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Dual reproductive cost of aging in male medflies: dramatic decrease in mating competitiveness and gradual reduction in mating performance

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Abstract

Although age-based effects on the reproductive success of males have been reported in several animal taxa the cost of aging on male mating success in lekking species has not been fully explored. We used the Mediterranean fruit fly, a lekking species, to investigate possible cost of aging on male reproductive success. We performed no choice and choice mating tests to test the hypothesis that aging does not affect the mating performance (mating success in conditions lacking competition) or the mating competitiveness (mating success against younger rivals) of males. The mating probability of older males decreased significantly when competing with younger males. Aging gradually reduced the mating performance of males but older males were still accepted as mating partners in conditions lacking competition. Therefore, older males are capable of performing the complete repertoire of sexual performance but fail to be chosen by females in the presence of young rivals. Older males achieved shorter copulations than younger ones, and female readiness to mate was negatively affected by male age. Older and younger males transferred similar amount of spermatozooids to female spermathecae. Females stored spermatozooids asymmetrically in the two spermathecae regardless the age of their mating partner. Aging positively affected the amount of spermatozooids in testes of both mated and non mated males. No significant differences were observed on the amount of spermatozooids between mated and non mated males.

Keywords

Male age; mating competitiveness; aging; sperm transfer; copulation duration; medfly; *Ceratitis capitata*

Introduction

Male mating success may be determined by several factors including age. As an individual grows older, the probability to die increases and to reproduce decreases (Kirkwood, 1977;

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Charlesworth, 2000, 2001; Kirkwood, 2002). Several relatively recent theoretical and empirical studies address the relationship between fitness and aging (Manning, 1985; Hansen and Price, 1995; Kokko, 1997, 1998), especially that of males (Kokko and Lindstrom, 1996; Beck and Powell, 2000). Most theories predict that age is an indicator of high genetic value. Older males bear higher parental experience and provide genetic fitness benefits to their offspring when chosen as mating partners (Brooks and Kemp, 2001). On the other hand, older males might be inferior to younger ones because of the energetic trade off between longevity and mating success, the accumulation of deleterious germ-line mutations, and the poorer adaptation to current conditions (Cordts and Partridge, 1996; Gosling et al., 2000). Most empirical studies on the effects of age on male mating performance have tested a narrow range of ages (testing relatively young ages) (Shelly et al., 2007; Neto et al., 2009; Perez-Staples et al., 2010), leaving unclear many aspects of the mating behaviour of old ages. To achieve matings, in lekking mating systems, older males should be capable of performing the full repertoire of sexual behaviour and also to compete against younger, more vigorous ones. Whether females are able to detect age related changes of their possible mating partners remains to be fully elucidated.

Studies on female preference for mating partners of different ages reveal great variability among species (Shelly et al., 2011; LaMunyon, 2001; Liedo et al., 2002; Schafer and Uhl, 2002; Radwan et al., 2005; Shelly et al., 2007; Avent et al., 2008; Fischer et al., 2008). Attractive male morphological traits are often positively correlated with age, and females that prefer age correlated characteristics indirectly favour the choice of older males. For example, in some species of mammals, older males are bigger and stronger and possess greater experience in courting and mating (Manning, 1985). In insects though, body size is fixed at adult emergence, hence as males get older they may become less attractive because of wear and tear (Jones and Kim, 1994) (wing damage, weak calling signals etc). Other factors, such as the nutritional status and previous mating experience, may interact with age and affect the sexual performance of males (Fischer et al., 2008; Aluja et al., 2009; Perez-Staples et al., 2010). Effects of age on male mating success may differ in insect species even within the same genus. For example, older *Drosophila pseudoscura* males were more successful in achieving copulations than younger ones whilst no effects of age on mating success were observed in males of *D. melanogaster* (Price and Hansen, 1998; Avent et al., 2008).

The Mediterranean fruit fly (medfly), *Ceratitidis capitata* (Diptera: Tephritidae) is a, well studied, insect species in terms of sexual behaviour (Eberhard, 2000; Sivinski et al., 2000; Yuval and Hendrichs, 2000). Male medflies form mating aggregations known as “leks” and emit a sex pheromone. Females are attracted to the leks, and after a complex process of courtship they choose their mating partner. Courtship begins soon after a female approaches a possible mating partner and usually includes male wing fanning, head rocking, abdomen curling and sound production (Prokopy and Hendrichs, 1979). Eventually the male attempts to mount the female, and if accepted the copulation lasts on average 2 – 3 h (Whittier et al., 1992) leading to sperm transfer (Yuval et al., 1996). Since medfly exhibits a non-resource based mating system, the female preference of a mating partner is likely based on characteristics related with male performance (pheromone quantity and quality, courtship behaviour) (see also Shelly et al., 2011) and somatic integrity (i.e., unscathed or broken wings) (Hunt et al., 1998; Hunt et al., 2004). Age is a factor that may interact with either male sexual advertisement or somatic integrity increasing or decreasing male sexual attractiveness, mating competitiveness and performance (Fischer et al., 2008). There are several studies on the factors determining the mating competitiveness of male medflies, mainly within the framework of Sterile Insect Technique (S.I.T.), where the quality of mass reared, sterilized males is assessed regularly (Calcagno et al., 1999; Liedo et al., 2002; Quilici et al., 2002; Shelly et al., 2007). Only relatively recently has the relationship between

sexual signalling (calling) and aging been studied (Papadopoulos et al., 2004). Additionally, Shelly and co-workers (2011) demonstrated that young females are capable of discriminating the age of their mating partners while older females are not. However, effects of age on male mating competition have been assessed only in a narrow range (i.e., until sexual maturity) leaving mating competitiveness of middle aged and older males unidentified.

Previous studies showed no effect of male Mediterranean fruit fly age on copulation duration in a narrow range of ages (males until 13 days old) (Liedo et al., 2002), whereas middle-aged *Drosophila pseudoscura* males copulated for longer and had shorter latency time (Avent et al., 2008). Copulation duration was affected by the quality of diet of *Anastrepha striata* males (Perez-Staples and Aluja, 2004). Studies on sperm transfer in medfly mainly focus on sperm competition, remating inhibition, fecundity, and offspring fitness (Mossinson and Yuval, 2003; Shelly et al., 2007). Nevertheless, the relationship between male age and spermatozoid transfer has not been addressed in this fly or other fruit flies.

We used the Mediterranean fruit fly that has been widely used as a model system for demographic research (Carey et al., 1998; Carey and Vaupel, 2005; Müller et al., 2009), to study the effects of aging on the mating performance of males in a lekking species. We assessed both the mating performance (mating success in conditions lacking competition) and mating competitiveness (mating success against younger rivals) and adopted no-choice and choice mating tests, respectively. In choice tests, females were allowed to choose between young and older males (10 to 50 days old), while in no choice tests young females were exposed to males of different ages (10 to 70 days old). To determine several aspects of male mating performance, in the absence of competition, we assessed the copulation characteristics (duration and latency time) in relation to age and the amount of spermatozooids transferred to females. We tested the hypotheses that mating performance is independent of male age and that aging does not affect male mating competitiveness. Thus, we predicted similar copulation duration, latency-to-mate time, and spermatozooids transfer between younger and older males. We also predicted that virgin females would not discriminate between young and old males

Materials and methods

Experimental conditions and flies used

The experiments were conducted in the laboratory of Entomology and Agricultural Zoology of the University of Thessaly during 2008 – 2009, at $25 \pm 1^\circ\text{C}$, $65 \pm 5\%$ R.H., and a photoperiod of L14:D10 with photophase starting at 0700 h. Light was provided by daylight fluorescent tubes and by natural light from four windows with the intensity inside the test cages ranging from 1500 to 2000 Lux.

Flies used in our experiments were obtained from field infested fruit (collected from the area of Volos, Greece) and were reared under laboratory conditions ($25 \pm 1^\circ\text{C}$) for 1 – 3 generations ($F_1 - F_3$). Details of the rearing method are given by Diamantidis and co-workers (2008). All flies were reared under the same conditions, thus differences in male body size or nutritional status were assumed negligible. Besides, according to Whittier and co-workers (1994) body size does not affect the mating frequency of medfly.

Mating tests

Two types of mating tests were performed. In “choice tests” the mating competitiveness (copulatory success in presence of younger males) of males of different ages (treatment) was tested against young (12 – 14 days old) sexually mature males (control). Additionally, the

effect of age on the mating performance (copulatory success in absence of younger males) of males was tested in “no choice tests”. In both choice and no choice tests females were virgin and sexually mature (12 – 14 days old). Females were maintained until the testing dates in 20 × 20 × 20 cm Plexiglas cages (maximum 20 individuals per cage), separated by sex to ensure that all were virgins when tested. Control and treatment males were individually maintained in cages (400 ml plastic transparent caps) until reaching the adequate testing age. Males (control and treatment) and females had free access to water and standard adult diet (yeast hydrolysate, sugar and water of a 4:1:5 ratio). Mortality was recorded in treatment males. One day before the choice test, treatment and control males were marked on the pronotum with a dot of red or green water colour paint, respectively, using the tip of a fine paintbrush. Additionally, the evening before the choice and no choice test days, females were placed in the Plexiglas cages (see above) designated for the mating tests to settle and adapt to the new environment. Water and adult food was provided *ad libitum* in the mating cages.

Choice test

At 0630, on the test day, 10 treatment males were placed in the Plexiglas cage (20 × 20 × 20 cm) along with 10 control males (12 – 14 days old) and the 10 females. Density of males is not expected to exert a significant effect on females choice as it was shown earlier in comparable experimental conditions (Whittier et al., 1994). Observations were conducted every 15 minutes from 0700 to 1600, and the number of control and treatment male copulations was recorded. Once a couple was formed it was removed from the cage to avoid repeated copulations, together with a randomly chosen male of the opposite category. This was done so that the ratio of treatment males to control males always remained constant at 1. Ten replicates of mating competitiveness tests were conducted for males 15, 20, 30, 40 and 50 days old.

No choice test

At 0630, on the test day, 10 males of predetermined age were placed in a Plexiglas cage with 10 virgin females, and were observed every 15 minutes from 0700 to 1600. Ten to twenty cages (replicates) ran for each age group. The number of copulations was recorded for each cage (replicate), and the newly formed mating couples were carefully removed and placed separately in different cages to complete the copulation. The latency time and the duration of each copulation were also recorded. Once the copulation had been concluded the male and female of each couple were dissected, and the number of spermatozooids in spermathecae and testes was estimated following the methodology given in Twig and Yuval (2005). Although the allocation of sperm to each spermatheca seems to be controlled by females we also tested whether the age of males affects the sperm storage. The number of spermatozooids in testes of males that failed to mate was also recorded. The procedure was concluded for male ages 10, 15, 20, 30, 40, 50 and 70 days.

Statistical analyses

Data analyses were performed using the SPSS 18.0 (SPSS Inc., Chicago, IL, U.S.A.) and the R 2.10 version (www.r-project.org). In both choice and no choice tests the effect of age on mating competitiveness and mating performance, respectively, was determined by parametric and logistic regression analyses. Moreover, we used the linear regression model to test the effect of age on the mating duration, the latency time, and the amount of spermatozooids transferred to females' spermathecae. The effect of age and mating status of males on the amount of spermatozooids in testes was assessed using the univariate analyses of variance (ANCOVA) with mating status as fixed factor and age as covariate. Finally, in an effort to quantify both the mating performance and mating competitiveness of male *medflies* we used a logistic regression model to simultaneously test the effect of mating

competitiveness and aging on male mating success, combining data of both choice and no choice experiments.

Results

Choice test

Aging negatively affects the mating competitiveness of males ($P < 0.05$, $R^2 = 0.97$), and as shown in Fig. 1 there was a sharp decline in mating competitiveness for males older than 20 days. Fifty days-old males achieved only $\approx 10\%$ of the total copulations relatively to young males.

No choice test

In absence of any competition against young males, male mating performance gradually decreased at older ages (Fig. 2). However, the mating success of males older than 50 days remained high and comparable to that of young males (12 – 15 days old). Males of the extreme age of 70 days old demonstrated a slightly reduced, though competent, ability to mate. Nevertheless, linear regression failed to determine significant effects of aging on the mating performance of males ($P > 0.05$, $R^2 = 0.45$) (Fig. 2).

Effect of aging and competition

The mating probability gradually decreased with age for males that did not experience competition by younger rivals (Fig. 3), but, there was a dramatic decrease for males older than 20 days under competitive conditions. Logistic regression analysis reveals that both aging (Wald test $t = 2.6$, $P < 0.05$) and competition (Wald test $t = 6.5$, $P < 0.01$) significantly reduced male mating success and that their interaction was also significant (Wald test $t = 4.9$, $P < 0.05$). The mating probability of a young male (≤ 20 days old) was around 50% regardless of competition. Aging alone reduces the mating probability to ≈ 30 and 24% for 50 and 70 days old males, respectively. Aging and competition against young males reduces the mating probability to approximately 5 and 1% for 50 and 70 days old males, respectively. The cost of aging on male mating competitiveness is estimated to 0.7, 18.0, 23.6, 25.4 and 23.2% for 20, 30, 40, 50 and 70 days old males respectively.

Effect of aging on copulation traits

A weak, though significant ($P < 0.05$, $R^2 = 0.570$), linear relationship between male age and mating duration was recorded in competition-free conditions (Fig. 4). Young males coupled for longer than older males. Linear regression revealed no effect of aging on the latency-to-mate time ($P > 0.05$, $R^2 = 0.285$). However, when males were grouped as younger or older than 30 days the latency time in ages older than 30 days was significantly longer ($t = 1.996$, $df = 178$, $P = 0.04$).

The number of spermatozooids transferred to female spermathecae varied independently of the age of males ($P > 0.05$, $R^2 = 0.074$) as shown in Fig. 5. Thus, older males (i.e., 50 and 70 days old) were capable of transferring similar numbers of spermatozooids in comparison to young males (i.e., 10 and 30 days old). Although spermatozooids were asymmetrically stored in the two spermathecae (Fig. 6), this relative asymmetry is not related to males' age ($P > 0.05$, $R^2 = 0.196$).

Age and spermatozooids amount in male testes

The quantity of spermatozooids in the testes of mated and non mated males was positively related with the age of males ($P < 0.05$, $R^2_{\text{mated}} = 0.7884$, $R^2_{\text{non mated}} = 0.8156$) (Fig. 7). ANCOVA revealed that age was a significant predictor of spermatozoid amount in testes (F

= 50.242, $df = 1$, $P < 0.01$). Mated and non mated males did not differ significantly in spermatozoid abundance ($F = 0.12$, $df = 263$, $P > 0.05$). Higher variability was reported in the number of spermatozoids in testes of older, non mated, males (40, 50, and 70 days old) that might be related with the relatively lower number of males (5, 4, 10, respectively) dissected at these ages.

Discussion

The results of our study demonstrate that (a) aging dramatically reduces the mating competitiveness of males, and gradually decreases their mating performance, (b) in absence of competition older males can still acquire a substantial proportion of copulations, (c) younger males copulate for longer than older ones, (d) age of males significantly affects the females readiness to mate, (e) aging does not affect the ability of males to transfer sperm cells to spermathecae, and (f) the amount of sperm cells increases with age in the testes of both mated and non mated males.

Older males lose the “mating contest” to the younger rivals probably during sexual signalling and/or courting behaviour, and therefore, achieve less copulations. Females are initially attracted by pheromone emitting males aggregated in leks, and accept or reject the copulation after a close-range courtship display. Previous studies have shown that medfly copulations almost always occur within leks and that solitary males achieve significantly fewer copulations (Hendrichs and Hendrichs, 1990; Shelly et al., 1993). Although not observed in this study, older males' participation in leks may be less frequent than younger ones' and this behaviour could reduce the probability of mating. Yuval and co workers (1998, 2002) found that leks are formed by males of high nutritional status and that malnourished males rarely participate in leks and are less likely to copulate. Furthermore, Niyazi and co workers (2008) proved that male territorial position within the lek affects their mating success. Males occupying the higher and closer to the light positions were visited more often by females and obtained more copulations. It is possible that the more vigorous, younger males manage to occupy the advantageous positions during sexual calling and/or defeat older males during aggressive interactions. However, this effect may appear more intense in laboratory conditions than in nature. The limited space of the Plexiglas cages could stimulate male – male interactions providing young males with a benefit. Nevertheless, male contest interactions seem to be very few in similar crowding conditions (Papadopoulos et al. 2009), and whether older or younger males win male battles is not yet determined. Moreover, Shelly et al. (2011) tested the effect of male age (until 40 days old) on female preference using field cages and found that female could discriminate against old males. Future studies should test the performance of older males in larger space (e.g., field cages) or preferably in nature.

Variation in sexual signalling (calling) intensity (Flath et al., 1993; Whittier et al., 1994; Shelly, 2000) and even non-calling males participating in leks have been reported for medfly (Shelly et al., 1994). The existence of non-calling males is not fully understood yet and may be caused by male – male aggression or by genetic factors (Shelly, 2000). It appears that age and time of the day can also affect the emission of pheromone (Flath et al., 1993; Papadopoulos et al., 1998; Papadopoulos et al., 2004; Zhang et al., 2006; Neto et al., 2009). It is known that frequently calling males usually achieve higher mating success (Shelly, 2000). Calling frequency, pheromone production and/or quality are likely to weaken with age. And, although this is not so obvious when calling in leks (because of mixed-age calling groups), individual pheromone quality and quantity may be evaluated by the female during courting. According to Shelly, (2000) pheromone composition (quality) does not seem to affect mating success of medflies. Nevertheless, calling frequency and thus pheromone production may decrease with age in male medflies, considering also the close association

between prevailing mortality risk and sexual signalling (Papadopoulos et al., 2004). It was recently shown that 10 and 40 day-old male medflies perform calling in a similar frequency and virgin females do not discriminate their sexual signals (Shelly et al., 2011) but whether pheromone production is reduced in older ages (>40 days) is not yet known.

Apart from the “quality” of sexual signalling, Shelly (2000) suggests that a combination of appropriate male courtship behaviour that is also synchronized to female's reaction is crucial for a successful copulation. Specific traits of courtship such as duration, angle of male – female facing and distance between the two sexes were found to vary among different medfly strains (Briceno and Eberhard, 2002; Briceno et al., 2007) and are likely to affect the acceptance or rejection of female mounting. Furthermore, interruption of buzzing songs during courtship results in less copulations (Briceno et al., 2002). Aging may negatively affect the elements that form a successful courtship. Additionally, it is possible that male morphological characteristics (body vigour, wing integrity, etc.) play a significant role for close-range behaviours such as courting. Older males are usually less vigorous than young males, as morphological disadvantages, like broken wings, accumulate because of wear and tear and this may reduce their attractiveness to females. Longer latency to mate times for older males might indicate poor sexual courting performance.

Aging may render old males inferior to younger ones in terms of mating performance but does not completely eliminate their ability to copulate. In conditions lacking competition, older males achieve more than 20% of the possible copulations even at the extreme ages of 70 days old. Although older males may perform less calling than younger ones (Papadopoulos et al., 2004; Neto et al., 2009), it appears that aging alone does not completely diminish their sexual performance. Female choosiness, may adjust to the level of male reproductive performance at the given circumstance. Previous studies have shown that maturation related receptivity of females depends mainly on their age, with younger and sexually mature virgin females being choosier and less receptive for mating (Shelly et al., 2011; Gray, 1999). However, virgin females used in our experiment still accepted older males as mating partners. Therefore, older males are capable of performing the complete repertoire of sexual performance but fail to be chosen by females in the presence of young rivals.

The process of copulation can be discerned in three phases: intromission, sperm transfer, and postcopulatory guarding before physical separation of the mating pair (Eberhard and Pereira, 1993; Field et al., 1999). Decrease of copulation duration in older ages can be attributed to shortening of one or more of these phases. Even if older male medflies are able to position the aedeagus at the inner end of the female bursa and transfer sperm with no delay as our results indicate, they may engage in shorter postcopulatory guarding due to age-related physical wear and tear (Field et al., 1999). It is widely accepted that copulation duration is regulated by females in tephritids (Fritz, 2009). After a female has fulfilled the requirement in sperm amount, she starts to walk, move and flick the wings, and tries to dislodge the male (Aluja and Norrbom, 2000) and young vigorous males are possibly more capable of remaining coupled with their mating partner than older counterparts. Though younger males lose valuable time by copulating longer in gaining more copulations, they probably increase their fitness by preventing female remating. Our results show that male age had a significant negative effect on copulation duration in the absence of conspecific males. This result agrees with data from two *Drosophila* species (Koref-Santibanez, 2001), but not with that of a recent study in *Anastrepha ludens* (Perez-Staples et al., 2010). Both previous studies only tested male ages up to 20 and 38 days respectively.

Our results clearly demonstrate that once accepted by females and achieving intromission, older males successfully transfer sperm to female spermathecae. Although male age affects

sperm transfer in other insect species (LaMunyon and Huffman, 2001; Jones et al., 2007; Hale et al., 2008) this does not seem to be the case for medfly. It should be noted though that we estimated the number of spermatozooids transferred to spermathecae and not sperm volume, per se. Future studies should shed light on the role of male age on both quantity and quality of accessory fluids, since they may also affect copulation duration and other important post copulatory characteristics (Chapman et al., 2000; Radhakrishnan and Taylor, 2007). Spermatozooids were asymmetrically stored in females' spermathecae and this asymmetry was not related to male age. Asymmetry of sperm storage has already been verified for medfly and other tephritids (Yuval et al., 1996; Taylor and Yuval, 1999; Taylor et al., 2000; Taylor et al., 2001; Harmer et al., 2006; Perez-Staples et al., 2007). Although, the significance of asymmetrical sperm allocation is not clear yet, this tendency appears to be controlled by the female and may serve postcopulatory paternity decisions (Perez-Staples et al., 2007).

Latency time has been used as an indicator of male attractiveness in insects (Shackleton et al., 2005). Previous studies in female receptivity of *Drosophila* species show that male latency to copulate increases with age and decreases with experience (Koref-Santibanez, 2001). Although the relation between male age and latency time is not clear in medfly, possible flaws in courting behaviour and/or morphological disadvantages may account for this increase in older ages.

Mated and unmated males bear similar amounts of sperm cells with a tendency of increase in relation to age. The resemblance in spermatozoid number is hardly surprising considering the relatively low amount of sperm cells transferred to female spermathecae during copulation. Moreover, it seems that spermatogenesis occurs throughout male lifespan in medfly resulting in continuous accumulation of sperm in testes.

Our results and that of another recent study (Shelly et al., 2011) demonstrate that male age is a significant predictor of mating success in medfly. Young female medflies were capable of discriminating males when their age differed by 30 days, however, older females were not (Shelly et al., 2011). The apparent female choice for younger males seems to return no direct fitness benefits in terms of offspring production (Shelly et al., 2011). The above findings are supported by our data as well, since sperm transfer to spermathecae is not related with male age. Nevertheless, future studies should elucidate possible indirect benefits of female preference and the mechanism of selecting younger individuals as mating partners should be further explored.

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Research highlights

- Aging dramatically reduces the mating competitiveness of males
- In absence of competition older males acquire a substantial proportion of copulas
- Younger males copulate for longer than older ones
- Females appear less eager to mate with older males
- Number of sperm cells increases with age in the testes of mated and non mated males

Figure 1.

Effect of aging on the mating competitiveness of male medflies. Choice tests included 10:10:10, young male: older male: young virgin female. In all tests, females used were sexually mature, virgin 12 – 14 d-old, while control (young) males were sexually mature, non mated 12 – 14 d-old. Ten replicates ran for each age class.

Figure 2.

Effect of aging on the mating performance of male medflies. No choice tests included 10 males caged with 10 mature virgin females (12 – 14 days old). Ten to 20 replicates ran for each age class.

Figure 3.

Mating probability at no choice (10:10) and choice tests (10:10:10) in relation to male age.

The mating probability is calculated by the formula $P = \exp(0,812 - 0,877*\chi_1 - 0,075*\chi_2 + 0,060*\chi_1\chi_2) / [1 + \exp(0,812 - 0,877*\chi_1 - 0,075*\chi_2 + 0,060*\chi_1\chi_2)]$ where $\chi_1 = 0$ for choice tests, $\chi_1 = 1$ for no choice tests and χ_2 is the age of males in days.

Figure 4. Copulation duration (A) and latency time (B) in relation to male's age. Twenty to 79 copulations were considered in each age class.

Figure 5.

Average number of sperm cells transferred to spermathecae in relation to male age. Fourteen to 45 females were dissected in each age class.

Figure 6.

Relative asymmetry ($|\text{Sperm cells in the left spermatheca} - \text{sperm cells in the right spermatheca}| / \text{total sperm cells in both spermathecae}$) of sperm cells transferred to spermathecae in relation to male's age. Fourteen to 45 females were dissected in each age class.

Figure 7.

Quantity of sperm cells in testes of males succeed in mating (solid circles), and those that did not mate (empty circles) during the testing period. Evaluation of sperm cells amount in testes was conducted one day after the mating test. Twelve to 44 and 5 to 16 males that successfully mated or did not were dissected in each age class respectively.