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Original Article

Early reproductive success in *Drosophila* males is dependent on maturity of the accessory gland

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In a number of insect taxa, male sexual maturity is not always directly attained at adult eclosion. Sexual maturity is often defined behaviorally as mating success, or physiologically as the point when gametes are produced. Here, we show that the sexual maturation process in males is more complex than previously described and that a measure of maturity also needs to include post-mating traits. In many species mating success is tightly linked with size of the accessory gland (AG). The AG is the production site of a complex cocktail of proteins that are transferred to the female along with the sperm, and which impact on important functions for male reproductive success. Using *Drosophila melanogaster* we conducted comprehensive behavioral assays of several aspects of male reproductive fitness and demonstrated that the AG has to attain full maturity for males to reach full reproductive competence. We tested males from shortly after, and up to 6 days, post adult eclosion, and considered both pre- and post-copulatory traits. Young males are less likely to gain a mating and show significantly less courtship behavior. Young males that managed to obtain a mating however were inferior in many post-copulatory traits like sperm competitiveness and effectiveness in suppressing female remating. We monitored male AG development over the first 6 days after eclosion and found it to significantly increase in size over the entire period.

Key words: **accessory gland, courtship behavior, *Drosophila melanogaster*, reproductive success, sex peptide (SP), sexual maturation.**

INTRODUCTION

Male reproductive success is determined by multiple interacting steps including pre- and post-mating traits, which range from physiological to behavioral ones. These traits need to be integrated with one another in order for males to first successfully gain a mating and then to fertilize the female ova. In young males these traits might display different ontogenies resulting in reduced performance with sexual maturation leading to attainment of full reproductive competence. In some insects sexual maturity is not automatically reached with adult eclosion and there is a time-lag before adults produce mature gametes (reviewed in Minelli et al. 2006). For example, in young hide beetles, *Dermestes maculatus*, males were found to transfer fewer sperm at mating than older beetles (Jones et al. 2007). However, in *Drosophila silvestris* males can produce sperm a few days after adult eclosion but do not perform courtship behavior. In this case it is the behavior that needs to mature before males successfully engage in matings (Boake and Adkins 1994). In a number of taxa mating success is associated with the size of the accessory gland (AG), which increases during early adulthood, for example, in stalked-eyed flies *Cyrtodiopsis dalmanni* (Baker 2003) and the firebug *Pyrrhocoris apterus* (Socha 2006). In the field cricket *Teleogryllus oceanicus* the AG increases in size within the first 20 days post adult eclosion with accompanying changes in seminal fluid protein abundance, while the testes actually reduce in size (Simmons et al. 2014). Hence this suggests that full sexual maturity is a process integrating diverse determinants of male reproductive success and that not one single measure fully captures this process. We here argue that a more comprehensive approach is needed to understand the process of sexual maturity and particularly post-mating traits need to be considered. Therefore, we measured a whole suit of traits like courtship behavior, sperm competition, and female post-mating responses to gain a fuller picture of the maturation process in male *Drosophila melanogaster*.

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In many insect species males mature more rapidly than females but in *Drosophila* the situation is often reversed (Pitnick et al. 1995). Across 42 *Drosophila* species the onset of reproduction in males ranged from day 0 to 19 after eclosion, whereas in females this range is markedly reduced (between day 1 to 8) (Pitnick et al. 1995). Pitnick and colleagues judged sexual maturity as the point when 80% of males on a given day succeeded in gaining a mating. This benchmark was reached at 2 days post eclosion for *D. melanogaster*. Nevertheless, *D. melanogaster* males can already successfully inseminate females when younger than 24 h post eclosion (Stromnae 1959). Both measures of fertility or mating success did not necessarily coincide with males achieving maximum fertilization competence and both ignored post-mating traits, which are crucial for male reproductive success (Fricke et al. 2010).

In polygamous species after a mating is accomplished males often face competition from rival male ejaculates (Simmons 2001). Sperm competition is a pervasive evolutionary pressure (Parker 1970) and often its intensity shapes both testis size and sperm morphology (reviewed in Simmons and Fitzpatrick 2012). Under sperm competition, sperm morphology is adapted to ensure high fertilization capacity (Miller and Pitnick 2002), whereby in *D. melanogaster*, for example, slow and long sperm successfully resist sperm displacement and stay in the fertilization set (Lupold et al. 2012). However, producing long sperm comes at the cost of delayed sexual maturity (Pitnick et al. 1995).

In addition to sperm numbers and morphology, the non-sperm component of an ejaculate is vital for mediating male competitive fertilization success (reviewed in Chapman 2001; Simmons and Fitzpatrick 2012). The non-sperm component of an ejaculate consists of seminal fluid proteins and in both vertebrates and invertebrates a large fraction of these proteins are produced in the AGs (reviewed in Poiani 2006). The functions of these proteins have been studied intensely in *D. melanogaster*, where males transfer more than 200 different seminal fluid proteins to the female during mating (Findlay et al. 2008; Findlay et al. 2009). A subset of those are produced in the male AG (Findlay et al. 2008) and we will here mainly consider those AG proteins—Acps (Adams and Wolfner 2007) and their function. Similar to other species, Acps play a crucial role in many post-copulatory traits in *D. melanogaster*. They are known to, for example, decrease the risk of sperm competition by decreasing female receptivity to remate (Chapman et al. 2003; Liu and Kubli 2003), trigger an egg laying boost (Herndon and Wolfner 1995; Heifetz et al. 2000), affect the storage and retention of sperm, and seem to cause displacement of stored sperm from previous mates (Harshman and Prout 1994; Neubaum and Wolfner 1999; Chapman et al. 2000; Prout and Clark 2000; Bloch Qazi and Wolfner 2003; Ravi Ram and Wolfner 2007). Since the production of Acps is of such importance for male reproductive success and post-mating competitiveness, the ability of the AG to produce Acps might be an important contributor to the sexual maturation process. In support of this idea, in male stalked-eyed flies (*C. dalmanni*) sexual maturity is linked with the size of the AGs (Baker 2003). Similarly in the firebug *P. apterus* the size of the AG increased for several days after eclosion and was positively linked with male mating success (Socha 2006). Further in *Drosophila* it is the size of the AG, not the testes, that responds to experimental evolution as demonstrated in 2 studies altering the risk and intensity of sperm competition in *D. melanogaster* (Linklater et al. 2007), and *D. pseudoobscura* (Crudgington et al. 2009). Taken together this highlights the significance of Acps for fertilization success and competiveness in a number of taxa (Simmons and Fitzpatrick 2012).

The AG in *D. melanogaster* are paired tube-like structures connected to the ejaculatory duct. Each gland consists of main cells, which secrete the majority of Acps and about 40 secondary cells, which contain large secretory vacuoles and are located at the tip of the gland. The secretory tissue is surrounded by a muscular sheath and a single layer of epithelium (Bertram et al. 1992). As evidence suggests that not only the composition but also the amount of Acps transferred is important (Kalb et al. 1993), we here test whether the AG is fully developed after eclosion. We hypothesize that ontogenetic changes occur in the AG during early adulthood and that these negatively affect a male's fertilization capacity. We used one particularly well known Acp—the Acp70A also called sex peptide (SP)—as an example to document functionality across male ages. SP was chosen because it induces very prominent phenotypical responses in the female. It reduces female receptivity for up to 4 days after mating and triggers an egg-laying boost (Liu and Kubli 2003). Furthermore, since the receipt of SP also reduces female fitness and lifetime reproductive success (Wigby and Chapman 2005) it possesses the potential for mediating sexual conflict between the sexes (Fricke et al. 2009).

We predict that developmental immaturity of the AG can be a major contributor to reduced male reproductive success in young males just a few hours after eclosion, particularly under competitive conditions. We hypothesize that the different determinants of male success mature at different rates and that only on maturation of all traits males will reach maximum reproductive capacity. Here we followed *D. melanogaster* males shortly after eclosion from the pupae up to 6 days of age and measured several components of male reproductive success to document the male sexual maturation process. We considered both pre- and post-mating traits to fully describe the developmental sequence of trait maturation whereupon males can achieve reproductive competence.

MATERIALS AND METHODS

Fly stocks

Wild-type flies

The Dahomey wild-type stock was originally collected in the 1970s in Dahomey, West Africa (now Benin) and cultured under laboratory conditions in mass culture population cages ever since. Our Dahomey stock flies were kindly provided by Tracey Chapman (University of East Anglia, United Kingdom) and maintained in our laboratory in population cages for more than a year prior to the experiment.

SP knockout flies

To generate SP knockout males SP^0 / *TM3,Sb,ry* males were crossed with virgin $\Delta 130$ / *TM3,Sb,ry* females. Male offspring of this cross with the genotype $SP^0/\Delta 130$ (SP^0) do not produce SP (Liu and Kubli 2003). To generate the corresponding control flies SP^0,SP^+ / *TM3,Sb,ry* males were mated to $\Delta 130$ / *TM3,Sb,ry* females to gain SP-producing $SP^0,SP^+/\Delta 130$ (SP^+) males. The $\Delta 130$ / *TM3,Sb,ry* stock was backcrossed for 3 generations into the Dahomey wild-type genotype and chromosomes 1, 2, and 4 of the SP^0 / *TM3,Sb,ry* and SP^0,SP^+ / *TM3,Sb,ry* stocks were backcrossed for 4 generations to control for genetic background. These stocks, as well as the *Sb* stock (see below) were kindly provided by Tracey Chapman.

Stubble flies

Flies from the Stubble (*Sb*) mutant stock can be phenotypically distinguished from wild-type flies by their shortened bristles on the thorax. *Sb* males were used as competitor males in the sperm competition experiment. *Sb*/*Sb* is lethal thus individuals with the *Sb* phenotype are heterozygous for the dominant *Sb* mutation. To control for genetic background the *Sb* stock was backcrossed into the Dahomey background for 4 generations.

Fly Culturing

All flies were cultured at 25 °C and 60% humidity at a 12:12 h dark–light cycle. The wild-type flies were kept in cages (19 × 32 × 22cm) at large population size with overlapping generations. Food was provided on a weekly basis by placing 3 glass bottles filled with 70 mL fresh standard sugar–yeast (SYA) food (100 g autolyzed yeast powder, 100 g dextrose, 20 g agar, 30 mL Nipagin [10% w/v solution], 3 mL propionic acid and 1 L water) in the cage. Mutant lines were cultured in glass bottles also containing 70 mL SYA food.

In order to generate wild-type flies for experiments 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) provided with a blob of live yeast paste were introduced into the cage and females were allowed to oviposit for 4–6 h. After incubating the plates over-night larvae were collected and allowed to develop under density controlled conditions (100 larvae per plastic vial [Ø 2.5 cm, height 8.4 cm] containing 7 mL of SYA food). Adult flies were collected as virgins on ice and kept in same sex groups of 20 individuals per vial. On 7 consecutive days we placed grape-juice-agar plates into the Dahomey wild-type cage to have 7 cohorts of larvae to generate males for the 7 age groups. Males were collected as virgins and allowed to mature until they were used in mating assays, which were conducted for all age groups on the same day. If necessary groups of males were flipped onto fresh food every 3 days prior to experiments.

Sb flies used for the sperm competition experiment were generated by mating 30 *Sb* females to 30 *Sb* males from the stock and allowing the females to oviposit on grape-juice agar plates for 6–8 h. Larvae were collected the next day and developed under density controlled conditions as well. The emerging adults were collected as virgins and sorted by genotype. *Sb* males were kept at a density of 20 per vial.

In order to generate SP^0 and SP+ males virgin *Δ130 / TM3,Sb,ry* females were put in a vial together with either SP^0 / *TM3,Sb,ry* or SP^0,SP^+ / *TM3,Sb,ry* males. Flies were allowed to interact and lay eggs for 24 h and afterwards the parents were removed and larvae were allowed to develop. SP^0 and SP^+ males were collected as virgins and kept in groups of 20.

We conducted several experiments to determine changes in male sexual maturity and reproductive success during young male ontogeny. In the first experiment we covered the first phase of adulthood in small intervals and focused on mating- and remating parameters. For the other experiments we tested fewer age groups and measured specific traits as a measure for male sexual maturity.

All matings were performed at 25 °C and 60% humidity directly after lights on. Experiments were conducted using glass vials (Ø 2.7 cm, height 10 cm) containing 7 mL of SYA food with additional ad libitum live yeast granules or paste. The time at which pairs were introduced to each other was recorded as was the starting time and the end of each mating. This allowed us to calculate latency (time between the introduction time and the start of a mating), as well as copulation duration.

Single mating short-term assay

In this first experiment we covered the first 6 days of male adulthood in small steps and therefore generated 7 age groups (16, 24, 48, 72, 96, 120, and 144 h post eclosion) with 34–40 wild-type males in each group. We measured female remating propensity and egg laying rate after a single mating opportunity as estimates of male reproductive success. Both traits are critically influenced by receipt of male seminal fluid proteins and particularly SP. Comparing values for these traits for the different male age groups with the baselines set by the SP^0 and the control males gives us an indication of the ability of young males to transfer a fully matured ejaculate.

For each age group virgin males were collected that eclosed up to 2 h before or after the defined time point. SP^0 and SP^+ males were all aged 144 h. Since the SP^0 males do not transfer any SP to the female during copulation their reproductive performance was used as an indicator of the amount of SP transferred by the wild-type males of different ages during copulation.

For the mating assay all males were individualized in glass vials 24 h before the start of the assay. After the acclimatization period 1 wild-type female (age 144 h post eclosion) was introduced into each vial and the couples were observed continuously for 3 h. Couples that did not mate within 3 h were removed from the experiment. After a copulation was completed the male was removed from the vial and the females were allowed to lay eggs for 24 h. Subsequently each female was transferred to a fresh glass vial containing a competitor wild-type male (age 144 h post eclosion) for an opportunity to remate. Females were observed for 1.5 h during which we recorded the number of females that remated. We also counted the number of eggs laid over the 24-h period in the vacated vials.

Single mating long-term assay

In a further set of experiments we tested the same 2 components of male reproductive performance over a longer time period after a single mating to test whether young males can maintain the female post-mating response over several days. We first measured productivity over 9 days in males from 4 age classes and in a second independent experiment male ability to reduce female remating propensity effectively over 4 days.

For the productivity assay virgin wild-type females were mated to males that were either 21, 48, 72, or 96 h old. Matings were performed as described above. From each male age group 30 successfully mated females were transferred to fresh food every 24 h and were allowed to lay eggs for 9 days. After counting eggs on day 1–5, 7, and 9, vacated vials were incubated for 11 days at standard conditions and the number of adult offspring eclosed from the pupae was counted.

For the remating long term assay virgin females that were all 96 h old were first mated to 24, 48, or 96-h old males. In total 300 females were mated and evenly spread over the 3 male age groups (n = 100). Females that did mate in the first mating were then given the opportunity to remate with a competitor male (wild-type, 4 days old) at different time points after the first mating. Four subsets of 25 mated females each from each male age group entered the remating assay either 24, 48, 72, or 96 h after the first mating. We observed pairs continuously for 1.5 h and scored whether a female remated or not within this period. Each female was only exposed to a second male once and then discarded. Females in treatments given a remating opportunity after 72 or 96 h were transferred to a fresh vial 48 h after the first mating.

Courtship behavioral assay

The intensity of courtship behavior shown by young males in comparison to older ones was analyzed in this assay. Fifty-five males each of the age of 21 h or 96 h after eclosion were observed for 30 min (spot-checks every 60 s) in a single mating vial while interacting with a 96-h old virgin female. Two observers each monitored 6 mating vials at once. The assay was carried out over 2 consecutive days between 9 AM and 11 AM starting directly after the lights came on. Individual males of both age groups were distributed equally and blind among the 2 observers and the 2 days. For every time point we recorded if the male was showing one of the following behaviors aimed towards the female: following, waving, tapping, licking, mounting or a combination of multiple behaviors simultaneously (for more details see Supplementary Table S1). If a male managed to mate with the female within the given observation period this couple was not observed any longer.

Sperm competition assay

In a further experiment 4 age groups (14, 48, 72, and 96 h post eclosion) of wild-type males were tested for their sperm defense ability. At first 2 groups of young males were generated: 11 h and 17 h post eclosion. But since only a small number of 11-h old males were able to successfully achieve a mating these 2 time points were combined and renamed as the 14-h age group to represent the midpoint. Since we observed lower mating rates in the younger age groups in the single mating assay, more young males were used here for the first mating in order to generate enough mated females for the remating assay. Therefore 99 males aged 14 h, and 46–50 males in the age groups 48, 72, or 96 h were used in this experiment. After mating those males once to wild-type virgin females (age 96 h) following the protocol as described above those females were given the opportunity to remate 24 h later. For the second mating we used *Sb* males as competitors (4 days old) as this allowed us to determine paternity. After a successful second mating females were transferred daily to new vials for 2 days. Vacated vials were incubated at standard conditions and after eclosion offspring were frozen and subsequently scored for paternity. We determined the number of offspring sired by the first male (P1) to estimate the ability of wild-type males of different ages to defend paternity under sperm competition. Since the *Sb* allele is homozygous lethal, *Sb* competitor males were all heterozygous for the *Sb* allele and therefore only half of their offspring bore the dominant *Sb* allele (females were homozygous for the wild-type allele). Paternity had thus to be calculated in the following way: we doubled the number of *Sb* offspring counted and corrected the number of wild-type offspring accordingly to gain the final numbers used for further analysis.

AG size measurement

In addition to performing the single mating short-term assay we also collected a subset of wild-type males from all age groups and froze them in order to dissect and measure the size of the AG. Furthermore we included an eighth age group with males of age 11 h post eclosion generated from the sperm competition assay. All males were virgin and not exposed to females prior to being frozen. Dissections were carried out in PBS (Phosphate Buffered Saline, tablets [Merck Millipore]) and the AGs were photographed under microscopic (Zeiss Axio Observer.Z1) amplification (50×). In order to keep the arms of the AGs straight and flat but to prevent squeezing and damaging the tissue a spacer (consisting of 3 layers of adhesive tape) was placed between the slide and cover slip. The area of the AGs was measured by drawing the outline of one arm of the AG and analyzing the size of the area measured in pixels with the software ImageJ (Wayne Rasband). For each AG only 1 arm was measured (we always choose the one that was fixed in a better position under the cover slide). In total the AGs of 17–21 males per age group were dissected and measured. Prior to performing the measurements the repeatability of the method was tested by measuring the same AG twice and analyzing the repeatability. As we raised larvae under standard density we expected no differences in body size between groups, however to confirm this we measured wing length as an index of body size as well. One wing from each male was cut off as close to the wing root as possible and placed on a slide with a drop of PBS and photographed under microscopic amplification (50×). Wing length was measured by drawing a line from the proximal junction of the 4th and 5th longitudinal vein to the intersection of the third longitudinal vein with the distal wing margin (described in Leibowitz et al. 1995) using ImageJ.

Data analysis

All tests were done using R (version 3.2.1) and RStudio (version 7.6) using the car package (Fox and Weisberg 2011). Graphs were created using R as well and SigmaPlot338 12.0 for Windows (Build 12.0.0.182, Systat Software, Inc. 2011). Results are reported as mean ± standard error (SE) throughout if not mentioned otherwise.

As data were not normally distributed we applied the nonparametric Kruskal–Wallis 1-way analysis of variance on ranks test when indicated. Further we used generalized linear models (glm) with the appropriate error structure and correction for over-dispersion via the quasi-extension if necessary. In detail we performed glms on the following data: mating rate using a binomial error structure, remating rate, egg–adult survival, sperm competition success and courtship intensity when comparing successful with unsuccessful males using a quasibinomial error structure and number of eggs laid by the female within the first 24 h using a quasipoisson error structure. We first analyzed the full models and tested significance of the factor age by excluding it and then comparing the full with the reduced model using an Analysis of Deviance. As we only used the SP mutant and their control males to provide baseline values for comparison, these data were not included in any of the analysis presented.

Data on eggs and eclosed adults from the single mating long term assay were analyzed using a generalized linear mixed model (glmm) with both time course and male age as fixed factors using the R package lme4 (Bates et al. 2015). For this analysis each female was assigned an individual identification number that was included in the model as a random factor. For the number of eggs laid and the number of eclosed adults a poisson error structure was used while a binomial error structure was used on the proportion of eclosed adults.

Before looking at differences between AG sizes among groups we checked for repeatability of the measurement method by using a linear regression on repeated measures of 55 glands (7–8 glands from each age group). As repeatability was high with 99% (multiple $r^2 = 0.996$, $P < 0.001$) we decided to measure an arm of every AG once for the main data set. Furthermore, we used a regression to test whether body size is dependent on age and whether differences in AG size depended on body size or age. Differences in AG size for the different age groups were analyzed by Kruskal–Wallis 1-way analysis of variance by ranks. To test when after eclosion the AG has reached its final size we applied a multiple comparison test for Kruskal–Wallis tests using the pgirmess package (Giraudoux 2016) to compare the AG size of the oldest age group (144 h) to all other groups.

RESULTS

Single mating short-term assay

Mating rate increased significantly with male age (G_6^2 = 95.12, $P < 0.001$, see Figure 1a). In particular, young males less than 48 h post eclosion performed less well compared with older males, where mating rates approached 100% in males older than 72 h. This reduced ability of young males to gain a first mating is also reflected in the fact that they needed on average 5 to 6 times longer to start a mating than males 96 h post eclosion or older (Supplementary Figure S1A). In general, latency to mating significantly decreased with increasing age (Kruskal–Wallis analysis $P < 0.001$, see Supplementary Figure S1A). Mating duration differed between the age groups in this experiment (Kruskal–Wallis analysis $P < 0.001$), but no clear pattern was visible (Supplementary Figure S1B). However, we found no effects of male age on the number of eggs laid within 24 h after the first mating (G_6^2 = 66.29, F = 1.87, P = 0.087, see Supplementary Figure S2A) or on egg–adult survival (G_6^2 = 86.89, F = 0.9, P = 0.5, see Supplementary Figure S2B).

Female propensity to remate depended significantly on the age of the first male (G_6^2 = 26.44, F = 4.27, $P < 0.001$, see Figure 1b). Only males older than 120 h post eclosion were effective in suppressing female remating with a pattern similar to that elicited by SP-transferring control males. In contrast, males less than 48 h post eclosion were ineffective at preventing females from engaging in a second mating, resulting in female remating rates resembling those for females not receiving SP at all (Figure 1b). This indicates that not a full complement of Acps was transferred. Overall this single mating assay showed that young males have difficulties gaining a mating, but when they do, egg fertilization success is comparable to mature males. Despite having functioning testes it seems that the AG shows delayed maturity in males younger than 48 h as young males could not suppress female remating.

Single mating long-term assay

No significant effect of male age could be found on the number of eggs laid on the observation days (days 1–5, 7 and 9) (G_3^2 = 2.83, P = 0.43, see Supplementary Figure S3A). However, there was a significant effect of time with an initial increase in egg numbers followed by a subsequent drop (G_1^2 = 340.26, $P < 0.001$, see Supplementary Figure S3A). This pattern was similar across all age groups as indicated by a marginally non-significant interaction term between age and time course (G_3^2 =6.76, P = 0.08). The mean total sum of eggs laid by the females over all observed days was not significantly different between the age groups (Kruskal–Wallis analysis P = 0.27, see Supplementary Figure S4).

The number of offspring also significantly decreased over time after a peak at day 3 (G_1^2 = 515.38, $P < 0.001$, see Supplementary Figure S3B). Male age had no significant effect (G_3^2 = 5.38, P = 0.15) on the number of adult offspring, but the rate of change was different across male age groups (male × time: G_3^2 = 53.64, $P < 0.001$, see Supplementary Figure S3B).

The difference in pattern between egg and adult counts is not only due to a reduced proportion of eclosed offspring with time (G_1^2 = 332.36, P = 0.01, see Figure 2) but particularly because younger males had a lower egg–adult survival rate (male age: G_3^2 = 11.19, $P < 0.001$) and there was a steeper reduction with time for the 2 younger than for the 2 older male age classes (male × time: G_3^2 = 53.45, $P < 0.001$). However, measured over the entire period this did not significantly alter the mean total sum of adult offspring produced across male age groups (Kruskal–Wallis analysis P = 0.21, see Supplementary Figure S4).

The long-term assay shows that even though younger males produced similar numbers of eggs than more mature males, young males cannot fertilize eggs as efficiently over several days.

Remating long-term assay

In this long-term assay female propensity to remate was scored over 4 days after the first mating for independent female groups. Female remating rate increased with time passed since the first mating (G_1^2 = 54.41, $P < 0.01$, see Figure 3), and was also significantly modulated by age of the first male (G_2^2 = 10.17, $P < 0.01$). The interaction term between the time passed and age of the first male however was not significant (G_2^2 = 0.62, P = 0.74). The remating behavior over all 4 time points was similar for females mated with the 2 older male age groups (48 and 96 h). In contrast, females mated with a 20-h old male showed a higher willingness to remate

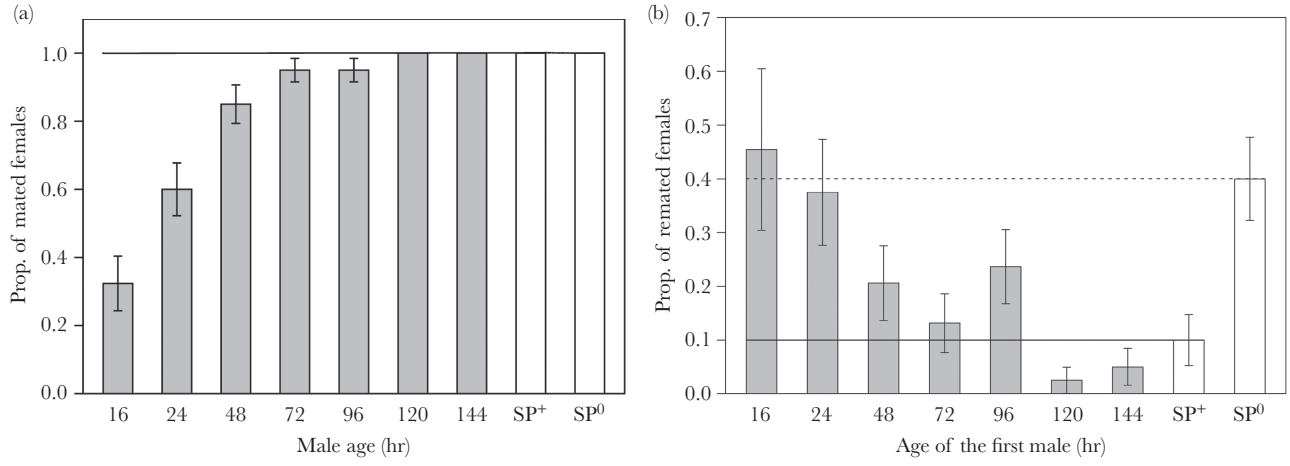

Figure 1

(a) Mating rate as proportion (±SE) of virgin wild-type females (age 144 h) that mated with males of different ages (ranging from 16 to 144 h) and either SP^+ (mating rate indicated by the black line) or SP^0 (indicated by the dashed, black line) males (age 144 h) within a 3-h observation period (n = 30 in each group) and (b) remating rate as the proportion (±SE) of females that remated 24 h later with a virgin wild-type competitor male (age 144 h) within a 1.5-h observation period after a first mating to the same males displayed in (a) in the single mating short-term assay.

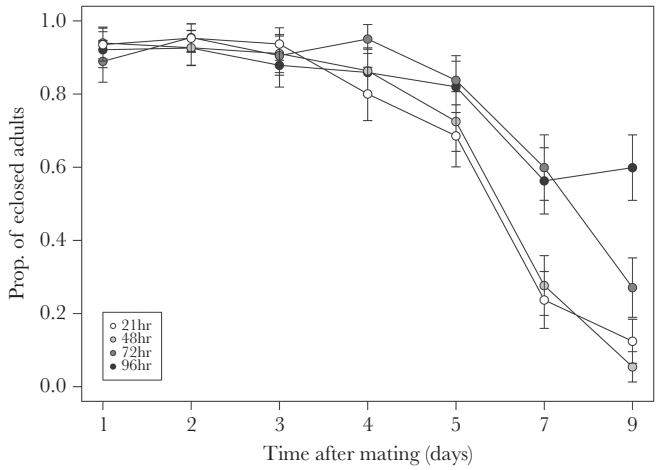

Figure 2
Proportion (±SE) of adult offspring eclosed on day 1–5, 7, and 9 after a single mating in the long-term assay with males ranging in age from (24–96 h).

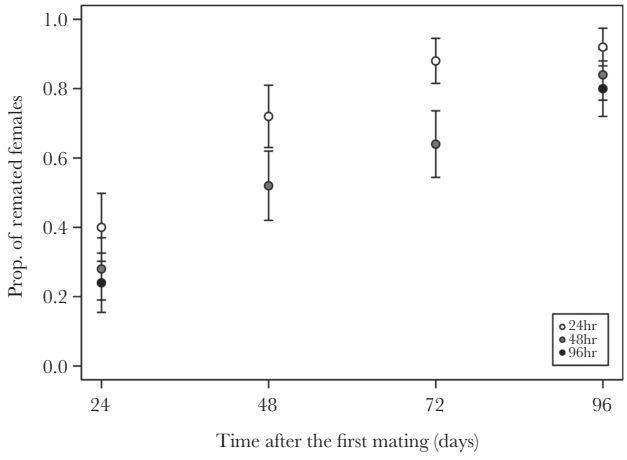

Figure 3
Remating rate of females mated to males ranging in age from 24 to 96 h post eclosion once. Females were given a chance to remate with a 96-h old competitor male either 24, 48, 72, or 96 h after a first mating (Please note: For time-points 48 and 72 h after the first mating the marker points indicating the remating rate for females previously mated to 48 and 72 h old males overlap so that just 1 marker point is visible in the figure).

throughout all time points. Thus young males are less efficient in preventing females from remating and this pattern persists over the entire time sampled.

Courtship behavioral assay

Very young males (21 h) direct significantly less courtship behavior towards a female than older males (96 h) ($\chi^2_1 = 147.47$, $P < 0.001$, see Figure 4a). Because there was great variability in the amount of courtship behavior young males showed, we further divided each age group into males that were successful versus unsuccessful in gaining a mating to test whether they differed in courtship intensity (Figure 4b). While overall courtship behavior was still significantly dependent on male age ($G^2_1 = 24.46$, $P < 0.001$), successful males invested more in courtship behavior than unsuccessful ones ($G^2_1 = 18.6$, $P < 0.001$). While young successful males displayed courtship at a rate similar to old males, unsuccessful young males showed particularly little courtship activity (age × success: $G^2_1 = 8.06$, $P < 0.01$, see Figure 4b).

Sperm competition assay

Age had a significant effect on male ability to defend paternity ($G^2_6 = 443.92$, $F = 5.15$, $P < 0.01$, see Figure 5) in our double mating experiment. Older males showed a 5 times higher sperm defense ability than young males, indicating that young males indeed do not transfer the full complement of Acps and are inferior in competitive conditions. For effects of male age on female mating and remating behavior and latency time (see Supplementary Figures S5 and S6A and Supplementary Table S2) and egg–adult survival (Supplementary Figure S7 and Supplementary Table S2) we found the same significant patterns as described above in the short-term single mating assay. The effect of male age on mating duration in contrast was not significant in this assay (Supplementary Figure S6B and Supplementary Table S2).

AG size measurement

Males of all age groups did not differ in body size and also AG size was not dependent on body size (Supplementary Figure S8 and Supplementary Table S2). We therefore excluded body size from further analysis.

Male AG size increased significantly over the first days after eclosion (Kruskal–Wallis analysis $P < 0.001$) and more than doubled between the ages of 11–144 h (Figure 6a). When comparing the AG size of 144 h old males to all other age groups, it becomes apparent that between 11 h and 72 h post eclosion the AG significantly differs in size compared with AGs of 96-h to 144-h old males (post hoc tests $P < 0.05$ for males younger than 96 h and $P > 0.05$ for males older than 72 h). The increase in AG size was accompanied by the glands becoming filled with a dark substance (Figure 6b), which potentially reflected the activity of the main cells in producing and secreting seminal fluid proteins into the lumen.

DISCUSSION

Our results show that after imaginal eclosion *D. melanogaster* males need 3 to 4 days to fully mature. We found deficits in pre- and post-copulatory, as well as in anatomical traits in young males. On the pre-copulatory level young males have problems to gain a first mating with a virgin female. In agreement with Pitnick et al. (1995) we also found that males needed to mature for 2 days to successfully gain a mating, when applying the 80% mating rate criterion. However, by considering additional determinants of male reproductive success we could show that even beyond this point young males had not reached full reproductive capacity. We found that the male AGs increased in size over the entire period measured, accompanied by improved success in several post-mating traits known to be mediated by Acps: for example, male sperm defense ability and suppression of female remating. We observed that the immaturity of the AG impedes males from reaching their full reproductive capacity until about 4 days after eclosion and full development of the AG seems to occur later than for behavioral and pre-mating traits.

Young males were quite unsuccessful in gaining a mating within the first 48 h after eclosion. The poor mating rates achieved by young males in combination with a long latency time when they managed to obtain a mating could be explained by low rates of courtship behavior young males display in comparison to older ones as shown in our courtship assay. Due to sparse courtship young males are less likely to persuade a female into mating. Alternatively (or additionally) females may resist more vigorously when courted by a young male (see also Long et al.

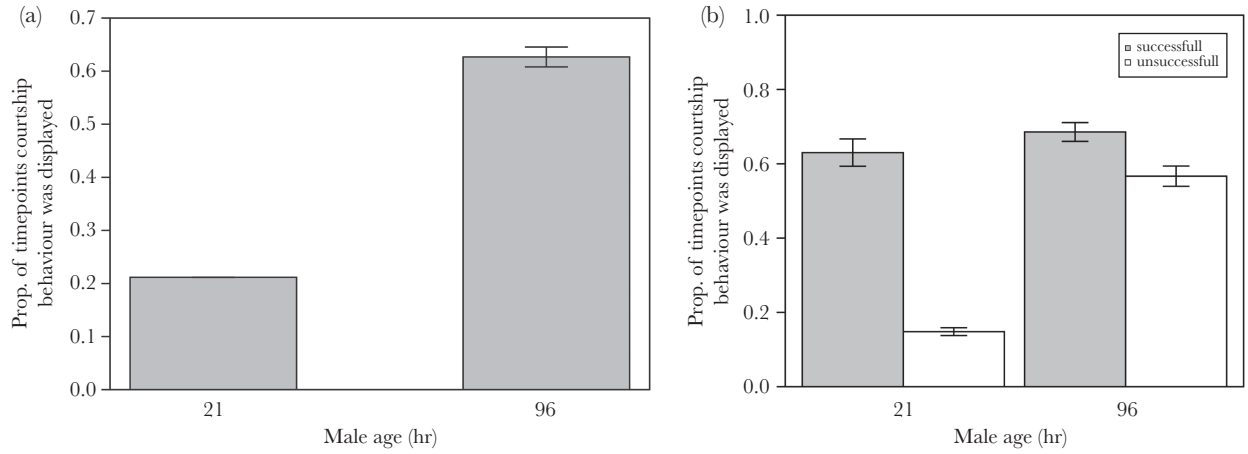

Figure 4
Courtship intensity of younger (21 h) versus older (96 h) males measured as (a) the proportion of time points where courtship behavior was displayed towards a virgin 96-h old female and (b) the proportion of courtship display shown by males successfully gaining a mating versus those being unsuccessful.

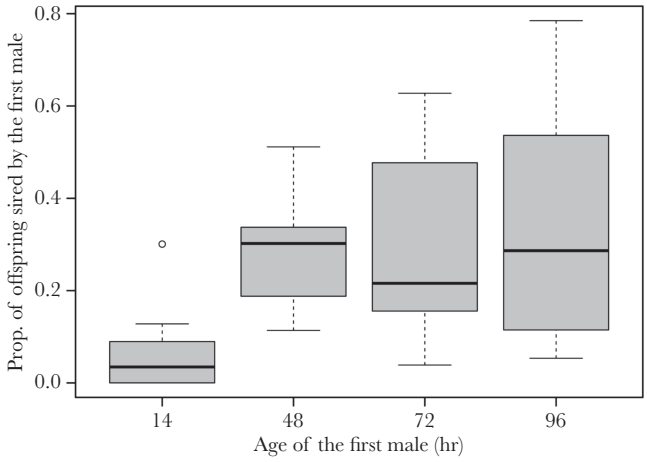

Figure 5
Boxplots of the median proportion of offspring sired by the first male (P1) (with 25% and 75% quartiles) out of 2. First males ranged in age from 14 to 96 h when competing against a second male in controlled double mating experiments set 24 h apart.

1980), but we didn't monitor the female response behavior in this assay. Also in *D. silvestris* males needed several days of behavioral maturation before they performed courtship behavior (Boake and Adkins 1994) and this despite that males started producing sperm about 2 days before they started courting. In our experiment we found strong variation in the ability of young males to court, but when young 21-h old males were capable of high courtship rates, then they were also successful in gaining a mating, while unsuccessful ones showed almost no courtship behavior at all. Male courtship behavior is complex and the neuronal circuits regulating this innate behavior are well described (reviewed in Billeter et al. 2006) with the neuronal circuitry already being in place at metamorphosis (Arthur et al. 1998). However, expression changes in courtship genes (Ruedi and Hughes 2009) and regulators of neuronal development (Lee et al. 2000) occur during early adulthood indicating that the system might not have fully matured at eclosion. Our data support this idea as we saw variation amongst young males with a large fraction failing to successfully court a virgin female.

Young males that successfully mated were inferior in several post-copulatory traits compared with older males: they could not suppress female remating as effectively in the short- or longer-term, and they were less successful at defending their paternity. All of these post-copulatory traits are mediated by Acps (reviewed in Chapman 2001; Wolfner 2009) and together these findings suggest that the ejaculate might not be optimally composed (Perry et al. 2013). Either, young males are not capable of transferring sufficient amounts of Acps or the composition of Acps is not balanced to result in maximal reproductive success. Development of the AG in *D. melanogaster* is determined during a critical phase in the late third larval instar and is under the control of the sex determination pathway (Chapman and Wolfner 1988). Expression of some Acp genes starts during the late pupal stages just before eclosion, for example, ovulin (Chapman and Wolfner 1988) and Acp57Dc (Cho et al. 2000), while others like Acp 95EF start after eclosion and increase to a peak 3 days after eclosion (DiBenedetto et al. 1990). Further, the secondary and main cells of the AG produce different subsets of Acps (DiBenedetto et al. 1990; Minami et al. 2012; Gligorov et al. 2013) and their development seems to be regulated partly by different genes (Xue and Noll 2002; Minami et al. 2012; Gligorov et al. 2013; Li et al. 2015). Hence, only when both cell types in the AG have fully matured can the AG effectively produce the full complement of Acps.

We indirectly tested the idea that young males transfer reduced amounts of Acps at mating by using SP knock out males as comparison to estimate SP amounts transferred by males of different ages. SP plays an important role in altering female remating behavior (Liu and Kubli 2003), in sperm competition (Fricke et al. 2009; Avila et al. 2010) and increases the number of eggs laid by females after mating (Liu and Kubli 2003). We found that males younger than 48 h elicited remating rates similar to the baseline set by SP^0 males, whereby 72 h or older males reduced female willingness to remate comparable to the SP transferring control males. Hence, for this trait it seems young males are not capable of transferring sufficient SP at mating. We did not find the same pattern for the increase in egg laying as young males were not significantly worse and did not differ from SP transferring control males. Here, another Acp, ovulin, could explain the initial boost as it increases egg laying in the short-term (Herndon and Wolfner 1995). High initial egg–adult survival rates after mating with young males show that

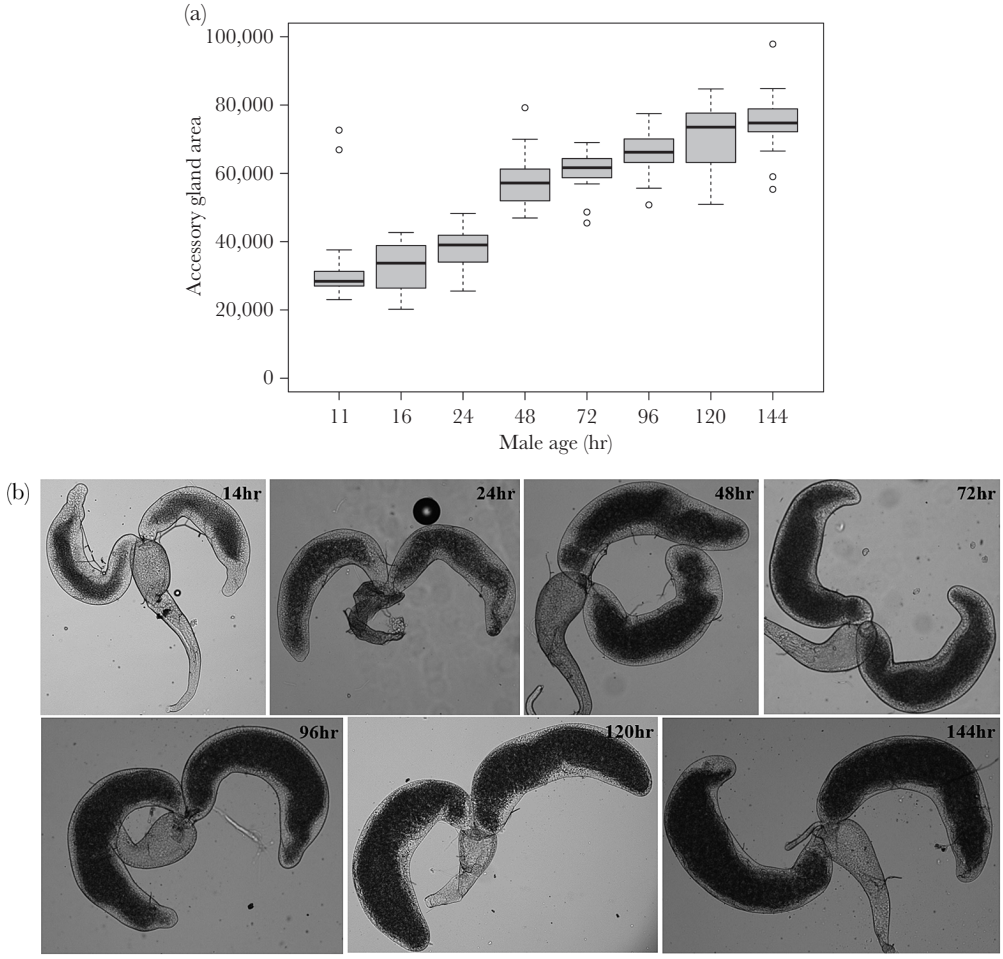

Figure 6
Male age dependent AG size was measured (as area in pixel) after organs were dissected from virgin males ranging in age from 16 to 144 h post eclosion. Presented (a) are boxplots with median size with upper (75%) and lower (25%) quartiles and (b) photographs of male AG taken at different time points after eclosion (14, 24, 48, 72, 96, 120, and 144 h) under microscopic amplification (50×). The paired AGs and the ejaculatory duct (ED) are visible on each picture.

sperm from young males is transferred, stored and able to fertilize eggs. The high egg–adult survival though was not maintained over a longer period of time shown by the single mating long-term assay. One possible explanation is that sperm of younger males is stored and used differently in the female reproductive tract. Together these results illustrate that young males do not transfer ejaculates optimally mediating male reproductive success.

Our argument that the AG is a vital component of male sexual maturation is supported by the observation that the gland itself is immature after eclosion and increases significantly in size within the first few days after adult eclosion. In *D. melanogaster* larger glands are more efficient at producing and transferring larger amounts of Acps (Wigby et al. 2009), thus indicating that the increase in AG size we observed is also accompanied by an accumulation of its products. Similarly in a number of insect taxa the AG increases in size after eclosion with a steady increase in total protein content, for example, in *Drosophila ananassae* and *Drosophila varians* (Hiremani and Shivanna 2010), *T. oceanicus* (Simmons et al. 2014), *P. apterus* (Socha et al. 2004), and *C. dalmanni* (Baker 2003). Hence, post-eclosion growth of the AG might be a common phenomenon important for male sexual maturation. As the AG is mainly important for post-reproductive traits, temporal differences in sexual maturation across taxa might be explained by the variation in the strength of polyandry and hence sperm competition. However, also abiotic factors can explain variation in sexual maturation as, for example, diet has a strong impact on male maturation (Minelli 2006).

CONCLUSION

We found strong effects of male age on several pre- and post-copulatory reproductive traits. This implies that several developmental processes important for reproduction are not completed when the male ecloses from the pupae. As our results indicate that less SP is transferred by very young males we propose that one important factor leading to the observed male sexual immaturity is transfer of an incomplete set of Acps during mating. While before the development of the testis was hypothesized to delay maturation (Pitnick et al. 1995) we here argue that AG development is another critical factor tightly linked with male sexual maturation. In line with this idea, *C. dalmanni* (Baker 2003) and *D. silvestris* (Boake and Adkins 1994) showed that testes maturation preceded AG-related behavioral maturation. Hence our findings might extend to other insect taxa and we need to comprehensively study the course of events to answer how males attain their full reproductive potential during sexual maturation.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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