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Physiological Maturation Lags Behind Behavioral Maturation in Newly Eclosed *Drosophila melanogaster* Males

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The accessory gland (AG†) produces seminal fluid proteins that are transferred to the female upon mating in many insects. These seminal fluid proteins often promote a male's post-copulatory reproductive success. Despite its crucial function many males eclose with a small gland not yet containing the full set of proteins. Thus, they need a physiological maturation period. Using *Drosophila melanogaster*, we tested whether this physiological maturation is linked to behavioral maturation in males and to what extent seminal fluid allocation patterns are influenced by physiological maturation. To that end, we measured AG protein content (as a proxy for physiological maturation) of young, immature males that were either successful in gaining a mating, but prevented from transferring seminal fluid proteins, or unsuccessful, thus using mating success as a proxy for behavioral maturation. Furthermore, we compared ejaculate allocation in immature and mature males in a single mating. Though mating success and gland maturation increase with male age, we found no evidence for a fine-tuned synchronization of behavioral and physiological maturation in males. This is especially surprising since we found reduced ejaculate allocation in very young, immature males, hinting at reduced fitness benefits from early matings in *D. melanogaster*.

INTRODUCTION

An insect's life can be divided into two distinct phases: a juvenile phase marked by growth and development and an adult phase dominated by reproduction. However, a sexual maturation period can be found in many species after adult eclosion [1-8]. Delayed sexual maturity might occur when attainment of full sexual competence requires additional resources that cannot be acquired during juvenile stages. Indeed, females of many insect species require additional protein intake for oogenesis (*e.g.,* in Diptera [9] and Hymenoptera [10]). Sexual maturation can be divided into two different classes: i) behavioral maturation, *i.e.,* the development and display of behavior that is related to mating [11,12], and ii) physiological maturation, *i.e.,* the development of mature eggs in

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†Abbreviations: AG, accessory gland; SFP, seminal fluid protein.

Keywords: Sexual maturation, accessory gland proteins, courtship, mating behavior, post-mating response, ejaculate allocation

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females [*e.g.*, 9,10] or sperm and non-sperm components of the male ejaculate [1,7,8]. As mating and courtship can pose considerable costs on males [13-15] and females [16-18], we would expect behavioral and physiological maturation to be synchronized. It would be adaptive to only exhibit costly mating behavior when physiological sexual maturity is reached, as only then mating behavior can produce maximum benefits. Here we tested this hypothesis using males of the fruit fly *Drosophila melanogaster*.

Seminal fluid proteins can be found in a large number of animal species – both invertebrates and vertebrates – and contribute to a male's reproductive success [19]. The accessory (reproductive) glands (AGs) found in insect males are often paired structures composed of secretory tissue (for *D. melanogaster* AGs, see Figure 1) and produce the majority of seminal fluid proteins (SFPs) that are transferred alongside the sperm to the female during mating [20]. In many insect species males eclose with small AGs that grow in the days following eclosion [*e.g.,* 8,21,22]. This growth is often accompanied by an increase in reproductive success, which is probably due to production of AG proteins, that make up the main part of the SFP cocktail in *D. melanogaster* [23]. Similar post-eclosion growth can be found in another part of the male *D. melanogaster* reproductive tract, the ejaculatory bulb, where growth is accompanied by an accumulation of secretions over the first 7 days after eclosion [24]. While the AG increases in size during the first week after eclosion, newly eclosed males (around 24 h post-eclosion) are inferior in a number of post-mating reproductive traits, that are strongly influenced by seminal fluid proteins, compared to older males (at least 96 h post-eclosion) [8]. Consequently, the authors hypothesized that immature males either allocate a smaller or an ill-composed ejaculate to their mating partners reducing their fitness gain per mating [8], leading us to investigate ejaculate allocation in maturing *D. melanogaster* males.

In a number of insect species, male mating rate and post-eclosion AG size/growth are positively correlated, *e.g.,* in the stalk-eyed fly *Cyrtodiopsis dalmanni* [3], the firebug *Pyrrhocoris apterus* [25] or the fruit fly *D. melanogaster* [8,26]. Thus, with increasing age, the male AG matures and mating rate increases. In young *D. melanogaster* males, mating success seems to be dependent on courtship effort [8]. Still, there is considerable variation in courting effort in young males of the same age [8], hence males seem to mature behaviorally at different rates. The question remains whether physiological maturation also proceeds at different rates, matching individual dynamics of behavioral maturation. Juvenile hormone [27] might influence male development of both, courtship behavior and AG growth, in *D. melanogaster* [28]. It changes the sensitivity of Or47b olfactory receptor neurons, which in turn influences male mating behavior [12]. The authors hypothesize that an increase in sensitivity of these neurons with age leads males to more efficiently locate and, as a consequence, more efficiently court females [12]. Thus, juvenile hormone does influence the behavioral aspect of sexual maturation in *D. melanogaster*. It also plays a vital role in the activation of seminal fluid protein synthesis in *D. melanogaster* [29], hence suggesting a crucial role in the physiological aspect of sexual maturation in males. Taken together, this led us to hypothesize that, possibly through the regulation of juvenile hormone, there might be a fine-tuned synchrony between AG development and behavioral maturation (see also [12]).

Very young *D. melanogaster* males that gain a mating are able to fertilize a female's eggs as shown by Ruhmann *et al*. [8], where egg-to-adult survival up to 3 days after a single mating is the same in females mated to very young (21 h) versus more mature (96 h) males. At the same time those young males are inferior to more mature males in post-mating traits (*e.g.,* suppression of female sexual receptivity and sperm defense ability), abilities that stem from the action of SFPs and thus maturation of the AG determines full sexual maturity in *D. melanogaster* males [8]. To test for a synchronization of behavioral and physiological sexual maturation in *D. melanogaster* males, we quantified the AG protein content of young males that either achieved a mating or not during a short (60 – 90 min) mating trial. We predicted that if male investment in courtship and AG maturation are linked, successful males should on average have a higher protein content in their AGs (before mating) than unsuccessful males. With the present experiments, we cannot distinguish whether young male mating success is determined by courtship activity or female choice, but previous work indicates that reduced courtship effort is the predominant mechanisms behind reduced mating success in very young *D. melanogaster* males [8]. Furthermore, we tested ejaculate allocation in maturing males to elucidate the mechanism behind a previously reported reduced post-copulatory success in young males [8].

MATERIAL AND METHODS

Flies

For all experiments a wildtype population of *D. melanogaster* was used, that had been collected in the 1970s in the Republic of Dahomey, Africa (now Benin). Ever since, this population has been maintained at large population sizes with overlapping generations. We kept the population at 25 °C, 60 % r.h., and a 12L:12D cycle (hereafter referred to as standard conditions). Flies were provided with fresh standard sugar-yeast-agar (SYA)

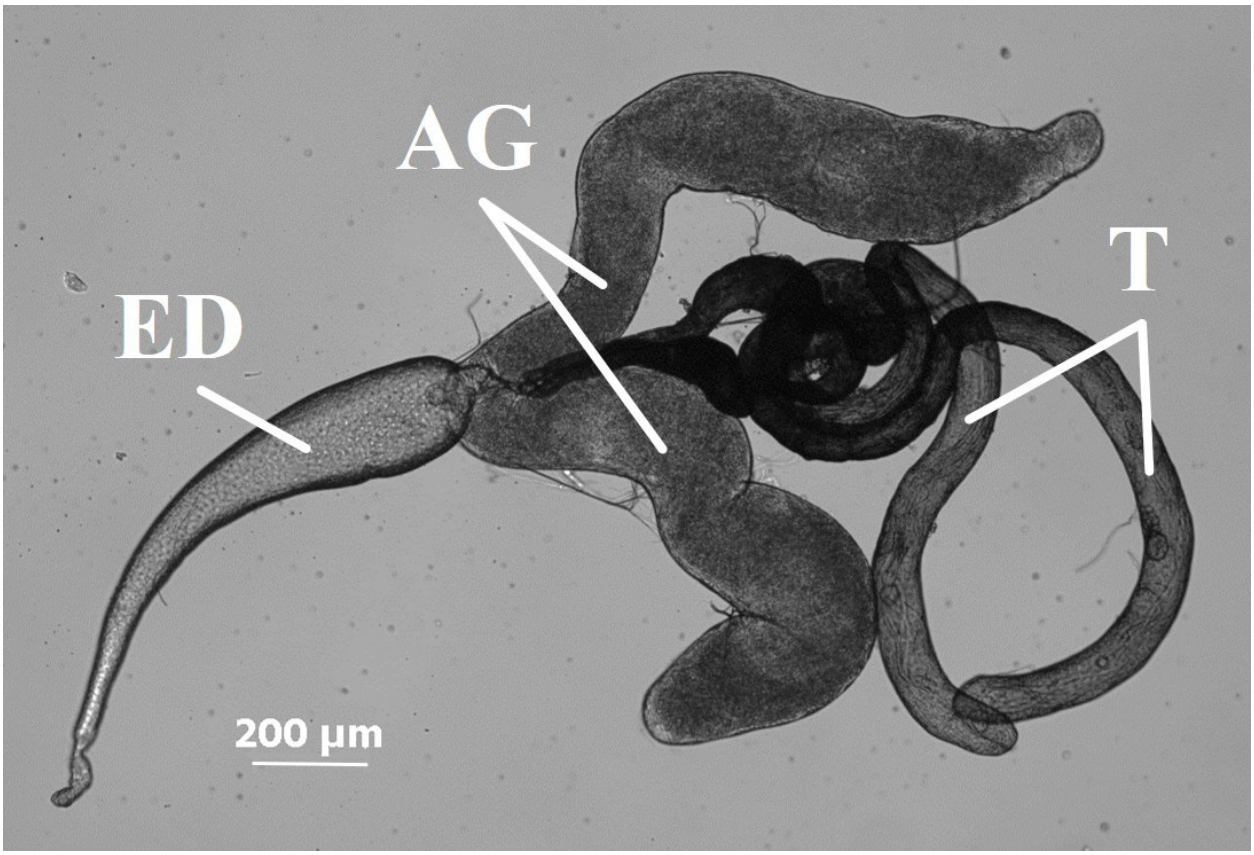

Figure 1. Microphotograph of a *Drosophila melanogaster* male reproductive tract. Picture was taken with the help of a Zeiss Axio Observer Z1 inverted microscope under 50× amplification. ED: ejaculatory duct, AG: accessory gland, T: testis.

medium (970 ml water, 100 g brewer's yeast, 50 g sugar, 15 g agar, 30 ml 10% Nipagin solution, and 3 ml propionic acid) once a week.

To obtain experimental flies, a grape-juice agar plate (550 ml water, 300 ml red grape juice, 25 g agar, and 21 ml 10 % Nipagin solution) with a small yeast patch was introduced into the population cage and flies were allowed to lay eggs for a couple of hours. Afterwards these plates were incubated under standard conditions (see above) for 24 h. We then collected first-instar larvae by gently taking them off the plate with the help of a dissection needle and transferring them, at a constant density of 100 individuals, to vials containing 7 ml of standard SYA medium supplemented with live yeast granules. We incubated larvae at standard conditions (see above) during development and flies were collected on ice within 8 hours after eclosion to ensure virginity. Where necessary (age less than 48 h post-eclosion), flies were collected within a narrower time window to ensure flies were of the appropriate age. If flies were kept for more than 24 h before the experiment, this was done in single sex groups of 20 individuals in standard SYA medium supplemented with live yeast granules and under standard conditions. When flies were kept longer than 4 days they were transferred to fresh vials every 3 – 4 days.

Mating Protocol

Mating experiments were done in the morning after lights on at standard conditions (see above) for experiment 1 and at room temperature (approximately 19 – 22 °C) for experiment 2. In the afternoon of the day before each mating trial, males were anaesthetized on ice and transferred individually into mating vials containing standard SYA medium supplemented with live yeast granules and kept at standard conditions. Upon the start of the mating trial a single virgin female (age: 5 d for experiment 1 and 7 d for experiment 2) was introduced into each mating vial while noting the start of each observation. We observed pairs continuously and the start of each mating as well as the end were noted. After the end of a mating, the pair was immediately separated. In experiment 1 we observed pairs for three hours for all trials, while in experiment 2 we observed them for 60 – 90 min, until approximately 50% of young males had mated.

Experiment 1: Maturation and Ejaculate Allocation Over Multiple Age Classes

The aim of this experiment was to determine ejaculate allocation for males of five different ages (14 – 16 h, 24 h, 48 h, 72 h and 96 h). We estimated ejaculate allocation by quantifying the protein content of the AGs from age-matched virgin and recently mated males. We reasoned that the average difference between the two male mating states determines the amount allocated to the female during mating.

To generate enough replicates, we performed three separate mating trials. At each trial males of each age class were given the chance to mate with a single virgin female. To compensate for differences in mating rate due to age (mating rate increases from 30% in 16 h old males to 95% in 96 h old males [8]) different numbers of males were set up (14 – 16 h: N = 20 – 40, 24 h: N = 38, 48 h: N = 27, 72 h and 96 h: N = 24 per trial), such that we would obtain approximately the same number of mated males for each age class in each mating trial for further protein quantification. Additionally, 23 virgin males per age class were also kept individually in mating vials with the exception that no female was introduced. After the end of a mating males were immediately flash frozen in liquid nitrogen and at the same time a virgin male of the corresponding age class was frozen as well. Only matings that lasted at least 7 min were considered successful and included in the protein quantification assay. Males were stored at -80 °C until dissection and further processing.

Experiment 2: Behavioral and Physiological Maturation

The aim of this experiment was to test, whether young males successful in gaining a mating were also more advanced in their physiological maturation. We compared AG protein content of successful males to same-aged but unsuccessful males (*i.e.,,* males not gaining a mating within the observation period). We deemed unsuccessful males to be less advanced in their behavioral maturation. At the same time, we aimed to gain a more exact estimate of the ejaculate allocation of young, immature (20 – 24 h) and older, fully mature (7 d) males. We consider 7-day old males to be fully mature: the expression of reproductive traits increases in the first days after eclosion, but then levels off for most traits around 4 days post-eclosion [8]. Furthermore, post-eclosion growth of the ejaculatory bulb plateaus around 7 days post-eclosion [24]; and, between 1 and 2 weeks post-eclosion senescence of reproductive traits begins [30, but see 31]. Since, in general, there might be differences in AG protein content of successful and unsuccessful males, we only used males that were successful in gaining a mating to estimate ejaculate allocation. From these males, we generated two groups: mated males that were flash-frozen after completing their copulation (uninterrupted), or that were interrupted within five minutes after the start of mating and then flash-frozen (interrupted). Flies were continuously observed such that we detected a pair that started mating at the latest within 1 – 2 min after the start. For those pairs in the interrupted group we then immediately started shaking the mating vials and pairs separated within 1 – 2 min. Thus, males mated with a female for a maximum of 4 min (usually considerably shorter) before being interrupted. For such short matings we expect SFP transfer to be negligible, since mating usually lasts about 20 minutes and SFP induced sperm displacement is only observed for matings longer than 4 min [32]. Additionally, the presence of a specific SFP, Acp36DE, for example is only detectable in the female reproductive tract when matings were interrupted after 10 min but not when interrupted after 5 min [33]. Hence, we are confident that our treatment prevented any significant transfer of SFPs.

Again, to generate enough replicates, repeated mating trials were performed on four consecutive days. At each mating trial males from both age classes were given the chance to mate with a single virgin female. We compensated for differences in mating rate due to male age [8] by setting up different numbers of males (20 – 24 h: N = 42 – 60 and 7 d: N = 31 – 39 per mating trial). Before starting the trials, we determined the subset of replicates where we disrupted mating and where we allowed males to continue until completion by labelling vials accordingly, thus males were randomly assigned to one or the other treatment. Males were flash frozen in liquid nitrogen according to their treatment (immediately after a mating was interrupted or after the natural end of a mating). Additionally, unsuccessful young males, those that did not achieve a mating within the observation period, were flash frozen immediately after the observation period. Males were stored at -80 °C until dissection and further processing.

Dissections and Sample Preparations

We pooled the AG pairs of three males per sample (experiment 1: N = 6 – 9 pools per age group and treatment; experiment 2: N = 12 per age group and treatment), to get enough starting material for protein quantification. Only males of the same age, treatment, and mating trial were pooled. During dissections, males were stored on dry ice and AGs were dissected on ice in a drop of phosphate buffered saline (Calbiochem, cat. no. 524650) containing protease inhibitors (Roche, cat. no. 05892970001). Three gland pairs were transferred into 150 µl phosphate buffered saline containing a protease inhibitor. For experiment 1 samples were homogenized immediately after dissection and then frozen at -80 °C

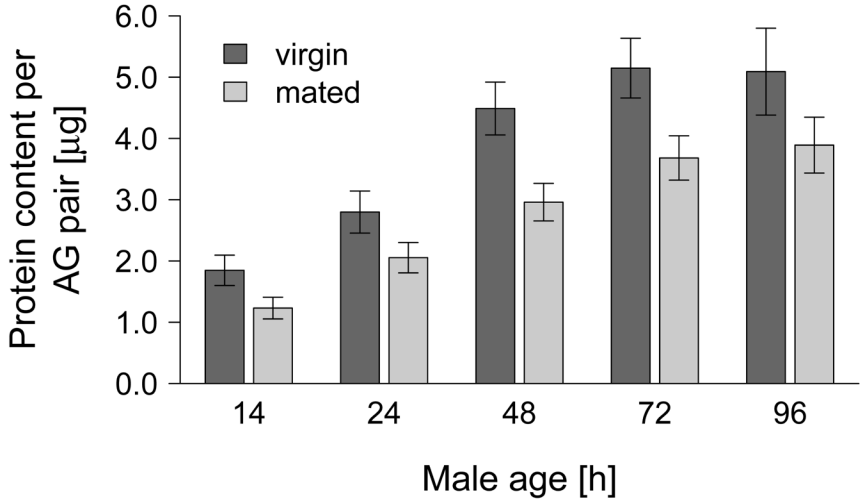

Figure 2. Total protein content of accessory glands from males of the indicated age classes. Males were flash frozen for subsequent dissection and protein quantification of the accessory gland either as virgins (dark grey) or immediately after mating with one virgin female (light grey). The graph depicts means ± SEs. SEs are based on variation between individual samples (pools of 3 males) and thus include the variation observed between different quantification runs. Protein content was significantly different between males of different age classes ($P_{4, 75} < 0.001$) and mating status ($P_{1, 75} < 0.001$), but there was no significant influence of the interaction between the two ($P_{4,71} = 0.4958$).

until protein quantification, while for experiment 2 gland samples were stored at -80 °C after dissection and homogenized immediately before protein quantification. Homogenization was done via sonication (Bandelin, Sonopuls HD 2070) with 4 cycles of 6 s sonication at 25% power with 20 s breaks on ice in between to avoid heating of the sample. Due to our sampling method the resulting samples do not only contain the gland content (SFPs), but also protein from the structural tissue of the gland. However, the *D. melanogaster* AG is a secretory tissue consisting only of a single layer of cells [34], thus we argue that the amount of protein coming from tissue rather than secreted SFPs is negligible.

Protein Quantification

Quantification of the samples' protein content was done via a Bradford assay [35]. For this purpose, we used Roti-Nanoquant (Roth, cat. no. K880.2) following the manufacturer's manual for 96 well culture plates and measured each sample in duplicates. As we could not process all samples of a given experiment in one quantification run, samples were divided into several runs. In order to avoid introducing systematic errors caused by potential differences between runs, time-treatment combinations were present in any given run in equal numbers. We used Bovine Serum Albumin (Roth, cat. no. 8076.2) to obtain a calibration curve for each quantification run.

Statistical Analyses

Data analyses were done in R version 3.4.0 [36] using packages *survival* [37] and *gplots* [38]. We used survival models with right-censored data to model mating rates of immature and mature males. Statistical differences between the two were assessed with a non-parametric log-rank test. All other data were analyzed with the help of generalized linear models (GLMs; [39]) using a gamma data distribution for time data (mating duration) and a Gaussian data distribution for the protein data. Significance of single terms was assessed on nested models using an F test.

RESULTS

Experiment 1: Maturation and Ejaculate Allocation Over Multiple Age Classes

The aim of this experiment was to determine ejaculate allocation for males of five different ages (14 – 16 h, 24 h, 48 h, 72 h and 96 h). Since there was considerable significant variation between the three protein-quantification runs (GLM with Gaussian distribution, run: N = 83, $F_{2, 75} = 53.08$, $P < 0.001$) we included run as a covariate. Protein content of the AGs (Figure 2) increased with male age ($F_{4, 75} = 34.92$, $P < 0.001$) and decreased after mating ($F_{1, 75} = 45.62$, $P < 0.001$). However, there was no significant difference in protein allocation between males of different ages (age ×

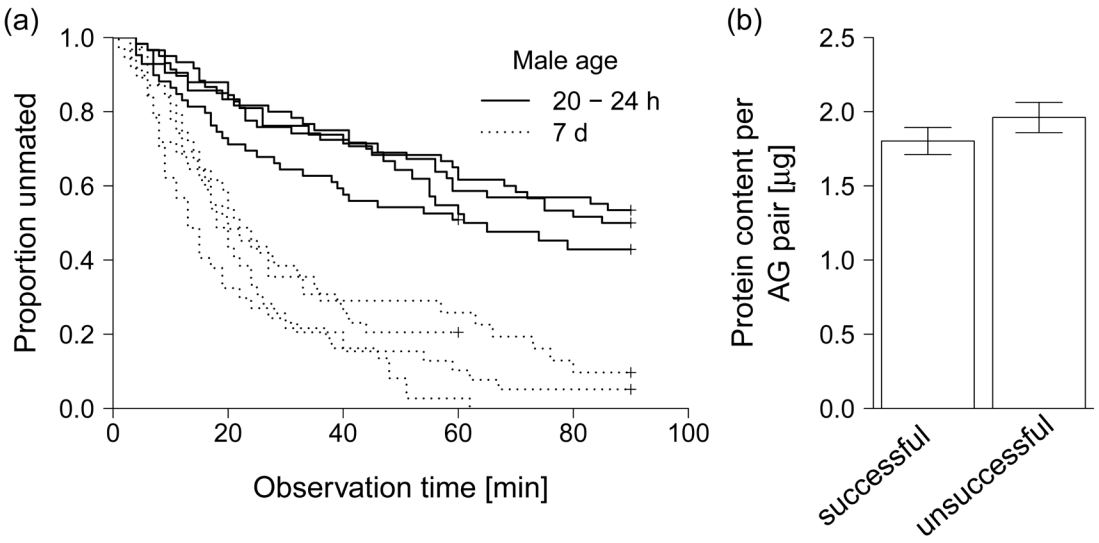

Figure 3. Mating success and accessory gland protein content. (a) Change in the proportion of unmated males over time for immature (solid lines) and fully mature (dotted lines) males. Though the mating trials were analyzed together (with male age having a significant influence on mating success, $P_{1, 364} < 0.001$), the four different trials are depicted separately here. (b) Protein content (mean ± SE) of accessory glands from immature (20 – 24 h post-eclosion) males that were either unsuccessful in gaining a mating during the observation period or successful, but had been interrupted to dislodge from their mating partner before the start of protein transfer. Differences between the two groups were not statistically different ($P_{1, 22} = 0.260$).

mating status: $F_{4, 71} = 0.85$, $P = 0.495$).

Experiment 2: Behavioral and Physiological Maturation

Mating rate and Duration

The aim of this experiment was to test, whether young males differ in their mating rate and duration from older, more mature males. Survival analysis shows that mature (7-day-old) males achieved a mating significantly faster than immature males ($N = 365$, $df = 1$, $\chi^2 = 108$, $P < 0.001$). By the time mating trials were terminated only about 40 – 50% of immature males but 80 – 100% of mature males had engaged in a mating (Figure 3a). Mating duration, however, was not significantly different (GLM with Gamma distribution, $N = 116$, $F_{1, 114} = 2.65$, $P = 0.106$) between young and old males (19.68 ± 0.45 min and 20.81 ± 0.54 min, respectively).

Mating Success and Accessory Gland Protein Content

The aim of this experiment was to test, whether young males successful in gaining a mating were also more advanced in their physiological maturation. Overall, there was considerable variation in the amount of protein in the AGs of immature males ranging from 1.17 to 2.48 µg per gland pair. As these estimates are calculated from measurements of three pooled AG pairs, we are likely to rather under- than overestimate variance in AG protein content. However, mating success in young males was not influenced by the amount of protein they had hitherto accumulated in their AGs (Figure 3b). AG protein content was not significantly different between successful and unsuccessful immature males (GLM with Gaussian data distribution, $N = 24$, $F_{1, 22} = 1.34$, $P = 0.260$).

Maturation and Ejaculate Allocation in Immature and Mature Males

The aim of this experiment was to compare ejaculate allocation between young, immature and older, mature males. Ejaculate allocation was strongly reduced in immature males (Figure 4). The change in protein content for interrupted versus uninterrupted males was significantly different between immature and mature males (GLM with Gaussian data distribution, $N = 48$, $F_{1, 44} = 13.60$, $P < 0.001$): Mature males allocated on average 0.98 µg of protein (30% of their total protein store) to a single mating, while immature males only transferred on average 0.24 µg of protein (13% of their total protein store) to the female.

DISCUSSION

Very young, immature *D. melanogaster* males do not exhibit their full reproductive potential [8], as has been shown in several other insect species [3,4,6,7]. This

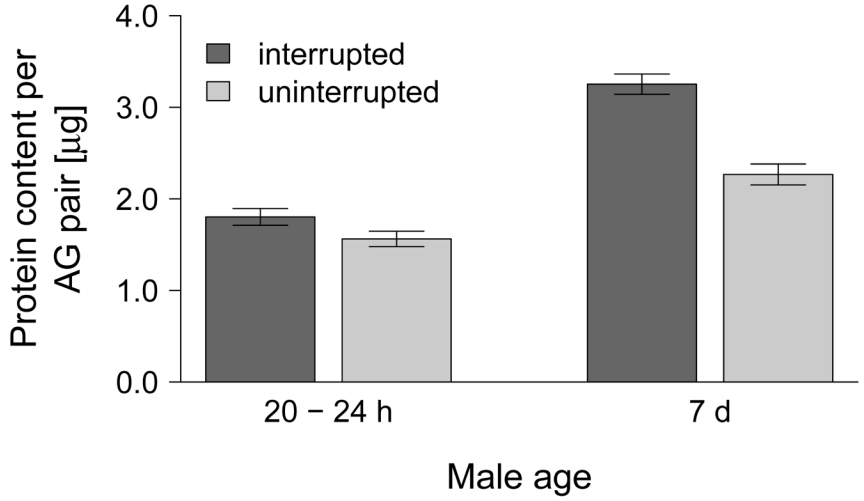

Figure 4. Total protein content of accessory glands from immature (20-24 h post-eclosion) or fully mature (7 days post-eclosion) males. Males were either prevented from protein transfer (interrupted, dark grey) or had just finished a natural-length mating (uninterrupted, light grey) with one virgin female before being flash frozen for subsequent dissection and protein quantification of the accessory glands. In contrast to males in Figure 2 all males had been successful in obtaining a mating. The graph depicts means ± SEs. Statistical analysis showed a significant influence of the interaction age × mating status on protein content ($P_{1, 44} < 0.001$).

disadvantage is not limited to mating rate, but is also observed in post-copulatory reproductive traits such as offspring eclosion rate, male ability to induce a female refractory period, and sperm competition success [8]. The expression of these post-copulatory reproductive traits is strongly influenced by the transfer of SFPs during mating [40]. Our experiments provide evidence for a reduced SFP transfer in very young, immature males compared to older, mature males. Despite this reduced ability to transfer a fully mature ejaculate, immature males still engage in mating and this is independent of inter-individual differences in post-eclosion AG growth.

Mating, especially courtship, is costly for males [13-15], thus we hypothesized that males synchronize behavioral and physiological sexual maturation in order to only invest in costly mating behavior when the fitness pay-off is optimal. As suggested by previously reported post-eclosion AG growth [8], our data provide evidence for the accumulation of proteins in the AG during the first days after eclosion. However, we did not find a fine-tuned synchrony of behavioral and physiological sexual maturation in *D. melanogaster* males. While AG growth and mating rate are both positively correlated with age [8], AG protein amount of same-aged males did not explain inter-individual variation in young male mating success. Thus, individual variation in maturation speed does not seem to be synchronized between behavioral and physiological sexual maturation. Analogous to our results, a study in the sand cricket *Gryllus firmus* demonstrated that behavioral and physiological sexual maturation of females are regulated independently [41]. However, in this species female oocyte (*i.e.,* physiological) maturation is accelerated by juvenile hormone while increase in female receptivity to mate (*i.e.,* behavioral maturation) is not influenced by its action. In contrast, in *D. melanogaster* AG protein synthesis and courtship behavior are both influenced by juvenile hormone [12,29], thus we expected a synchronized maturation of both, but did not find that. One potential reason might be that downstream of juvenile hormone different compounds might alter dynamics of physiological and behavioral maturation, thus uncoupling these two processes. Additionally, other factors might explain inter-individual differences in AG post-eclosion growth. One possibility might be nutrition acquired during larval stages, with high condition males having more reserves at eclosion and thus being able to fill up their AG quicker than low condition males. Due to genetic differences in nutrition requirements and/or uptake, males in our experiment might have differed in condition and hence physiological maturation rate after eclosion. Male condition influences ontogeny with high condition males displaying an earlier reproductive peak in the neriid fly *Telostylinus angusticollis* [42]. Access to yeast during adulthood influences expression of SFP mediated post-copulatory reproductive traits in *D. melanogaster* [43], suggesting a role for nutrition in seminal fluid production. If nutrition influences SFP production early in a male's life, males might

show different physiological maturation trajectories independent of the action of juvenile hormone and thus independent of behavioral maturation.

Our estimates of protein content in males with uninterrupted and interrupted matings highlights that young, immature males transfer smaller ejaculates in a single mating. While fully mature males allocated about 30% of their total protein store to the female, which is in agreement with a previous study [44], young males allocated considerably less protein to the female. Our second experiment shows a four-fold increase in seminal fluid protein transfer in older, fully mature males (7 days old) compared to very young, immature males (not more than 1 day old), and our first experiment, though not statistically significant, supports this result. In the latter, we only found an approximately two-fold increase in protein transfer in older, more mature males (48 – 96 h old) compared to very young, immature males (14 – 24 h old), which was not statistically significant. Still, we conclude that overall young, immature males transfer a smaller (in terms of amount of SFPs) ejaculate than fully mature males for two reasons. First, the effects in both experiments go in the same direction, thus at least qualitatively supporting each other. Second, the second experiment allowed for a stronger statistical test since we tested fewer groups and chose more extreme phenotypes (mature males were 3 days older than the oldest males in the first experiment), which might explain differences in statistical significance between the two experiments. Hence, we are confident that immature males do not transfer a full ejaculate, and this is biologically relevant. Even relatively small reductions in SFP transfer in sexually mature males – a reduction in mating duration from 17 to 14 min, using mating duration as a proxy for SFP transfer [32] – resulted in a measurable decline in female post-mating response with direct effects for male fitness [45]. Therefore, we conclude that the here demonstrated two- to four-fold reduction in protein transfer in immature males might well explain the lower post-copulatory performance of immature *D. melanogaster* males [8]. While a small ejaculate is disadvantageous, we cannot exclude that an immature male's ejaculate is also ill-composed further contributing to the reproductive phenotype observed in young *D. melanogaster* males. In the field cricket *Teleogryllus oceanicus*, for example, relative abundance of several seminal fluid proteins change as males get older [7] and hence warrants further investigations in the fly.

Surprisingly, mating duration in young males was approximately the same as in older, more mature males. This indicates that once a young male engaged in a mating, female choice did not act against this male to cause a pre-mature termination of the copulation and thus protein transfer. Furthermore, there are indications that mating duration determines the amount of SFPs transferred to the female during mating in *D. melanogaster* [32,45], thus it is surprising that despite equal mating durations young males transfer a smaller ejaculate. This suggests that there might be physical constraints acting on protein transfer in young, immature males. This was also suggested for males of the lycaenid butterfly, *Jalmenus evagoras*, where mating duration increases for second and third matings despite the fact that males transfer a smaller spermatophore (ejaculate mass) during these matings [46]. In summary, our experiments clearly show that young, immature males transfer a smaller ejaculate during mating. We suggest that this is at least one mechanism leading to young males not receiving the same fitness gain per single mating than fully mature males as previously reported [8].

From an evolutionary perspective, it seems puzzling that immature males engage in costly courtship behavior while still receiving sub-optimal fitness gains from a mating. Male ability to repress female remating increases from 55% for young males (16 h) to 95% for mature males (6 days post-eclosion) [8]. In addition to this increased risk of encountering sperm competition through increased female remating rates, young males are also inferior to more mature males in defending their sperm against a competitor: young males (14 h) only sire 5% of a female's offspring while older males (4 days) sire 25% of offspring when being first to mate with a double-mated female [8]. In the absence of competition, however, young males (21 h) receive as many offspring from a single mating as more mature males (4 days) [8]. Clearly, more data are needed to fully understand the cost-benefit relationship of matings in immature *D. melanogaster* males. Though young males gain less from a single mating than fully mature males, benefits might still outweigh the costs of mating. In our study, males were only given the choice to mate with a young, fertile, virgin female and pay the costs or forego the chance of mating. In this situation, it might benefit males to take the opportunity even though their fitness gain per mating is still sub-optimal, particularly if they encounter sperm competition. However, until that female remates they still have exclusive access to her ova. Young males might have altered their mating decision when encountering a previously mated female and have invested less in courting and mating with her due to the imminent risk of sperm competition. Hence, the cost-benefit balance for young males to engage in courtship and mating might be dependent on a multitude of factors and so far we do not know how variation in these might affect a male's overall reproductive success and fitness.

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Data Availability Statement: The datasets generated and analyzed during the current study are available in the Mendeley Data repository (http://dx.doi.org/10.17632/mntv23hfwt.1).

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