Insect science

Insect Science (2017) 00, 1–9, DOI 10.1111/1744-7917.12446

ORIGINAL ARTICLE

Male age and strain affect ejaculate quality in the Mexican fruit fly

Mariana Herrera-Cruz[1], Solana Abraham[2,3], Nicolas Nuñez-Beverido[4,5], Norma Flores-Estévez[4], Martha Reyes-Hernández[4], Mayvi Alvarado[5] and Diana Pérez-Staples[4]

[1]*Cátedra CONACYT– Facultad de Medicina y Cirugía, Universidad Autónoma "Benito Juárez" de Oaxaca, Oaxaca, México;* [2]*Laboratorio de Investigaciones Ecoetológicas de Moscas de la Fruta y sus Enemigos Naturales (LIEMEN), PROIMI, Tucumán, Argentina;* [3]*CONICET, Argentina;* [4]*INBIOTECA, Universidad Veracruzana, Xalapa, Veracruz, Mexico and* [5]*Instituto de Neuroetología, Universidad Veracruzana, Xalapa, Veracruz, Mexico*

Abstract Aging in all organisms is inevitable. Male age can have profound effects on mating success and female reproduction, yet relatively little is known on the effects of male age on different components of the ejaculate. Furthermore, in mass-reared insects used for the Sterile Insect Technique, there are often behavioral differences between mass-reared and wild males, while differences in the ejaculate have been less studied. The ejaculate in insects is composed mainly of sperm and accessory gland proteins. Here, we studied how male age and strain affected (i) protein quantity of testes and accessory glands, (ii) the biological activity of accessory gland products injected into females, (iii) sperm viability, and (iv) sperm quantity stored by females in wild and mass-reared *Anastrepha ludens* (Diptera: Tephritidae). We found lower protein content in testes of old wild males and lower sperm viability in females mated with old wild males. Females stored more sperm when mated to young wild males than with young mass-reared males. Accessory gland injections of old or young males did not inhibit female remating. Knowledge of how male age affects different ejaculate components will aid our understanding on investment of the ejaculate and possible postcopulatory consequences on female behavior.

Key words accessory glands; age; *Anastrepha ludens*; mass-rearing; sperm; Tephritidae

Introduction

Age often has an important effect on the reproductive success of males. Male age-related reductions in sperm quality and quantity and fertilization success have been observed in invertebrates and vertebrates, including humans (reviewed by Johnson & Gemmell, 2012). In insects, the effect of male ageing on sperm traits has been studied in various species (García-Gonzalez & Simmons, 2005; Hale *et al.*, 2008; Papanastasiou *et al.*, 2011). In the mosquito *Aedes aegypti*, the number of sperm in virgin males varied with age, with a maximum between 10 and 20 d and then a decline after 20 d (Ponlawat & Harrington, 2007). On the contrary, in *Drosophila pectinata*, older males transferred greater number of sperm (Santhosh & Krishna, 2013), and in *Drosophila melanogaster* older males exhibited higher sperm viability, a measure of sperm quality (Decanini *et al.*, 2013). While sperm viability was not affected by male age in the hide beetle *Dermestes maculatus* (Hale *et al.*, 2008). Sperm viability is affected by levels of sperm competition and female mating status in crickets (Simmons *et al.*, 2007; Thomas & Simmons, 2007), showing that it is a plastic trait, susceptible to modifications depending on the context. In tephritids, sperm storage by females and sperm number decline with male age in the Mediterranean fruit fly *Ceratitis capitata* and the Queensland fruit fly *Bactrocera tryoni*, respectively (Taylor *et al.*, 2001; Pérez-Staples

Correspondence: Diana Pérez-Staples, INBIOTECA, Universidad Veracruzana, Av. de las Culturas Veracruzanas 101, Col. E. Zapata, Xalapa, Veracruz Cp. 91090, Mexico. Tel/Fax: +52 228 8422773; email: diperez@uv.mx

et al., 2008; but see Papanastasiou *et al.*, 2011). However as far as we know, sperm viability has only been studied in *C. capitata* (Twig & Yuval, 2005), and there have been no studies in pest tephritids of male age on sperm viability.

Both the quality and quantity of sperm and accessory gland proteins (AGPs), the 2 main ejaculate components, may vary with male age and affect their ability to modulate females response after copulation. However, the effect of male age on AGPs has received less attention (but see Santhosh & Krishna, 2013). AGPs in particular have been found to impact female oviposition, host location, female remating behavior, sperm use and longevity among other factors (reviewed in Avila *et al.*, 2011; Perry *et al.*, 2013). In *Drosophila bipectinata*, the amount of AGPs increases with age, with older males transferring greater amount of proteins (Santhosh & Krishna, 2013). There is evidence that the effect of AGPs on female behavior can be affected by external factors, such as strain, diet, fertility, and hormonal treatment of males (Abraham *et al.*, 2012). However, no studies have tested the interaction of male age directly on the biological function of AGPs.

In general, older males have reduced sperm traits in terms of quality and quantity, and lower fertilization success (Johnson & Gemmell, 2012). However, older males could invest more in current reproduction compared to younger males because they have reduced potential for future reproduction (Benowitz *et al.*, 2013). In that case, we would expect older males to invest more in terms of AGPs and sperm compared to young males. For example, *Drosophila pseudoobscura* females prefer to mate with old males, and such females produced more offspring, a direct benefit possibly due to increased investment in sperm or seminal fluids (Avent *et al.*, 2008). This increased investment could be in the form of protein content in the testes and male accessory glands, higher sperm viability or increased sperm numbers.

The Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae) is a long-lived species, with an average life expectancy of approximately 50 d under laboratory conditions (Carey *et al.*, 2005). Mass-reared males can start mating as soon as 6 d old, while wild males are considered sexually mature and can mate from 10 d onwards (Dickens *et al.*, 1982; Reyes-Hernández & Pérez-Staples, 2017). In wild males, a previous study found older sexually experienced males (36 d old) to have increased mating success compared to 18-d-old males, although no benefits in terms of fecundity or fertility for their mates were found (Pérez-Staples *et al.*, 2010). Insemination success is affected by male age, young males from 0 to 20 d old are in reproductive onset, then reproductive maturity (where males reached the highest reproductive success) is reached between 25 and 30 d, and finally senescence begins at 30 d, when insemination success begins to decrease (Harwood *et al.*, 2015). However, there is not a clear atrophy of reproductive organ size at these ages, and even at 57 d of age males have more sperm in their seminal vesicles than younger 9-d-old males (Reyes-Hernández & Pérez-Staples, 2017).

Knowledge of *A. ludens* reproduction (and age relationships) is crucial because the Sterile Insect Technique (SIT) is an important control tool for this species (Gutierrez-Sampeiro *et al.*, 1993). In SIT mass-reared sterile males are released in the field to compete with wild males for wild females (Knipling, 1955, 1959). For SIT to be effective, sterile mass-reared males should be sexually competitive against wild males and they should transmit sperm to render the female infertile as well as a full ejaculate to inhibit the female from remating. Nevertheless, we do not know if mass-rearing produces males that have lower ejaculate quality compared to wild males and if male age affects the ejaculate. For example, in wild *A. ludens*, female remating is not affected by male age (Abraham *et al.*, 2016a), while in mass-reared flies females mated with young males who were 6 d old were less likely to remate compared with females mated with older males (Reyes-Hernández & Pérez-Staples, 2017). A previous study determined that AGPs of young males were not responsible for inhibiting females from remating (Abraham *et al.*, 2014), however, male age and its effects on AGPs was not tested.

In this study, we determined for wild and mass-reared *A. ludens* of different ages (young and old): (i) the protein content in male accessory glands and testes; (ii) the mating probability of females injected with AGPs; (iii) sperm viability; and (iv) sperm quantity stored by females.

Materials and methods

Study insects

Fertile mass-reared *A. ludens* adults were obtained from the Moscafrut facility in Metapa de Domínguez, Chiapas, Mexico. Flies were obtained as pupae sent by air to Xalapa, Veracruz. Wild flies were recovered from infested oranges collected at Tuzamapan, and the area surrounding Xalapa. Fruits were taken to the laboratory and placed in 30 cm × 50 cm × 15 cm plastic trays with soil. Larvae migrated from the fruit to the soil where they pupated. After 7–10 d, the soil was sieved, and recovered pupae were placed in 30 cm × 30 cm × 30 cm cages at 26 ± 2 °C and 80% ± 10% RH until adult emergence. Artificial light was provided under a 12 : 12 photoperiod,

with half the lights going off or on at 6:00 and the other half at 6:30 to simulate dawn or dusk. Natural light was also provided from a window. Experiments were carried out at the Instituto de Biotecnología y Ecología Aplicada, Universidad Veracruzana, Xalapa, Veracruz, Mexico.

On the day of emergence, flies were sorted by sex and transferred to 30 cm × 30 cm × 30 cm cages in groups of 200–300 adults. Both sexes were provided with water and a diet consisting of sugar and hydrolyzed yeast (Yeast Hydrolyzated Enzymatic, ICN Biomedicals®, Santa Ana, CA, USA) in a ratio of 3 : 1.

Quantitative estimation of accessory gland and testes proteins

The following ages were used for virgin males: young (8–12 d old) and old (42–45 d old) mass-reared flies, and young (13–17 d old) and old (64–72 d old) wild flies. Protein content of accessory glands and testes were analyzed. Males were dissected in the morning in cold saline solution (NaCl 0.9%, PISA®, Guadalajara, Jalisco, Mexico), and glands or testes were transferred into a centrifuge tube (1.5 mL) with saline solution and protease inhibitors according to the manufacturers specifications (Roche® Complete Protease inhibitor cocktail, Sigma-Aldrich, Darmstadt, Germany) and gently crushed with a microsize tissue grinder for 1 min to release the content. For each sample, 35 accessory glands (i.e., from 35 males), of each male category were pooled and placed in 35 μL of saline. Forty testes (i.e., 20 pairs = 20 males), were pooled and placed in 40 μL of saline. The samples were centrifuged at 10 000 × *g* at 4 °C for 1 min (Hermle Z 300K centrifuge, Franklin, WI, USA), and the pellet was discarded. Protein was quantified from the supernatant using the Quick Start Bradford Protein Assay (Bio-Rad, USA). For the standard curve, we used serial dilutions of Bovine Serine Albumin (0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 mg/mL), using saline solution as diluent. The final volume for each standard and sample were 20 μL, to which 1 mL of 1× dye reagent was added. After at least 5 min of incubation at room temperature, the absorbance of each sample was measured at 595 nm in a spectrophotometer (GENESYS, model Genesys 10, Rochester, NY, USA) and the protein concentration was determined using the standard curve created by plotting the absorbance values *versus* their concentration. Two readings were taken for each sample and then averaged. Three independent replicates were carried out for each age and condition except for wild young males were only 2 replicates were obtained. Replicates for mass-reared flies were from batches obtained from the MoscaFrut Factory at least 15 d apart.

Injections of AGP homogenates

Preparation and injections General methodology followed Abraham *et al.* (2012, 2014). Accessory glands were dissected from live *A. ludens* males immersed in saline solution (NaCl 0.9%, PISA®). Glands were transferred into a 30 mm × 6 mm glass micro homogenizer with saline and gently crushed to release the accessory glands products. This aqueous extract was placed in an Eppendorf tube (1.5 mL) and centrifuged at 12 000 r/m at 4 °C for 1 min (Hermle Z 300K centrifuge). The resulting supernatant was placed in crushed ice and used for injections on the same day it was extracted. The dose of AGPs injected was 0.2 male equivalent (Jang, 1995; Radhakrishnan & Taylor, 2007; Abraham *et al.*, 2012, 2014). In our case, 20 *A. ludens* males of each treatment were dissected and the glands were homogenized in 100 μL of saline to obtain a 0.2 male equivalent concentration in 1 μL of solution. Injections were done immediately after extractions to avoid protein degradation.

For injections, sexually mature virgin females were used. Injections were done under a dissecting microscope (Leica S8AP0, Germany) with a 40× lens. Females were injected with 1 μL of solution containing the AGPs through the intersegmental membrane near the third abdominal sclerite. A Hamilton 1 μL microsyringe (Microliter™ #7001; Hamilton Co., Reno, NV, USA) was used to inject females. To rule out the effect of the injection treatment on the sexual receptivity of the female, the same amount of saline was injected into control females ("saline females"). After injections, females were transferred to 750 mL plastic containers in groups of 20–30 individuals with adult diet and water.

Treatments Forty-eight hours after the injection of females, we evaluated the receptivity of females from all treatments. In all experiments, we used virgin females injected only with saline ("saline females") as controls. As additional controls, we also used punctured females, that is, females that were punctured with the Hamilton syringe but were not injected with any substance, and virgin intact females, which were not subjected to any type of treatment.

For mass-reared flies we evaluated the receptivity of mass-reared females (10–17 d old) injected with AGPs from young (10–17 d old) or old (42–47 d old) mass-reared males. For wild flies, we evaluated the receptivity of wild females (14–27 d old) injected with AGPs from young (14–27 d old), or old (64–69 d old) wild males.

Evaluation of female receptivity Two days after injections, 1 female from each treatment and 1 young virgin

male of the same strain as the female (i.e., mass-reared females with mass-reared males [10–17 d old] and wild females with wild males [14–27 d old]) were placed in 500 mL plastic containers at 16:00 h in the laboratory at 24 °C. Thus, all females regardless of their age were placed with young males. Containers were checked for copulating pairs continuously for 6 h after releasing flies. Number of copulating pairs was recorded. Matings began at dusk and continued during dark. Observations were carried out with a dim red lamp. This procedure was repeated twice for mass-reared flies and 3 times for wild flies.

Sperm viability

Mating pairs of *A. ludens* were obtained the evening before dissection. In all cases, young virgin females were used (15–17 d old for mass-reared females and 19–24 d old for wild females) and male age varied according to the strain (mass-reared males: young 15–17 d old [$n = 21$], old 50–53 d old [$n = 20$]; wild males: young 21–26 d old [$n = 8$], old 61–68 d old [$n = 10$]).

At the end of copulations, males were discarded, and females were maintained singly with water and food. The following day, approximately 15–20 h after the end of copulations, the 3 spermathecae were dissected out of the females in a drop of 20 μL of PBS solution (1×) and dyed with LIVE/DEAD viability/cytotoxicity stain (L-7011 Molecular Probes, Eugene, OR, USA). Red and green dyes were previously diluted with PBS 1× (1 : 50 dilution for green dye and 1 : 20 dilution for red dye). Dilutions were done the same day of the dissection and were protected from light. The spermathecae were crushed, and a drop of 10 μL of green dye was added and incubated in dark chamber for 7.5 min. Then, a drop of 2.5 μL of red dye was added and incubated in a dark chamber for 2.5 min. The sample was covered with a 22 mm × 22 mm cover slip and immediately observed under a fluorescent microscope (Leica USA Microsystems DM750) at 40× magnification. The proportion of live/dead sperm were counted in 50 fields, first with the green filter for live sperm and then with the red filter for dead sperm for each field at a time. Any sperm fluorescing both red and green (bicolor sperm) were considered dead. This procedure was carried out at room temperature (20–22 °C approximately), but the live/dead stain was maintained at 25–30 °C.

Sperm quantity

Mating pairs were obtained as above using males of the same ages as in sperm viability: mass-reared males: young 15–17 d old ($n = 66$), old 52–54 d old ($n = 60$); wild males: young 21–23 d old ($n = 75$), old 60–66 d old ($n = 66$). Females were always virgin, young and of the same strain: mass-reared females (14–17 d old); wild females (19–25 d old). The time at which copulations began and ended was noted for calculation of copula duration. Experiments were replicated 3 times for a total of 267 pairs. After mating, females were dissected the following morning in saline solution. Spermathecae and the ventral receptacle were dissected and mounted on a slide. Following Taylor *et al.* (2001), the total number of sperm was calculated by observing 20 random fields in a contrast phase microscope (Iroscope series 300, Model MG-11, Irapuato, Mexico) and then extrapolating to the rest of the slide.

Statistical analyses

Protein content Data for protein content of accessory glands and testicles was analyzed with a Generalized Linear Model (GLM) with a normal distribution and Identity as a link, using replicate and male condition as independent variables. Post hoc comparisons were carried out through Contrasts.

Injections The number of copulating pairs was analyzed with χ^2 tests of homogeneity. For a comparison among more than 2 treatments, the sequential Bonferroni method (Rice, 1989) was applied after χ^2 tests.

Sperm viability To analyze sperm viability, a GLM with a binomial distribution and a logit link was performed on number of live sperm (as the number of successes) and total sperm as the number of trials. Two outliers were deleted, and 2 additional females mated with wild old males were not included in the analysis because they did not store any sperm in their storage organs. To test differences between groups, post hoc contrasts were used.

Sperm numbers Probability of sperm stored was analyzed by likelihood ratio Chi-square. Sperm stored by females was analyzed by Least Squares Regression, after ln transforming sperm numbers, where age, strain and the interaction between age and strain were the independent variables. Replicate was not significant and was excluded from the final model. A linear regression was used to analyze sperm number and copula duration. All statistical analyses were carried out in JMP version 9 (SAS Insitute Inc, 2010).

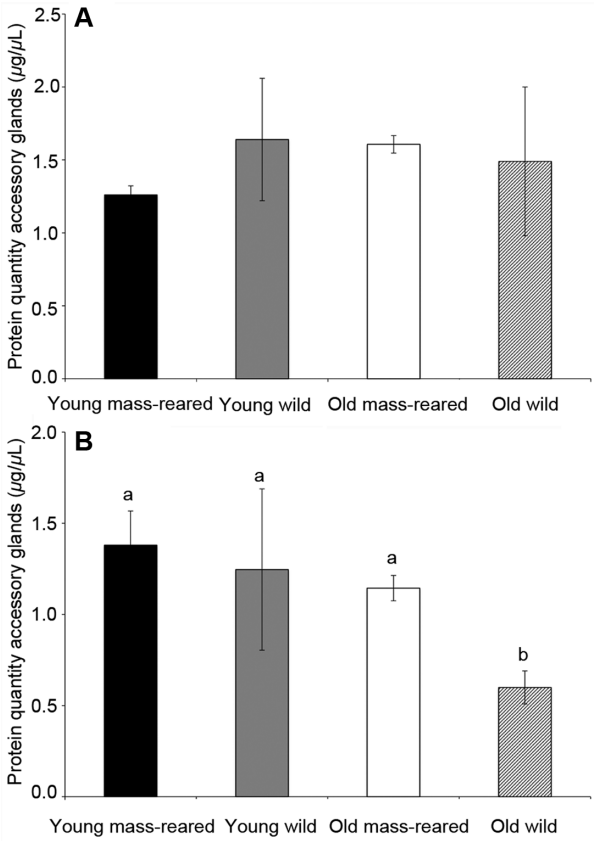

Fig. 1 Mean (± SE) protein quantity in *Anastrepha ludens* male accessory glands (A) and testes (B) according to strain (mass-reared or wild) and age. Different letters above bars indicate significant differences ($P < 0.05$) using Contrasts. Each treatment represents 1 μL of saline per 1 μL of accessory glands or testes. Each age and condition represent 3 replicates.

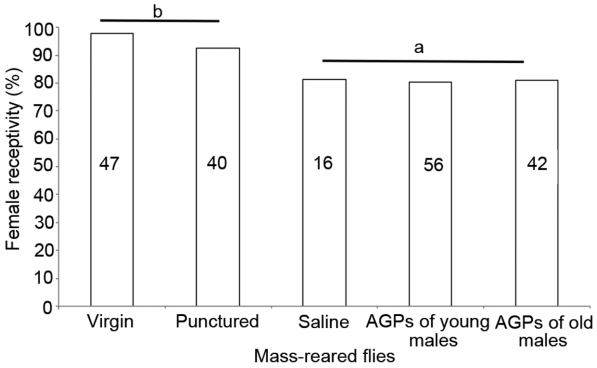

Fig. 2 Receptivity of *Anastrepha ludens* mass-reared females of different categories: virgin, punctured or injected with saline as controls, and injected with AGPs of young or old males. Numbers within bars represent sample size. Different letters over bars indicate significant difference ($P < 0.001$) after the sequential Bonferroni method (Rice, 1989) following χ^2 tests.

Results

Quantitative estimation of accessory gland proteins

Accessory glands of all males had a mean (±SE) quantity of 1.47 ± 0.131 μg/μL of protein, while testes had a mean quantity of 1.079 ± 0.125 μg/μL of protein. There was no significant difference in the protein content of accessory glands according to male condition (GLM male condition $\chi^2 = 1.23$, df = 3, $P = 0.744$; replicate $\chi^2 = 0.724$, df = 2, $P = 0.696$) (Fig. 1A). However, old wild males had significantly less protein in the testes compared to other males (male condition $\chi^2 = 12.81$, df = 3, $P = 0.005$; replica $\chi^2 = 4.490$, df = 2, $P = 0.106$). Post hoc contrasts revealed significant differences in the protein content in testes between old mass-reared males and old wild males (a strain effect within old males), between young and old wild males (an age effect within wild males), and between young mass-reared males and old wild males, but no significant differences between young males (wild or mass-reared) or between old and young mass-reared males (Fig. 1B).

Injections of AGPs in mass-reared and wild flies

Mass-reared flies There were no significant difference in female receptivity between virgin or punctured females ($\chi^2 = 1.422$, df = 1, $P > 0.05$) nor between injected females (saline, AGP old, AGP young males) ($\chi^2 = 0.009$, df = 2, $P > 0.05$). There were significant differences between control noninjected and injected females ($\chi^2 = 9.468$, df = 1, $P = 0.006$). That is, there was a reduction of receptivity due to the effect of injection technique but not to the effect of AGPs (Fig. 2).

Wild flies There were no differences in female receptivity between the 2 categories of control noninjected females ($\chi^2 = 0.229$, df = 1, $P > 0.05$) nor the 3 categories of injected females ($\chi^2 = 3.88$, df = 2, $P > 0.05$). There were differences between control noninjected (virgin and punctured) and injected females ($\chi^2 = 5.65$, df = 1, $P = 0.034$). Once again, there was a reduction of receptivity due to the effect of the injection technique but not to the effect of AGPs (Fig. 3).

Sperm viability assay

There was a significant effect of male condition on sperm viability stored in the 3 spermathecae ($\chi^2 = 76.632$,

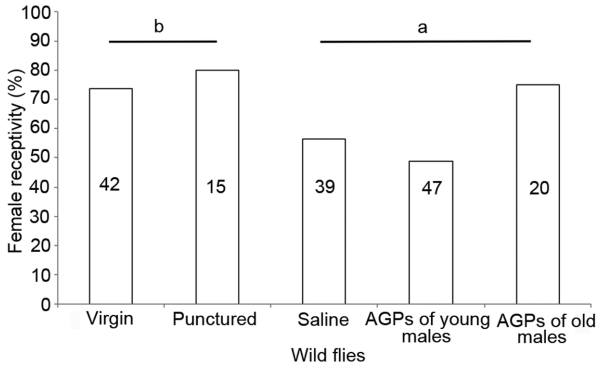

Fig. 3 Receptivity of *Anastrepha ludens* wild females of different categories: virgin, punctured or injected with saline as controls, and injected with AGPs of young, or old males. Numbers within bars represent sample size. Different letters over bars indicate significant difference ($P < 0.001$) after the sequential Bonferroni method (Rice, 1989) following χ^2 tests.

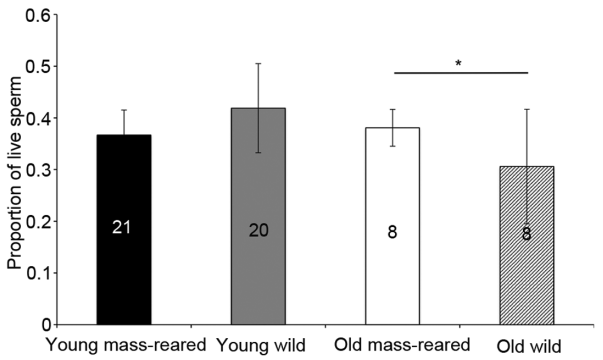

Fig. 4 Spermathecal sperm viability (mean proportion ± SE) in *Anastrepha ludens* young females mating with either mass-reared or wild males of different ages. Numbers within bars represent sample size. Asterisk over bar indicates significant differences using post hoc contrasts.

df = 3, $P < 0.0001$). Post hoc contrasts revealed females mating with old wild males had lower sperm viability compared to females mating with old mass-reared males (Fig. 4). There were no significant differences between females mating with old or young mass-reared flies, between young or old wild males nor between females mating with either young wild or young mass-reared males.

Sperm stored

There was a weak positive, but significant, relationship between copula duration and sperm stored in the 3 spermathecae and the ventral receptacle ($R^2 = 0.021$, $F = 0.018$, df = 1,265, $P = 0.018$). All of the older males transferred sperm, while 5.67% of females mated with young males did not store any sperm ($\chi^2 = 10.436$, $P = 0.001$). There was no effect of strain on likelihood to transfer sperm ($\chi^2 = 0.026$, $P = 0.872$). Of the females that did store sperm, there was a significant effect of strain ($F = 22.23$, df = 1,255, $P < 0.0001$) but not age ($F = 0.772$, df = 1,255, $P = 0.380$) on ln transformed total sperm stored, and there was a significant interaction between age and strain ($F = 6.952$, df = 1,255, $P = 0.009$) (Fig. 5). Post hoc Tukey HSD test revealed that females mating with young wild males stored more sperm than females mating with young mass-reared males. Females mating with young wild males also stored more sperm than females mating with old mass-reared males. Females mating with old wild males stored more sperm than females mating with young mass-reared males (Fig. 5). All other comparisons were nonsignificant.

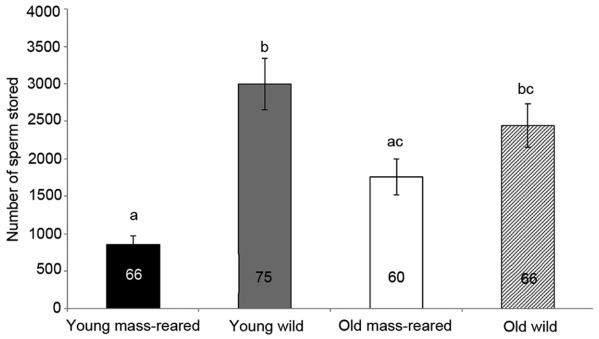

Fig. 5 Sperm numbers (mean ± SE) stored in the spermathecae and ventral receptacle by young *Anastrepha ludens* females mated to old or young wild or mass-reared males. Females were of the same strain as males. Numbers within bars represent sample size. Different letters over bars represent significant differences.

Discussion

Age and strain effects on accessory glands and testes

We found that old wild males had lower protein quantity in their testes compared to young wild and old mass-reared males. That is, there was an age effect (within wild flies) and a strain effect (within old flies), whereas protein amount in accessory glands was not affected by age or strain. In other insects, such as *D. pectinata*, the amount of protein in the male accessory glands transferred to females does increase with age (Santhosh & Krishna, 2013), while in *D. melanogaster* older males have fewer

proteins (Rezaei *et al.*, 2015). In comparison, the overall amount of protein found in the accessory glands of *A. ludens* was quite low compared to drosophilids (Ravi Ram & Ramesh, 2002).

In a previous study in *A. ludens,* we found that accessory gland size had a more gradual increase with male age compared to testes, and accessory gland size more closely followed male mating success than testes size (Reyes-Hernández & Pérez-Staples, 2017). Here, male age seemed to have a higher effect on testes and its contents than on male accessory glands and its contents, indicating that there can be differential investment in ejaculate components. Indeed, in *D. melanogaster*, males can even tailor specific proteins in their ejaculate, strategically reducing or maintaining peptides depending on the context (Wigby *et al.*, 2009; Sirot *et al.*, 2011). As accessory glands grow, males could be investing more in protein production, keeping a relative constant μg/μL amount as they age. However, it is unknown if the specific type of proteins found in *A. ludens* male accessory glands vary with age.

Protein amount in the male accessory glands did not vary with male age for wild or mass-reared flies. Similarly, there was no effect of male age or strain on the biological effect of male AGPs, as injections of AGPs into *A. ludens* did not inhibit their receptivity (see also Abraham *et al.*, 2014). In contrast, injection of aqueous extracts of AGPs into females inhibit female receptivity in a great number of species (Radhakrishnan & Taylor, 2007; Yamane *et al.*, 2008a,b; Shutt *et al.*, 2010; Abraham *et al.*, 2012; Brent & Hull, 2014; Yu *et al.*, 2014). The impact of male age on AGPs in *A. ludens* should be evaluated on other female behaviors, such as oviposition or feeding.

Age and strain effects on sperm

Sperm viability decreased in old wild males, compared with their mass-reared counterparts. In the cricket *T. oceanicus*, sperm viability in the male spermatophore increased with male age (García-González & Simmons, 2005), while in *D. maculatus* sperm viability was not affected by male age (Hale *et al.*, 2008). In the yellow dung fly *Scathophaga stercoraria*, male age did not influence either sperm viability in the testes or sperm resistance to degradation inside the female tract (Bernasconi *et al.*, 2002). One possibility is that sperm viability decreased with male age due to sperm senescence (White *et al.*, 2008), or sperm of wild old males were less capable of resisting female-mediated processes of sperm degradation (Degrugillier, 1985; Birkhead *et al.*, 1993; Bernasconi *et al.*, 2002). Lower sperm viability could also be related to lower protein stores in the testes.

Sperm viability differed from previous findings in other Diptera species. For example, in *C. capitata*, sperm viability of more than 80% was registered (Twig & Yuval, 2005). In *D. melanogaster*, Snook and Hosken (2004) found sperm viability close to 30%, while Radhakrishnan and Ferdorka (2011) found sperm viability as high as 90%. Our estimate of sperm viability is close to that provided by Snook and Hosken (2004) if moribund sperm (fluorescing green and red) are considered dead. On the contrary, if we consider moribund sperm as live, our estimate is nearer to that provided by Radhakrishnan and Ferdorka (2011). In general, researchers included bicolor sperm as dead sperm (Bernasconi *et al.*, 2002; Shafir *et al.*, 2009; Radhakrishnan & Fedorka, 2011).

There seems to be an interesting trade-off between sperm viability and sperm numbers stored by females. Here we found that, despite lower protein content in the testes and lower sperm viability, sperm stored by females mating with old males did not decrease. This is consistent with a previous study that found older males had more sperm in their seminal vesicles than younger males (Reyes-Hernández & Pérez-Staples, 2017). In *B. tryoni,* the number of sperm stored by females increased with male age and then declined (Pérez-Staples *et al.*, 2008). While we did not see this decline, females mating with young mass-reared males stored fewer sperm than females mating with young wild males. Likewise, for their first mating, young sterile males have lower sperm numbers than wild males (Abraham *et al.*, 2016b). This has implications for the efficiency of SIT as ideally, mass-reared males should have comparable numbers of sperm to wild males.

In summary, wild males experienced a steeper deterioration as they aged compared to mass-reared males. Old wild males had fewer proteins in their testes, and lower sperm viability compared with their younger counterparts and compared to old mass-reared males. The fact that this effect was more evident in wild males compared to mass-reared males suggests that there is decreased variance in ejaculate components in highly regulated mass-rearing conditions. Old mass-reared males were able to maintain and preserve quality and quantity attributes of the ejaculate, while old wild males were not. The only attribute that remained unchanged both with age and strain, was the amount of protein in the male accessory glands. To better understand how male condition can affect the ejaculate and its effect on female behavior, it will be useful to investigate the components and molecular identity of the seminal fluid in *A. ludens*.

Acknowledgments

We thank Dina Orozco and the staff at the MoscaFrut factory for providing files. We are thankful to Wilbert Poot and Clara Córdova for help throughout the study. We thank Enrique Alarcón and Antonio Andrade (INBIOTECA) for access to equipment. Funding was provided by CONACyT Mexico Ciencia Básica grant No. 179741 awarded to DPS. SA was supported by a postdoctoral grant from CONICET, Argentina, MHC by a CONACyT postdoctoral scholarship, and NNB and MHR by a CONACyT masters and doctoral scholarship, respectively. DPS thanks Kavita Isvaran for hosting her at the Indian Institute of Science (Bangalore, India), during her sabbatical.

Disclosure

The authors state that they have no involvement, financial or otherwise, that might potentially bias this work.

References

Abraham, S., Cladera, J., Goane, L. and Teresa Vera, M. (2012) Factors affecting *Anastrepha fraterculus* female receptivity modulation by accessory gland products. *Journal of Insect Physiology*, 58, 1–6.

Abraham, S., Nuñez-Beverido, N., Contreras-Navarro, Y. and Pérez-Staples, D. (2014) Female receptivity in *Anastrepha ludens* (Diptera: Tephritidae) is not modulated by male accessory gland products. *Journal of Insect Physiology*, 70, 41–48.

Abraham, S., Contreras-Navarro, Y. and Pérez-Staples, D. (2016a) Female age determines remating behavior in wild Mexican fruit flies. *Journal of Insect Behavior*, 29, 340–354.

Abraham, S., Contreras-Navarro, Y., Núñez-Beverido, N., Lara, L., Ovruski, S. and Pérez-Staples, D. (2016b) The male ejaculate as inhibitor of female remating in two tephritid flies. *Journal of Insect Physiology*, 88, 40–47.

Avent, T.D., Price, T.A.R. and Wedell, N. (2008) Age-based female preference in the fruit fly *Drosophila pseudoobscura*. *Animal Behaviour*, 75, 1413–1421.

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D. and Wolfner, M.F. (2011) Insect seminal fluid proteins: identification and function. *Annual Review of Entomology*, 56, 21–40.

Bernasconi, G., Hellriegel, B., Heyland, A. and Ward, P.I. (2002) Sperm survival in the female reproductive tract in the fly *Scatophaga stercoraria* (L.). *Journal of Insect Physiology*, 48, 57–63.

Benowitz, K.M, Head, M.L., Williams, C.A., Moore, A.J. and Royle, N.J. (2013) Male age mediate reproductive investments and response to paternity assurance. *Proceedings of the Royal Society B*, 280, 20131124.

Birkhead, T.R., Møller, A.P. and Sutherland, W.J. (1993) Why do females make it so difficult for males to fertilize their eggs? *Journal of Theoretical Biology*, 161, 51–60.

Brent, C.S. and Hull, J.J. (2014) Characterization of male-derived factors inhibiting female sexual receptivity in *Lygus hesperus*. *Journal of Insect Physiology*, 60, 104–110.

Carey, J.R., Liedo, P., Muller, H.G., Wang, J.L., Senturk, D. and Harshman, L. (2005) Biodemography of a long-lived tephritid: reproduction and longevity in a large cohort of female Mexican fruit flies, *Anastrepha ludens*. *Experimental Gerontology*, 40, 793–800.

Decanini, D.P., Wong, B.B.M. and Dowling, D.K. (2013) Context-dependent expression of sperm quality in the fruitfly. *Biology Letters*, 9, 20130736.

Degrugillier, M.E. (1985) *In vitro* release of housefly *Musca domestica* L. (Diptera: Muscidae) acrosomal material after treatments with secretions of female accessory glands and micropyle cap substance. *International Journal of Insect Morphology and Embryology*, 14, 381–391.

Dickens, J.C., Solis, E. and Hart, W.G. (1982) Sexual development and mating behavior of the Mexican fruit fly, *Anastrepha ludens*. *Southwestern Entomologist*, 7, 9–15.

García-González, F. and Simmons, L.W. (2005) Sperm viability matters in insect sperm competition. *Current Biology*, 15, 271–275.

Gutierrez-Samperio, J., Reyes, J. and Villaseñor, A. (1993) National plan against fruit flies in Mexico. *Fruit Flies: Biology and Management* (eds. M. Aluja & P. Liedo), pp. 419–423. Springer, New York.

Hale, J.M., Elgar, M.A. and Jones, T.M. (2008) Sperm quantity explains age related variations in fertilization success in the hide beetle. *Ethology*, 114, 797–807.

Harwood, J.F., Chen, K., Liedo, P., Müller, H.G., Wang, J.L., Morice, A.E. and Carey, J.R. (2015) Female access and diet affect insemination success, senescence and the cost of reproduction in the male Mexican fruit fly *Anastrepha ludens*. *Physiological Entomology*, 40, 65–71.

Jang, E.B. (1995) Effects of mating and accessory gland injections on olfactory-mediated behavior in the female Mediterranean fruit fly, *Ceratitis capitata*. *Journal of Insect Physiology*, 41, 705–710.

JMP (2010) Ver. 9.0. SAS Institute, Inc. Cary, NC. USA.

Johnson, S.L. and Gemmell, N.J. (2012) Are old males still good males and can females tell the difference? *BioEssays*, 34, 609–619.

Knipling, G.F. (1955) Possibilities of insect control of eradication through the use of sexually sterile males. *Journal of Economical Entomology*, 48, 459–462.

Knipling, E.F. (1959) Sterile-male method of population control. *Science*, 130, 902–904.

Papanastasiou, S.A., Diamantidis, A.D., Nakas, C.T., Carey, J.R. and Papadopoulos, N.T. (2011) Dual reproductive cost of aging in male medflies: dramatic decrease in mating competitiveness and gradual reduction in mating performance. *Journal of Insect Physiology*, 57, 1368–1374.

Pérez-Staples, D., Harmer, A.M.T., Collins, S.R. and Taylor, P.W. (2008) Potential for pre-release diet supplements to increase the sexual performance and longevity of male Queensland fruit flies. *Agricultural Forest Entomology*, 10, 255–262.

Pérez-Staples, D., Martínez-Hernández, M.G. and Aluja, M. (2010) Male age and experience increases mating success but not female fitness in the Mexican fruit fly. *Ethology*, 116, 778–786.

Perry, J.C., Sirot, L. and Wigby, S. (2013) The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution*, 28, 414–422.

Ponlawat, A. and Harrington, L.C. (2007) Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, 44, 422–426.

Radhakrishnan, P. and Taylor, P.W. (2007) Seminal fluids mediate sexual inhibition and short copula duration in mated female Queensland fruit flies. *Journal of Insect Physiology*, 53, 741–745.

Radhakrishnan, P. and 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.

Ravi Ram, K. and Ramesh, S.R. (2002) Male accessory gland secretory proteins in *nasuta* subgroup of *Drosophila*: synthetic activity of *Acp*. *Zoological Science*, 19, 513–518.

Reyes-Hernández, M. and Pérez-Staples, D. (2017) Mating senescence and reproductive organ size in the Mexican fruit fly. *Physiological Entomology*, 42, 26–35

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*. *Zoological Science*, 32, 16–24.

Rice, W.R. (1989) Analyzing tables of statistical tests. *Evolution*, 43, 223–225.

Shafir, S., Kabanoff, L., Duncan, M. and Oldroyd, B.P. (2009) Honey bee (*Apis melifera*) sperm competition in vitro - two are not less viable than one. *Apidologie*, 40, 556–561.

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

Shutt, B., Stables, L., Aboagye-Antwi, F., Moran, J. and Tripet, F. (2010) Male accessory gland proteins induce female monogamy in anopheline mosquitoes. *Medical and Veterinary Entomology*, 24, 91–94.

Snook, R.R. and Hosken, D.J. (2004) Sperm death and dumping in *Drosophila*. *Nature*, 428, 939–941.

Simmons, L.W., Denholm, A., Jackson, C., Levy, E. and Madom, E. (2007) Male crickets adjust ejaculate quality with both risk and intensity of sperm competition. *Biology Letters*, 3, 520–522.

Sirot, L.K., Wolfner, M.F. and Wigby, S. (2011) Protein-specific manipulation of ejaculate composition in response to female mating status in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 9922–9926.

Taylor, P.W., Kaspi, R., Mossinson, S. and Yuval, B. (2001) Age-dependent insemination success of sterile Mediterranean fruit flies. *Entomologia Experimentalis et Applicata*, 98, 27–33.

Thomas, L.M. and Simmons, L.W. (2007) Male crickets adjust the viability of their sperm in response to female mating status. *The American Naturalist*, 170, 190–195.

Twig, E. and Yuval, B. (2005) Function of multiple sperm storage organs in female Mediterranean fruit flies (*Ceratitis capitata*, Diptera: Tephritidae). *Journal of Insect Physiology*, 51, 67–74.

Wigby, S., Sirot, L.K., Linklater, J.R., Buehner, N., Calboli, F.C.F., Bretman, A., Wolfner, M.F. and Chapman, T. (2009) Seminal fluid protein allocation and male reproductive success. *Current Biology*, 19, 751–757.

White, J., Wagner, R.H., Helfenstein, F., Hatch, S.A., Mulard, H., Naves, L.C. and Danchin, E. (2008) Multiple deleterious effects of experimentally aged sperm in a monogamous bird. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 13947–13952.

Yamane, T., Miyatake, T. and Kimura, Y. (2008a) Female mating receptivity after injection of male-derived extracts in *Callosobruchus maculatus*. *Journal of Insect Physiology*, 54, 1522–1527.

Yamane, T., Kimura, Y., Katsuhara, M. and Miyatake, T. (2008b) Female mating receptivity inhibited by injection of male-derived extracts in *Callosobruchus chinensis*. *Journal of Insect Physiology*, 54, 501–507.

Yu, J.F., Cong, L., Xu, J., Liu, J.H. and Ye, H. (2014) Male accessory gland secretions modulate female post-mating behavior in the moth *Spodoptera litura*. *Journal of Insect Behavior*, 27, 105–116.

Manuscript received September 21, 2016
Final version received December 23, 2016
Accepted January 11, 2017