

Age related changes in male accessory gland and female fitness are independent of rearing temperatures in *D. melanogaster*

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Abstract: Age specific variations in mating, fecundity and reproductive performance occur in many organisms. *Drosophila melanogaster* represents a good model system for age-related studies and testing direct fitness benefits gained when females mate with specific male phenotypes. However, how male age related changes in male accessory glands and fitness traits are affected by rearing temperatures, has not been examined. Here we examined age-related mating of *D. melanogaster* in three age classes reared at three different temperatures. In all the rearing temperatures, young males with larger accessory glands containing a small number of large cells produced greater quantity of Acps than old males. Furthermore, young males transferred a greater quantity of Acps and sperms to mated females and as a result females that mated with young males produced significantly greater number of eggs and sperms than female mated with old males. This result was found to be similar in all the three temperatures in *D. melanogaster*. Together our data indicate that male age related changes in the accessory gland and sperm traits are independent of rearing temperatures.

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1. Introduction

The phenotype of an organism is the result of complex interactions between the organism's genotype and its environment during development. This means that a given genotype can give rise to a variety of phenotypes depending on both intrinsic and extrinsic conditions (West-Eberhard, 2003). Age is one of the important intrinsic factors that influence the physiology and reproduction of animals (Delisle, 1995; Jones et al., 2000; Moore and Moore, 2001). Age specific variation in mating, fecundity and reproductive performance are also known to occur in animals (Walter, 1988). Recently, the occurrence of age-specific fecundity and ovariole number were reported in *D. bipectinata* (Somashakar and Krishna, 2011), and *D. ananassae* (Prathibha et al., 2011). The authors found that middle-aged females had significantly greater ovarioles number and fecundity than young and old females. Similarly, temperature is another important external factor that has profound effects on the physiology, ecology, and fitness of ectotherm (Dillon et al., 2009). As an individual undergoes aging, its physiology will also be affected as well as the quality of gametes. Whether such age related changes independent of rearing temperature or has not been studied. Previous studies showed that male ageing is often associated with reduction in both quality and quantity of sperms. In spite of this, in many taxa, females paradoxically prefer to mate with relatively old males (Simmons, 2001).

Drosophila melanogaster has long been used as a model species to investigate age-related male mating success. During the past decade, model organisms including *D. melanogaster* have been used extensively to study aging. These model systems have been particularly useful for genetic studies of aging because of their small genome size, short generation time, and short life span compared with mice and humans. In addition, *Drosophila* species exhibit complex behaviours, many of which decline with age. Small ectotherm such as in *Drosophila*, cannot endogenously regulate body temperature so it must rely on behaviour to maintain body temperature within physiological permissive range. Furthermore, there is no parental care in *Drosophila*, and females receive only seminal fluid, which contains accessory gland secretions and sperm, from the male (Gillot, 2003). Following copulation, mated females exhibit dramatic physiological changes resulting from Acps. Post-mating changes include the stimulation of oviposition, egg laying, and reductions in female receptivity and sperm storage (Gillott, 2003). Therefore, *Drosophila* represent a good model systems for testing direct fitness benefits gained when females mate with the preferred male phenotype. However, the age effect on accessory gland and sperm traits in different rearing temperatures has not been studied. Hence the present study aimed to answer whether age related changes in accessory gland and sperm traits of male and its effect

on female fitness is independent of rearing temperatures.

2. Materials and Methods

The experimental stock was established from progenies of 50 isofemale lines of *D. melanogaster* that were collected in Mysore, India. These progenies were mixed together, and 20 males were placed together with 20 females in each culture bottle. Crosses were maintained at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with 70% relative humidity on a medium of wheat cream agar. Flies were kept in a 12-h light: 12-h dark cycle for three generations to acclimatize to laboratory conditions. At 4th generation, eggs were collected from this stock using Delcours' procedure (1969). One hundred eggs were transferred into a vial containing wheat cream agar medium and these vials were reared separately at $15^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $29^{\circ}\text{C} \pm 1^{\circ}\text{C}$. When adults started emerging, virgin females and unmated males were isolated within 3 hours of their eclosion and were aged until they were used in their respective rearing temperatures. The experiments were conducted at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Assignment of age classes

For assigning age classes to males, the longevity (number of days from eclosion until death) of unmated males was determined by maintaining individuals in a vial until their death. Flies were transferred to a clean vial each week. Fifty replicates were performed, and the mean longevity was 61 ± 2 days for the temperature $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 65 ± 2 days for the temperature $15^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 58 ± 2 days, for the temperature $29^{\circ}\text{C} \pm 1^{\circ}\text{C}$. We also studied male mating activities from eclosion until death. Similar results were obtained for all the three rearing temperatures. Males showed low levels of activity on day 1 after eclosion. During days 2–54, however, males performed all mating activities and mated with females. After day 55th, male mating activities declined greatly. "Based on these data, male age classes were defined as: young (2–3 days), middle-aged (27–28 days), and old (52–53 days).

The first set of emerged flies was aged 52–53 days (to obtain old males). When these flies were 25 days old the next set of flies was isolated and aged 27–28 days (to obtain middle-aged males). When these males were 25 days old (and the old set was 50 days old) the next set of flies was isolated and aged 2–3 days (to obtain young males). This procedure allowed us to culture and maintain young, middle-aged, and old males under the same conditions and to conduct experiments using these three sets of flies at the same time. Most (99%) cross-sectional studies on aging have used this protocol.

Effect of age on the accessory gland in three rearing temperatures

Young, middle-aged, or old unmated males were etherized, and accessory glands were dissected Medium A (Ashburner 1970). Tissues were fixed in 1 N HCl for 5 min. Accessory glands were photographed using a digital camera (Supporting figure S1). The shape of the accessory gland was generally that of an 's', 'c', or 'j'. Each gland was therefore divided into smaller areas consisting of triangles, trapeziums, and rectangles (Supporting figure S1). The area of each geometrical form was then calculated (Ravi Ram and Ramesh 2002) and the sum of these areas represented the size of the gland (cm^2). The actual area of the gland was calculated by dividing these values by the magnification. Soon after taking these photographs, accessory glands were transferred into 2% lactoaceto orcein stain for 20 min. Glands were then gently opened with fine entomological needles and squashed between a glass slide and a coverslip. Acetic acid (45%) was used to spread the main cells of the accessory glands into a single layer. The total number of main cells in each accessory gland was counted, and main-cell sizes were measured. For cell-size measurements, the length of 50 randomly selected main cells was measured using a micrometre. Fifty accessory glands were analysed for each age class (young, middle-aged, and old). Separate experiments were carried out for all the three rearing temperatures.

Effect of age on the quantity of Acps in three rearing temperatures

Accessory glands were dissected from young, middle-aged, or old males in insect saline using entomological needles. Males of each age class were either unmated or had recently copulated (<5 min before they were sacrificed). Glands were fixed in 95% ethanol. Fixed glands were placed on a glass slide, and the membrane was removed using a fine needle and a stereomicroscope (Supporting figure S2). The isolated secretions were washed in methanol/chloroform (1:1) and dried at 37°C for 15 min. Approximately 100 μL sample buffer (0.625 M Tris-HCl, pH 6.8, 1% SDS, 1% β -mercaptoethanol, and 10% glycerol) was added to each sample to dissolve the glands and secretions. Twenty pairs of accessory glands from each age class (10 mated and 10 unmated males for each trials) were collected and, total Acps was estimated using the Bradford method (Bradford, 1976). Fifty trials were run each male age class (young, middle aged and old). Separate experiments were carried out for all the rearing temperatures.

Bradford method

Approximately 50 μL of Acps (obtained as described above) were mixed with 5 mL Bradford reagent, which was generated by adding 100 mg Coomassie Brilliant Blue G-250 (in 50 mL 95% ethanol) to 100 mL 85% phosphoric acid and then diluting the mixture to 1 L with distilled water. The

solution was allowed to stand for 5 min to develop color. The quantity of proteins in each sample was determined by measuring optical density at 595 nm of the solution using a spectrophotometer. Bovine serum albumin was used as the standard. Fifty trials were run for each male age class (young, middle-aged, and old). Separate experiments were run for all the rearing temperatures.

Eggs and progeny produced by female that mated with males of different ages

Individual mated females were collected and placed into a vial. Every 24 h a female was placed into a new vial, and this procedure was repeated until the death of the fly. The total number of eggs and emerged progeny were counted. Separate experiments were performed for all the rearing temperatures.

Effect of male age on copulation duration, quantity of Acps, and sperm count

A young, middle-aged, or old unmated male was placed in an Elens-Wattiaux mating chamber (Elens-Wattiaux, 1964) with a virgin female (5–6 days old). The pair was observed for 1 h. Pairs that did not mate were discarded. If mating occurred, the copulation duration was recorded. Soon after mating, the reproductive organ of the female was dissected in 20 μ L Beadle-Ephrussi Saline (128.3 mM NaCl, 4.7 mM KCl, 23 mM CaCl₂) (Ephrussi-Beadle, 1936). Because sperm could dissociate into the solution, 20 μ L lactoaceto orcein was added to the slide without draining the saline. The number of sperm was then counted using an Olympus CX21 microscope. The quantity of Acps was measured for the mated males as described above. Fifty trials were run for each male age class. Separate experiments were run for all the rearing temperatures.

Statistical analysis

One way ANOVA followed by Tukey's post hoc test carried out all the above parameters studied using the SPSS 10.1 programme.

3. Results

Analysis of accessory gland traits of unmated males, the number of main cells and the size of the accessory gland increased with age, whereas the quantity of Acps and the size of the main cells decreased with age (Table 1). This result was similar in all the rearing temperatures studied. Except the size of main cell all other traits showed significant variation between male age classes. Tukey's post hoc test showed that quantity of Acps and size of main cell were significantly greater in young male than in old males. The size of accessory gland and main cell number were significantly lower in young male compared to old males.

In all three rearing temperatures, the duration of copulation generally increased with age. In contrast, the quantity of Acps, the amount of Acps, sperm transferred to the mated female and the fecundity and fertility of the mated female decreased with the age of the male (Table 2, Figure 2a and b). The amount of Acps transferred to the mated female as well as the fecundity and fertility of the mated female varied significantly with male age class. No significant variation in copulation duration was measured between males of different ages. Tukey's post hoc test showed that the quantity of transferred Acps and sperms transferred to mated female was significantly greater in young male than those of old and middle aged males. Female mated with young male laid significantly greater number of eggs and produced greater progeny than female mated with either middle aged or old males.

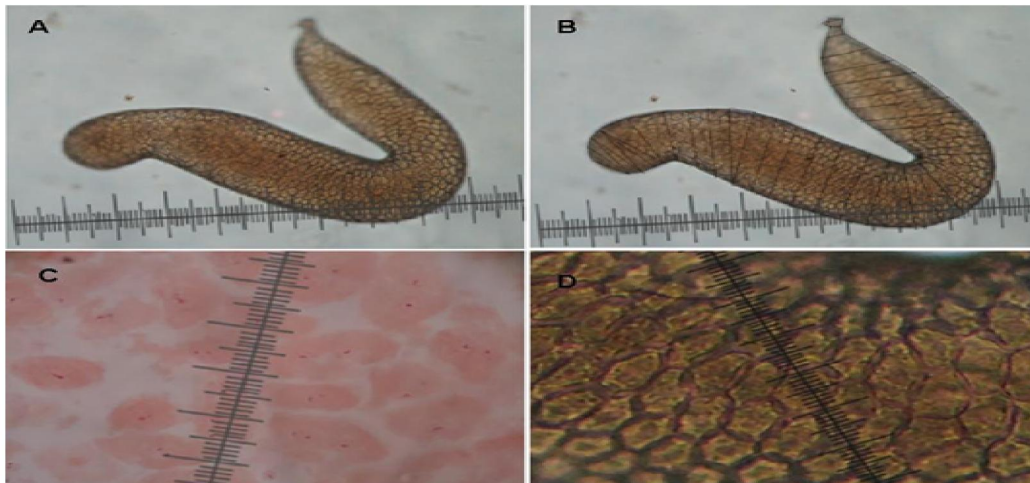


Figure S1. Measurement of cell number, cell size and gland size. (A. Accessory gland lobe. B. Marked accessory gland lobe for measuring the size of the gland. C and D. Measurement of cell size of accessory gland lobe.



Figure S2. Measurement of quantity of Acps using secretion alone A. Secretion with membrane. B. secretion without membrane.

Table 1. Effect of age on the accessory gland in three rearing temperatures of *D. melanogaster*.

Rearing temperatures	Parameters (Accessory glands)	Male age classes			F value
		Young (2-3 days)	Middle (27-28 days)	Old (52-53days)	
15°C±1°C	Quantity of Acps of unmated males (µg/pair of glands)	18.96±1.45 ^a	16.25±0.91 ^b	15.96±1.42 ^c	42.38**
	Main cell number	1232±6.51 ^a	3912±10.2 ^b	4590±14.6 ^c	11.12**
	Main cell size (in mm)	0.00641±0.0002 ^a	0.00486±0.0001 ^b	0.00190±0.0001 ^c	100.93***
	Size of accessory gland(in mm)	0.350±0.00016 ^a	0.354±0.00015 ^a	0.486±0.0002 ^b	29.32**
22 °C±1°C	Quantity of Acps in unmated males (µg/pair of glands)	19.63±1.47 ^b	17.73±1.34 ^b	16.93±0.92 ^a	58.13**
	Main cell number	1364± 8.86 ^a	2592±10.2 ^b	3502±20.88 ^c	175.57***
	Main cell size (in mm)	0.00675±0.0001 ^a	0.00475±0.0002 ^b	0.00286±0.0001 ^c	88.11***
	Size of accessory gland(in mm)	0.339±0.00010 ^a	0.392±0.00050 ^a	0.391±0.0008 ^b	3.502*
29 °C±1°C	Quantity of Acps in unmated males (µg/pair of glands)	16.85±0.95 ^a	16.46±0.94 ^b	15.45±1.34 ^c	18.52**
	Main cell number	1408±4.4 ^a	2920±12.1 ^b	4513±13.0 ^c	199.72***
	Main cell size (in mm)	0.00751±0.0001 ^a	0.00612±0.00014 ^b	0.00201±0.0001 ^c	660.45***
	Size of accessory gland (in mm)	0.395±0.00011 ^a	0.462±0.00021 ^b	0.346±0.0001 ^b	53.23**

*** Significance at $p < 0.001$; ** Significance at $p < 0.001$; *Significance at $p < 0.05$ level.

Different letters indicate significance at 0.05 level by Tukey's post hoc test.

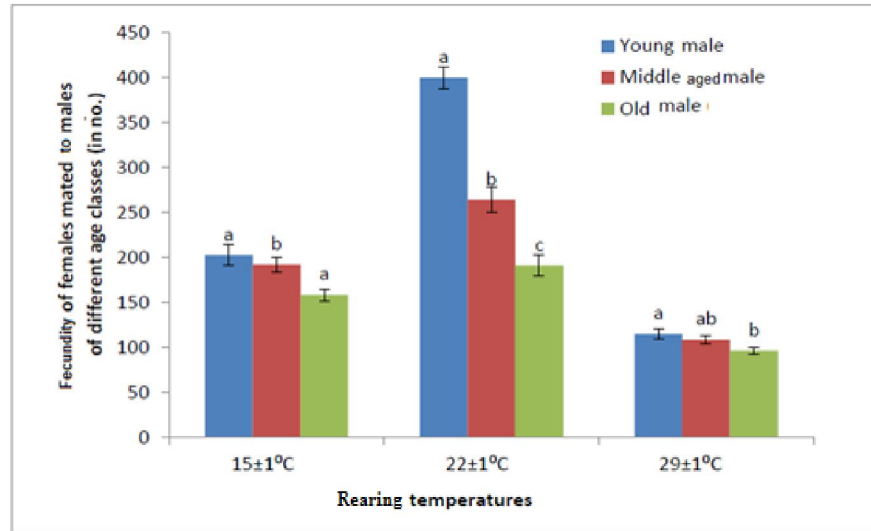


Figure 2a. Male age effect on fecundity of females mated to males of different age classes in *D. melanogaster* reared at three different temperatures. F value for 15°C±1°C: 49.32; F value for 22°C±1°C, 69.56; F value for 29°C±1°C, 43.21. ** Significant at $p < 0.001$ for all three temperatures. Different letters indicate significance at 0.05 level by Tukey's post hoc test.

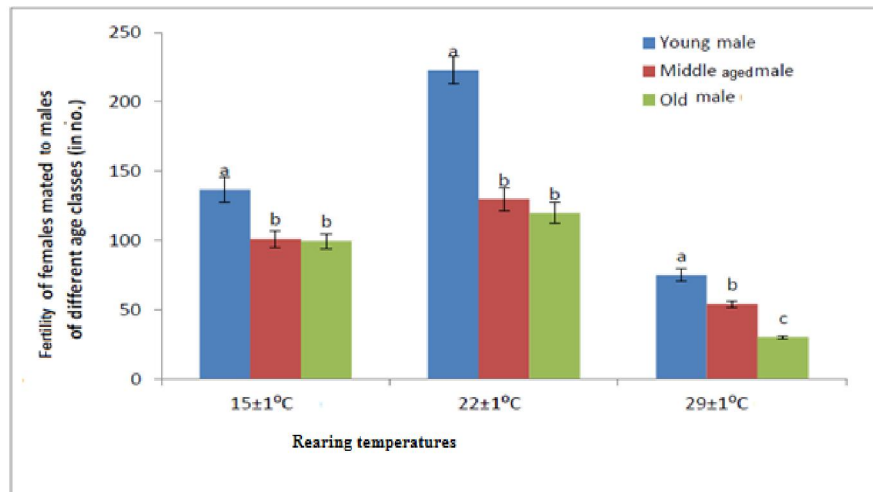


Figure 2b. Progeny numbers (fertility) of females mated to different age classes of male in three different rearing temperatures in *D. melanogaster*. F value for 15°C±1°C: 8.92; F value for 22°C±1°C, 42.56; F value for 29°C±1°C, 58.21. ** Significant at $p < 0.001$ for all three temperatures. Different letters indicate significance at 0.05 level by Tukey's post hoc test.

4. Discussion

In animals with internal fertilization, females receive not only genes but also a variety of seminal fluid products in male ejaculation including Acps and sperms. Therefore the quantity and quality of sperms and Acps received from a male can affect the female fitness and reproduction (Leviton and Peterson, 1995; Drnevich et al., 2001). Thus, the female may directly benefit from mating with preferred male if ejaculate quantity and quality co-varies with a male trait used by females in mating (Andersson, 1994).

Careful analysis of Table 1 revealed that young males with large accessory glands containing a small number of large main cells produced greater quantities of Acps than old males. This indicates that male age affects the structure and function of accessory glands in *D. melanogaster*. Monsma et al., (1990) in *D. melanogaster* and Ravi Ram and Ramesh (2002) in *D. nasuta* have indicated that the quantity of Acps is also influenced by the secretory activity of main cells in the accessory gland. The effect of age on the Acps and sperms observed in *D. melanogaster* was found to be

similar in all the three rearing temperatures studied. This suggests that that male age related changes in quantity of Acps was independent of rearing temperatures. Our result supports the work of Daunt et al. (1999), who found while working with iteroparous breeders that age related variation in reproductive performance was not the result of environmental effects.

That the structure, pattern, synthesis, and accumulation of Acps are similar in different species

of *Drosophila* (Wagstaff and Begun 2005), but not the quantity of accessory gland secretion (Chen, 1984; 1996; Wolfner, 1997; Ravi Ram and Ramesh, 1999; 2001). The quantity of Acps is also influenced by body size and larval density (Chapman et al. 1995). It was also presumed that quantity of Acps is influenced by its structural parameters such as the size of the accessory glands or number of main cells and size of main cells in the accessory glands.

Table 2. Effect of age on copulation duration, accessory gland and sperm traits in three rearing temperatures of *D. melanogaster*.

Rearing temperatures	Parameters	Male age classes			F-value
		Young (2-3 days)	Middle (27-28 days)	Old (52-53days)	
15°C±1°C ^a	Copulation duration (in minute)	21.8±0.122	24.03±0.131	25.06±0.141	0.794 ^{NS}
	Quantity of Acps of mated male (µg/pair of glands)	12.48±0.12 ^a	12.12±0.112 ^a	11.28±0.112 ^b	11.78 ^{**}
	Transferred quantity of Acps (µg/pair of glands)	5.46±0.101 ^a	4.06±0.100 ^b	3.46±0.101 ^c	88.42 ^{***}
	Sperm transferred (in number)	4914±108 ^a	3165±108 ^b	1243±108 ^c	80.32 ^{**}
22 °C±1°C ^b	Copulation duration (in minute)	19.2±0.122	20.88±0.132	21.92±0.126	1.01 ^{NS}
	Quantity of Acps of mated males (µg/pair of glands)	13.84±0.12 ^a	12.32±0.117 ^b	11.98±0.116 ^b	44.31 ^{**}
	Transferred quantity of Acps (µg/pair of glands)	5.82±0.102 ^a	5.42±0.101 ^b	4.73±0.109 ^c	13.31 ^{**}
	Sperm transferred (in number)	6440±100 ^a	4776±120 ^b	3999±90 ^c	91.49 ^{***}
29 °C±1°C ^a	Copulation duration (in minute)	21.8±0.122	24.03±0.131	25.06±0.141	0.15 ^{NS}
	Quantity of Acps of mated male (µg/pair of glands)	12.49±0.11 ^a	11.83±0.111 ^a	11.58±0.12 ^b	32.53 ^{**}
	Transferred quantity of Acps (µg/pair of glands)	5.23±0.101 ^a	4.01±0.101 ^b	3.53±0.110 ^c	6.64 [*]
	Sperm transferred (in number)	5441±101 ^a	3777±102 ^b	3014±101 ^c	90.71 ^{***}

*** Significant at p<000.1; ** Significant at p < 0.001; *Significant at p<0.05; NS- Insignificant at 0.05 level (p>0.05).

Different letters indicate significance at 0.05 level by Tukey's post hoc test.

[Difference in quantity of Acps between mated and unmated male was considered as a transferred quantity of Acps: this data was used to calculate mean and SE of transferring quantity of Acps.

Ex. Quantity of Acps of Unmated trials, 1, 2,3,.....,50.

Quantity of Acps of Mated trials, 1,2,3,.....,50

Difference in quantity of Acps between mated male and unmated male, 1,2,3,.....,50]

Ravi Ram and Ramesh (2003) found in *D. nasuta*, a lack of relation between the size of the accessory glands, main cell number of accessory glands and quantity of Acps synthesis. However our results suggests that the main cells of accessory gland

and size of accessory glands are an important factor known to influence the quantity of Acps produced. Studies of Monsma et al., (1990) in *D. melanogaster* and Ravi Ram and Ramesh (2001) in *D. nasuta* have shown that quantity of Acps synthesis may depend on

the secretory activity of cells in the accessory gland. Therefore, in the present study on *D. melanogaster*, the difference in the quantity of Acps proteins in unmated, young, middle-aged and old males could be from the variation in main cell size of accessory glands and the differential activity of Acps genes with male age. Studies have also found that the Pox genes namely paired (*prd*) are responsible for controlling cell proliferation and differentiation of secretory cells. The expression of the *prd* gene in secretory cells of newly eclosed flies was high but the level gradually reduced with increasing of male age (Xue et al., 2001).

In our study the same pair of flies used to analyse copulation duration was used to quantify Acps and sperm. Table 2, Figure 2a and b, shows that, compared with middle-aged and old males, young males transferred greater quantities of Acps and sperm to females. This suggests that, in *D. melanogaster*, the amount of Acps that is transferred to a female declines with male age. As a result, females that mated with a young male produced a greater number of eggs and progeny than females that mated with middle-aged or old males (Figure 2 and b). Jones et al., (2007) studied age-related sperm transfer and sperm competition in male Hide beetles (*Dermestes maculatus* De Geer) and found that young, middle-aged, and old males could all successfully copulate but that middle-aged and old males were more likely to transfer sperm and compared with young males. In contrast, *D. melanogaster* young males successfully transferred relatively greater quantities of sperm to mated females. In *D. ananassae* (Prathibha et al., 2001) and *D. bipunctinata* (Somashekar et al., 2011) females that mate with older males produce more eggs and progeny than females that mate with middle-aged or young males. However, they did not analyse Acps or sperm in their studies. Thus, these studies on *D. melanogaster* suggest that male age has a significant influence on the structure and quantity of Acps that is independent of rearing temperature. Females mated with young males obtained fitness benefits, and this was also independent of rearing temperatures.

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