

## Article

# Sperm allocation strategies in a sperm heteromorphic insect

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Handling editor: Anders Møller

Received on 4 June 2019; accepted on 29 September 2019

## Abstract

Theories predict that in polyandrous species, the focal male should increase sperm allocation per mate in the presence of rivals to gain greater share of paternity, but in the presence of additional mates, he should reduce sperm allocation per mate to save sperm for insemination of more mates. However, empirical findings are often inconsistent and reasons behind are unclear. Furthermore, many studies use copulation duration as an estimate of the number of sperm transferred. Yet, empirical evidence for such assumption is largely lacking. Here, we used a sperm heteromorphic insect *Ephestia kuehniella* whose males produce two types of sperm, eupyrenes (fertile) and apyrenes (nonfertile), to test these postulations. We allowed focal males to detect chemical and acoustic but no tactile cues from rivals or additional mates both before and during mating and measured copulation duration and sperm allocation in successive copulations. We demonstrate that males transfer significantly more eupyrenes per mate in the presence of rivals and that the sperm allocation pattern persists in successive copulations under this condition. However, males do not adjust apyrene allocation in response to rivals probably because apyrenes play a relatively minor role in male reproductive success. Contrary to a previous study, focal males do not respond to additional mates most likely due to the lack of tactile cues in the present study. We reveal that sperm allocation is not a function of copulation duration in this insect for spermatophore formation and delivery occupy most of copulation duration and sperm transfer is complete near the end of copulation.

**Key words:** Sperm allocation, sperm competition, copulation duration, socio-sexual environment

Animals with sexual reproduction are predicted to adjust their physiology and behavior in response to their socio-sexual environments (Wilson et al. 2014) because the dynamics of such surroundings may significantly affect their fitness (Mohorianu et al. 2017). Over the past few decades, two prominent models have been developed to predict how and why male animals react to different socio-sexual settings. The sperm competition model (Parker 1970; Parker et al. 1997, 2013; Parker and Pizzari 2010) envisages that a focal male should increase sperm allocation per mate in the presence of rivals to gain greater share of paternity. The sperm economy model (Wedell et al. 2002; Abe and Kamimura 2015), on the other hand,

predicts that in the presence of additional mates (further copulation opportunity), the focal male should reduce sperm allocation per mate to save sperm for insemination of more mates. Various studies have attempted to test these two models with mixed findings (e.g., Garbaczewska et al. 2013; Xu and Wang 2014; Esfandi et al. 2015; Sal Moyano et al. 2016; Kelly and Gwynne 2017; Pardo et al. 2018) but reasons behind discrepancies between studies are often unclear.

In the study of sperm competition, sperm competition risk refers to the probability for a focal ejaculate to compete with at least one rival ejaculate while sperm competition intensity relates to competition between different ejaculates within a focal female (Parker and

Pizzari 2010). The number of males nearby (sperm competition risk) is thus different from the number of competing ejaculates (sperm competition intensity) and introduction of more males to a focal male should not affect sperm competition intensity (Engqvist and Reinhold 2005). So far, there is no evidence that ejaculate size changes when the number of rivals nearby is more than one (Bretman et al. 2010; Kelly and Jennions 2011). Furthermore, *Ephesia kuehniella* males transfer similar number of sperm to virgin and once copulated females, suggesting that males do not adjust ejaculate allocation based on sperm competition intensity in this species (Esfandi et al. 2015). In the present study, we manipulated socio-sexual environments to test the effects of sperm competition risk and probability of further copulation on sperm allocation.

Many investigations into the impact of socio-sexual environments on male investment strategies have only examined the first copulation following an exposure to a particular socio-sexual situation (e.g., Wang et al. 2008; Bretman et al. 2009; Wigby et al. 2009; Price et al. 2012; Garbaczewska et al. 2013; Jarrige et al. 2015; Ullah et al. 2017) probably due to logistical constraints. Evidence from *Drosophila melanogaster* suggests that the impact of such exposure may quickly diminish (Bretman et al. 2012; Rouse and Bretman 2016; Mohorianu et al. 2017) after the removal of the socio-sexual setting. Yet, it is not clear whether the impact on sperm allocation strategies can persist over successive copulations following both pre- and during-mating exposure to the socio-sexual situation. Knowledge generated from such study would help us understand sperm allocation strategies and develop ways for potential manipulations of these strategies.

Various studies on sperm competition assume that copulation duration and the number of sperm transferred are positively correlated (e.g., Alonso-Pimentel and Papaj 1996; Prokop and Vaclav 2005; Wang et al. 2008; Mazzi et al. 2009; Wigby et al. 2009; Bretman et al. 2009, 2010, 2012, 2014; Mason et al. 2016; Rouse and Bretman 2016). However, empirical evidence for such relationship is rare (Garcia-Gonzalez and Gomendio 2004). Many studies have raised doubt about the above assumption (e.g., Gilchrist and Partridge 2000; Hosokawa and Suzuki 2001; Schofl and Taborsky 2002; Lüpold et al. 2011; Weir et al. 2011; Price et al. 2012; Garbaczewska et al. 2013; Jarrige et al. 2015, 2016). These disagreements may be associated with whether a spermatophore (a protein capsule containing a mass of seminal fluid and sperm) is formed during copulation, whether sperm transfer occurs throughout copulation or only in some stage of copulation, and whether males prolong copulation as a form of postcopulatory mate-guarding. Therefore, understanding the mechanisms of sperm transfer can help determine the functions of copulation duration.

The Mediterranean flour moth, *Ephesia kuehniella* (Lepidoptera: Pyralidae), is a well-established model for studying sperm allocation strategies in response to socio-sexual environments. For example, adults may communicate using chemical, acoustic, or tactile cues (Calvert and Corbet 1973; Pérez and Zhantiev 1976; Corbet and Lai-Fook 1977; Trematerra and Pavan 1995; Xu and Wang 2014; Esfandi et al. 2015), allowing males to perceive the presence of nearby conspecific adults with or without physical contact. Both sexes copulate multiply during their lifetime (Xu and Wang 2009a, 2009b). The male produces and transfers a spermatophore into the female's bursa during copulation (Xu and Wang 2010a), and the last male that copulates with a mated female has sperm precedence (Xu and Wang 2010a, 2010b). Like other lepidopterans and many flies (Swallow and Wilkinson 2002; Till-Bottraud et al. 2005), *E. kuehniella* males produce two types of sperm, apyrenes that have a nucleus and can fertilize eggs, and

apyrenes that have no nucleus and cannot fertilize eggs (Xu and Wang 2010a).

The function of apyrene sperm in insects is still under debate. For example, Cook and Gage (1995) and Cook and Wedell (1999) propose that they act as cheap filler to deceive females about their sperm load and delay female receptivity. However, recent work by Konagaya and Watanabe (2015) and Thorburn et al. (2018) does not support the cheap filler hypothesis. In a latest study, Sakai et al. (2019) demonstrate that apyrene sperm are necessary for migration of eupyrene sperm from the bursa to the spermatheca. Through examination of the first copulation, Xu and Wang (2014) reported that focal males adjust allocation of both types of sperm as predicted by sperm competition and sperm economy models when all three cues (chemical, acoustic, and tactile) from rivals or additional mates are present during copulations. However, focal males do not alter allocation of either type of sperm if they only detect chemical and acoustic cues from conspecific adults during copulations (Esfandi et al. 2015). So far, it is unknown whether the pre- and during-mating exposures to the non-tactile cues trigger focal males to change their sperm allocation strategies in the first and subsequent copulations. It is also unclear whether sperm allocation is a function of copulation duration in this species.

In the present study, we investigated whether and how socio-sexual environments affected sperm allocation in *E. kuehniella* by exposing focal males to conspecific adults without physical contact for 24 h before copulation and then during successive copulations. Because *E. kuehniella* males ejaculate >60% of their lifetime sperm of both types in their first four copulations regardless of socio-sexual environments (Esfandi 2017), we tested sperm allocation patterns and copulation durations in each of their first four copulations. We hypothesize: 1) the pre- and during-mating noncontact exposure to rivals elicits focal males to increase sperm allocation per mate and the same exposure to additional mates causes focal males to reduce sperm allocation per mate over four successive copulations, and 2) sperm allocation is a function of copulation duration.

## Materials and Methods

### Insect colony maintenance and environmental conditions

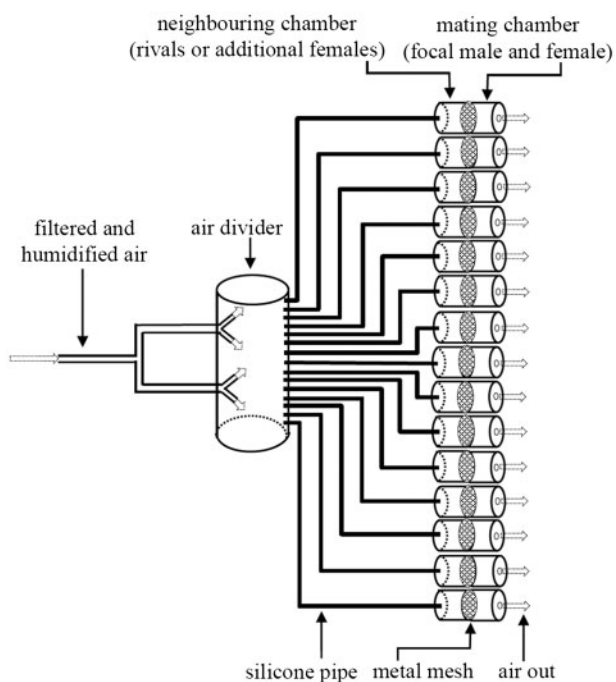
Newly emerged adults from >500 larvae, collected in Foxton, New Zealand, were maintained in 15 transparent plastic cylinders (8 cm diameter × 10 cm height) for egg laying. The cylinder was covered with a plastic lid that had a hole (3 cm diameter) in the middle covered with two layers of cloth mesh (2.8 aperture per mm). Eggs laid between the second and fifth oviposition days were collected daily from cylinders and transferred into Petri dishes (8.5 cm diameter × 1.5 cm height) for hatching. Two-hundred newly hatched larvae collected from Petri dishes were transferred into an aforementioned cylinder containing 50 g of a standard diet (43.5% wholemeal wheat flour, 43.5% maize meal, 3.0% yeast, and 10% glycerine). Two crumpled paper towels (25 × 25 cm) were placed into each cylinder for pupation. Fifteen cylinders were set up. Mature pupae were collected from the paper towels, sexed, and weighed individually using an electronic balance (Mettler Toledo AG135, Switzerland) with a readability of 0.00001 g. Pupal weight was categorized into three groups: light (<mean - 1 SD), average (mean ± 1 SD), or heavy (>mean + 1 SD). To minimize the impact of body weight on copulation duration and sperm ejaculated, we randomly selected pupae from the average body weight category for experiments. They were then individually kept in glass vials (2 cm diameter × 7.5 cm height)

for adult emergence. All adults were virgin and one day old at the beginning of the experiments.

The breeding colony was maintained and experiments were conducted at  $25 \pm 1^\circ\text{C}$  and  $60 \pm 10\%$  relative humidity with a photoperiod of 14: 10 h (L: D). All experiments were carried out during the scotophase and a red light (Sylvania, F36W/Red, Holland) was used for illumination.

### Experimental setup and data collection

For each treatment (see below), we used a device consisting of 15 identical experimental containers and an air divider for experiments (Figure 1). Each experimental container was made of two identical transparent plastic cylinders (8 cm diameter  $\times$  10 cm length) connected to each other with a Parafilm on external walls. The two cylinders were separated with a metal mesh (2.8 apertures per mm) in between, allowing free air movement but preventing insects in two separate cylinders from physical contact. One cylinder was used as a mating chamber for keeping focal insects and another as a neighboring chamber for accommodating rival males or additional females. The mating chamber had a lid at the end with a hole (3 cm diameter) in the middle that was covered with a fabric (cloth) mesh (2.8 apertures per mm). The air from a compressed air tap was filtered through activated charcoal, measured via an airflow meter, humidified by passing through distilled water, and then blown into the air divider, a bigger transparent plastic cylinder (15 cm diameter  $\times$  20 cm height), from which the air was equally divided into 15 silicone pipes (0.5 cm diameter), each of which was connected to a neighboring chamber. The air was blown through the neighboring chamber to the mating chamber and then out through the hole at the end of the mating chamber. Therefore, the insects in the mating chamber could perceive the presence of insects in the neighboring chamber by chemical and acoustic cues without physical contact. The air speed was set to allow the air in all 15 experimental containers to be replaced once per minute.



**Figure 1.** A diagram of the experimental device.

We set up three treatments using the aforementioned experimental device: 1) male-biased (+M)—one focal male and one focal female in the mating chamber and five males in the neighboring chamber, with the focal male having been exposed to five males in the neighboring chamber for 24 h before paired with the focal female; 2) female-biased (+F)—one focal male and one focal female in the mating chamber and five females in the neighboring chamber, with the focal male having been exposed to five females in the neighboring chamber for 24 h before paired with the focal female, and 3) control (CONT)—one focal male and one focal female in the mating chamber and no insect in the neighboring chamber, with the focal male having been kept individually for 24 h before paired with the focal female. Fifteen replicates were performed for each treatment. To avoid the effect of chemical residues left on experimental containers, we made and used three identical sets of 15 containers, each for one treatment.

The focal male treated as above was paired with an individually kept 1-day-old virgin female once a day until his fourth copulation. Pairing started one hour before the onset of the scotophase and each pair was observed continuously for mating behavior until one hour into the photophase. Each replicate was clearly numbered, and the sample identity and treatment level were revealed during data analysis. Copulation duration (period between the connection and disconnection of genitalia) of each of the four matings was recorded, and the focal female was removed from the chamber immediately after termination of copulation and dissected to count the number of eupyrene and apyrene sperm in the spermatophore using the method described by Koudelová and Cook (2001).

### Sperm allocation and copulation duration in response to socio-sexual environments

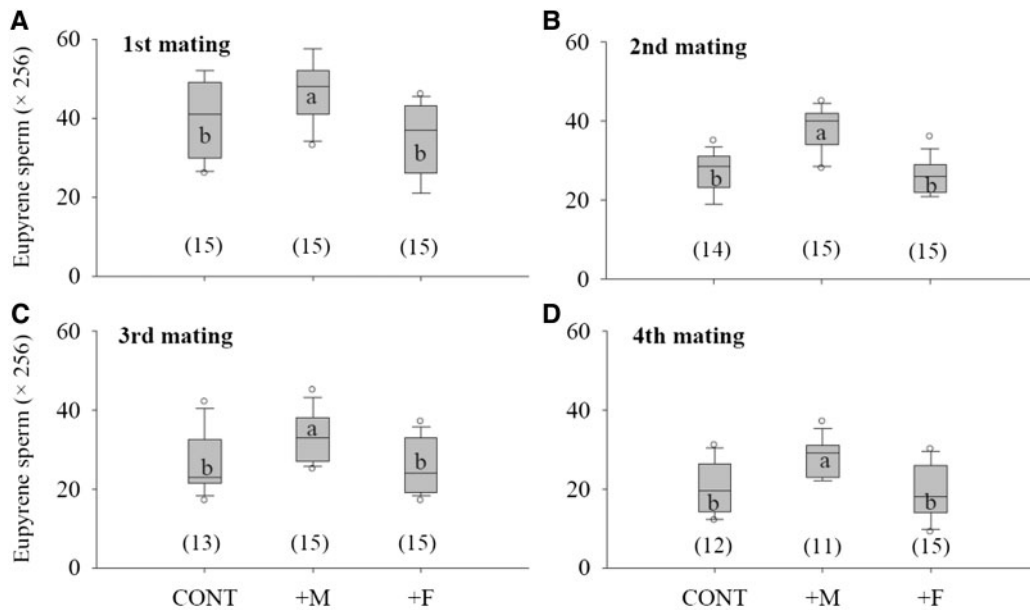
We compared males' sperm allocation and copulation duration in their first four copulations to test whether perceived sperm competition risk (+M) and further copulation opportunity (+F) affected sperm allocation and copulation duration, and whether the effect persisted in successive copulations. We then analyzed the relationship between copulation duration and sperm allocation in the first four copulations to determine whether these two parameters were correlated in any way.

### Statistical analysis

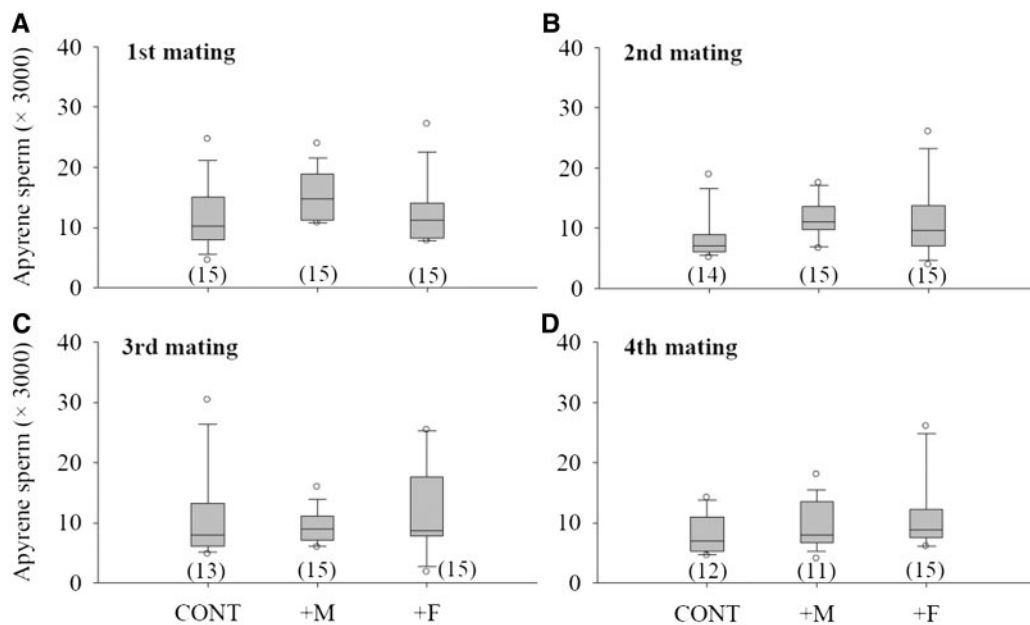
A Shapiro-Wilk test (UNIVARIATE procedure) was used to test the normality of data. Data on sperm transfer and copulation duration (Figures 2, 3 and 5) in each copulation were normally distributed after being  $\ln(x)$  transformed and thus the differences between treatments were analyzed using analysis of variance (ANOVA) followed by a Tukey's post-hoc test for multiple comparisons. A full mixed effect model (MIXED procedure) was used to test the effects of treatment and mating frequency on the number of sperm ejaculated with both factors and their interaction as fixed effects and male ID as a random effect (Figure 4). Those data were also  $\ln(x)$  transformed to achieve normality of data distribution before analysis. A Spearman's rank correlation (RANK procedure and CORR procedure) was applied to detect the relationships between the number of eupyrene and apyrene sperm and copulation duration (Table 1). All data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

### Results

In the present study, most males achieved at least four copulations but a few copulated fewer times. Our results demonstrate that +M males ejaculated significantly more eupyrene sperm than +F and



**Figure 2.** Number of eupyrene sperm ejaculated in the first four matings. For each mating, boxes with the same letters are not significantly different (ANOVA:  $P > 0.05$ ). 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 in a box shows the median score; the '└' and '┘' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum or maximum scores. Numbers in brackets under box plots are sample sizes.



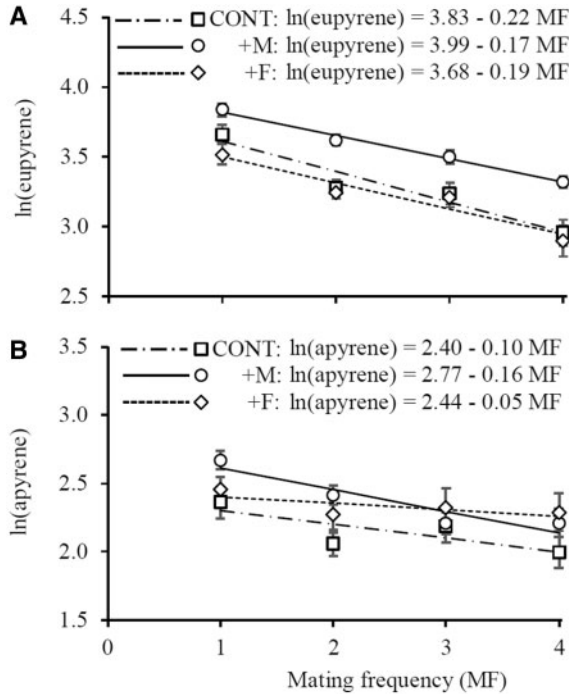
**Figure 3.** Number of apyrene sperm ejaculated in the first four matings. There was no significant difference among treatments (ANOVA:  $P > 0.05$ ). For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line in a box shows the median score; the '└' and '┘' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum or maximum scores. Numbers in brackets under box plots are sample sizes.

CONT males in each of the first four copulations (1st mating:  $F_{2,42} = 7.20$ ,  $P = 0.0020$ ; 2nd mating:  $F_{2,41} = 22.55$ ,  $P < 0.0001$ ; 3rd mating:  $F_{2,40} = 6.42$ ,  $P = 0.0038$ ; 4th mating:  $F_{2,35} = 8.54$ ,  $P = 0.0010$ ) with no significant difference between +F and CONT males in any of the four copulations (Figure 2). However, the mean number of apyrene sperm ejaculated was not significantly different between treatments in any of the first four successive copulations

(1st mating:  $F_{2,42} = 2.78$ ,  $P = 0.0737$ ; 2nd mating:  $F_{2,41} = 2.89$ ,  $P = 0.0672$ ; 3rd mating:  $F_{2,40} = 0.04$ ,  $P = 0.9582$ ; 4th mating:  $F_{2,35} = 1.57$ ,  $P = 0.2231$ ) (Figure 3).

We show that the number of both eupyrene and apyrene sperm significantly decreased with increasing mating frequency (CONT:  $F_{1,38} = 46.18$ ,  $P < 0.0001$  for eupyrene, and  $F_{1,38} = 4.22$ ,  $P = 0.0469$  for apyrene; +M:  $F_{1,40} = 36.87$ ,  $P < 0.0001$  for

eupyrene, and  $F_{1, 40} = 30.62, P < 0.0001$  for apyrene; +F,  $F_{1, 38} = 46.18, P < 0.0001$  for eupyrene) (Figure 4). There was no significant interaction between the treatment and mating frequency ( $F_{2, 222} = 0.79, P = 0.4545$  for eupyrene;  $F_{2, 222} = 1.59, P = 0.2062$  for apyrene), indicating that the sperm decline rate over successive copulations remained the same between treatments (Figure 4).



**Figure 4.** Relationship between the number of sperm ejaculated and mating frequency: (A) eupyrenes ( $\times 256$ ) and (B) apyrene sperm ejaculated ( $\times 3,000$ ). All data were used for analysis but only means ( $\pm SE$ ) were presented.

The mean copulation duration was not significantly different between treatments in any of the first four successive copulations (1st mating:  $F_{2, 42} = 1.48, P = 0.1714$ ; 2nd mating:  $F_{2, 41} = 1.47, P = 0.2426$ ; 3rd mating:  $F_{2, 40} = 1.12, P = 0.3374$ ; 4th mating:  $F_{2, 35} = 1.30, P = 0.2859$ ) (Figure 5). Furthermore, we reveal that copulation duration and eupyrene allocation were not significantly correlated regardless of socio-sexual settings ( $P > 0.05$ ) except for the fourth copulation where copulation duration and eupyrene sperm number in +F were significantly positively correlated ( $P = 0.0444$ ) (Table 1). Similarly, copulation duration and apyrene allocation were not significantly related regardless of socio-sexual settings in any of the first four copulations ( $P > 0.05$ ) (Table 1).

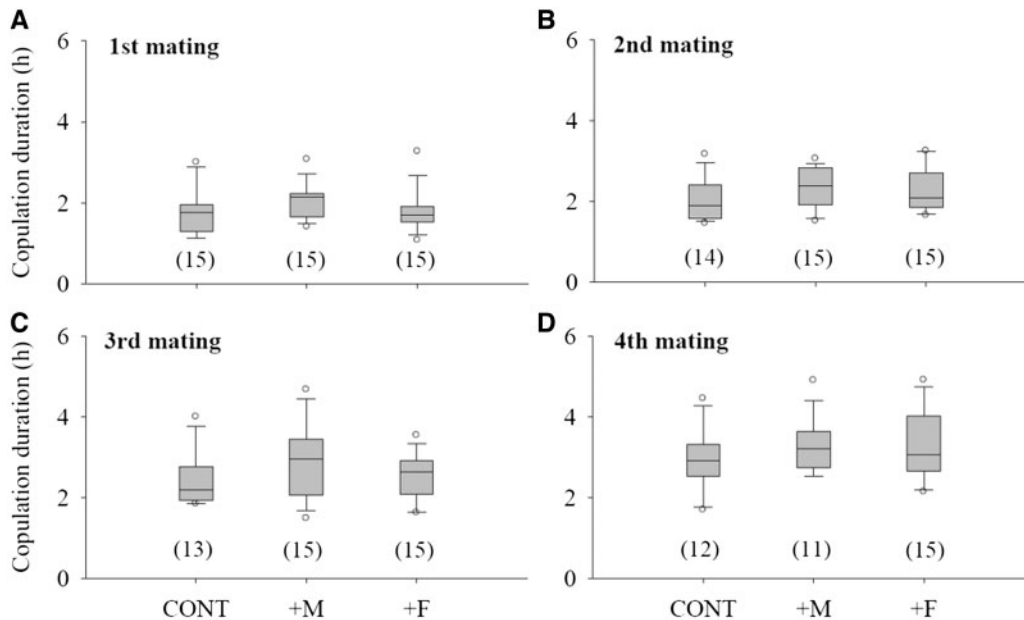
**Discussion**

The present study shows that *E. keuhniella* males allocated more eupyrene (fertile) sperm per mate after pre- and during-mating non-contact exposure to rivals where focal males perceived chemical and

**Table 1.** Spearman’s rank correlation coefficients of eupyrene and apyrene sperm ejaculated in relation to copulation duration

Sperm	Treatment	Copulation duration			
		1st mating	2nd mating	3rd mating	4th mating
Eupyrene	CONT	0.2948 <sup>ns</sup>	-0.1978 <sup>ns</sup>	0.0250 <sup>ns</sup>	0.0140 <sup>ns</sup>
	+M	-0.2901 <sup>ns</sup>	0.4707 <sup>ns</sup>	0.2590 <sup>ns</sup>	-0.0364 <sup>ns</sup>
	+F	0.0546 <sup>ns</sup>	-0.1436 <sup>ns</sup>	-0.1101 <sup>ns</sup>	0.5252 <sup>*</sup>
Apyrene	CONT	0.0018 <sup>ns</sup>	-0.0022 <sup>ns</sup>	-0.1271 <sup>ns</sup>	-0.2281 <sup>ns</sup>
	+M	0.1360 <sup>ns</sup>	0.2645 <sup>ns</sup>	0.0751 <sup>ns</sup>	-0.3242 <sup>ns</sup>
	+F	-0.2359 <sup>ns</sup>	-0.0786 <sup>ns</sup>	0.1360 <sup>ns</sup>	0.5058 <sup>ns</sup>

<sup>ns</sup> not significantly different ( $P > 0.05$ ) and, <sup>\*</sup>significantly different ( $P < 0.05$ ): eupyrene or apyrene sperm number versus copulation duration among treatments in each of the first four copulations.



**Figure 5.** Copulation duration in the first four matings. There was no significant difference among treatments (ANOVA:  $P > 0.05$ ). 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 in a box shows the median score; 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. Numbers in brackets under box plots are sample sizes.

acoustic cues from rivals. However, the focal males do not respond to sperm competition risk if they perceive the same cues during but not before mating (Esfandi et al. 2015). In two other species, lesser wax moth *Achroia grisella* (Jarrige et al. 2015) and *D. melanogaster* (Bretman et al. 2011), males' exposure to at least two of three cues (chemical, acoustic and tactile) from rivals before mating is essential for a response to sperm competition risk to occur. On the other hand, without pre-mating exposure to rivals, males require all three cues during mating to respond to sperm competition risk in *D. melanogaster* (Garbaczewska et al. 2013), *E. kuehniella* (Xu and Wang 2014) and a crab *M. edwardsii* (Pardo et al. 2018). These discoveries strongly suggest that the types of cues males receive from rivals and the time when males detect rivals play an essential role in triggering their response to sperm competition risk across taxa.

Our current data demonstrate that males subject to both pre- and during-mating noncontact exposures to rivals retained the strength of the impact on sperm allocation for most of their reproductive life. The findings in the present study are contrary to those from studies on *D. melanogaster* where the impact of pre-mating exposure to sperm competition risk quickly declines after the rivals are removed (Bretman et al. 2012; Rouse and Bretman 2016; Mohorianu et al. 2017). However, many studies on this topic only examine the first copulation (e.g., Wang et al. 2008; Wigby et al. 2009; Price et al. 2012; Garbaczewska et al. 2013; Jarrige et al. 2015; Ullah et al. 2017), and thus cannot test how long such impact can last in a male's lifetime. Consequently, we still do not know how long the impact of pre-mating exposure to sperm competition risk would remain in other species.

We do not support the notion made in other studies that males transfer more seminal fluid and sperm to increase female fecundity and fertilize more offspring (e.g., Wang et al. 2008; Price et al. 2012; Afaq and Omkar 2017; Ullah et al. 2017). For instance, male *E. kuehniella* do not transfer nutrients to females to increase egg production (Xu and Wang 2009a) and females produce the same number of offspring (fertility) after receiving between 3, 400 and 29, 000 eupyrene sperm from one copulation (Xu and Wang 2009b) into which our current data fall in any given copulation. Furthermore, in a butterfly *Melitaea cinxia* (Duploux et al. 2018) spermatophore size has no effect on female reproductive outputs. Based on these studies, we suggest that the increase of eupyrene is not for production of more offspring *per se*, but to win the sperm competition game because the paternity of offspring is determined by the relative number of competing sperm in females from different males (Parker et al. 1997; Parker and Pizzari 2010).

Contrary to Xu and Wang (2014), the current study did not find any response by focal males to additional mates. The discrepancies between the two studies on the same species may be associated with the types of cues males receive from additional mates, i.e., without tactile cues from additional mates, males do not adjust their sperm allocation in *E. kuehniella* regardless of exposure time. However, in *D. melanogaster* (Garbaczewska et al. 2013) and *M. edwardsii* (Pardo et al. 2018), males do not alter sperm allocation even if they perceive all three cues from additional mates during mating. The reasons behind are unknown. In their study of an orthopteran *Deimacrida rugose*, Kelly and Gwynne (2017) report that males that have noncontact exposure to females before mating increase rather than reduce sperm allocation. They speculate that this might be due to females prolonging copulations when their perceived future mating opportunities are low.

Our current study provides empirical evidence that copulation duration was generally not correlated with the number of either type of sperm transferred regardless of socio-sexual environments.

Although many studies assume that the number of sperm transferred is positively correlated with copulation duration without actually counting the sperm (e.g., Alonso-Pimentel and Papaj 1996; Prokop and Vaclav 2005; Wang et al. 2008; Bretman et al. 2009, 2010, 2012, 2014; Mazzi et al. 2009; Wigby et al. 2009; Mason et al. 2016; Rouse and Bretman 2016), empirical evidence for such relationship is still very limited. For example, golden egg bug *Phyllomorpha laciniata* males increase both copulation duration and sperm transferred in response to sperm competition risk (Garcia-Gonzalez and Gomendio 2004). On the contrary, increasing empirical studies question the generalization about the positive relationship between copulation duration and sperm allocation (e.g., Gilchrist and Partridge 2000; Hosokawa and Suzuki 2001; Schoff and Taborsky 2002; Lüpold et al. 2011; Weir et al. 2011; Price et al. 2012; Garbaczewska et al. 2013; Jarrige et al. 2015, 2016; Duploux et al. 2018). Based on the current and previous studies, we suggest that sperm allocation is not a function of copulation duration in *E. kuehniella* and probably many other species.

The discrepancies between studies on the relationship between copulation duration and sperm numbers may be related to sperm transfer mechanisms in different organisms. For instance, males may prolong their copulations as a form of postcopulatory mate-guarding in response to sperm competition risk because sperm transfer is completed in the early stage of a copulation in *D. melanogaster* (Gilchrist and Partridge 2000), a stink bug *Megacopta punctatissima* (Hosokawa and Suzuki 2001) and a fire bug *Pyrrhocoris apterus* (Schoff and Taborsky 2002). Males of these insects do not form a spermatophore during copulation and they deliver their ejaculate directly to or near the sperm storage site (spermatheca) in females, allowing males to complete sperm transfer in relatively short time and provide them with the opportunity to perform post-insemination mate-guarding (Gilchrist and Partridge 2000; Hosokawa and Suzuki 2001; Schoff and Taborsky 2002), or may permit males to adjust copulation duration according to the number of sperm (Garcia-Gonzalez and Gomendio 2004) or the amount of accessory gland products (Abraham et al. 2011; Pérez-Staples et al. 2014) they transfer. However, copulation in Lepidoptera and many other arthropods involves the formation of a spermatophore, transfer of ejaculate into it, and then delivery of the entire capsule into the bursa, from where sperm move to the spermatheca (Xu and Wang 2010a). This sperm delivery mechanism does not allow males to complete sperm transfer in the early stage of a copulation. For example, in stalk-eyed fly *Cyrtodiopsis whitei* sperm transfer occurs in the second half of a copulation (Lorch et al. 1993). In *E. kuehniella*, a copulation involves a process of making a spermatophore, and completing ejaculate transfer and delivering the spermatophore into the bursa near the end of the copulation (Xu 2010). The lengthy process of sperm delivery involving spermatophore formation may leave little room for copulation duration adjustment or postcopulatory mate-guarding. Rather, males may be able to adjust sperm transfer rate with consistent copulation duration in response to sperm competition risk as reported in Jarrige et al. (2015). This may explain the lack of a relationship between copulation duration and sperm allocation in some insects with spermatophores.

In conclusion, our results strongly support the sperm competition prediction where males increase eupyrene sperm allocation per mate if they are exposed to chemical and acoustic cues from rivals both before and during copulation. We provide strong evidence that the impact of sperm competition risk can persist for most of males' reproductive life under this condition. However, males do not adjust eupyrene sperm allocation in response to the above socio-sexual

setting probably due to the relatively minor role apyrene sperm play in males' reproductive success and strategic resource allocation. The lack of support for sperm economy model is probably because the present study does not allow the focal males to detect tactile cues from additional mates. We reveal that there is no relationship between copulation duration and sperm allocation in this moth, suggesting that males are able to adjust sperm transfer rate in response to sperm competition risk. We propose that understanding the mechanisms behind sperm transfer is important for unveiling the functions of copulation duration.

## Acknowledgments

We thank five anonymous reviewers for their constructive comments on earlier versions, which have significantly improved the article. This work was supported by a Massey University Doctoral Research Scholarship and a Massey University Research Fund.

## Authors' contribution

K.E carried out experiments and collected data. All authors contributed to design, data analysis and preparation of the article.

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