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The impact of ageing on male reproductive success in *Drosophila melanogaster*

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ABSTRACT

Male reproductive ageing has been mainly explained by a reduction in sperm quality with negative effects on offspring development and quality. In addition to sperm, males transfer seminal fluid proteins (Sfps) at mating; Sfps are important determinants of male reproductive success. Receipt of Sfps leads to female post-mating changes including physiological changes, and affects sperm competition dynamics. Using the fruit fly *Drosophila melanogaster* we studied ageing males' ability to induce female post-mating responses and determined the consequences of male ageing on their reproductive success. We aged males for up to 7 weeks and assayed their ability to: i) gain a mating, ii) induce egg-laying and produce offspring, iii) prevent females from remating and iv) transfer sperm and elicit storage after a single mating. We found that with increasing age, males were less able to induce post-mating responses in their mates; moreover ageing had negative consequences for male success in competitive situations. Our findings indicate that with advancing age male flies transferred less effective ejaculates and that Sfp composition might change over a male's lifetime in quantity and/or quality, significantly affecting his reproductive success.

1. Introduction

Ageing is characterized by progressive declines in a number of bodily functions that accumulate in an increased risk of death. This decline is accompanied by reduced reproduction and fertility. Three phases of ageing can often be discerned in a number of model organisms, like *Drosophila*, whereby during the first, the ageing phase, performance decreases rapidly, while during the late-phase performance stabilizes at low levels to decline significantly ~2 weeks before death in a 'death spiral' (Shahrestani et al., 2012; Mueller et al., 2016). In general, a decrease in reproductive capacity in males is due to senescence of the soma, often expressed as lower mating success, and of the reproductive tissues, causing impaired fertility (reviewed in Johnson and Gemmell, 2012). Explanations for decreased male fertility with age have focused mainly on diminished sperm quality (reviewed in Radwan, 2003; Pizzari et al., 2007; Reinhardt et al., 2015). However, not only sperm and/or spermatogenic tissues age. We expect the entire ejaculate including its non-sperm components to show signs of senescence, contributing to poor reproductive success in older males. Here we focus on this aspect, investigating several consequences of ageing on male reproductive success in *Drosophila melanogaster*, a species where the link between male reproductive success and the non-sperm components of the ejaculate is well established.

Sperm of older males are predicted to have accumulated a larger number of mutations (Radwan, 2003; Pizzari et al., 2007; Reinhardt et al., 2015) resulting in decreased offspring viability or quality. Data from a number of organisms are consistent with this prediction (Price and Hansen, 1998; Jones et al., 2000; Jones and Elgar, 2004; Karl and Fischer, 2013), although there are exceptions (Schäfer and Uhl, 2002; Fricke and Maklakov, 2007; Avent et al., 2008; Krishna et al., 2012; Verspoor et al., 2015). It was further observed that older males sired fewer offspring when in competition over fertilization e.g. in the cellar spider *Pholcus phalangioides* (Schäfer and Uhl, 2002), the hide beetle *Dermestes maculatus* (Jones et al., 2007) and the bulb mite *Rhizoglyphus robini* (Radwan et al., 2005). This reduced sperm competitive ability may be due to altered sperm quality or other characteristics, like the non-sperm component. For example, in the red junglefowl *Gallus gallus* older males achieve lower paternity share (McDonald et al., 2017). In this species sperm velocity can be influenced by the male through allocating a larger ejaculate (Cornwallis and O'Connor, 2009). A recent study reported sperm velocity dependent variation in the red junglefowl seminal fluid proteome (Borziak et al., 2016) and, interestingly, a

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distinct seminal proteomic signature in older males. This suggests that the non-sperm components of the ejaculate might also change with age in ways that could affect male competitiveness. Since success in sperm competition is a key determinant of male fitness (Simmons, 2001; for an example in *D. melanogaster* see Fricke et al., 2010) it is important to extend the study of male reproductive ageing to such non-sperm components of the ejaculate.

In *D. melanogaster*, non-sperm components of the ejaculate are vital for male fertilization success and play important roles in sperm competition (reviewed in Chapman, 2001; Avila et al., 2011; Simmons and Fitzpatrick, 2012). Along with sperm, a male transfers about 200 different seminal fluid proteins (Sfps) to a female (Findlay et al., 2008, 2009). Many Sfps are produced in the male's accessory glands (AG) (reviewed in Avila et al., 2011). The AGs are two tube-like lobes connected to the ejaculatory duct. Their proteins are secreted by the glands' ~1000 "main cells" and 40 "secondary cells"; the latter are located at the tip of the gland and contain large secretory vacuoles (Bertram et al., 1992). As males age, some AG secondary cells can be actively shed and even be transferred to females during mating (Leiblich et al., 2012). Here, we tested ageing *D. melanogaster* males' ability to elicit known female post-mating responses to such non-sperm components of the ejaculate and tested whether this reduced ability contributes to diminished reproductive or competitive success of ageing *D. melanogaster* males.

The fact that Sfps in *D. melanogaster* play a crucial role in many post-copulatory traits (reviewed in Avila et al., 2011) prompted us to examine whether there were age dependent changes in their amount or action. Sfps for example, decrease the risk of sperm competition by decreasing female receptivity to remating (Chapman et al., 2003; Liu and Kubli, 2003), affect the storage and retention of sperm (Harshman and Prout, 1994; Neubaum and Wolfner, 1999; Chapman et al., 2000; Prout and Clark, 2000; Bloch Qazi and Wolfner, 2003; Wong et al., 2008; Avila et al., 2010; Ravi Ram and Wolfner, 2007) and boost egg production and laying by the female (Herndon and Wolfner, 1995; Heifetz et al., 2000; Chapman et al., 2003; Liu and Kubli, 2003) which increases the number of offspring a male can sire with a single mating (Fricke et al., 2009; Fricke and Chapman, 2017). These female post-mating changes are accompanied by a remodelling of the female uterus into a mated conformation (Adams and Wolfner, 2007; Kapelnikov et al., 2008a, 2008b; Mattei et al., 2015) which is suggested to aid in sperm entry into storage (Adams and Wolfner, 2007; Mattei et al., 2015). At least one seminal protein, Acp36DE, is necessary for these uterine conformational changes (Avila and Wolfner, 2009). Since Sfps play such an important role in male reproductive success and post-mating competitiveness, we investigate whether they are affected by a male's ageing. To that end, we measured whether males' age affects their mates' post-mating responses. We focused on responses elicited by the sex peptide (SP), which heightens a male's reproductive success through a variety of means (Fricke et al., 2009; Fricke and Chapman, 2017), including reducing female receptivity for up to ~4 days after mating, boosting egg-laying (Chen et al., 1988; Chapman et al., 2003; Liu and Kubli, 2003) and regulating the efficient release of stored sperm (Avila et al., 2010). The other Sfp we examined was ovulin, which increases ovulation rate by mated females (Heifetz et al., 2000; Rubinstein and Wolfner, 2013), but only exerts short-term effects on the female and has so far no known effect on sperm competition outcomes.

In this study, we examined the consequence of male ageing over a substantial part of male lifespan (from 4 days to 7 weeks post-eclosion) on the male's ability to induce female post-mating responses. Upon noticing that there were age-dependent changes in the intensity of female post-mating responses induced by the male, we used an enzyme-linked immunosorbent assay (ELISA, Sirot et al., 2009) directly quantify the amounts of two Sfps (SP and ovulin) transferred in single-matings by males of different ages. We combined these measures with tests of age-dependent male reproductive success after a single mating as well as in competitive situations.

2. Materials and methods

2.1. Fly stocks

2.1.1. Wild-type flies and Stubble (Sb) flies

The wild-type Dahomey stock has been maintained at large population size in cages with overlapping generations since it was collected in the 1970s in Dahomey, West Africa (now Benin). Hence we put no constraint on adult lifespan and allowed the flies to reproduce throughout their entire lifespan. The population was fed once a week by introducing three glass bottles into the population cage with 70 mL fresh standard sugar-yeast (SYA) food (Bass et al., 2007). Our Dahomey stock flies were kindly provided by Prof. Tracey Chapman (University of East Anglia, UK) and have been maintained in our laboratory in population cages for several generations prior to the experiments. Further we used flies with the *Stubble* (*Sb*) mutation, back-crossed into the wild-type Dahomey genetic background for four generations to increase genetic variability and have the mutation in a comparable genetic background. *Sb*, a dominant mutation that causes an easily-scored short-bristle phenotype, is recessive-lethal, hence the *Sb* males used in our assays were heterozygous for this mutation. *Sb* males were used in a direct competition assay as competitor males, enabling us to determine offspring paternity and collect fitness measures for our focal males from different age classes.

2.1.2. Sex peptide (SP) knockout flies

Following the protocol in Liu and Kubli (2003), flies that lacked SP (SP^0) were heterozygous for SP^0 (an allele with two mutations in SP, one a stop codon), and Δ130, a deletion that removes the SP gene; each SP allele was carried in stock over *TM3,Sb,ry*. Each line was backcrossed to Dahomey (3 generations for Δ130/*TM3,Sb,ry*; 4 for SP^0/*TM3,Sb,ry*) to control for genetic background and increase vigor. The SP knockout flies were kindly provided by Prof. Tracey Chapman. SP^0 males served as an age-matched baseline to compare to the strength of SP-elicited female post-mating responses by the wild-type males.

2.1.3. Fly culturing

Flies were maintained and all experiments were conducted in a constant climate room at 25 °C and 60% humidity at a 12:12 h dark – light cycle. Mutant flies were kept in glass bottles containing 70 mL of SYA food and regularly flipped onto fresh food.

To generate flies used in the experiments we allowed the parent generation to oviposit on grape-juice-agar plates [50 g agar, 600 mL red grape juice, 42.5 mL Nipagin (10% w/v solution) and 1.1 L water] supplemented with live-yeast paste. Following 24 h of incubation, larvae were collected and 100 were transferred into a standard vial (diameter 2.5 cm, height 8.4 cm, containing 7 mL of SYA food) to develop under density controlled conditions. Throughout our experiments we added additional ad libitum live yeast granules or paste to our vials. To reduce variability in male age for our experiments we restricted wild-type Dahomey females to oviposit for 3–5 h. At eclosion, adults were collected as virgins and 20 individuals were kept in same sex groups in standard vials until the beginning of the experiment. If flies were kept for longer than 4 days prior to the experiment they were transferred to fresh food every 3–4 days.

2.2. Mating assays

We designed our experiments to use virgin rather than previously-mated males (see Jones and Elgar, 2004 for such an approach), interpreting data from the latter comes with its own set of challenges: because *D. melanogaster* males transfer ~one third of their seminal proteins in each mating (Ravi Ram et al., 2005) and need at least four matings in short succession to largely-empty their Sfp stores (Hihara, 1981), using a previously mated male would mean that his ejaculate would be a mix of old and fresh ejaculatory components. Moreover, the