-producing cells, and the categories and function of Sfps (*11*, *17*, *48–50*), flies clearly represent a powerful system for developing the basic tools for healthy male reproductive ageing.

**References**


1. S. A. Kidd, B. Eskenazi, A. J. Wyrobek, *Fertil. Steril.* **75**, 237–248 (2001).
2. T. E. Reed *et al.*, *Am. Nat.* **171**, E89–E101 (2008).
3. E. V. Zubkova, M. Wade, B. Robaire, *Fertil. Steril.* **84**, 1191–1198 (2005).
4. S. L. Johnson, J. Dunleavy, N. J. Gemmell, S. Nakagawa, *Ageing Res. Rev.* **19**, 22–33

(2015).

5. M. Sarner, *New Sci.* **236**, 28–32 (2017).

6. M. Koppik, C. Fricke, *Mol. Ecol.*, 6704–6716 (2017).

7. B. T. Preston, M. S. Jalme, Y. Hingrat, F. Lacroix, G. Sorci, *Ecol. Lett.* **14**, 1017–1024 (2011).

8. S. L. Johnson *et al.*, *Proc. R. Soc. B Biol. Sci.* **285**, 20172174 (2018).

9. K. Borziak, A. Álvarez-Fernández, T. L. Karr, T. Pizzari, S. Dorus, *Sci. Rep.* **6**, 35864 (2016).

10. J. C. Perry, L. Sirot, S. Wigby, *Trends Ecol. Evol.* **28**, 414–422 (2013).

11. B. R. Hopkins, I. Sepil, S. Wigby, *Curr. Biol.* **27**, R404–R405 (2017).

12. F. W. Avila, L. K. Sirot, B. A. Laflamme, C. D. Rubinstein, M. F. Wolfner, *Annu. Rev. Entomol.* **56**, 21–40 (2011).

13. L. K. Sirot, A. Wong, T. Chapman, M. F. Wolfner, *Cold Spring Harb. Perspect. Biol.* **7** (2015), doi:101101/cshperspect.a017533.

14. K. Gilany, A. Minai-Tehrani, E. Savadi-Shiraz, H. Rezadoost, N. Lakpour, *J. Reprod. Infertil.* **16**, 61–71 (2015).

15. B. R. Hopkins, F. W. Avila, M. F. Wolfner, in *Encyclopedia of Reproduction* (2018; https://app.dimensions.ai/details/publication/pub.1099672946), pp. 137–144.

16. L. Guarente, C. Kenyon, *Nature*. **408**, 255–262 (2000).

17. C. Wilson, A. Leiblich, D. C. I. Goberdhan, F. Hamdy, *Curr. Top. Dev. Biol.* **121**, 339–375 (2017).

18. H. Ruhmann, M. Koppik, M. F. Wolfner, C. Fricke, *Exp. Gerontol.* **103**, 1–10 (2018).

19. M. Koppik, H. Ruhmann, C. Fricke, *J. Insect Physiol.* **111**, 16–24 (2018).

20. N. Prowse, L. Partridge, *J. Insect Physiol.* **43**, 501–512 (1997).

21. I. Kvelland, *Hereditas*. **53**, 281–306 (1965).

22. M. S. Snoke, D. E. L. Promislow, *Heredity (Edinb).* **91**, 546–556 (2003).

23. E. R. Churchill, C. Dytham, M. D. F. Thom, *Sci. Rep.* **9**, 2167 (2019).

24. L. Xue, M. Noll, *Proc. Natl. Acad. Sci. U. S. A.* **97**, 3272–3275 (2000).

25. I. Sepil *et al.*, *Mol. Cell. Proteomics*. **18**, S46–S58 (2019).

26. G. Findlay, X. Yi, M. MacCoss, W. Swanson, *PLoS Biol.* **6**, e178 (2008).

27. N. Takemori, M. T. Yamamoto, *Proteomics*. **9**, 2484–2493 (2009).

28. O. Lung *et al.*, *Genetics*. **160**, 211–224 (2002).

29. A. Wong, S. N. Albright, M. F. Wolfner, *Proc. Natl. Acad. Sci.* **103**, 18644–18649 (2006).

30. B. A. LaFlamme, K. Ravi Ram, M. F. Wolfner, *PLoS Genet.* **8**, 30–32 (2012).

31. Y. Heifetz, L. N. Vandenberg, H. I. Cohn, M. F. Wolfner, *Proc. Natl. Acad. Sci.* **102**, 743–748 (2005).

32. J. Labbadia, R. I. Morimoto, *Annu. Rev. Biochem.* **84**, 435–464 (2015).

33. L. Holman, *Funct. Ecol.* **23**, 180–186 (2009).

34. J. Cheng *et al.*, *Nature*. **456**, 599–604 (2008).

35. G. Ben-David, E. Miller, J. Steinhauer, *Spermatogenesis*. **5562** (2015), doi:10.1080/21565562.2015.1006089.

36. J. P. Moatt, C. Dytham, M. D. F. Thom, *Physiol. Behav.* **131**, 111–114 (2014).

37. L. Wang, C. J. McLeod, D. L. Jones, *Cell Cycle*. **10**, 2628–2634 (2011).

38. K. Reinhardt, R. Naylor, M. T. Siva-Jothy, *PLoS One*. **6**, e22082 (2011).

39. Hihara, Fuyuo, *Zool. Mag.* **90**, 307–316 (1981).

40. T. Finkel, *Nat. Med.* **21**, 1416–1423 (2015).

41. I. Sepil, P. Carazo, J. C. Perry, S. Wigby, *Sci. Rep.* **6**, 1–9 (2016).

42. S. J. Broughton *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **102**, 3105–3110 (2005).

43. J. E. Wilkinson *et al.*, *Aging Cell*. **11**, 675–682 (2012).

44. S. L. Johnson, N. J. Gemmell, *BioEssays*. **34**, 609–619 (2012).

45. W. B. Neaves, L. Johnson, J. C. Porter, C. R. Parker, C. S. Petty, *J. Clin. Endocrinol. Metab.* **59**, 756–763 (1984).

46. S. Ueishi, H. Shimizu, Y. H. Inoue, *Cell Struct. Funct.* **34**, 61–69 (2009).

47. C. J. McLeod, L. Wang, C. Wong, D. L. Jones, *Curr. Biol.* **20**, 2100–2105 (2010).

48. E. C. Rettie, S. Dorus, *Spermatogenesis*. **2**, 213–223 (2012).

49. E. Bonilla, E. Y. Xu, *Mol. Hum. Reprod.* **14**, 137–142 (2008).

50. A. K. Tiwari, P. Pragya, K. Ravi Ram, D. K. Chowdhuri, *Theriogenology*. **76**, 197–216 (2011).

## Figures

**Figure 1:** Decline in reproductive performance in response to male age and mating activity. **(A)** Experimental design. “U” stands for unmated and “F” stands for frequently mated males. **(B)** Number of offspring emerging from a single mating, excluding infertile matings (age and mating interaction: $\chi^2_1$= 98.668; p= 0.0005) (n= 73-135). **(C)** The proportion of infertile matings (age and mating interaction: $\chi^2_1$= 11.32; p= 0.0035) (n= 110-137). **(D)** Female latency to remate when exposed to two standard males 2 days after a single mating to an experimental male (age and mating interaction: $\chi^2_1$= 34.886; p< 0.0001) (n= 60-70). **(E)** Paternity share of the experimental first male when competing with the ejaculate of a standardized second male (age and mating interaction: $\chi^2_1$= 219.34; p= 0.0234) (n= 37-55). **(F)** Number of eggs laid by females mated to spermless experimental males (*binomial*: age: $\chi^2_1$= 1.2509; p= 0.263; mating: $\chi^2_1$= 0.609; p= 0.435; age and mating interaction: $\chi^2_1$= 0.027; p=0.87; *count:* age: $\chi^2_1$= 83.106; p= 0.003; mating: $\chi^2_1$= 102.38; p= 0.0008; age and mating interaction: $\chi^2_1$= 12.774; p= 0.237) (n= 30-42). **(G)** Female latency to remate when exposed to two standard males 2 days after a single mating to a spermless experimental male (age and mating interaction: $\chi^2_1$= 13.648; p= 0.0002) (n= 25-35). Results are shown as means ± SEM. Shaded areas are confidence intervals at 0.15 level. Differences at p < 0.05 within mating groups and age categories are represented as different letters.

**Figure 2:** The seminal fluid proteome responds differentially to ageing in U versus F males (n=4 replicate experiments per group) **(A)** Heatmap of the abundance of the 117 Sfps detected in accessory gland and ejaculatory duct samples. The abundance of 40 out of 117 Sfps exhibit a significant differential response to age and mating after multiple test correction. The annotation classification of each Sfp is indicated. **(B)** Line plots showing the change in standardized Sfp abundance for each Sfp with age. The Sfp average change in abundance for unmated and frequently mated males is depicted with lines marked ‘U’ and ‘F’ respectively (age and mating interaction: L Ratio$^2_1$= 163.856; p< 0.0001). **(C)** Principal component analyses of the seminal fluid proteome in male reproductive tissues (age and mating interaction: L Ratio$^2_1$= 34.949; p< 0.0001). **(D)** Heatmap of the abundance of 117 seminal fluid proteins transferred to females during mating. None of the

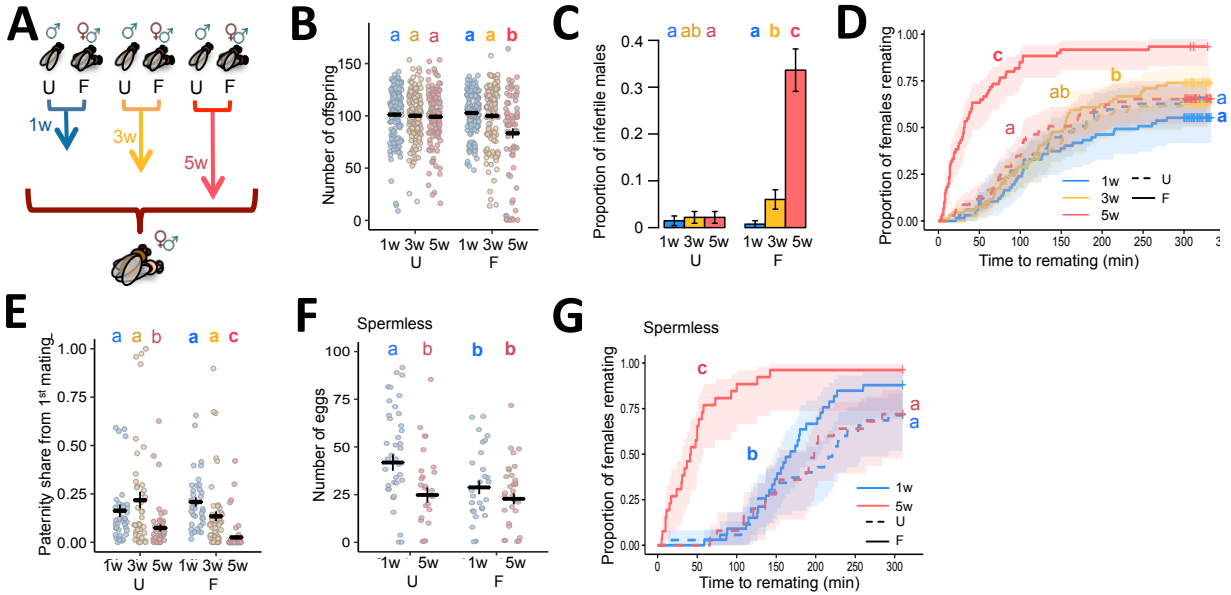
individual 117 Sfps exhibited a significant interaction between age and mating group after false discovery rate multiple test correction. Two ejaculatory duct specific Sfps were transferred in significantly higher quantities in response to age independent of mating activity (CG17242, CG5162) and ten Sfps were transferred in significantly higher quantities in response to frequent mating independent of age (Acp26Aa, CG10587, CG17472, CG3097, CG34002, Est-6, NLaz, Regucalcin, Sfp24F, Sfp65A). The annotation classification of each Sfp is indicated. **(E)** Line plots showing the standardized abundance of Sfps transferred with age (age and mating interaction: L $Ratio^2_1$= 130.595; p< 0.0001). **(F)** Principal component analyses of the seminal fluid proteome transferred to females (age and mating interaction: L $Ratio^2_1$= 11.485; p= 0.003). Differences at $p < 0.05$ within mating groups and age categories are represented as different letters. **(G)** Representative Acp62F, Acp26Aa and Semp1 Western blots, in 1w and 5w old unmated (U) and frequently mated (F) males. The abundance of each protein is predicted from the proteomic data and illustrated as a heatmap. Each lane is an individual male. All blots are shown in Supplemental Figure S4.

**Figure 3:** Ageing and frequent mating impacts sperm production and transfer. **(A)** Average number of mature germline cysts per testis (as measured by sperm individualization) (age: $\chi^2_1$= 223.78; p< 0.0001; mating: $\chi^2_1$= 1.334; p= 0.119; age and mating interaction: $\chi^2_1$= 0.772; p= 0.496) (n= 16-22). **(B)** Seminal vesicle area ($mm^2$) (age and mating interaction: $F^2_1$= 68.494; p< 0.0001) (n= 49-79). **(C)** Proportion of males with no evidence of sperm within the seminal vesicle (age and mating interaction: $\chi^2_1$= 1; p< 0.0001) (n= 47-51). **(D)** Sperm viability 10 minutes after removal from the seminal vesicles (male age: $\chi^2_1$= 0.004; p= 0.985; mating group: $\chi^2_1$= 102.69; p= 0.002; interaction between male age and mating group: $\chi^2_1$= 15.921; p= 0.223) (n= 36 - 51). **(E)** Number of GFP fluorescent sperm heads in female sperm storage organs 90 minutes after mating starts (seminal receptacle and the two spermatheca) (*binomial*: age and mating interaction: $\chi^2_1$= 13.417; p= 0.0012; *count*: age and mating interaction: $\chi^2_1$= 726.46; p= 0.0062) (n= 22-43). Results are shown as means ± SEM. Differences at $p < 0.05$ within mating groups and age categories are represented as different letters. “U” stands for unmated and “F” stands for frequently mated males.

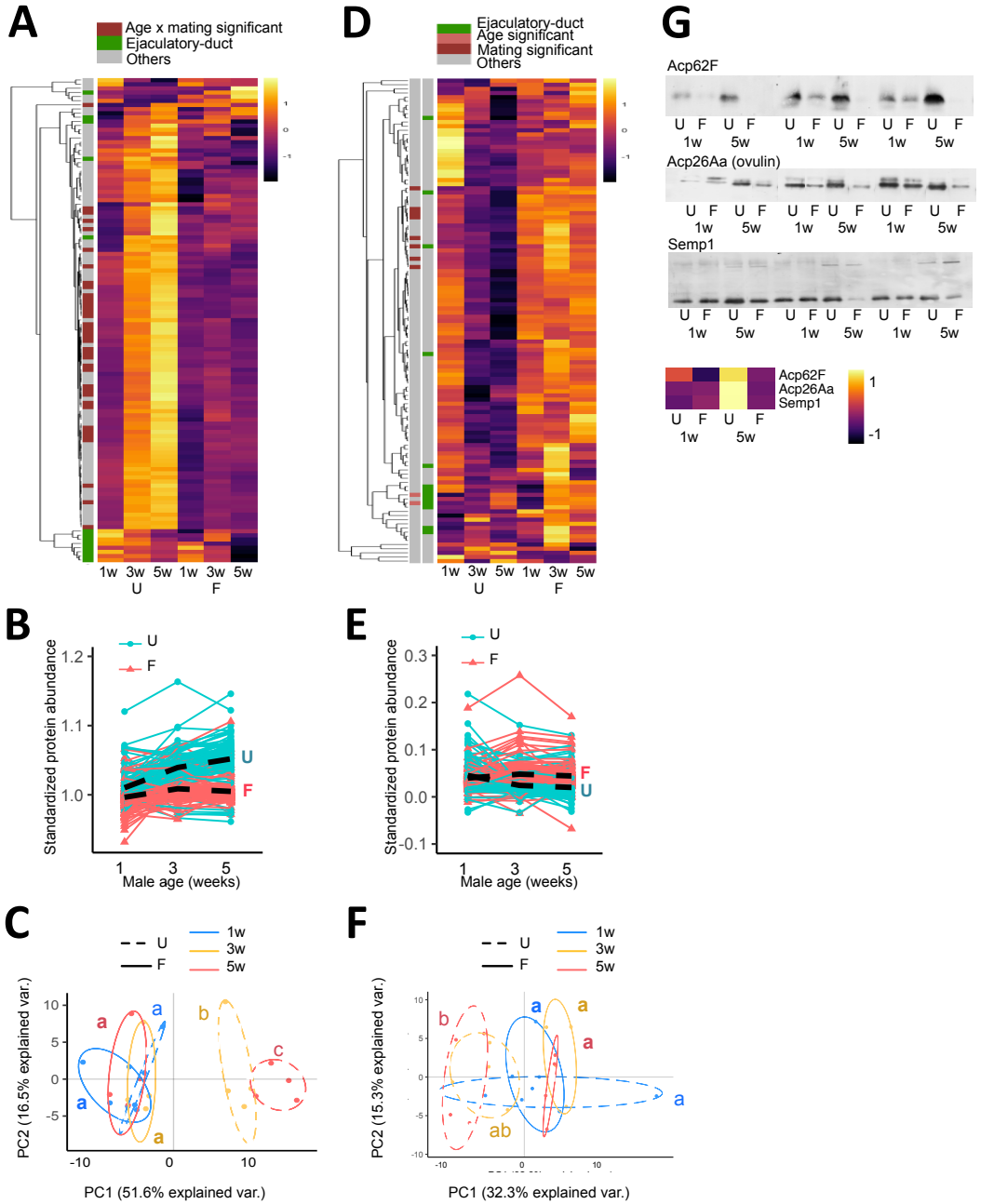
**Figure 4:** Schematic summary of the impacts of ageing on the ejaculate.

**Figure 5:** Manipulation of the insulin signaling pathway delays reproductive ageing in Old-F males. **(A)** The proportion of infertile matings (*U:* age: $\chi^2_1$= 10.413; p= 0.001; line: $\chi^2_1$= 0.437; p= 0.508; age and line interaction: $\chi^2_1$= 1.941; p= 0.164; n= 95-202) (*F:* age: $\chi^2_1$= 115.83; p< 0.0001; line: $\chi^2_1$= 5.433; p= 0.0198; age and line interaction: $\chi^2_1$= 3.684; p= 0.055; n= 94-189). (**B)** Female remating latency (*U:* age: $\chi^2_1$= 28.927; p< 0.0001; line: $\chi^2_1$= 3.602; p= 0.058; age and line interaction: $\chi^2_1$= 0.660; p= 0.416; n= 92-198) (*F:* age and line interaction: $\chi^2_1$= 4.727; p= 0.03; n= 91-186). “U” stands for unmated and “F” stands for frequently mated males. “C” stands for control and “A” stands for ablated lines. Results are shown as means ± SEM. Shaded areas are confidence intervals at 0.15 level. Differences at p < 0.05 within lines and age categories are represented as different letters.

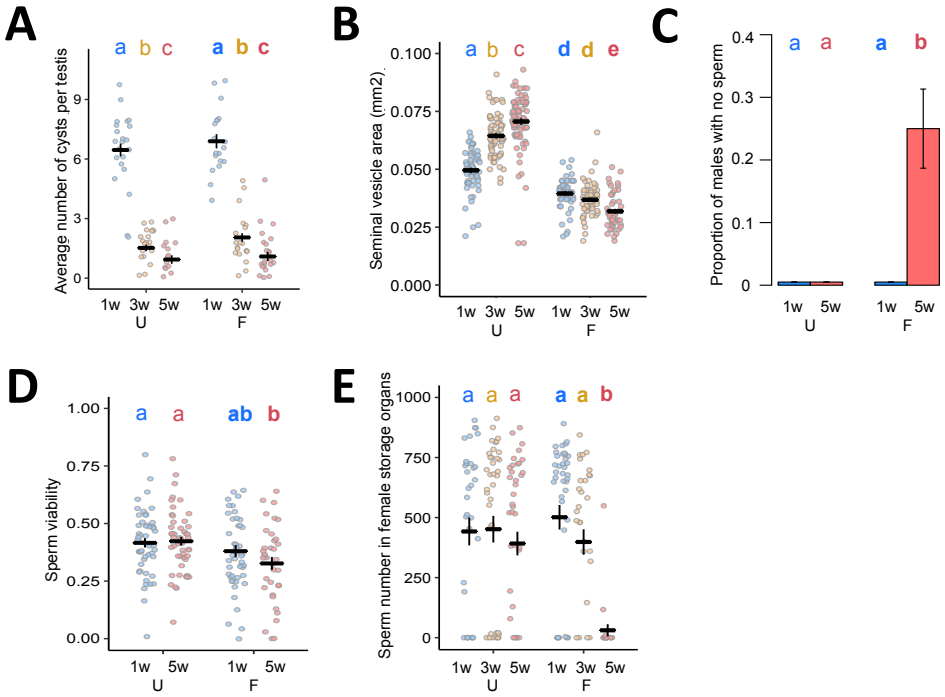
# Figure 1


A
U F U F U F
1w
3w
5w
B
Number of offspring
a a a a a b
1w 3w 5w 1w 3w 5w
U F
C
Proportion of infertile males
a ab a a b c
1w 3w 5w 1w 3w 5w
U F
D
Proportion of females remating
Time to remating (min)
1w
3w
5w
U
F
E
Paternity share from 1st mating
a a b a a c
1w 3w 5w 1w 3w 5w
U F
F
Spermless
Number of eggs
a b b b
1w 5w 1w 5w
U F
G
Spermless
Proportion of females remating
Time to remating (min)
1w
5w
U
F

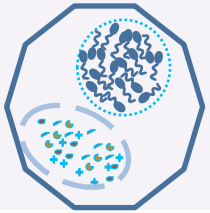
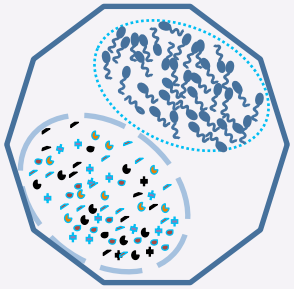
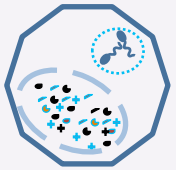
# Figure 2

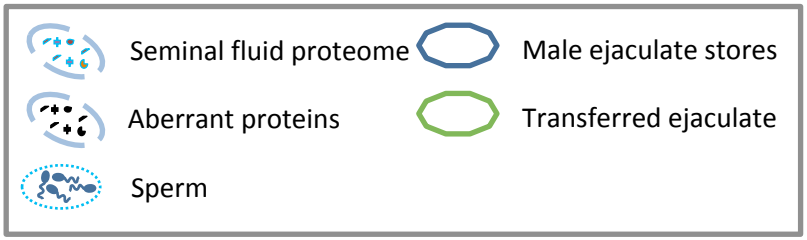

A
Age x mating significant
Ejaculatory-duct
Others
1w 3w 5w 1w 3w 5w
U F
D
Ejaculatory-duct
Age significant
Mating significant
Others
1w 3w 5w 1w 3w 5w
U F
G
Acp62F
Acp26Aa (ovulin)
Semp1
U F U F U F U F U F U F
1w 5w 1w 5w 1w 5w
Acp62F
Acp26Aa
Semp1
1
-1
B
Standardized protein abundance
Male age (weeks)
U
F
E
Standardized protein abundance
Male age (weeks)
C
PC2 (16.5% explained var.)
PC1 (51.6% explained var.)
1w
3w
5w
F
PC2 (15.3% explained var.)
PC1 (32.3% explained var.)

# Figure 3

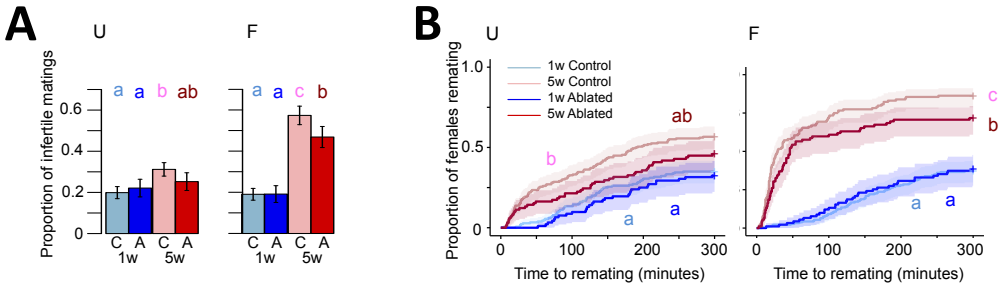

A
Average number of cysts per testis
a b c a b c
1w 3w 5w 1w 3w 5w
U F
B
Seminal vesicle area (mm2)
a b c d d e
1w 3w 5w 1w 3w 5w
U F
C
Proportion of males with no sperm
a a a b
1w 5w 1w 5w
U F
D
Sperm viability
a a ab b
1w 5w 1w 5w
U F
E
Sperm number in female storage organs
a a a a a b
1w 3w 5w 1w 3w 5w
U F

# Figure 4

| Male status | Male sperm and seminal fluid stores | Transferred ejaculate | Fertility | Seminal proteome composition | Seminal stimulation of females |
|---|---|---|---|---|---|
| Young |  | | High (standard + competitive) | Baseline | High (fecundity + refractoriness) |
| Old (Unmated) |  | | High (standard)<br>Low (competitive) | Altered | Low (fecundity)<br>High (refractoriness) |
| Old (Frequently mated) |  | | Low (standard + competitive) | Baseline | Low (fecundity + refractoriness) |



# Figure 5


A
U
F
Proportion of infertile matings
0.6
0.4
0.2
0
a a b ab
a a c b
C A C A
1w 5w
B
Proportion of females remating
1.0
0.5
1w Control
5w Control
1w Ablated
5w Ablated
ab
b
a
a
c
b
a a
Time to remating (minutes)
0 100 200 300