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ORIGINAL ARTICLE

Male larval experience of cues from adult rivals alters lifetime sperm investment patterns in a sperm heteromorphic moth, *Ephestia kuehniella*

Junyan Liu, Xiong Zhao He and Qiao Wang

School of Agriculture and Environment, Massey University, Palmerston North, New Zealand

Abstract Male animals may adjust their resource allocations for reproduction and other fitness functions in response to cues from rivals. For instance, adult males increase their investment in sperm for a higher paternity share when they perceive sperm competition risk in their surroundings. In nature, both juveniles and adults may coexist spatially and temporally. Yet, it is not clear how juvenile males of different ages respond to cues from adult rivals and fine-tune their lifetime investment in sperm production and ejaculation in any insect. Here we used the Mediterranean flour moth, *Ephestia kuehniella*, which produces both fertile eupyrene and infertile apyrene sperm, to explore this question. We demonstrate that the late, but not early, instar larvae are sensitive to adult male cues. As a response, they produce more sperm before emergence and their resultant adults have shorter mating latency and ejaculate more sperm in the first few matings. When the juvenile stage produces more eupyrenes, the adult stops making these sperm, but regardless of the number of apyrenes produced during the juvenile stage, the adult continues to make them. These findings suggest that the number of spermatogonia for eupyrenes may be limited and that for apyrenes may be flexible. Our results show that the insect does not trade off survival, mating frequency, body size, or testis size for sperm production in response to adult males during the larval stage. Knowledge created in the present study offers insight into the stage-dependent sensitivity of juvenile males to cues from adult rivals and subsequent lifetime resource allocations.

Key words immature stage; Lepidoptera; spermatogenesis; sperm allocation; sociosexual environment

Introduction

Animals often fine-tune their physiology and behavior in response to their sociosexual environment to gain a competitive advantage (Pigliucci, 2005; Bretman *et al.*, 2011; Kasumovic & Brooks, 2011; Taborsky, 2016; Dore *et al.*, 2018). For example, adult males increase their investment in sperm for a higher paternity share when they perceive sperm competition risk from rivals (Parker, 1970; Wedell *et al.*, 2002; Parker & Pizzari, 2010). Previous studies indicate that juvenile males also can detect their future sperm competition risk from the presence of other juveniles and subsequently adjust their resource allocations (Gage, 1995; He & Miyata, 1997; Yamane & Miyatake, 2005; McNamara *et al.*, 2010; Kasumovic & Brooks, 2011; Taborsky, 2016; Liu *et al.*, 2021, 2022). In nature, conspecific young and adults may coexist regularly at a given time and space, adding adult cues to juvenile sociosexual surroundings (Chapman *et al.*, 2007; Nehring & Müller, 2009; Bjørnstad *et al.*, 2016; Arbaiza-Bayona *et al.*, 2022). To date, only a few studies have investigated

Correspondence: Qiao Wang, School of Agriculture and Environment, Massey University, Private Bag 11222, Palmerston North 4100, New Zealand. Tel: +64 6 3504831; email: q.wang@massey.ac.nz

the impact of adult males on resource allocations in juvenile male insects, including investment in testes (Bailey *et al.*, 2010; Bretman *et al.*, 2016) and ejaculation in the first mating (Gray & Simmons, 2013; Simmons & Lovegrove, 2017). Yet, it is unknown whether sensitivity of juvenile males to cues from adult males is stage dependent and whether these cues affect their lifetime sperm production and ejaculation in any insect.

Sperm heteromorphic insects such as most lepidopterans start producing larger nucleate eupyrene sperm and smaller anucleate apyrene sperm during juvenile stages (Garbini & Imberski, 1977; Friedländer *et al.*, 2005). Eupyrenes fertilize eggs while apyrenes may assist in success of sperm competition (Cook & Wedell, 1999; Wedell *et al.*, 2009; Mongue *et al.*, 2019) and fertilization (Holman & Snook, 2008; Sakai *et al.*, 2019; Hague *et al.*, 2021). So far, it is not clear whether and how juvenile males adjust their expenditure in these sperm of different functions according to the timing of their experience of adult males. Furthermore, changes in resource allocations may result in trade-offs between spermatogenesis and other life traits (Ramm & Stockley, 2009; Devigili *et al.*, 2015; Simmons *et al.*, 2017; Paschoal & Zara, 2022). However, previous studies suggest that juvenile social environment has little impact on juvenile survival (Woodroffe & Macdonald, 2000; Berger *et al.*, 2015; Cannarsa *et al.*, 2015), body size at maturity (Gage, 1995; Lemaître *et al.*, 2011; Bretman *et al.*, 2016; Hobson *et al.*, 2020), and resultant adults' mating frequency (Rutledge & Uetz, 2014; Liu *et al.*, 2022) and longevity (McNamara *et al.*, 2010). Yet, none of these studies has explored the effect of juvenile experience on all these traits in any single species, making it difficult to determine whether adjustment of investment in sperm would alter resource allocation to all other traits.

Our study species, the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is a polygamous insect and a serious pest of stored products in the world. It is an ideal model for investigations into how males alter their resource allocations to traits of different functions after their larvae of different stages are exposed to conspecific adult males because its reproductive behavior and life history strategies have been well studied (Calvert & Corbet, 1973; Xu *et al.*, 2007; Xu & Wang, 2009a, 2010a, 2010b, 2010c, 2011, 2013, 2014; Esfandi *et al.*, 2015, 2020). This moth obtains all resources for survival and reproduction from larval feeding and its adults do not feed (Calvert & Corbet, 1973). It becomes sexually mature at emergence and starts mating at the onset of the first scotophase (night) (Xu *et al.*, 2008), and the quantities of sperm available determine their desire for mating (Norris & Richards, 1933). Before ejaculation, apyrene bundles disassociate, while eupyrenes remain aggregated in bundles of 256 spermatozoa (Garbini & Imberski, 1977; Koudelová & Cook, 2001). Since *E. kuehniella* has multiple and overlapped generations all year round (Richardson, 1926; Liu, personal observation), both immature and adult stages can occur simultaneously. Furthermore, Liu *et al.* (2020) demonstrate that male adults emit both acoustic and chemical cues, which are used as signals of sperm competition risk. We predict that juvenile males of this moth may sense these adult cues and adjust their resource allocation strategies as a response.

In the current study, we exposed *E. kuehniella* larvae of different stages to adult males. We recorded their survival to adulthood, and dissected half of the treated males at emergence and measured their testis size and sperm number. We paired each of the remaining treated males with a virgin female daily and counted the number of sperm ejaculated. We also recorded mating latency of the first mating, and lifetime mating frequency and longevity of these paired males. This design allowed us to test stage-dependent sensitivity of juvenile males to adult male cues and subsequent resource allocations, providing insight into lifetime reproductive investment of juvenile males in response to adult cues.

Materials and methods

Insects and environmental conditions

We collected more than 2000 *E. kuehniella* larvae together with their original food (a mixture of wheat and corn flour) from Turks Poultry, Foxton, New Zealand. We then transferred these into 20 transparent plastic cylinders (8 cm diameter and 10 cm length) covered with cotton gauze (2.8 apparatus per mm^2), each with 100 larvae and about 100 g of their original food, and maintained them in the laboratory. We randomly collected 300 newly eclosed adults ($\approx$ 1 : 1 sex ratio) from all cylinders and introduced them into a transparent plastic cage (28 cm length, 28 cm width, and 24 cm height), lined with a plastic sheet on the bottom for egg collection. We collected eggs daily for 10 d and incubated them in Petri dishes (8.5 diameter and 1.5 cm length). We inoculated 200 neonate larvae onto 50 g of standard diet (*ad libitum*), consisting of 3% yeast, 10% glycerine, 43.5% maize meal, and 43.5% whole meal wheat flour (Liu *et al.*, 2020) in a cylinder mentioned above. Under this rearing condition, the sex ratio at emergence $\approx$ 1 : 1 (Liu, personal observation). We maintained 10 such cylinders as our laboratory colony. We kept the colony and conducted all experiments at

25 ± 1 °C, 60% ± 10% relative humidity, and a photoperiod of 10 h dark (scotophase) and 14 h light (photophase). Under this condition, larval and pupal stages last about 29 and 8 d, respectively.

Treatments

We randomly selected 800 neonate larvae from the colony and evenly transferred 200 larvae into each of four above-mentioned cylinders with 50 g of standard diet. To determine whether and how *E. kuehniella* males adjusted their lifetime investment in reproduction and survival after their larvae of different stages were exposed to adult males, we set up three treatments and one control as follows: (1) ELE (early larval exposure)—immediately after the transfer of neonate larvae, we introduced 10 adult males into the cylinder and allowed them to stay for 5 d, after which time, we removed all adults; (2) LLE (late larval exposure)—15 d after the transfer of neonate larvae, we introduced 10 adult males into the cylinder and allowed them to stay for 5 d, after which time, we removed all adults; (3) CLE (complete larval exposure)—immediately after the transfer of neonate larvae, we introduced 10 adult males into the cylinder and replaced them with 10 new ones once every 5 d until pupation, after which time, we removed all adults, and (4) CON (control)—no adult males in the cylinder. All adult males used at the onset of exposure were newly emerged, virgin and randomly selected from the breeding colony. The cylinders were placed in four separate environment chambers (Percival Scientific I-36VL, Perry, the USA) with identical environmental conditions as the laboratory colony.

Immature survival, body size, and testis size and sperm count at emergence

After the larvae reached the final (sixth) instar, we started observing pupation in these cylinders daily in the following 8 d (sampling time for body size) and recorded the total number of pupae from each cylinder. We individually weighed male pupae using an electronic dual range balance with readability of 0.00001 g (Mettler Toledo AG135, Greifensee, Switzerland) and considered pupal weight as the index of body size (Xu & Wang, 2020). We placed weighed male pupae individually in glass vials, stuffed cotton wool on the opening of the vials and numbered each vial. We then maintained these pupae in their original environment chambers and recorded the total number of emerged males from the vials.

Immediately after eclosion, we randomly selected five newly emerged males (< 2 h after eclosion) per day from each cylinder for 6 d (sampling time) and froze them at −20 °C. We then dissected these males to extract their testes, and measured testis size with the aid of a stereomicroscope (Leica MZ12, Wetzlar, Germany) connected with imaging software (CellSens® GS-ST-V1.7, Olympus, Tokyo, Japan). Because the testis shape of this species is spherical (Nowock, 1973), we calculated its size as volume $= 4/3\pi r^3$, where $\pi = 3.14$ and $r =$ radius of the testis (Liu *et al.*, 2021). We determined its radius r using the mean diameter from three measurements across the organ's central axis divided by two (Raichoudhury, 1936; Gage, 1995). We then quantified eupyrene and apyrene sperm using the methods detailed in Koudelová and Cook (2001) and Liu *et al.* (2022). Briefly, we placed the testis into a drop of Belar saline solution on a cavity slide and tore it apart using a fine needle. To evenly disperse eupyrene sperm bundles and disassociate apyrenes, we gently rotated the solution for 30 s. We counted the number of eupyrene sperm bundles on the slide under a phase-contrast microscope (Olympus BX51, Tokyo, Japan) at 40× magnification and calculated the total number of eupyrenes as the total number of eupyrene bundles multiplied by 256 (each bundle has 256 eupyrene sperm). We then thoroughly flushed the sample off from the cavity slide into a glass vial and diluted it with 30-mL distilled water. We gently rotated the vial for 30 s to allow even dispersal of apyrenes in the vial and then pipetted eight 10-μL subsamples from the vial and dropped them separately onto a microscope slide. After air dry, we counted the number of apyrene sperm under the phase-contrast microscope at 100× magnification and calculated the total number of apyrene sperm for each male as the mean number of apyrenes per 10 μL multiplied by the dilution factor (i.e., 3000). Thirty males were tested for each treatment and control.

Mating latency, lifetime mating frequency and longevity

At the onset of the first scotophase, we randomly selected and individually paired five newly emerged males (< 2 h of eclosion) per day for 6 d (sampling time) from each treatment and control with 1-d-old-virgin females randomly selected from the breeding colony that had been singly housed in glass vials since the pupal stage. Each pair was confined in a mating chamber (transparent plastic cylinder, 6.5 cm diameter and 8.5 cm length) with the lid covered by cotton gauze (2.3 apparatus per mm^2). We monitored the chambers and observed the mating behavior continuously until the end of each copulation under

10 red light tubes (Sylvania, F36W/Red, Holland) and recorded the mating latency (time between introduction of both sexes and their genital connection) of 28, 26, 27, and 28 males for ELE, LLE, CLE, and CON, respectively.

Given that a male requires 24-h refractory period to produce a full spermatophore again after each copulation (Xu & Wang, 2009b), we introduced a 1-d-old virgin female to the focal male in the mating chamber at the onset of the second scotophase following emergence. We repeated this procedure until the focal male died. We inspected the mating pair once every 15 min until copulation cessation and immediately removed the mated female from the mating chamber. We recorded the lifetime mating frequency and longevity of 28, 26, 27, and 28 focal males for ELE, LLE, CLE, and CON, respectively.

Lifetime sperm ejaculation

To determine the lifetime number of sperm ejaculated by each focal male, we dissected all mated females from the above experiment and extracted the spermatophores from their bursa copulatrix. In total, we dissected 175, 146, 157, and 183 mated females for ELE, LLE, CLE, and CON, respectively. We placed a spermatophore into a droplet of Belar saline solution on a cavity slide and ruptured it using a fine needle to release sperm under the stereomicroscope. We then counted the number of eupyrene and apyrene sperm under the phase-contrast microscope using the methods described above.

Statistical analysis

All analyses were carried out using SAS 9.13. Rejection level was set at $P < 0.05$. We used a generalized linear model (GENMOD procedure) followed by a CONTRAST statement to compare the difference in pupation and emergence rate between treatments as an estimate of juvenile survival. Data on the ln(x)-transformed testis size, number of sperm counted at emergence, square-rooted mating latency, total number of lifetime sperm ejaculated, and longevity were normally distributed (Shapiro–Wilk test, UNIVARIATE procedure), and thus analyzed using a linear mixed-effect model (MIXED procedure) (Davies & Gray, 2015; Liu *et al.*, 2021) followed by a Tukey's test for multiple comparisons between treatments. Data on the body size and mating frequency were not normally distributed (Shapiro–Wilk test, UNIVARIATE procedure) and thus analyzed using a generalized linear mixed models (GLMMIX procedure) with a Poisson distribution in the model followed by a Tukey's test for multiple comparisons between treatments. Because our experimental design was pseudoreplicated, we treated the treatment as a fixed factor and replicate nested into sampling time as a random factor in the models (Millar & Anderson, 2004; Harrison *et al.*, 2018).

We used a two-sample t-test to compare the difference between the number of sperm counted at emergence and lifetime number of sperm ejaculated for each treatment. An exponential functional model (Archontoulis & Miguez, 2015) was used to fit the data on the cumulative percentage of eupyrene and apyrene sperm ejaculated over successive matings, i.e., cumulative percentage of sperm ejaculated $= \alpha \times [1 - \exp(-\beta \times \text{mating order})]$, where α ($= 1$) is the maximum percentage of cumulative sperm ejaculated, and β is the increasing rate of sperm cumulation. We used the nonoverlapped 83.4% confidence limits (83.4% CLs) of the cumulative sperm number to determine the statistical significance between treatments (Julious, 2004).

Results

Immature survival, body size, and testis size and sperm count at emergence

We obtained 106, 94, 89, and 86 male pupae and 102, 90, 83, and 85 male adults from ELE, LLE, CLE and CONT, respectively, with no significant difference between treatments (for number of pupae, $x_3^2 <$ /sup > =4.67, $P = 0.1975$; for number of adults, $x_3^2 <$ /sup > = 3.96, $P = 0.2653$). Body size (Fig. 1A) and testis size (Fig. 1B) were also not significantly different between treatments (for body size, $F_{3,266} = 0.60$, $P = 0.6145$; for testis size, $F_{3,87} = 0.31$, $P = 0.8192$). However, the number of eupyrene and apyrene sperm counted at emergence was significantly higher in LLE and CLE than in ELE and CON ($F_{3,87} = 15.59$, $P < 0.0001$ for eupyrenes; $F_{3,87} = 12.26$, $P < 0.0001$ for apyrenes) (Fig. 2). There was no significant difference in sperm count between LLE and CLE, or between ELE and CON ($P > 0.05$).

Mating latency, lifetime mating frequency and longevity

Males from LLE and CLE had significantly shorter mating latency than those from ELE and CON ($F_{3,78} = 55.41$, $P < 0.0001$) (Fig. 3). However, the presence of adult males during the larval stage had no significant effect on male mating frequency (mean $\pm$ SE $= 6.25 \pm 0.28$, 5.62 ± 0.30, 5.81 ± 0.27, and 6.54 ± 0.24 times for ELE, LLE, CLE, and CON, respectively) ($F_{3,78} = 0.67$,

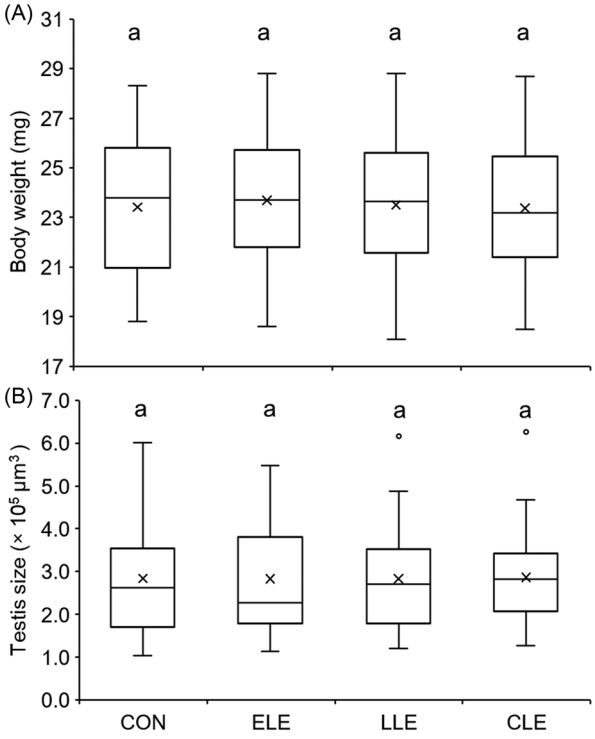

Fig. 1 Effect of larval exposure to male adults on body size (A) at pupal stage and testis size (B) at emergence in *E. kuehniella*. CON, ELE, LLE, and CLE denote nonexposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and "×" in each box show the median score and means, respectively; the '⊥' and 'T' are the lower and upper whiskers representing scores outside the 50% middle; the dots are the outliers of maximum scores. The same letters on the top of the boxes indicate no significant differences between treatments ($P > 0.05$).

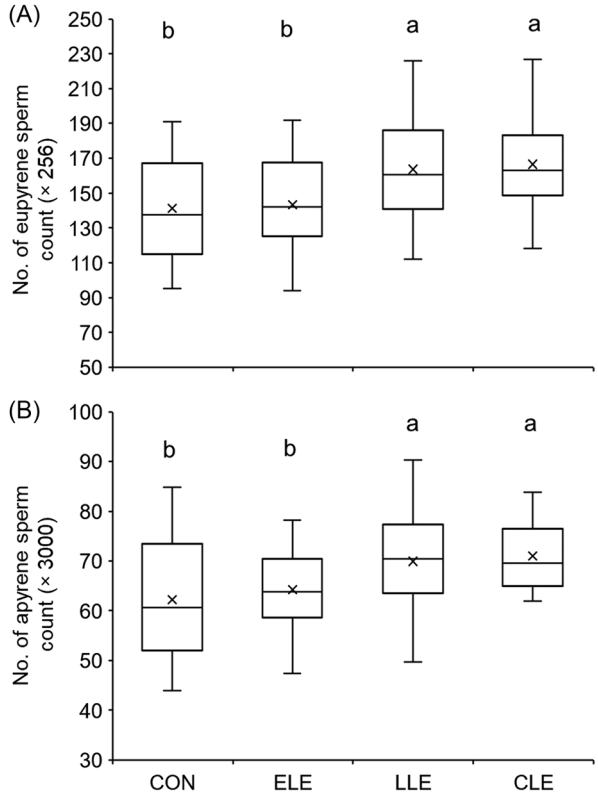

Fig. 2 Effect of larval exposure to male adults on eupyrene count (A) and apyrene count (B) at emergence in *E. kuehniella*. CON, ELE, LLE, and CLE denote nonexposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and "×" in each box show the median score and means, respectively; the '⊥' and 'T' are the lower and upper whiskers representing scores outside the 50% middle. The different letters on the top of the boxes indicate significant differences between treatments ($P < 0.05$).

$P = 0.5718$) and longevity (mean ± SE = 9.93 ± 0.39, 8.77 ± 0.43, 9.37 ± 0.45 and 9.54 ± 0.34 d for ELE, LLE, CLE, and CON, respectively) ($F_{3,78} = 1.48$, $P = 0.2253$).

Lifetime sperm ejaculation

Our findings indicate that males in all treatments ejaculated similar number of eupyrene ($F_{3,78} = 0.90$, $P = 0.4466$) (Fig. 4A) and apyrene sperm ($F_{3,78} = 2.09$, $P = 0.1080$) (Fig. 4B) in their lifetime. However, the number of eupyrene sperm ejaculated in lifetime was significantly higher than that counted at emergence in ELE and CON ($t_{56} = 2.29$, $P = 0.0260$ for ELE; $t_{56} = 2.93$, $P = 0.0049$ for CON) while these were similar in LLE and CLE ($t_{54} = -0.87$, $P = 0.3892$ for LLE; $t_{53} = -0.03$, $P = 0.9774$ for CLE) (Fig. 5A). In all treatments, the number of apyrene sperm ejaculated in lifetime was significantly higher than that measured at emergence ($t_{56} = 9.27$, $P < 0.0001$ for ELE; $t_{54} = 8.64$, $P < 0.0001$ for LLE; $t_{53} = 10.30$, $P < 0.0001$ for CLE; $t_{56} = 8.65$, $P < 0.0001$ for CON) (Fig. 5B). The cumulative percentage of both eupyrenes (Fig. 6A) and apyrenes (Fig. 6B) ejaculated over successive matings increased significantly faster in LLE and CLE than in ELE and CON (nonoverlapping 83.4% CLs).

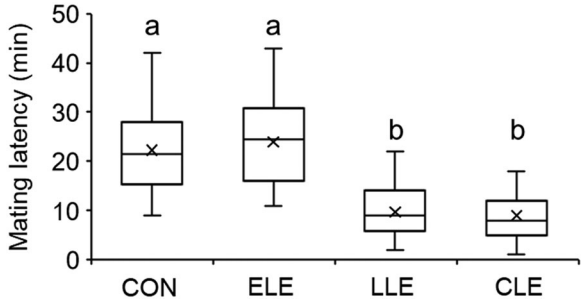

Fig. 3 Effect of the presence of adult males during the larval stage on the first mating latency of *E. kuehniella*. CON, ELE, LLE, and CLE denote nonexposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and "×" in each box show the median score and means, respectively; the '⊥' and 'T' are the lower and upper whiskers representing scores outside the 50% middle. The different letters on the top of the boxes indicate significant differences between treatments ($P < 0.05$).

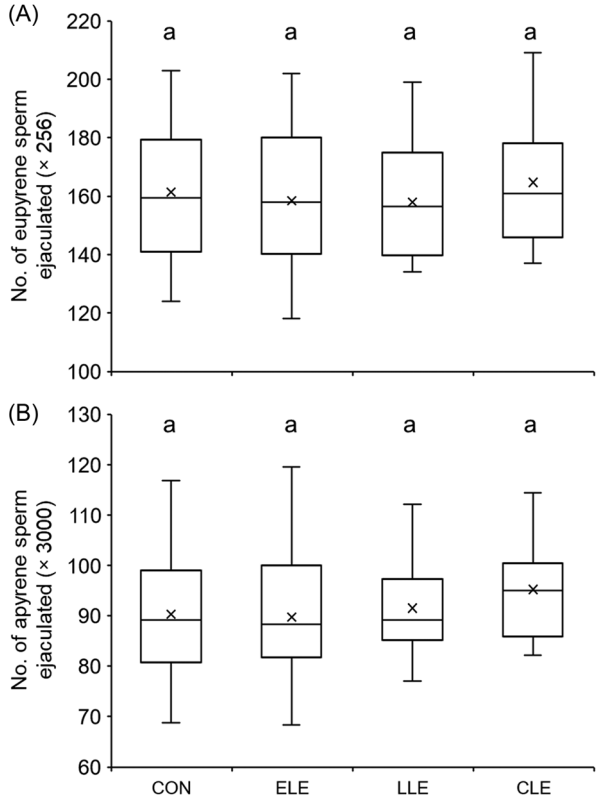

Fig. 4 Effect of the presence of adult males during the larval stage on eupyrene (A) and apyrene (B) sperm ejaculated during the lifetime of *E. kuehniella*. CON, ELE, LLE, and CLE denote nonexposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and "×" in each box show the median score and means, respectively; the '⊥' and 'T' are the lower and upper whiskers representing scores outside the 50% middle. The same letters on the top of the boxes indicate no significant differences between treatments ($P > 0.05$).

Discussion

The present study shows that after exposure to conspecific adult males during the late or entire larval stage, *E. kuehniella* males carried more eupyrenes (fertile and nucleate sperm) and apyrenes (infertile and anucleate sperm) at emergence (Fig. 2) and had shorter mating latency (Fig. 3). Adults from the larvae unexposed or only exposed to adult males during the early larval stage continued to produce eupyrenes after emergence while they kept on making apyrenes after emergence regardless of their larval experience (Fig. 5). Compared to adults from the larvae unexposed or only exposed to adult males during the early larval stage, those from the larvae exposed during the late or entire larval stage transferred more eupyrenes and apyrenes in their early life (Fig. 6). However, larval exposure to adult males had no effect on immature survival, body, and testis size (Fig. 1), and longevity, mating frequency and lifetime number of sperm ejaculated in resultant adults (Fig. 4). These findings indicate that *E. kuehniella* larvae adjust their lifetime sperm expenditure depending on whether and when they experience the cues from conspecific adult males.

Earlier studies have examined insect juvenile response to the cues of conspecific adult males but have not determined whether their sensitivity to these cues is stage dependent and whether these cues affect sperm production (Bailey *et al.*, 2010; Gray & Simmons, 2013; Bretman *et al.*, 2016; Simmons & Lovegrove, 2017). In the present study, we demonstrate for the first time that the late instar larvae of *E. kuehniella* could respond to sperm competition risk signaled by adult males, leading to higher sperm production before emergence (Fig. 2) and shorter mating latency in their resultant adults (Fig. 3). The lack of response to adult cues by younger larvae may be attributed to the fact that testes start forming their shape only when the larvae reach the fourth instar (about 15 d old) (Liu *et al.*, 2022, 2023), allowing them to adjust their sperm production from this stage on. The shorter mating latency induced by adult male cues implies that intramale competition risk also reduces mate selectivity by males.

Previous studies report that larval exposure to juvenile rivals increases eupyrene counts both at emergence

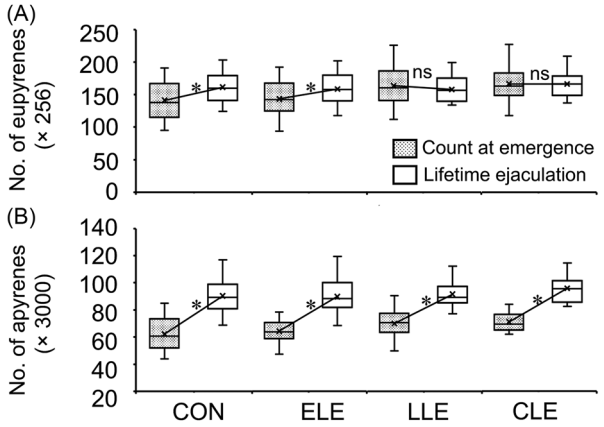

Fig. 5 Effect of exposure to adult males during the larval stage on the number of eupyrenes (A) and apyrenes (B) counted at emergence and during lifetime ejaculation in *E. kuehniella*. CON, ELE, LLE, and CLE denote nonexposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. Each box plot shows the median line and the upper and lower quartiles, i.e., the range where 25% of scores fall above and 25% fall below the median; the line and "×" in each box indicate the median score and means, respectively; the '⊥' and 'T'are the upper and lower whiskers showing the maximum and minimum scores, respectively. For each treatment, lines between boxes with "*" and "ns" indicate significantly different ($P < 0.05$) and not significantly different ($P > 0.05$), respectively.

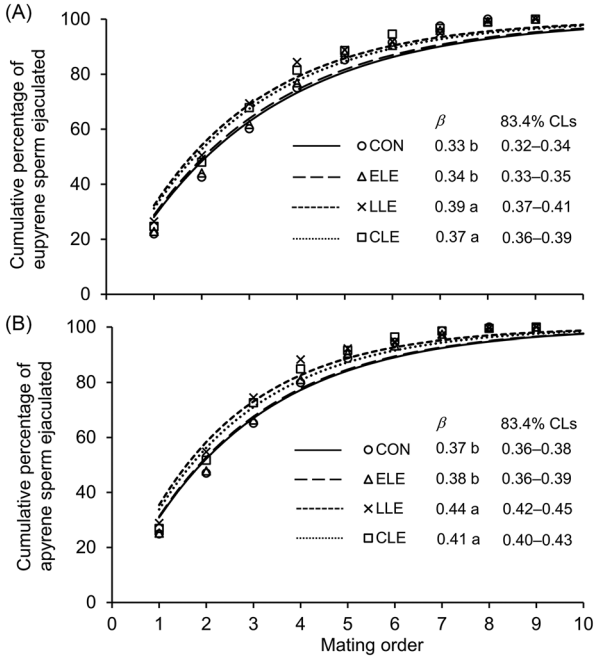

Fig. 6 Effect of exposure to adult males during the larval stage on cumulative eupyrenes (A) and apyrenes (B) ejaculated by *E. kuehniella* males over successive matings. CON, ELE, LLE, and CLE denote nonexposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. Cumulative percentage of sperm ejaculated $= \alpha \times [1 - \exp(-\beta \times \text{mating order})]$, where α ($= 1$) is the maximum percentage of cumulative sperm ejaculated, and β is the increasing rate of sperm cumulation. The increasing rate β with different letters is significantly different (nonoverlapping 83.4% CLs).

(Liu *et al.*, 2022) and in lifetime ejaculates (Liu *et al.*, 2023) in *E. kuehniella*. However, our current study indicates that the higher sperm production during the juvenile stage in response to adult males (Fig. 2) did not translate into greater sperm ejaculation during the lifetime of resultant adults (Fig. 4). These findings reveal that male larvae respond to the cues from juvenile rivals and adult males differently, implying the existence of distinct cues in juvenile and adult males. We suggest that the cues from juvenile rivals may signal the future sperm competition risk while those from adult males may indicate the immediate risk. Accordingly, after the larvae detect the immediate risk, they allocate all available resources for eupyrene production during the juvenile stage, whereas, if they perceive the future risk, they spread their resource allocations for eupyrene production across juvenile and adult stages.

Further comparison of sperm counts at emergence and in lifetime ejaculates reveals two clear patterns. First, whether adults could continue producing eupyrenes depended on their larval experience (Fig. 5). These findings suggest that (1) the number of spermatogonia for producing eupyrenes may be limited (Witalis & Godula, 1993; Jarrige *et al.*, 2015; Mari *et al.*, 2018) so that the adults cannot manufacture more eupyrenes if their juveniles have used most or all of them, and (2) the number of cells for producing apyrenes may be less limited (Silberglied *et al.*, 1984; Cook & Gage, 1995; Liu *et al.*, 2023), allowing adults to produce more apyrene throughout their life regardless of their larval experience. Production of more apyrenes throughout lifetime may be important to gain advantages in sperm competition and fertilization success (Cook & Wedell, 1999; Holman & Snook, 2008; Wedell *et al.*, 2009; Sakai *et al.*, 2019; Hague *et al.*, 2021). Second, adults carrying more sperm at emergence ejaculated more in their first few matings (Fig. 6). We suggest that earlier ejaculation of more sperm may contribute to a greater reproductive success as demonstrated in other animals (Shackleton *et al.*, 2005; Hosken *et al.*, 2008; Wensing *et al.*, 2017; Burke & Holwell, 2021) and support the sperm competition game model (Parker & Pizzari, 2010).

Our study indicates that *E. kuehniella* did not trade off their survival, mating frequency, body size and testis size for sperm production in response to the cues from male adults during the larval stage (Figs. 1 and 4). We suggest that under our rearing conditions with *ad libitum* food supply (also see Bhavanam *et al.*, 2012; Liu *et al.*, 2022), the larvae have sufficient resources to adjust spermatogenesis without compromising juvenile survival, adult longevity, body size, and mating frequency, which are essential traits for male fitness (Honěk, 1993; Blanckenhorn, 2000; Komo *et al.*, 2020; Kappeler, 2021). Although sperm production and testis size are positively correlated in some species (review in Vahed & Parker, 2012), various studies demonstrate that in response to sperm competition risk, testis size has no significant effect on sperm production in *E. kuehniella* (Liu *et al.*, 2022) and other animals (Byrne *et al.*, 2002; Gay *et al.*, 2009; Fitzpatrick *et al.*, 2012; Bretman *et al.*, 2016; Liao *et al.*, 2019; Hobson *et al.*, 2020). The lack of correlation between testis size and sperm production is probably because animals can dedicate varying portions of testis volumes to spermatogenesis and other functions in response to sperm competition environment (Lüpold *et al.*, 2020). It could also be possible that juvenile males have a higher spermatogenesis rate (Ramm & Schärer, 2014; Firman *et al.*, 2018) when detecting potential future rivals.

In conclusion, larval sensitivity to the cues from conspecific adult males is age specific in *E. kuehniella*, probably related to the stage of testis development. After detecting adult male cues by older male larvae with developed testes, they produce more sperm before emergence and their resultant adults start mating earlier and ejaculate more sperm in their first few matings, enhancing their reproductive success. Adults stop producing eupyrenes if their immatures raise eupyrene production as a response to adult cues, but they continue producing apyrenes regardless of their larval experience. These findings suggest that the number of spermatogonia for production of fertile sperm may be limited but that for producing infertile sperm may be flexible. Under our rearing conditions with *ad libitum* food supply, *E. kuehniella* do not trade off their survival, mating frequency, body size, and testis size for sperm production in response to the cues from male adults during the larval stage. It would be worth testing whether any trade-off could occur under food-stressed conditions. The knowledge generated here provides insight into stage-dependent sensitivity of juvenile males to adult male cues and subsequent lifetime resource allocations.

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We declare we have no conflict of interest associated with this publication.

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