

Article

Larval social cues influence testicular investment in an insect

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Abstract

Socio-sexual environment can have critical impacts on reproduction and survival of animals. Consequently, they need to prepare themselves by allocating more resources to competitive traits that give them advantages in the particular social setting they have been perceiving. Evidence shows that a male usually raises his investment in sperm after he detects the current or future increase of sperm competition because relative sperm numbers can determine his paternity share. This leads to the wide use of testis size as an index of the sperm competition level, yet testis size does not always reflect sperm production. To date, it is not clear whether male animals fine-tune their resource allocation to sperm production and other traits as a response to social cues during their growth and development. Using a polygamous insect *Ephestia kuehniella*, we tested whether and how larval social environment affected sperm production, testis size, and body weight. We exposed the male larvae to different juvenile socio-sexual cues and measured these traits. We demonstrate that regardless of sex ratio, group-reared males produced more eupyrenes (fertile and nucleate sperm) but smaller testes than singly reared ones, and that body weight and apyrene (infertile and anucleate sperm) numbers remained the same across treatments. We conclude that the presence of larval social, but not sexual cues is responsible for the increase of eupyrene production and decrease of testis size. We suggest that male larvae increase investment in fertile sperm cells and reduce investment in other testicular tissues in the presence of conspecific juvenile cues.

Key words: body weight, immature stage, pyralidae, sperm production, social environment, testis size

Socio-sexual environment can influence animals' fitness in reproduction and survival (Mohorianu et al. 2017; Alberts 2019). Such effects can lead to their adjustment of behavior and physiology to maximize their fitness gain (Wilson et al. 2014; Mirth et al. 2021). Hence, animals can prepare themselves by allocating more resources to the traits that are competitive and beneficial in the particular social setting they have been perceiving. For example, a male raises his investment in sperm after he detects the current or future increase of sperm competition because relative sperm numbers can predict his

paternity share (Parker 1970; Parker et al. 1997; Simmons 2001; Parker and Pizzari 2010; Lüpold et al. 2020). Although larger males usually have more sperm (Pitnick 1996; Hatala et al. 2018; Chung et al. 2019; Xu and Wang 2020), the social environment experienced by juvenile males does not appear to affect their body size in some insects (Gage 1995; Hosken and Ward 2001; Allen et al. 2011; Bretman et al. 2016) and mammals (Hobson et al. 2020).

The fact that males increase investment in sperm in response to raising sperm competition leads to the wide use of testis size as an

index of the level of sperm competition (Lüpold et al. 2020). Yet, there is evidence that testis size does not increase at higher sperm competition levels in some insects (Crudgington et al. 2009; Gay et al. 2009; Bretman et al. 2016; Chechi et al. 2017) and vertebrates (Byrne et al. 2002; Fitzpatrick et al. 2012; Liao et al. 2019; Hobson et al. 2020). The lack of positive relationship between testis size and sperm production could be attributed to at least 2 reasons: 1) animals can dedicate varying portions of testis volumes to spermatogenesis and other functions in response to sperm competition environment (Lüpold et al. 2020) and 2) adult testis mass can decrease after a mating (Simmons et al. 2000; Greenway et al. 2020) or due to senescence (Rosa et al. 2019). Therefore, measurement of testis size does not always reflect sperm production.

So far, most studies on insect sperm investment have focused on males' response to sperm competition environment during the adult stage (e.g., Simmons et al. 2007; Moatt et al. 2014; Larsdotter-Mellström and Wiklund 2015; Simmons and Lovegrove 2017; Lymbery et al. 2019; Esfandi et al. 2020). This is probably because adults can detect sperm competition levels using sex-specific cues in their surroundings (e.g., Bretman et al. 2011; Uzsák et al. 2014; Baker et al. 2019; Liu et al. 2020) and adjust their sperm investment accordingly (Wedell et al. 2002; Parker and Pizzari 2010; Lüpold et al. 2020). However, insect juveniles can also communicate using various cues, such as non-sex specific aggregation pheromones, trail pheromones, and defensive sounds (e.g., Yack et al. 2001; Duthie et al. 2003; Scott et al. 2010; Fitzgerald et al. 2019). Furthermore, female pupae of some species release sex pheromones that can be detected by conspecific male pupae (Pontier and Schweisguth 2015) or adults (e.g., Choi et al. 2007; Estrada et al. 2010). Hence, male insects should be able to detect their socio-sexual situations during immature stages. This can allow juvenile males to predict future sperm competition levels and subsequently adjust their resource allocation (Gage 1995; Allen et al. 2011; Kasumovic and Brooks 2011; Gray and Simmons 2013).

Because most resource allocation to traits making up the adult body (e.g., Oberlander 1985; Nijhout and Emlen 1998; Moczek and Nijhout 2004; Rolff et al. 2019; Mirth et al. 2021) and immunity (e.g., Barnes and Siva-Jothy 2000; Cotter et al. 2004; Triggs and Knell 2012) takes place during larval or nymphal stages in insects, these juveniles can adjust their resource allocation to traits of different functions in response to socio-sexual cues, leading to potential trade-offs between different body traits (Nijhout and Emlen 1998; Simmons and Emlen 2006; Luecke and Kopp 2019). To date, only a few studies have investigated how male insects fine-tune their investment in reproduction during growth and development as a response to potential sperm competition risk. For example, in some holometabolous species, adult males have larger testes (Gage 1995; Stockley and Seal 2001; Johnson et al. 2017) or ejaculate more sperm in the first mating (Gage 1995; He and Miyata 1997; McNamara et al. 2009) after their larvae are exposed to stronger conspecific social cues (more juveniles are present in the vicinity). There are also reports that hemimetabolous adult males ejaculate more sperm during their first mating if their nymphs are reared with conspecific male nymphs (Allen et al. 2011) or with adult songs of conspecific males (Gray and Simmons 2013). Yet, it is still not clear whether the socio-sexual settings during growth and development affect sperm production and result in any detectable trade-off between body size, testis size, and sperm number. Answers to these questions would provide insights into adaptive responses of juvenile males to their socio-sexual environment.

In the present study, we used a polygamous insect, *Ephestria kuehniella*, to investigate whether and how the socio-sexual contexts experienced by male juveniles affected their investment in body size, testis size, and sperm production. *Ephestria kuehniella* larval stage lasts 29–31 days and pupal stage takes 8–9 days, with larvae having 6 instars (instars 1–3 \approx 14–15 days and instars 4–6 \approx 15–16 days) (Brindley 1930; Liu J, personal observation). Adults of this species do not feed and thus all their resources are obtained during the larval stage (Norris and Richards 1932). Females start producing sex pheromones at the pupal stage (Calvert and Corbet 1973). Like most lepidopterans (reviewed in Swallow and Wilkinson 2002), *E. kuehniella* males produce 2 types of sperm, larger fertile eupyrenes (nucleate) and smaller infertile apyrenes (anucleate), which can be easily distinguished (Garbini and Imberski 1977; Koudelová and Cook 2001). Prior to ejaculation, apyrene sperm bundles dissociate and become motile whereas eupyrene sperm remain in bundles (Koudelová and Cook 2001; Liu J, personal observation). Both types of sperm migrate to the spermatheca but only eupyrenes can fertilize eggs (Friedländer and Gitay 1972; Xu and Wang 2010). Apyrene sperm may delay the renewal of female receptivity (Cook and Wedell 1999; Wedell et al. 2009), protect eupyrene sperm against a hostile female reproductive tract (Holman and Snook 2008) or facilitate eupyrene migration from the bursa to the spermatheca (Sakai et al. 2019). Due to their different functions, eupyrenes evolve faster than apyrenes in response to selection pressures (Fitzpatrick et al. 2020).

Based on the empirical studies and theoretical predictions outlined above, we postulate that males kept together with other males during juvenile stages should be smaller with larger testes and more sperm than those reared individually or with females. To test this hypothesis, we prepared hundreds of larvae and reared them singly or in group with different sex ratios. We then weighed mature pupae, and on emergence, dissected male adults, measured testis size, and counted the sperm in their testes. Our design allowed us to determine whether the number of sperm produced was the function of testis size and/or body size in response to socio-sexual environment during growth and development in *E. kuehniella*.

Materials and Methods

Insect sampling and rearing

We collected *E. kuehniella* larvae by hand from chicken feed at Turks Poultry, Foxton, New Zealand. We allowed them to feed on their original food and develop to adults in the laboratory. We randomly selected and introduced about 300 newly emerged adults into a transparent plastic cage (28 cm length \times 28 cm width \times 24 cm height) lined with porous plastic sheets on the bottom for egg laying. We established a laboratory colony using larvae that hatched from these eggs. Briefly, we introduced 200 neonate larvae into a transparent plastic cylinder (8 cm diameter \times 10 cm height) with 50 g standard diet (ad libitum; Bhavanam et al. 2012) consisting of maize meal, whole meal wheat flour, glycerine, and yeast with a ratio = 43.5:43.5:10.0:3.0. We covered the cylinder with 2 layers of cloth mesh. We maintained 10 cylinders of the colony, from which we randomly collected about 300 newly emerged adults and transferred them into the aforementioned plastic cage for egg laying. To generate an experimental line, we randomly collected 1,000 neonate larvae from the eggs laid in the cage and reared them individually in 2-mL transparent micro-centrifuge tubes with a hole in the lid made by an insect pin for ventilation. We provided 0.25 g standard diet per larva in the experimental line. We kept the insect colony and experimental larvae at $25 \pm 1^\circ\text{C}$,

$60 \pm 10\%$ RH, and 10:14 h (dark:light) and carried out experiments under these environmental conditions.

Juvenile socio-sexual settings

Because sex can be determined through visible testes in male abdomens of the fourth instar larvae (Brindley 1930; Liu J, personal observation), we randomly selected newly molted fourth instar larvae from the experimental line and transferred them into glass vials (2 cm diameter \times 7.5 cm height) to form 3 treatments (Figure 1A): 1) SM—1 male was maintained in a glass vial from the fourth instar larva to adult emergence; 2) 6M—6 males were kept in a glass vial from the fourth instar larvae to adult emergence; and 3) 1M5F—1 male and 5 females were reared in a glass vial from the fourth instar larvae to adult emergence. All vials were provided with standard diet of 0.25 g per larva and covered with cotton wool. We only used insects from vials where all individuals successfully developed to adults for data collection. We used all males from these vials for measurements (see below), that is, the male from each SM vial, the male from each 1M5F vial, and all 6 males from each 6M vial. In total, we measured 32 adult males from treatment SM and 30 adult males for each of the other 2 treatments.

Effects of juvenile socio-sexual settings on body size and testis size

We individually weighed mature male pupae from all 3 treatments with an electronic dual range balance (Mettler Toledo AG135, Switzerland) and returned them to their original vials immediately after weighing. We used pupal weight as body size as reported in many insects including moths (e.g., Jiménez-Pérez and Wang 2004; Xu and Wang 2013, 2020).

Immediately after emergence, we individually transferred adult males into 2-mL transparent micro-centrifuge tubes, clearly labeled each tube, and killed them at -20°C in a freezer. We then individually dissected all males to extract their testes and measured testis volume under a stereomicroscope (Leica MZ12, Germany) connected with a digital camera (Olympus SC30, Japan) operated by an adequate imaging software (CellSens[®] GS-ST-V1.7, Olympus, Japan). Because *E. kuehniella* testes are fused into a single spherical organ (Nowock 1973; Liu J, personal observation), we determined its radius using the mean diameter from 2 measurements across the organ's central axis (Figure 1B) (Raichoudhury 1936; Gage 1995) divided by 2 and calculated the testis volume (size) using the formula $4/3\pi r^3$, where $\pi=3.14$ and r = radius of the testis.

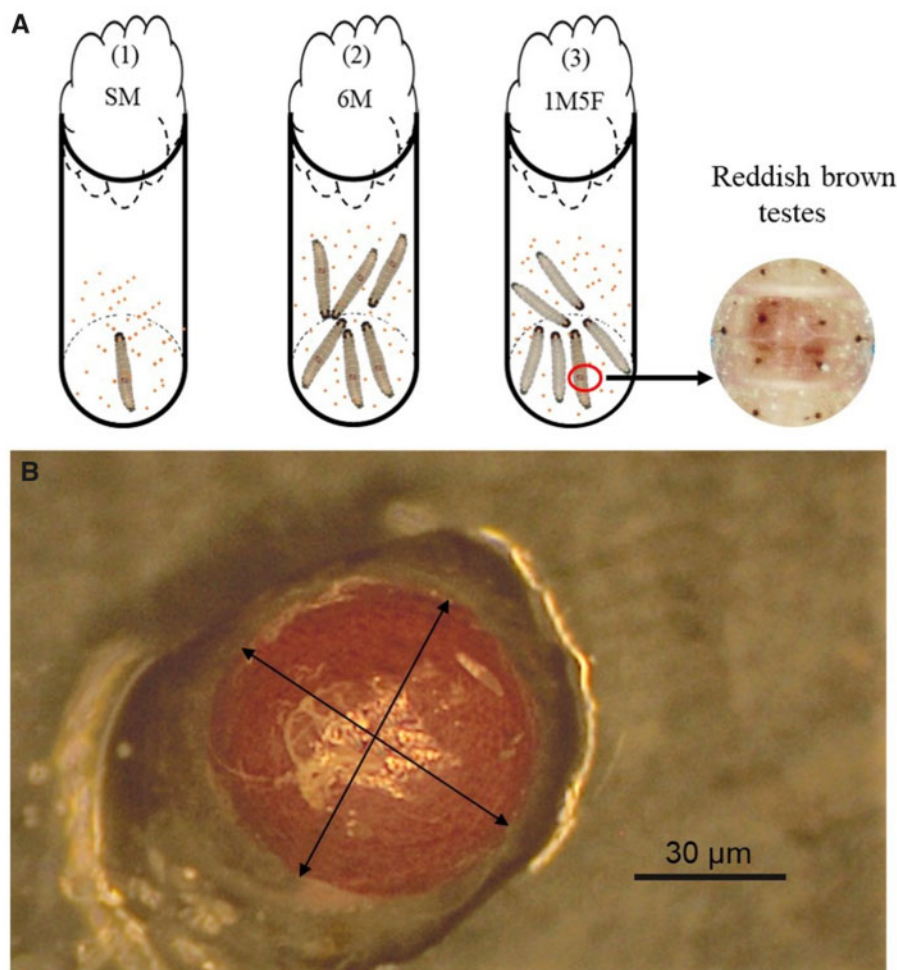


Figure 1. Treatment setups (A) and testis measurement (B) for *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, 6 males together from the fourth instar larvae to adult emergence, and 1M5F, 1 male and 5 females together from the fourth instar larvae to adult emergence.

Effects of juvenile socio-sexual settings on sperm production

After measurement of testis size, we placed the testis into a drop of Belar saline solution on a cavity slide and tore it apart completely using a fine needle tip and then gently rotated the cavity slide for ~30 s to evenly disperse eupyrene bundles and dissociated apyrenes. We counted the number of eupyrene bundles under a phase-contrast microscope (Olympus BX51, Japan) at 40× magnification and calculated the total number of eupyrenes as the total number of eupyrene bundles multiplied by 256 because each bundle contains 256 eupyrenes in *E. kuehniella* (Garbini and Imberski 1977). We then thoroughly washed the sample off the cavity slide and diluted it with distilled water to 30 mL in a glass vial. We gently rotated the vial for about 30 s to allow even dispersal of apyrenes in the vial and then took 8 10- μ L subsamples from the vial using a Gilson autopipette. We placed these subsamples apart from each other on a microscope slide and allowed them to air dry. We counted the number of apyrene sperm of all 8 subsamples under the phase-contrast microscope at $\times 100$ magnification and calculated the mean number per 10 μ L as the sum of apyrene sperm in 8 subsamples divided by 8. We then calculated the total number of apyrene sperm for each male as the mean number of apyrenes per 10 μ L multiplied by the dilution factor (3,000) (Koudelová and Cook 2001).

Statistical analysis

We calculated the residuals of data and tested the residual distribution (Shapiro–Wilk test, UNIVARIATE procedure) after fitting the data to a general linear model. We showed that data on body size and eupyrene number were normally distributed and those on testis size and apyrene number became normally distributed after $\ln(x)$ -transformed. As our experimental design was pseudoreplicated, we analyzed the data using a linear mixed-effects model (Millar and Anderson 2004; Harrison et al. 2018) with treatment as a fixed factor and replicate nested into vial (male source) as a random factor (Davies and Gray 2015; Harrison et al. 2018). We then used a Tukey's studentized range (HSD) test for multiple comparisons between treatments. All analyses were done with SAS 9.4 (SAS Inc., USA).

Results

Effects of juvenile socio-sexual settings on body size and testis size

Our results show that socio-sexual cues during immature stages had no significant effect on male body size ($F_{2,29} = 2.69$, $P = 0.0847$) (Figure 2A). We found that adult males that developed from group-reared juveniles had significantly smaller testes than those from singly reared ones ($F_{2,29} = 4.60$, $P = 0.0183$) (Figure 2B). However, juvenile sex ratio (6 males or 1 male + 5 females) had no significant effect on testis size ($F_{1,29} = 0.18$, $P = 0.6704$) (Figure 2B).

Effects of juvenile socio-sexual settings on sperm production

We demonstrate that the testes of males from the group-reared juveniles (6 males and 1 male + 5 females) produced significantly more eupyrene sperm than those from singly reared ones (1 male) ($F_{2,29} = 11.52$, $P = 0.0002$) (Figure 3A). However, testes in all treatments produced a similar number of apyrene sperm ($F_{2,29} = 1.47$, $P = 0.2458$) (Figure 3B). The number of eupyrene and apyrene produced did not vary with sex ratio during the immature stages

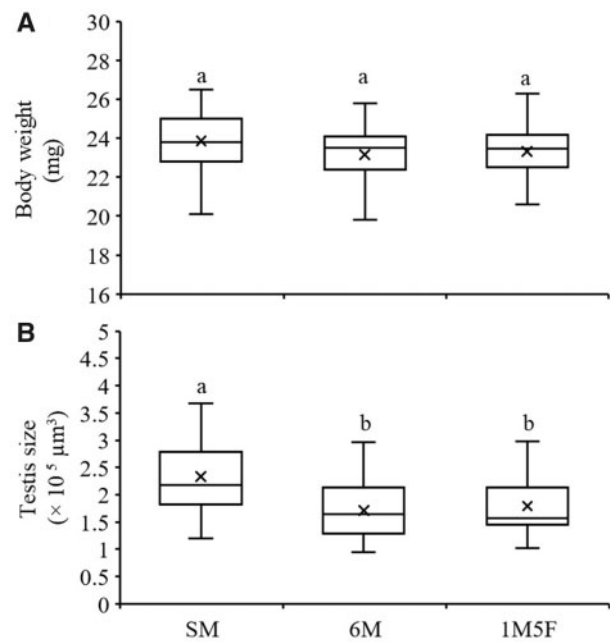


Figure 2. Effect of socio-sexual environment during immature stages on the body weight (A) and testis size (B) of *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, 6 males together from the fourth instar larvae to adult emergence, and 1M5F, 1 male and 5 females together from the fourth instar larvae to adult emergence. Each box plot shows the median line and the upper and lower quartiles, that is, the range where 25% of scores fall above and 25% fall below the median; the “x” and line in a box indicate the mean and median scores, respectively; the “T” and “⊥” are the upper and lower whiskers showing the maximum and minimum scores, respectively. For each parameter, boxes with different letters are significantly different ($P < 0.05$).

(6 males or 1 male + 5 females) ($F_{1,29} = 0.02$, $P = 0.8896$ for eupyrene; $F_{1,29} = 1.77$, $P = 0.1938$ for apyrene) (Figure 3).

Discussion

We found significantly more eupyrene (fertile) sperm in the testes of adults that developed from group-reared larvae than from singly reared ones, suggesting that the presence of juvenile cues could be an indicator of sperm competition risk and males increase resource allocation to eupyrene production when their young are maintained in groups. Evidence shows that most spermatogenesis takes place during immature stages in *E. kuehniella* (Garbini and Imberski 1977) and other lepidopteran insects (Swallow and Wilkinson 2002). This would provide opportunities for males to adjust their investment in sperm production based on their social contexts during their growth and development. A few earlier studies (Gage 1995; He and Miyata 1997; McNamara et al. 2009) also draw similar conclusions. However, these authors determine the impact of juvenile cues by counting the number of sperm in males' first ejaculates, which may not represent the total number of sperm produced by males. Hence, our current findings provide the first evidence of the impact of juvenile cues on sperm production in an insect. The present study shows that males did not increase investment in apyrene production in response to the presence of larval cues. This may be because apyrenes play a minor role in sperm competition relative to eupyrenes (Cook and Gage 1995; Thorburn et al. 2018; Esfandi et al. 2020) and the increased resource allocation to eupyrene production leaves

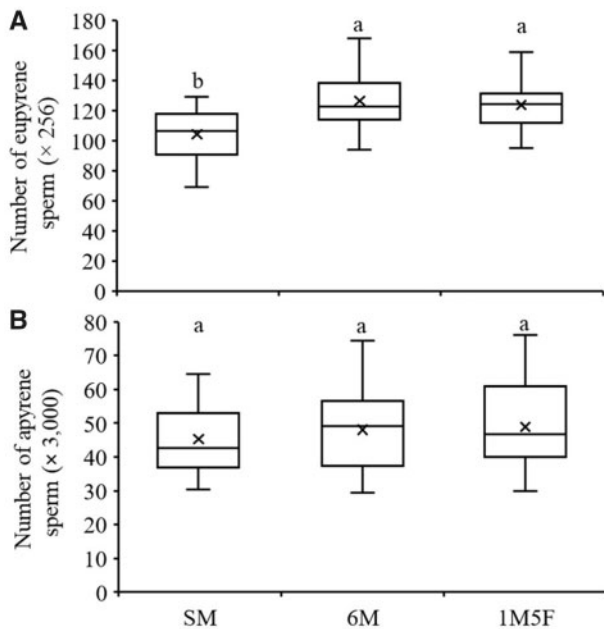


Figure 3. Effect of socio-sexual environment during immature stages on the total number of eupyrene (A) and apyrene (B) sperm in testes of *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, 6 males together from the fourth instar larvae to adult emergence, and 1M5F, 1 male and 5 females together from the fourth instar larvae to adult emergence. Each box plot shows the median line and the upper and lower quartiles, that is, the range where 25% of scores fall above and 25% fall below the median; the 'x' and line in a box indicate the mean and median score, respectively; the "T" and "⊥" are the upper and lower whiskers showing the maximum and minimum scores, respectively. For each parameter, boxes with different letters are significantly different ($P < 0.05$).

less resource to produce more apyrenes. Furthermore, the last male sperm precedence is common in many insect species (Simmons 2001) including *E. kuehniella* (Xu and Wang 2010). The sperm from the last male can displace some sperm from the previous male to dominate paternity in some moths (e.g., Cook et al. 1997; Xu and Wang 2010). However, the degree of last male sperm precedence may depend on the number of sperm ejaculated by both the first and second males. Therefore, production of more eupyrene sperm during immature stages may benefit males regardless whether they mate with virgin or mated females.

Previous studies demonstrate that testis size increases with the increase of juvenile density and suggest that larger testes produce more sperm (Gage 1995; Stockley and Seal 2001; Johnson et al. 2017). However, our data show that although group-reared males produced significantly more eupyrenes than singly reared males, they had significantly smaller testes than singly reared males in *E. kuehniella*. Insect testes consist of both sperm cells and gland tissues (e.g., Nowock 1973; Verson 1889; Wolf 1991; White-Cooper et al. 2009) and have functions other than sperm production (Simmons and Fitzpatrick 2012; Ramm and Schärer 2014; Parker 2016), such as production of sex hormones (review in de Loof 2006). Therefore, males may be able to donate varying portions of testis volumes to spermatogenesis and other functions in response to sperm competition environment (Lüpold et al. 2020). Because a resource used for 1 trait may not be used for another, potential trade-offs between traits of different functions may occur (Nijhout and Emlen 1998; Moczek and Nijhout 2004; Luecke and Kopp 2019). Based on the results from the present study and current knowledge

about testicular components and functions, we suggest that in response to the presence of conspecific social cues *E. kuehniella* male larvae may increase investment in fertile sperm cells and reduce investment in other tissues of the testes. Furthermore, body weight remained the same across treatments in the present study, suggesting that *E. kuehniella* young provided with plentiful food and space do not trade-off their body weight with reproductive traits. Similar conclusions are reached in other insects (Gage 1995; Hosken and Ward 2001; Bretman et al. 2016). In future studies, it may be worth testing how larval cues affect resource investment in testicular (Lüpold et al. 2020), immune (Barnes and Siva-Jothy 2000; Cotter et al. 2004; Triggs and Knell 2012), and pre-copulatory (Simmons and Emlen 2006) functions.

According to Corbet (1971) and Mudd (1983), *E. kuehniella* larvae use chemical and tactile cues to communicate for population density regulation. Numerous studies demonstrate that immature stages of many holometabolous insect species use non-sex-specific chemical or acoustic cues for various purposes. For example, juveniles communicate using aggregation pheromones for feeding in moths (Fitzgerald et al. 2019) and locating pupation sites in moths (Duthie et al. 2003; Kwadha et al. 2019) and beetles (Kojima et al. 2014). Larvae employ trail pheromones for survival in moths (Crump et al. 1987; Fitzgerald and Pescador-Rubio 2011), butterflies (Fitzgerald and Underwood 1998), and sawflies (Flowers and Costa 2003). Caterpillars use acoustic cues to communicate for territorial defence (Yack et al. 2001; Scott et al. 2010). Although none of the above studies reports that those cues could alter investment in reproduction, we propose that chemical, acoustic, and tactile cues used by the larvae may provide reliable information about the future sperm competition levels, supporting Kasumovic and Brooks' (2011) prediction that cues used by immature insects may result in anticipatory developmental plasticity as a future mating strategy.

Several studies report that some lepidopterans including *E. kuehniella* start producing female sex pheromones at the pupal stage (Calvert and Corbet 1973; Choi et al. 2007) and the pheromones released by female pupae of moths (Duthie et al. 2003) and butterflies (Estrada et al. 2010) can attract conspecific adult males. However, little is known about whether juvenile males of any holometabolous insect adjust investment in reproduction as a response to those sex-specific cues. Our results demonstrate that larval sex ratio did not affect testis size and sperm production, suggesting that testicular investment in *E. kuehniella* juvenile males only responds to the presence of social, but not sexual cues, during their growth and development. However, in a hemimetabolous insect, males can respond to sex ratio during the immature stage, adjusting ejaculation allocation during the adult stage (Allen et al. 2011). Further studies are thus warranted to determine whether holometabolous and hemimetabolous males have different resource allocation strategies in response to their juvenile socio-sexual environment.

In the present study, we have tested whether and how larval social cues affect sperm production, testis size, and body weight in *E. kuehniella*. We demonstrate that regardless of larval sex ratio, group-reared males produce smaller testes but more eupyrene sperm than singly reared ones and that body weight and apyrene numbers remain the same across treatments. We conclude that the presence of non-sexual larval social cues is responsible for the increase of eupyrene production and decrease of testis size. We suggest that male larvae increase investment in fertile sperm cells and reduce investment in other testicular tissues in the presence of conspecific cues.

Authors' Contributions

J.L., Q.W., and X.Z.H. conceived and designed the study. J.L., Y.Z., and X.-L.Z. collected the data. All authors contributed to data analysis and manuscript preparation.

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Conflict of Interest

The authors declare no conflict of interest.

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