Combined cues of male competition influence spermatozoal investment in a moth

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Abstract
1. Male animals usually raise their sperm allocation after detecting sperm competition risk. To date, only a few studies have investigated the cues used by males to sense and respond to rivals. Yet, it is still largely unknown whether males respond to single or combined cues and whether they can increase their lifetime spermatozoal investment after a perception of rival cue(s).

2. Here we postulate that males increase ejaculation and production of sperm after detecting combined cues from rivals, but such response quickly diminishes after the cues are removed. We exposed newly emerged and virgin focal males of the moth *Ephestia kuehniella* to various rival cues and then permanently removed the cues. We introduced a virgin female to an exposed focal male and an unexposed focal male, respectively, per day and counted the number of sperm transferred by the focal males in each mating and recovered in their body after death.

3. We demonstrate that males significantly increased their lifetime sperm allocation and production after premating detection of either single (acoustic or chemical) or combined cues (acoustic + chemical or acoustic + chemical + tactile) from their rivals with combined cues (acoustic + chemical + tactile) somewhat strengthening such response in eupyrene production.

4. The number of sperm ejaculated by males significantly decreased over successive matings in their lifetime regardless of whether they were exposed to rival cues, but the decline was significantly faster in rival-cue-exposed males than in unexposed ones. This suggests that the increase in spermatogenesis cannot fully compensate for that of sperm expenditure in response to rival cues.

5. We show that 10-hr premating rival exposure was sufficient to maximize males’ response in sperm ejaculation and production. The impact of the rival perception on sperm transfer persisted for most of males’ reproductive life, suggesting that the moth males have a long-term memory of sperm competition risk experienced in the early adulthood.
1 | INTRODUCTION

When males of polygamous species perceive the presence of rivals, they often raise their ejaculate allocation per mate for a higher paternity share (Bretman, Gage, & Chapman, 2011; Gage & Baker, 1991; Parker, 1970; Parker, Ball, Stockley, & Gage, 1997; Parker & Pizzari, 2010; Wedell, Gage, & Parker, 2002). For example, following premating exposure to rivals, the moth Ephestia kuehniella males increase their sperm ejaculation (Esfandi, He, & Wang, 2019). Similar response to rivals has also been reported in many other species such as the butterfly Pieris napi (Larsdotter-Mellström, Eriksson, Janz, Nylin, & Carlsson, 2016), the cricket Teleogryllus oceanicus (Bailey, Gray, & Zuk, 2010) and fruit flies Drosophila melanogaster (Bretman, Fricke, & Chapman, 2009; Rouse, Watkinson, & Bretman, 2018) and D. pseudoobscura (Maguire, Lizé, & Price, 2015). However, sperm are not cheap (Dewsbury, 1982; Pitnick & Markow, 1994; Savalli & Fox, 1999; Xu & Wang, 2009a) and socio-sexual surroundings are fluctuating rapidly in nature (Bretman, Gage, et al., 2011; Pizzari, 2017). It would thus be important for males to detect the cues that carry correct information on their current and future sperm competition environment and to adjust their sperm allocation accordingly in a timely manner.

Animals use acoustic, olfactory, tactile and/or visual cues to communicate for various purposes (Alcántara-Alcover, Artacho-Ramirez, Zamora-Álvarez, & Martínez, 2014; Cocroft & Rodríguez, 2005; Frommen, 2020; McKinney, Vernier, & Ben-Shahar, 2015; Romer & Lewald, 1992; Schiestl, 2010; Sweeney, Jiggins, & Johnsen, 2003; Tunstall & Warr, 2012; Yew et al., 2009). Many animal species need to detect more than one cue simultaneously before responding to a social environment (Acquistapacca, Aquiloni, Hazlett, & Gherardi, 2002; Bro-Jørgensen, 2010; Gray, Bailey, Poon, & Zuk, 2014; Partan & Marler, 1999; Uetz & Roberts, 2002; Zabierek & Gabor, 2016). In some cases, combined cues can trigger a stronger response (Partan & Marler, 1999). So far, only a few studies have investigated the cues used by males to detect rivals and to react. For example, males may use either chemical (Aragón, 2009; Carazo, Font, & Alftan, 2007; delBarco-Trillo & Ferkin, 2004; Larsdotter-Mellström et al., 2016) or acoustic (Bailey et al., 2010; Rebar & Greenfield, 2017) cues from their rivals to perceive and respond to sperm competition environment. However, to respond to socio-sexual situations, D. melanogaster males need to detect two of acoustic, chemical and tactile cues from rivals (Bretman, Westmancoat, Gage, & Chapman, 2011) and D. pseudoobscura males require both chemical and tactile cues (Maguire et al., 2015). Both studies suggest that males do not respond to single cues and visual cues are not important in rival detection. Yet, in most mating systems, it is unclear whether individual rival cues can cause a response to sperm competition environment and whether combined cues can enhance the response.

Findings from D. melanogaster demonstrate that the impact of a rival exposure may quickly diminish (Bretman, Westmancoat, Gage, & Chapman, 2012; Mohorianu et al., 2017; Rouse & Bretman, 2016) after the removal of sperm competition risk. This suggests that either the fly males can rapidly adjust their sperm allocation in response to changes of sperm competition environment or they only have a short memory of an exposure to rivals. In the moth E. kuehniella, males do not adjust their sperm allocation if they have no premating exposure to rivals but are subject to the presence of rivals from the first mating until death (Esfandi, He, & Wang, 2015). However, when the moth males have both premating and lifetime exposure to rival cues, they raise their sperm ejaculation for most of their reproductive life (Esfandi et al., 2019). It is still unknown whether moth males have a long memory of the premating rival experience or they also rapidly reduce their sperm allocation after the removal of premating rival cues.

Most studies on the impact of sperm competition environment on sperm allocation strategies have only tested the first mating following an exposure to a particular socio-sexual setting (e.g. Bretman et al., 2009; Garbaczew ska, Billiter, & Levine, 2013; Jarrige, Riemann, Goubauld, & Schmoll, 2015; Price, Lizé, Marcello, & Bretman, 2012; Ullah, Sugimoto, Kongchuesin, Konvipasruang, & Gotoh, 2017; Wibly et al., 2009). To date, only a few studies have examined the first few successive matings (Bretman et al., 2012; Larsdotter-Mellström & Wiklund, 2015; Rouse & Bretman, 2016; Wylde, Crean, & Bonduriansky, 2020). These studies may determine whether males increase allocation of ‘ready’ sperm for one or a few matings by accelerating the last stages of sperm maturation, a phenomenon called sperm priming (Bozynski & Liley, 2003; Cattelan & Pilastro, 2018; Chung, Jennions, & Fox, 2019). The sperm priming process is different from spermatogenesis which needs longer period and occurs before sperm priming in each mating (Evans, 2009). Therefore, to demonstrate whether sperm production also increases after exposure to rivals, we need to count the total number of sperm produced in the lifetime of both rival-exposed and unexposed males, including all sperm that are ejaculated and that are recovered in their body after death.

Traditional knowledge on sperm production reveals that most lepidopteran insects stop producing eupyrenes (fertile sperm) after pupation (Friedländer, 1997; Lachance & Olstad, 1988; review in Friedländer, Seth, & Reynolds, 2005) but several recent studies on lepidopterans indicate that spermatogenesis continues into the adult stage in response to certain stimuli such as larval diapause (Bebas, Cymborowski, Kazek, & Polanska, 2018) and...
adult overwintering (Hiroyoshi, Yoshimura, Ibawuchi, Reddy, & Mitsushashi, 2017). Yet, it is unclear whether lepidopteran males might adjust sperm production in response to sperm competition during the adult stage.

The Mediterranean flour moth, E. kuehniella (Lepidoptera: Pyralidae), is an ideal model insect for the study of the function and impact of rival cues on sperm allocation and production because its reproductive behaviour and life-history strategies are well investigated (e.g. Calvert & Corbet, 1973; Corbet & Lai-Fook, 1977; Esfandi, He, & Wang, 2015, 2019; Pérez & Zhantiev, 1976; Xu & Wang, 2009a, 2009b, 2010a, 2010b, 2014, 2020). Adults become sexually mature soon after emergence and mating initiates only in the scotophase, particularly the second half of the scotophase (Xu, Wang, & He, 2008). In the present study, adult males live for 9.4 ± 0.2 days and inseminate 6.1 ± 0.3 females in their lifetime. Males produce and transfer a spermophore into the female’s bursa during copulation (Xu & Wang, 2010a). Similar to other lepidopterans and many flies (Swallow & Wilkinson, 2002; Till-Bottraud, Joly, Lachaise, & Snook, 2005), E. kuehniella males produce both eupyrene sperm that can fertilize eggs and apyrene sperm that cannot fertilize eggs (Xu & Wang, 2010a). Some studies suggest that E. kuehniella males produce an ultrasound to persuade females for mating during courtship (Salehi, Rajabpour, Rasekh, & Farkhari, 2016; Trematerra & Pavan, 1995) but whether the ultrasound also functions as a cue of rivalry is unknown. Furthermore, Barth (1937) and Corbet and Lai-Fook (1977) speculate that E. kuehniella males may release a male courtship pheromone from their hairpencils. However, the existence and function of the pheromone are still unknown for this moth, although similar structures of other lepidopteran species produce male sex pheromones (Mori, Aki, & Kido, 1993; Nishida, Baker, & Roelofs, 1982; Teal & Oostendorp, 1995). Previous work shows that E. kuehniella males can detect rivals with (Xu & Wang, 2014) or without physical contact (Esfandi et al., 2019), suggesting that either acoustic, chemical, tactile or combined cues are used for communications between males.

In the present study, we carried out a series of experiments using E. kuehniella to examine how males responded to single and combined rival cues. Based on the knowledge outlined above, we proposed to test two hypotheses: (a) either acoustic or chemical cue from rivals can trigger males to increase ejaculation and production of sperm but combined cues enhance such response and (b) males’ response to rival cues quickly diminishes after the cues are removed. We exposed newly emerged virgin males to the following rival cues for 10 hr before mating and then removed the cues permanently: (a) acoustic cue only, (b) chemical cue only, (c) combined acoustic and chemical cues, (d) combined acoustic, chemical and tactile cues and (e) no rival cues (control). We offered each rival-cue-exposed and control male a virgin female per day until they died. We dissected each mated female to count sperm ejaculated and each dead male to count sperm remaining in his body at death. These experiments allowed us to record sperm ejaculation per mating and lifetime sperm production in response to single and combined rival cues.

2 | MATERIALS AND METHODS

2.1 | Insects and environmental conditions

We collected E. kuehniella larvae from Turks Poultry, Foxton, New Zealand in December 2018, and maintained them with their original food until adult emergence in the Entomology and IPM Laboratory of Massey University. We introduced about 300 males and 300 females into a transparent plastic cage (28 cm length × 28 cm width × 24 cm height) lined with porous plastic sheets on the bottom for oviposition. We then introduced 232 newly laid eggs (>200 larvae) onto 50 g standard diet (3.0% yeast, 10% glycerine, 43.5% whole meal wheat flour and 43.5% maize meal) in each of 10 transparent plastic cylinders (8 cm diameter × 10 cm height) covered with cloth meshes (2.8 apparatus per mm). We placed a piece of white paper (8 cm diameter) folded four times in the cylinder for pupation. We collected mature pupae from the cylinders, and weighed them using an electronic dual range balance (Mettler Toledo AG135) with an accuracy of 0.00001 g. We categorized pupal weight as light (<mean – 1 SD), average (mean ± 1 SD) and heavy (>mean + 1 SD), and used the adults that emerged from average weight pupae for experiments to minimize the potential effect of body weight. The colony was maintained and all experiments carried out at 25 ± 1°C and 70 ± 10% relative humidity, with a photoperiod of 14:10 hr (light:dark).

2.2 | Premating treatment of focal males

We manufactured a series of devices for premating treatment of focal males (Figure 1). A basic device was made of a transplant plastic cylinder (6.5 cm diameter × 17.0 cm length) covered with an airtight plastic lid at each end and separated into two chambers, the left chamber and the right chamber, by double-layer metal meshes (2.8 apparatus per mm). We made a hole (0.5 cm diameter) in the middle of each lid through which we inserted a plastic Y-tube (0.5 cm diameter) and sealed the gap between the tube and lid using the glue-gun glue. We placed the device horizontally on the bench top during all treatments. The air from a compressed air tap was filtered through activated charcoal, measured with an airflow meter and humidified by passing through distilled water before blowing into the cylinder through one arm of the Y-tube at the left end and out from one arm of the Y-tube at the right end (Figure 1). We set the air speed to replace the air in the cylinder once per minute. We used each device only once to avoid potential contaminations.

We set up five treatments to allow newly emerged and virgin focal males to perceive the following cues from five newly emerged rivals or their extractions before mating: (a) acoustic cue only (+A), (b) chemical cue only (+C), (c) acoustic and chemical cues (+A+C), (d) acoustic, chemical and tactile cues (+A+C+T) and (e) no rivals (CONT). In treatment +A, we introduced a focal male into the left chamber, turned the air tap on and then transferred five rivals...
into the right chamber so that the focal male could hear but not smell or touch the rivals. For treatment +C, we individually placed one focal male and five pieces of filter paper (1.5 cm width \times 5 cm length) containing pheromone extracts from five newly emerged males in the six cells made of the aforementioned metal mesh in the right chamber. This way the focal male could smell but not hear or touch the rival cues. We extracted the male pheromone according to Romel, Scott-Dupree, and Carter (1992) and Stanley, Chandrasekaran, Preetha, and Subaharan (2018). Briefly, we gen
tically clipped the abdominal tip of a newly emerged male (<1 hr old) and excised the three terminal abdominal segments with microscissors. We placed excised segments of five males into a conical glass vial containing 1 ml dichloromethane at 25°C for 1 hr. We then put five pieces of the filter paper into the vial to absorb all supernatant, after which, we removed them from the vial and exposed them to the air for 10 min for dichloromethane to evaporate fully, before placing them in the cells. In treatment +A+C, we transferred six males individually into the six metal mesh cells in the right chamber and used all males as focal males after exposure. This treatment allowed focal males to hear and smell but not touch rivals. In treatment +A+C+T, we introduced six males into the right chamber, allowing them to hear, smell and touch each other, and used all males as focal males after exposure. In CONT, we placed one focal male in the left chamber and none in the right chamber. In treatment +A and CONT, one arm of each Y-tube was blocked with a cork. In treatments +C, +A+C and +A+C+T, one arm of each Y-tube was connected with a silicon tube to facilitate air circulation between the two chambers. Because most mating initiates in the second half of the scotophase (Xu et al., 2008), all focal males were exposed to the rival cue(s) for 10 hr (5 hr before the onset of the scotophase and 5 hr after the onset of the scotophase) prior to the following experiments.

### 2.3 Sperm ejaculation and production

To test the function and impact of rival cues, we made a device consisting of 15 identical mating chambers (transparent plastic cylinders,
6.5 cm diameter × 17.0 cm length) for each treatment. The air from a compressed air tap was filtered, measured and humidified as mentioned above before blowing into the air divider, a large transparent plastic cylinder (15 cm diameter × 20 cm height), from which the air was equally divided into 15 silicone tubes (0.5 cm diameter), each of which was connected to a mating chamber through an airtight plastic lid at one end of the chamber. The air blew out through a hole (1 cm diameter) covered with the aforementioned metal mesh at the other end of the mating chamber. We set the air speed to replace the air in all 15 mating chambers once per minute.

We introduced a 1-day-old virgin female and a focal male into a mating chamber immediately after the focal male’s 10-hr exposure to rival cue(s) or control to allow 5 hr for mating to occur. We removed the female immediately after the termination of copulation and dissected her to count the number of eupyrene and apyrene sperm transferred by the focal male according to Cook and Wedell (1996) and Koudelová and Cook (2001). We then introduced a 1-day-old virgin female per day to the focal male in the mating chamber 5 hr after the onset of the next scotophase until the focal male died. Each mated female was dissected to count the sperm as above. The number of sperm from dissected females was considered the number of sperm ejaculated. We dissected the dead focal male to count the number of eupyrene and apyrene sperm remaining in testes, seminal vesicle and vas deferens. We found sperm from all mated females and dead males. The total number of sperm produced was calculated as the sum of the total number of sperm ejaculated plus the number of sperm recovered from dead males. There were 21, 22, 21, 20 and 22 replicates (focal males) for treatments +A, +C, +A+C, +A+C+T and CONT, respectively.

2.4 | Statistical analysis

All data were normally distributed (Shapiro–Wilk test, UNIVARIATE procedure). In order to test how treatment affected the total number of sperm ejaculated and produced in lifetime, we analysed the data using a linear mixed effect model (MIXED procedure) with the treatment and the number of females a male mated as fixed factors in the model. Because six focal males were in the same device in treatments +A+C and +A+C+T, we also included the replicate identity as a random factor in the model. A CONTRAST statement was applied to perform the multiple comparisons between treatments.

We performed repeated measures analyses using a linear mixed effect model (MIXED procedure) to test whether males’ response to rival cues quickly diminished after the cues were removed. In the analysis, we included treatment, mating frequency and their interaction as fixed factors in the model and a subject effect of focal male in the statement of ‘REPEATED/TYPE = cs SUBJECT = focal_male’ after the model. A CONTRAST statement was then used to compare the slopes of regression lines of sperm ejaculation over successive matings between treatments. Because our data showed that the influence of treatment on the number of sperm ejaculated disappeared after the fourth mating, we compared the number of sperm ejaculated between treatments in each of the first four matings using the CONTRAST statement after removing the mating frequency and interaction factors from the linear mixed effect model.

We analysed the number of eupyrene and apyrene sperm separately. All analyses were done using SAS 9.13.

3 | RESULTS

3.1 | Effects of the number of cues from rivals on focal males’ lifetime sperm ejaculation and production

Our data show that compared to control males, males subject to premating exposure to rival cues ejaculated significantly more eupyrene in their lifetime ($F_{4,79} = 9.77, p < 0.0001$; Figure 2A). Males exposed to rival cues before mating produced significantly more eupyrene sperm in their lifetime than control males ($F_{4,79} = 123.35, p < 0.0001$), with males exposed to all three cues producing the highest number of eupyrenes (Figure 3A). Premating exposure to rivals also triggered
males to ejaculate \( F_{4,79} = 34.34, p < 0.0001 \); Figure 2B) and produce \( F_{4,79} = 127.22, p < 0.0001 \); Figure 3B) significantly more apyrene sperm in their lifetime than control males.

### 3.2 Effects of the number of cues from rivals on focal males’ sperm allocation in successive copulations

The number of eupyrene and apyrene sperm ejaculated by focal males significantly decreased over successive copulations in all treatments and the control \( F_{1,511} = 542.38, p < 0.0001 \) for eupyrenes; \( F_{1,520} = 571.97, p < 0.0001 \) for apyrenes; Figure 4). In comparison of slopes of regression lines, we show that the number of eupyrene sperm ejaculated over time declined significantly faster in rival-cue-exposed males than in control males \( F_{4,101} = 9.45, p < 0.0001 \); Figure 4A). A similar trend was also found in apyrene ejaculation over successive matings \( F_{4,101} = 12.46, p < 0.0001 \) but less obvious (Figure 4B).

Looking into all matings over focal males’ lifetime, we found that the impact of rival exposure on sperm transfer disappeared after the
FIGURE 6 Mean (±SE) number of apyrene sperm ejaculated in the first four matings (A–D, respectively) after a premating exposure to rival cues in male Ephesia kuehniella. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical, and tactile cues. 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 ‘x’ show the median score and means, respectively; the ‘L’ and ‘T’ are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum or maximum scores. For each mating, boxes with different letters are significantly different in mean between treatments (p < 0.05).

fourth mating (p > 0.05). Following the analysis of how treatment affected the number of sperm ejaculated in the first four matings, we reveal that males ejaculated similar number of eupyrenes in their first mating irrespective of whether or not they were exposed to rival cues ($F_{4,101} = 1.13, p = 0.3468$; Figure 5A). Regardless of the type and number of rival cues to which males were exposed, they transferred significantly more eupyrenes to their mates than the unexposed control in the second, third and fourth matings ($F_{4,96} = 9.01, p < 0.0001$ for the second mating; $F_{4,91} = 3.71, p = 0.0076$ for the third mating; $F_{4,78} = 6.42, p = 0.0002$ for the fourth mating; Figure 5B–D). Similar patterns occurred for apyrene ejaculation in the second, third and fourth matings ($F_{4,96} = 9.64, p < 0.0001$ for the second mating; $F_{4,91} = 6.26, p = 0.0002$ for the third mating; $F_{4,79} = 5.17, p = 0.0009$ for the fourth mating; Figure 6B–D) but males exposed to single cues from rivals (acoustic or chemical) also transferred significantly more apyrenes in their first mating ($F_{4,101} = 4.47, p = 0.0023$; Figure 6A).

4 | DISCUSSION

The present study indicates that E. kuehniella males increased their lifetime sperm transfers following a premating exposure to either individual (acoustic or chemical) or combined (acoustic + chemical or acoustic + chemical + tactile) cues from rivals (Figure 2). In contrast, D. melanogaster males respond to the presence of rivals after detecting any two of the acoustic, chemical and tactile cues from rivals (Bretman, Gage, et al., 2011) while for the same reaction to occur, D. pseudoobscura males require combined chemical and tactile cues from rivals (Maguire et al., 2015). The findings from the moth and flies hitherto suggest that the type and number of cues required for male insects to detect and respond to their rivals may have evolved in response to ecological and physiological differences between species across orders. Because acquisition and processing of information from the surroundings often involve costs in energy and time, animals should be selected to make their decisions based on the trade-off between the costs and the risk of making wrong decisions (Schneebberger & Taborsky, 2020). Fruit fly adults often live in aggregation, continue to feed and have a long longevity (Partridge & Farquhar, 1981). Most activities including mating occur in the morning and dusk (Allada & Chung, 2010; Cusumano et al., 2009). These features suggest that the fly adults would detect a lot of noise from their social environment, need multiple cues from rivals before making correct decisions on sperm allocations and have enough resources in terms of energy and time to process multiple cues. However, adults of many moth species, such as E. kuehniella, live solitarily, mate during the night, feed little and have a short longevity. It would thus be advantageous for moth males to make decisions upon detecting any one cue from the rivals.

In many insect species, spermatogenesis initiates in immature stages and continues into the adult stage (e.g. Kuroda, 1974; Malawey, Mercati, Love, & Tomberlin, 2019; Ponlawat & Harrington, 2007). However, various studies suggest that lepidopteran males often stop producing eupyrene sperm after pupation (Chaudhury & Raun, 1966; Friedländer, 1997; Lachance & Olstad, 1988; Witalis & Godula, 1993; review in Friedländer et al., 2005; Mari, Gigliolli, Nanya, & Portela-Castro, 2018). There are a few exceptions, though, for example, in the moths Calpodes ethlius (Lai-Fook, 1982), Achroia grisella (Fernandez-Winkller & Cruz-Landim, 2008) and Galleria mellonella (Bebas et al., 2018) and a butterfly Polygonia c-aureum (Hiroyoshi et al., 2017), spermatogenesis still occurs during the adult stage following certain stimuli such as larval diapause (Bebas et al., 2018) and adult overwintering (Hiroyoshi et al., 2017). Prior to the present study, it was not clear whether adult moth males might adjust sperm production reacting to experience in sperm competition during the adult stage. Through examining the total number of sperm ejaculated during focal males’ lifetime and recovered from dead focal males, we demonstrate that rival-exposed E. kuehniella adult males increased lifetime sperm production after exposure to rivals during the early adulthood (Figure 3). This finding strongly suggests that sperm competition risk can stimulate spermatogenesis during the adult stage in a lepidopteran species.

Our data indicate that while a single cue causes an increase in eupyrene investment (Figures 2 and 3), combined cues (+A+C+T) seem to strengthen the response in eupyrene production (Figure 3A). Studies on other animals such as Drosophila spp. (see results in Bretman, Gage, et al., 2011; Maguire et al., 2015) and the spider Schizocosa ocreata (Uetz, Clark, Kane, & Stoffer, 2019) also suggest that combined cues may enhance males’ response to sperm competition environment. According to the backup signal hypothesis, the receivers should obtain more certain information on their socio-sexual environment and adjust their resource allocation to
reproduction with more confidence because detection of increasing number of cues carrying the same message may have synergistic impact on males’ response (Dore et al., 2018; Partan & Marler, 1999). However, there is no evidence that combined cues could enhance production of apyrene in their lifetime (Figure 3B), probably because apyrenes play relatively minor roles in sperm competition (Konagaya & Watanabe, 2015; Mongue, Hansen, Gu, Sorensen, & Walters, 2019; Sakai et al., 2019; Thorburn, Knell, & Parrett, 2018).

We show that the number of sperm ejaculated by focal males decreased over successive matings in all treatments and the control (Figure 4). Because E. kuehniella adults do not feed, our findings fit the model on reproductive output declines with age of adults having fixed resources obtained during the immature stages (Begon & Parker, 1986). Furthermore, males in many different taxa may also suffer from a reduction in the quantity of their sperm with age regardless of whether adults feed (Fricke & Maklakov, 2017; Vega-Trejo, Fox, Iglesias-Carrasco, Head, & Jennions, 2019). However, the decline in eupyrene sperm transfer over time went faster in all treatments than in the control although apyrene decline rate in two treatments was similar to that in the control (Figure 4). The faster decrease in sperm transfer in treatments could result from significantly more sperm expenditure during the first few matings (Figures 5 and 6). We suggest that both sperm priming and production are involved in the process, but the increase in spermatogenesis is not enough to fully compensate for that of sperm expenditure.

After 10-hr premating exposure (Figures 5 and 6) or 24-hr premating + lifetime exposure (Esfandi et al., 2019) to rival cues, E. kuehniella males allocated significantly more sperm in their first few matings. This indicates that 10-hr exposure is enough to trigger males to maintain raised sperm allocation for most of their reproductive life (first four matings) where they ejaculate about 60% of their lifetime sperm (present study; Esfandi et al., 2019). Our findings support the notion that insects’ brain has a long memory of an exposure to a socio-sexual environment (Dion, Monteiro, & Nieberding, 2019). However, D. melanogaster males maintain their response to sperm competition risk for 1 and 12 hr following 24 and 36-hr premating exposure to rival cues, respectively (Rouse & Bretman, 2016), suggesting that the fly brain can control both short and long memory periods (Guven-Ozkan & Davis, 2014) and exposure period is important for the duration of memory. Rouse et al. (2018) explain that plasticity should allow a male to react to rapid changes in the sperm competition environment through short-term memory and guard against reversion of behaviour when sperm competition risk in the vicinity is still high after the immediate risk has been removed.

We propose that the difference in male longevity and lifetime mating frequency between the fly and the moth may underlie the discrepancy in rival exposure period and memory duration. E. kuehniella males live for an average of 9 days and inseminate an average of six females in their lifespan (present study) while D. melanogaster males survive for about 60 days and inseminate >60 females in their lifetime (Partridge & Farquhar, 1981). For insects whose adult males live a long life and mate many times, such as D. melanogaster, it would be advantageous to regulate both short and long memory in response to rapid dynamics of socio-sexual situations (Rouse et al., 2018). However, short-lived insects whose males can only mate a few times in their lifespan, such as E. kuehniella, may have limited room to change and reverse their resource allocation rapidly in response to sperm competition levels. Therefore, they would benefit from long memory of a rival exposure. Furthermore, E. kuehniella has limited dispersal ability (Rees, 2004) and thus sperm competition environment is less likely to change rapidly. As a result, it should be relatively safe for males to maintain their response to the sperm competition level detected in their early adulthood.

Our findings demonstrate that the focal males ejaculated similar number of eupyrenes in their first mating in all treatments and the control (Figure 5) while Esfandi et al. (2019) reveal that males ejaculated significantly more eupyrenes in their first mating after exposure to rival cues. We attribute the divergence of these two studies to the duration between rival cue detection and sperm ejaculation. In Esfandi et al. (2019), it was more than 26 hr (24-hr exposure to rivals + mating latency) while in the present study it was less than 13 hr (10-hr exposure to rivals + mating latency). Because males constantly release sperm from testes into vas deferens and then into the sperm storage site, the duplex (e.g. Proshold, 1991; Thorson & Riemann, 1977), the newly and increasingly produced sperm after detection of rival cues would take time to arrive at storage site. Therefore, the number of sperm at the duplex at the first mating should be greater in Esfandi et al. (2019) than in the present study and males just ejaculate what they have in the storage after detecting the rival cues. However, the number of apyrenes ejaculated (Figure 6) at the first mating was not as consistent as that of eupyrenes. The reasons behind are not clear.

In the present study, we have tested how focal males of a moth respond to single and combined cues from rivals and discussed ecological implications in relation to dynamics of socio-sexual environment. We conclude that (a) males raise their sperm allocation and production after detecting either acoustic or chemical cues from their rivals with combined cues sometimes strengthening such response, and (b) males can remember the sperm competition risk for most of their reproductive life following one premating exposure to rival cues.

**ACKNOWLEDGEMENTS**

We thank Kay Sinclair for her technical assistance. We particularly thank the Senior Editor Dr Charles Fox and the Editor Dr Christine Miller and two anonymous reviewers for their constructive comments and suggestions, which have improved the paper significantly. This work was supported by a China Scholarship Council-Massey University PhD Scholars Programme (CSC No. 201806660018) to J.L., a Guangxi University Foundation Strengthening Program Postgraduate Overseas Research Project to Y.Z., a Guangxi Scholarship Fund of Guangxi Education Department to X.-L.Z., and Massey University Research Funds to Q.W. and X.Z.H.


