Resource Allocations of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) in Response to Socio-Sexual Environment during Immature and Adult Stages

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Resource Allocations of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) in Response to Socio-Sexual Environment during Immature and Adult Stages

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Abstract

Animals adjust their investment in different life history traits according to their surroundings to maximise their fitness. Using a polygamous insect, the Mediterranean flour moth *Ephestia kuehniella* Zeller, which produces fertile eupyrene and infertile apyrene sperm, I investigated resource allocation strategies employed by males in response to socio-sexual cues during the adult and juvenile stages. I demonstrate that adult males raised their lifetime production and ejaculation of both eupyrenes and apyrenes after detecting either acoustic or chemical cues from adult rivals with combined cues strengthening such response, and that rival-experienced males could remember the sperm competition risk for most of their reproductive life. I manipulated juvenile socio-sexual settings and then examined their sperm production and ejaculation as well as survival, body and testis size, and mating behaviour. I provided the first evidence that juvenile social cues from conspecific larvae, pupae or adults had lasting impacts on lifetime sperm production and allocation. Adults from group-reared larvae, regardless of sex ratio, had smaller testes but produced more eupyrenes at emergence than from singly reared ones, and that body size and apyrene numbers remained the same across treatments. Male pupae had similar testis size but increased production of both eupyrenes and apyrenes at emergence in response to cues from conspecific pupae irrespective of sex. Late instar male larvae were able to detect cues from adult rivals and subsequently produced more sperm of both types at emergence, but adult cues had no effect on body and testis size. Juvenile socio-sexual environment had significant effects on sperm production and ejaculation during adult stage. My study indicates that after their late instar larvae were exposed to juvenile or adult rivals, adults produced and ejaculated more eupyrenes and apyrenes in their lifetime and had shorter mating latency. However, rival exposure had no effect on males' mating frequency and longevity. Knowledge generated here enhances our understanding of how males of a polygamous insect calibrate their resource investment in response to dynamic social environment.

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I would like to dedicate this thesis to my beloved parents.

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CHAPTER 1

General Introduction

1.1 Introduction

A fundamental assumption in most evolutionary theories is that individuals cannot maximise both reproduction and survivorship indefinitely (Kirkwood, 1977; Stearns, 1989; Roff, 2002). As the future survival is uncertain, individuals may prioritise their resource allocation to reproduction, resulting in trade-offs with other life-history traits, such as growth and somatic maintenance (Bonduriansky et al., 2008; Ghalambor et al., 2010; Maklakov & Immler, 2016). These trade-offs are often associated with highly variable environmental conditions, especially the rapidly changing socio-sexual environment (Kasumovic et al. 2008). Through phenotypic plasticity, animals can modulate their reproductive traits, strategies, and investment according to their socio-sexual surroundings throughout development and aging process to maximise their fitness (Pigliucci, 2005; Kasumovic & Brooks, 2011; Taborsky, 2016; Dore et al., 2018; Westneat et al., 2019).

The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is an ideal model species for investigating how individuals adjust their resource allocation to reproduction in response to the socio-sexual environment (e.g., Xu & Wang, 2010a, 2014; Esfandi et al., 2015, 2020). This is owing to the ease of mass-rearing along with its short developmental period, simple diet, early sexual maturation, high levels of promiscuity in both sexes, high female fecundity, polygamy, and non-feeding during adult stage (Calvert & Corbet, 1973; Sedlacek et al., 1996; Rees, 2004; Xu et al., 2007; Xu & Wang, 2009a, 2009b; Sadeghi et al., 2018). Furthermore, as a lepidopteran insect, *E. kuehniella* males produce two types of sperm, infertile apyrenes and fertile eupyrenes (Garbini & Imberski, 1977; Koudelová & Cook, 2001; Friedländer et al., 2005), allowing evaluation of differential resource allocations to these sperm in response to socio-sexual environment.

1.2 Relevance of the study

In nature, animals unintentionally or intentionally generate cues throughout their lifetime (Kasumovic & Brooks, 2011; Dore et al., 2018). Conspecifics detecting those cues can adjust their behaviour and physiology to maximise their fitness (Parker, 1998; Danchin et al. 2004; Parker & Pizzari, 2010; Bretman et al., 2013; Corbel et al., 2022). For example, when males face an increased sperm competition risk, they often increase their resource allocations to reproduction, developing larger testes and producing ejaculates of greater sperm numbers, to raise the probability for a focal ejaculation to compete against at least one rival ejaculation (Parker, 1970; Parker et al., 1997; Vahed & Parker, 2012). Because manufacturing sperm is costly (Dewsbury, 1982; Lemaître et al., 2020), increased investment in sperm may cause trade off with other life traits (Ramm & Stockley, 2009; Devigili et al., 2015; Paschoal & Zara, 2022).

Prior to response to sperm competition risk, animals need to ensure the cues they detect reflect socio-sexual situations accurately and reliably (Auld et al., 2010; Dore et al., 2020). At the adult stage, such detection may involve the input of chemical (delBarco-Trillo & Ferkin, 2004; Lane et al., 2015; Larsdotter-Mellström et al., 2016a) and/or acoustic (Bateman & MacFadyen, 1999; Dunn et al., 2015; Charlton & Reby, 2016; Rebar & Greenfield, 2017) stimuli from their conspecific rivals. For instance, Bretman et al. (2011a) and Maguire et al. (2015) demonstrate that male flies need to sense at least two types of cues from rivals to respond to the sperm competition environment. Yet, in most mating systems it is not clear whether a single rival cue can elicit a response, whether combined cues can strengthen the response, and how these rival cues affect sperm allocation and production in a male's lifetime.

Animals' social experience during their juvenile stages may also influence resource allocations to reproduction and survival (West-Eberhard, 2003; Kasumovic & Brooks, 2011; Taborsky, 2016; Lange et al., 2021). Studies show that male insects increase their investment in testes (Gage, 1995; Stockley & Seal, 2001; Johnson et al., 2017) and sperm allocation in the first mating (Yamane & Miyatake, 2005; McNamara et al., 2010) if their larvae are reared in high density, indicative of future sperm

competition risk. However, it is still not clear (1) how larval and pupal social cues affect investment in testes and sperm production before emergence; (2) how juvenile socio-sexual settings influence lifetime sperm production and allocation, and (3) whether exposure to similar social-sexual settings by different juvenile stages lead to diverse lifetime sperm expenditure.

Conspecific young and adults may co-exist spatially and temporarily (e.g., Harris & Moore, 2005; Chapman et al., 2007; Magellan & Magurran, 2009; Lemaître et al., 2011; Lee et al., 2013; Prounis et al., 2015; Bayoumy et al., 2021; Ham et al., 2022). Therefore, cues from adult males may indicate sperm competition risk to juvenile males, influencing their resource allocations to future growth, reproduction, and survival (Kasumovic & Brooks, 2011; McDowall et al., 2019). To date, only a few studies have examined how the presence of adult rival cues during juvenile stages alters reproductive investment in testes (Bailey et al., 2010; Bretman et al., 2016) and ejaculation in the first mating (Gray & Simmons, 2013; Simmons & Lovegrove, 2017). Nonetheless, it remains to be ascertained whether juvenile sensitivity to adult male cues is stage dependent and whether these cues affect lifetime sperm production and ejaculation.

1.3 Aims and objectives

The aim of my thesis study is to address the above questions and provide insight into adaptive resource allocations by males as responses to the socio-sexual cues present at different life stages using *E. kuehniella* as a model organism, with five objectives:

(1) To test whether and how adult males adjust their investment in lifetime reproduction in response to single (acoustic or chemical) and combined cues (acoustic + chemical, or acoustic + chemical + tactile) from rivals;

(2) To examine whether and how larval social environment affects sperm production and its trade-offs with testis and body size;

(3) To determine whether pupal social environment influences sperm production and testis size;

(4) To investigate whether and how males adjust their investment in lifetime reproduction and other life-history traits in response to juvenile socio-sexual experience; and

(5) To explore whether the adult rival cues present at different larval stages affect testicular investment at emergence and sperm production and allocation patterns in lifetime.

1.4 Literature review

In this section, I review the current knowledge on male reproductive resource allocations in response to socio-sexual environment relevant to my studies on *E. kuehniella*.

1.4.1 General biology of *Ephestia kuehniella*

The taxonomic classification of *E. kuehniella* is:

Order: Lepidoptera

Superfamily: Pyraloidea

Family: Pyralidae

Subfamily: Phycitinae

Genus: *Ephestia*

Species: *kuehniella*

The life cycle of *E. kuehniella* includes four stages: egg, larva, pupa, and adult (Figure 1.1). The eggs are white and oval-shaped (Figure 1.1A) (Brindley, 1930; Kamel, 1969), and hatch within 5 days at 25 ± 1°C, 60 ± 10% relative humidity (Tarlack et al., 2014; personal observation). The newly hatched larvae are cream or pink in

colour and covered with sparse hairs. The testes are visible through the cuticle on the dorsal side of the abdomen from the 4th instar onward (Figure 1.1B) (Brindley, 1930). Larval stage lasts 29–31 days, with six instars (14–15 days for 1st–3rd instars and 15–16 days for 4th–6th instars) (Brindley, 1930; von Gierke, 1932). The width of head capsule can be used to determine larval instars (Athanassiou, 2006). Larvae use chemical and tactile cues for population density regulation (Corbet, 1971; Mudd, 1983). The pupae are pale green at the early stage and become dark about one day before emergence (Karalius & Buda, 1995; Hill, 2002). Male and female pupae can be determined based on the visible reddish-brown testes on male dorsal side of the abdomen (Figure 1.1C). Pupal stage takes 8–9 days (Brindley, 1930), during which time, females emit sex pheromones (Calvert & Corbet, 1973).

Because *E. kuehniella* adults do not feed, this species obtains all resources for reproduction and somatic maintenance during the larval stage (Norris & Richards, 1932). Adults have pale grey bodies with grey-black-zigzag patterns on forewings (Figure 1.1D). Male and female adults live for 7–11 and 6–10 days, respectively (Brindley, 1930; Tarlack et al., 2014). Sex of this moth can be determined by the terminal abdomen with an ovipositor in females and a pair of claspers in males (Figure 1.1E) (Hill, 2002). Males have hairpencils near claspers (Figure 1.1F), which are believed to release a male courtship pheromone (Barth, 1937; Corbet & Lai-Fook, 1977). Males produce an ultrasound when fanning wings (Trematerra & Pavan, 1995; Salehi et al., 2016), which can be detected by nearby conspecific adults (Pérez & Zhantiev, 1976; Salehi et al., 2016). Vision appears negligible in adult communications (Traynier, 1968; Ono, 1981).

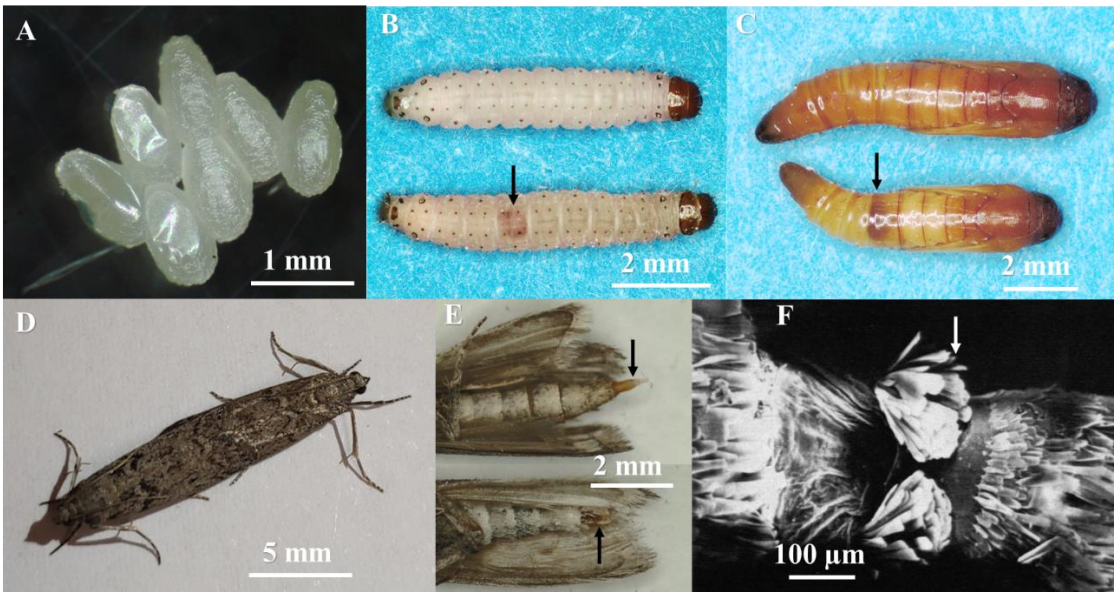

Figure 1.1 Life stages of *E. kuehniella*: (**A**) eggs, (**B**) mature larvae [top, female; bottom, male with visible testes (black arrow) on the dorsal side of abdomen], (**C**) pupae [top, female; bottom, male with visible testes (black arrow) on the dorsal side of abdomen], (**D**) adults (left, female; right, male), (**E**) terminal abdomen of adults [top, female with an ovipositor (black arrow); bottom, male with a pair of claspers (black arrow)], and (**F**) male hairpencils [white arrow; adapted from Corbet & Lai-Fook (1977)].

1.4.2 Reproductive biology of *Ephestia kuehniella* with special reference to males

The moth has multiple overlapped generations year-round (Richardson, 1926). Adults emerge throughout the 24-hr cycle with a peak three hours before the scotophase (Xu et al., 2008), and become sexually mature a few hours after emergence and start mating during the first scotophase (Calvert & Corbet, 1973; Xu et al., 2008). In the lifespan males can mate with up to nine different females (Xu & Wang, 2009a).

The male internal reproductive system mainly comprises a pair of fused testes and a pair of vasa deferentia attached to each testis (Figure 1.2). Each vas deferens consists of a swollen upper portion, a seminal vesicle, and a narrow lower tubular portion. The vasa deferentia empty into the paired duplex, each portion of which is

continuous with an elongate accessory gland. Posteriorly the two portions of the duplex join the unpaired ejaculatory duct or simplex (Norris & Richards, 1932; Riemann et al., 1974).

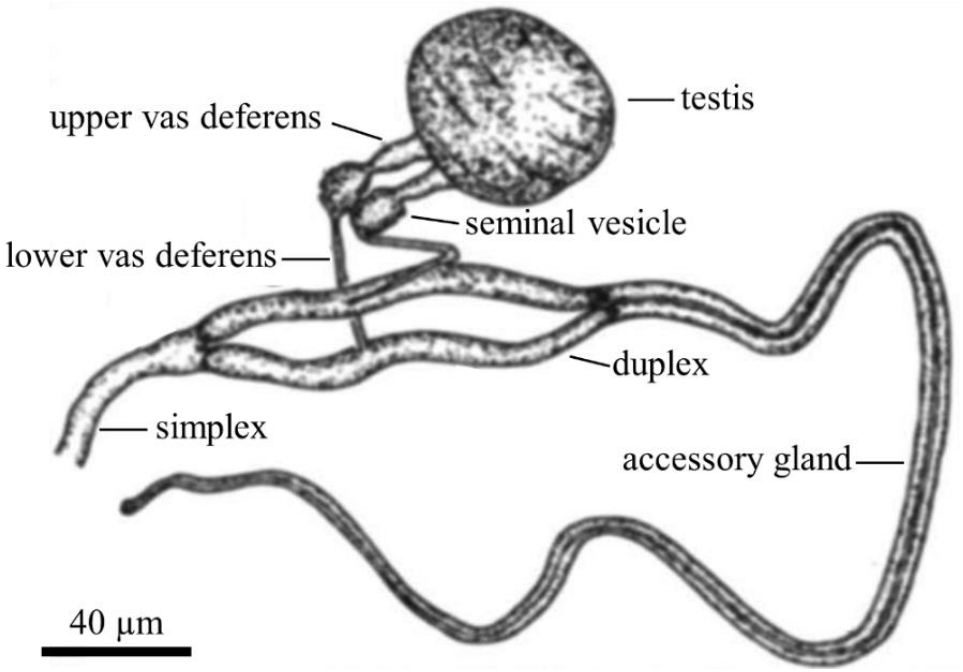

Figure 1.2 Reproductive system of *E. kuehniella* males [Adapted from Riemann et al. (1974)].

The spherical testes with both spermatogenic and non-spermatogenic tissues (Nowock, 1973; Wolf, 1991) start to grow and develop in the early larval stage (Marec et al., 1993). Production of larger fertile eupyrenes (nucleate) and smaller infertile apyrenes (anucleate) (Figure 1.3A) begins in the last larval stage and the early pupal stage, respectively (Garbini & Imberski, 1977). Prior to ejaculation, apyrene sperm bundles disassociate and become motile while eupyrene sperm remain aggregated in bundles of 256 spermatozoa (Garbini & Imberski, 1977; Koudelová & Cook, 2001) (Figure 1.3B).

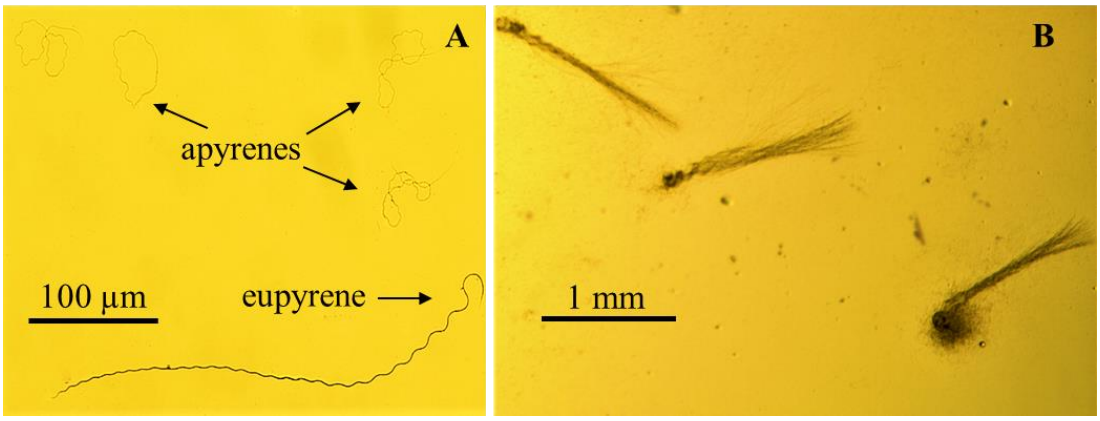

Figure 1.3 Eupyrene and apyrene sperm (**A**), and eupyrene sperm bundles (**B**).

The quantity of sperm available determines the desire for mating (Norris & Richards, 1933). During copulation, males produce a spermatophore (Figure 1.4), transfer ejaculate into it, and then deliver the entire capsule into the female's bursa, from where both types of sperm move to the spermatheca but only eupyrenes can fertilize eggs (Xu & Wang, 2010a) and elicit oviposition (Xu & Wang, 2011). The number of sperm from one mating is more than necessary for fertilization of the full egg load of a female (Trematerra, 1997). This species has the last male sperm precedence (Xu & Wang, 2010a). Males require a 24-hr inter-mating interval to produce another spermatophore (Xu & Wang, 2009b).

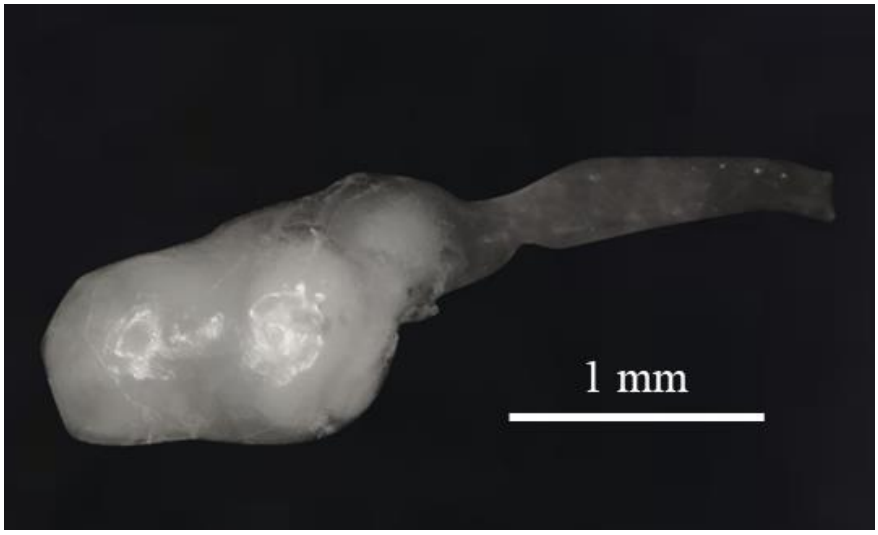

Figure 1.4 Spermatophore produced by a *E. kuehniella* male during mating.

Xu & Wang (2014) report that *E. kuehniella* males increase allocation of both types of sperm to a mate in the first mating when all three cues (acoustic, chemical, and tactile) from rivals are present during copulations. However, males do not adjust allocation of either type of sperm if they only detect acoustic and chemical cues from conspecific adults during copulations (Esfandi et al., 2015). If focal males are allowed to detect acoustic and chemical but no tactile cues from rivals both before and during mating, males ejaculate significantly more eupyrenes but do not adjust apyrene allocation (Esfandi et al., 2020). These discoveries suggest that acoustic, chemical, tactile or combined cues are used for communications between males and these cues affect receivers' sperm allocations to some extent.

1.4.3 Spermatogenesis and sperm dichotomy in Lepidoptera

In Lepidoptera, most species produce two distinct spermatozoa: fertile eupyrenes and infertile apyrenes, except in two species of the primitive Micropterigidae (Sonnenschein & Hauser, 1990; Friedländer et al., 2005). It is long thought that spermatogenesis occurs in immature stages, but males stop producing eupyrenes after reaching adulthood (Chaudhury & Raun, 1966; Lachance & Olstad, 1988; Witalis &

Godula, 1993; Friedländer, 1997; Friedländer et al., 2005; Jarrige et al., 2015; Mari et al., 2018). However, in several lepidopteran species, such as the lesser wax moth *Achroia grisella* Fabricius (Fernandez-Winckler & Cruz-Landim, 2004; Fernandez-Winckler & da Cruz-Landim, 2008), greater wax moth *Galleria mellonella* L. (Bebas et al., 2018) and Asian comma butterfly *Polygonia c-aureum* L. (Hiroyoshi et al., 2017), eupyrene spermatogenesis may continue into the adult stage due to certain stimuli such as larval diapause (Bebas et al., 2018) and adult overwintering (Hiroyoshi et al., 2017). It is yet unknown whether and how socio-sexual experience gained at the adult and/or juvenile stages affects spermatogenesis.

Although apyrene sperm cannot fertilize eggs, males produce and ejaculate significantly more apyrenes than eupyrenes (Silberglied et al., 1984; Swallow & Wilkinson, 2002; Friedländer et al., 2005). Apyrenes are thought to play important roles in the success of sperm competition and fertilization. For instance, they may function as fillers to deceive females about their sperm load, discouraging the females from copulating with other males (Cook & Wedell, 1999; Wedell et al., 2009; Mongue et al., 2019), and assist eupyrenes migration from female bursa copulatrix to spermatheca (Sakai et al., 2019; Chen et al., 2020) via their own active motility or activating motility of eupyrenes (Osanai et al., 1987; Hayashi, 1998). More recent studies suggest that the roles of apyrenes may cease after both types of sperm arrive at the spermatheca (Konagaya et al., 2020; Hague et al., 2021). Due to their different functions, eupyrenes may evolve faster than apyrenes in response to selection pressures (Fitzpatrick et al., 2020).

1.4.4 Socio-sexual environment and sperm competition

A socio-sexual environment is defined as the composition of individuals of the same species surrounding the individuals of interest (West-Eberhard, 2003; Kasumovic et al., 2008). It can have profound impacts on individual physiology and behaviour (Moore et al., 1997; Shuster & Wade, 2003; Bailey & Moore, 2018; Flintham et al., 2018; Leech et al., 2021). For example, it can provide males with information on the presence of rivals, helping them assess sperm competition risk and adjust resource allocations to

reproduction and survival (English et al., 2017; Dore et al., 2018; Westneat et al., 2019; Magris, 2021).

When one female copulates with multiple males within the same reproductive episode, sperm competition occurs, i.e., ejaculates from different males compete to fertilize a given set of ova (Parker 1970). Although the outcome of sperm competition may be affected by sperm morphology and velocity (Morrow & Gage, 2000; Snook, 2005; Gomendio & Roldan, 2008; Ramm et al., 2014) and seminal fluid proteins (Wigby et al., 2009; Sirot, 2019; Ramm, 2020; Polak et al., 2021), the most important determinant appears to be sperm number (Tomkins & Simmons, 2000; Kelly & Jennions, 2011; Parker et al., 2012; Rowe et al., 2022).

1.4.5 Responses to conspecific rival cues in adult males

In response to various conspecific rival cues, males may produce and ejaculate a greater number of sperm in the first mating, such as in mammals (Kilgallon & Simmons, 2005; delBarco-Trillo & Ferkin, 2004, 2007), fishes (Fraser & Stacey, 2002; Evans et al., 2003; Fitzpatrick, 2020), birds (Martin et al., 1974; Pizzari et al., 2003; Birkhead & Montgomerie, 2020), and insects (Gage, 1991; Gage & Baker, 1991; Gage & Barnard, 1996; Simmons et al., 2007; Larsdotter-Mellström & Wiklund, 2009; Esfandi et al., 2020; Noguera, 2022). Furthermore, many studies show that the impact of rival exposure on sperm allocation can last in the first few successive matings (e.g., Harris & Moore, 2005; Bretman et al., 2012; Larsdotter-Mellström & Wiklund, 2015; Rouse & Bretman, 2016; Wylde et al., 2020). Yet, it remains unknown whether rival-cue(s) exposure affects males' sperm investment over their lifespan.

The main types of cues insect males can detect and respond to sperm competition environment are the sound, smell, and tactile, while vision may be trivial (Greenfield, 2016). For example, *Drosophila* males respond to the sperm competition situations after detecting at least two types of cues (chemical, acoustic and tactile) from rivals (Bretman et al., 2011a; Maguire et al., 2015). In moths, males can emit acoustic (Spangler, 1987; Trematerra & Pavan, 1995; Skals & Surlykke, 1999; Jia et al., 2001;

Nakano et al., 2008) or chemical cues (Nishida et al., 1982; Teal & Oostendorp, 1995; Kalinova et al., 2009; Davie et al., 2010; Kindl et al., 2011; Hosseini et al., 2016; Stanley et al., 2018) during sexual communication. However, it is not clear whether these cues function as a signal of rivalry and how males would respond to them. Moreover, for most animal taxa, it is not clear if only one type of rival cues is enough to trigger a response to sperm competition environment and if combining more cues can enhance the response, i.e., redundant multimodal signals intensify the strength of a signal, leading to an increased response of the receiver (Partan & Marler, 1999; Dore et al., 2018).

1.4.6 Responses to conspecific juvenile rivals in immature males

There is growing interest in exploring how juvenile males allocate their resources to cope with the dynamic socio-social environment during growth and development (Bretman et al., 2011b; Kasumovic et al., 2011; Taborsky, 2016; Firman et al., 2018). Among vertebrates, much evidence shows that juvenile males can adjust their sperm production when perceiving cues from other developing peers (Long & Montgomerie, 2006; Evans & Magurran 1999; Ramm & Stockley, 2009; Dziminski et al., 2010; Firman et al., 2013). However, it has not yet determined in any insect species whether and how juveniles can alter their sperm production as a response to juvenile socio-sexual settings although they communicate via acoustic and/or chemical cues during the larval and/or pupal stages (Gilbert, 1976; Deinert et al., 1994; Kotaki & Fujii, 1995; Yack et al., 2001; Choi et al., 2007; Mankin et al., 2009; Estrada et al., 2010; Scott et al., 2010; Fletcher, 2015; Pontier & Schweisguth, 2015; Álvarez et al., 2018; Dolle et al., 2018; Thurman et al., 2018; Fitzgerald et al., 2019; Geoffrey et al., 2021).

Some studies show that in response to increased sperm competition risk young males grow larger testes for greater sperm production or mate more frequently later in life (evidence in vertebrates: Harcourt et al., 1981; Kusano et al., 1991; Stockley et al., 1997; Prado & Haddad, 2003; Pitcher et al., 2005; Fitzpatrick et al., 2009; Soulsbury, 2010; evidence in insects: Gage, 1995; Stockley & Seal, 2001; Johnson et al., 2017). However, other studies on vertebrates (Byrne et al., 2002; Fitzpatrick et al., 2012;

Firman et al., 2013; Liao et al., 2019) and insects (Gay et al., 2009; Simmons & Buzatto, 2014; McNamara et al., 2016; Bretman et al., 2016; Chechi et al., 2017; Kapila et al., 2021) contradict the above findings on testis size. Explanations of these discrepencies include (1) males increase the testis efficiency rather than size in response to sperm competition environment during sexual maturation (Rowe & Pruett-Jones, 2011; Ramm & Schärer, 2014; Giannakara et al., 2016; Parker, 2016; Firman et al., 2018); (2) testes suffer aging and degeneration over time, especially in insects (Ward & Simmons, 1991; Fernandez-Winckler & Cruz-Landim, 2004; Linklater et al., 2007; Rosa et al., 2019; Hiroyoshi et al., 2021), and (3) males dedicate varying parts of testis volumes to spermatogenesis and other functions (e.g., De Loof, 2006; Simmons & Fitzpatrick, 2012; Ramm & Schärer, 2014; Parker, 2016) in response to sperm competition environment (Lüpold et al., 2020).

In several insect species, after male juveniles are reared in higher density or with other male juveniles, their adults ejaculate more sperm during their first mating (Gage, 1995; He & Miyata, 1997; Yamane & Miyatake, 2005; McNamara et al., 2010; Allen et al., 2011; Katsuki et al., 2013). Yet, it is unclear whether juvenile males can adjust resource allocation to lifetime sperm production and ejaculation in response to their juvenile peers. Furthermore, more investment in reproduction may compromise on survival due to resource trade-offs (van Voorhies, 1992; Martin & Hosken, 2004; Ferkau & Fischer, 2006; Bonduriansky & Brassil, 2005; Bonduriansky et al., 2008; McNamara et al., 2008; Oliver & Cordero, 2009; Cornwallis et al., 2014; Metzler et al., 2016; Mautz et al., 2019; Duxbury et al., 2018; Jehan et al., 2020). However, it is still unknown how juvenile males can manage resource allocations for sperm production, mating frequency and adult longevity in response to juvenile socio-sexual experience.

1.4.7 Responses to adult rivals in juvenile males

Evidence shows that the presence of adult rivals can affect resource allocations in juvenile males. For example, after their juveniles are exposed to adult rivals, adult crickets *Teleogryllus oceanicus* Le Guillou have larger testes at emergence (Bailey et al., 2010) and ejaculate more sperm in their first mating (Gray & Simmons, 2013;

Simmons & Lovegrove, 2017) but adult cockroaches *Nauphoeta cinerea* Olivier ejaculate more sperm in their first mating with no change in the testis size (Harris & Moore, 2005). Nonetheless, these studies have not determined whether adult male cues affect sperm production of juvenile males and lifetime sperm production and ejaculation of resultant adults, and whether there is trade-off between reproduction and other life history traits (e.g., juvenile survival, adults' body size and longevity).

Individuals at different stages of development should have varied sensitivities to environmental cues to strategically allocate resources to reproduction and survival (Fawcett & Frankenhuis, 2015; Walasek et al., 2021, 2022). The existence of 'sensitive periods' has been recognised in humans and mammals for decades (e.g., Illingworth & Lister, 1964; Rice & Barone, 2000). In contrast, the window of sensitivity for insects is poorly investigated; it remains unclear whether juvenile sensitivity to social cues is stage dependent (Gage, 1995; Stockley & Seal, 2001; McNamara et al., 2010; Katsuki et al., 2013; Bretman et al., 2016; Johnson et al., 2017).

CHAPTER 2

Combined Cues of Male Competition Influence Spermatozoal Investment in *Ephestia kuehniella*

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Junyan Liu
Name and title of main supervisor:	Professor Qiao Wang
In which chapter is the manuscript/published work?	Chapter 2
What percentage of the manuscript/published work was contributed by the student?	80

Describe the contribution that the student has made to the manuscript/published work:

The candidate conceived and designed the study, conducted laboratory work, and analysed the data. The candidate wrote the first draft of the manuscript, with changes and corrections completed with input from supervisors (co-authors).

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1 Doctoral Research Committee July 2022

Abstract

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). Here I 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. I exposed newly emerged and virgin focal males of the moth *Ephestia kuehniella* to various rival cues and then permanently removed the cues. I 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. I 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. 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 of spermatogenesis cannot fully compensate for that of sperm expenditure in response to rival cues. I show that 10-hr premating rival exposure was sufficient to maximise 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.

2.1 Introduction

When males of polygamous species perceive the presence of rivals, they often raise their ejaculate allocation per mate for a higher paternity share (Parker, 1970; Gage & Baker, 1991; Parker et al., 1997; Wedell et al., 2002; Parker & Pizzari, 2010; Bretman et al., 2011b). For example, following premating exposure to rivals, the moth *E. kuehniella* males increase their sperm ejaculation (Esfandi et al., 2020). Similar response to rivals has also been reported in many other species such as the butterfly *Pieris napi* (Larsdotter-Mellström et al., 2016b), the cricket *T. oceanicus* (Bailey et al., 2010), and fruit flies *D. melanogaster* (Bretman et al., 2009; Rouse et al., 2018) and *D. pseudoobscura* (Maguire et al., 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 et al., 2011b; 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 (Romer & Lewald, 1992; Sweeney et al., 2003; Cocroft & Rodriguez, 2005; Yew et al., 2009; Schiestl, 2010; Alcántara-Alcover et al., 2014; McKinney et al., 2015). Many animal species need to detect more than one cue simultaneously before responding to a social environment (Partan & Marler, 1999; Acquistapace et al., 2002; Uetz & Roberts, 2002; Bro-Jørgensen, 2010; Gray et al., 2014; 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 (delBarco-Trillo & Ferkin, 2004; Carazo et al., 2007; Aragón, 2009; Larsdotter-Mellström et al., 2016b) 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 et al., 2011a) 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 et al., 2012; Rouse & Bretman, 2016; Mohorianu et al., 2017) 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 et al., 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., 2020). 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; Wigby et al., 2009; Price et al., 2012; Garbaczewska et al., 2013; Jarrige et al., 2015; Ullah et al., 2017). 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 et al., 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 et al., 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 (Lachance & Olstad, 1988; Friedländer, 1997; review in Friedländer et al., 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 et al., 2018) and adult overwintering (Hiroyoshi et al., 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; Pérez & Zhantiev, 1976; Corbet & Lai-Fook, 1977; Xu & Wang, 2009a, 2009b, 2010a, 2010b, 2014, 2020; Esfandi et al., 2015, 2020). Adults become sexually mature soon after emergence and mating initiates only in the scotophase, particularly the second half of the scotophase (Xu et al., 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 spermatophore into the female's bursa during copulation (Xu & Wang, 2010a). Similar to other lepidopterans and many flies (Swallow & Wilkinson, 2002; Till-Bottraud et al., 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 (Trematerra & Pavan, 1995; Salehi et al., 2016) but whether the ultrasound also functions as a cue of rivalry is unknown. Furthermore, Barth (1937) and Corbet & 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 (Nishida et al., 1982; Mori et al., 1993; Teal & Oostendorp, 1995). Previous work shows that *E. kuehniella* males can detect rivals with (Xu & Wang, 2014) or without

(Esfandi et al., 2020) physical contact, suggesting that either acoustic, chemical, tactile or combined cues are used for communications between males.

In the present study, I 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, I proposed to test two hypotheses: (1) either acoustic or chemical cue from rivals can trigger males to increase ejaculation and production of sperm but combined cues enhance such response, and (2) males' response to rival cues quickly diminishes after the cues are removed. I exposed newly emerged virgin males to the following rival cues for 10 hr before mating and then removed the cues permanently: (1) acoustic cue only, (2) chemical cue only, (3) combined acoustic and chemical cues, (4) combined acoustic, chemical and tactile cues, and (5) no rival cues (control). I offered each rival-cue-exposed and control male a virgin female per day until they died. I dissected each mated female to count sperm ejaculated and each dead male to count sperm remaining in his body at death. These experiments allowed me to record sperm ejaculation per mating and lifetime sperm production in response to single and combined rival cues.

2.2 Materials and methods

2.2.1 Insects and environmental conditions

I 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. I 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. I 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^2). I placed a piece of white paper (8 cm diameter) folded four times in the cylinder for pupation. I collected mature pupae from the cylinders, and weighed them using an

electronic dual range balance (Mettler Toledo AG135, Greifensee, Switzerland) with an accuracy of 0.00001 g. I categorised 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 minimise the potential effect of body weight. The colony was maintained and all experiments carried out at 25 ± 1°C and 60 ± 10% relative humidity, with a photoperiod of 14:10 hr (light:dark).

2.2.2 Premating treatment of focal males

I manufactured a series of devices for premating treatment of focal males (Figure 2.1). 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^2). I made a hole (0.5 cm diameter) in the middle of each lid through which I inserted a plastic Y-tube (0.5 cm diameter) and sealed the gap between the tube and lid using the glue-gun glue. I 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 2.1). I set the air speed to replace the air in the cylinder once per minute. I used each device only once to avoid potential contaminations.

I 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: (1) acoustic cue only (+A), (2) chemical cue only (+C), (3) acoustic and chemical cues (+A+C), (4) acoustic, chemical and tactile cues (+A+C+T), and (5) no rivals (CONT). In treatment +A, I 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, I individually placed one focal male and five pieces of filter paper (1.5 cm width × 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. I extracted the male pheromone according to Romel et al. (1992) and Stanley et al. (2018). Briefly, I gently clipped the abdominal tip of a newly emerged male (<1 hr old) and excised the three terminal abdominal segments with microscissors. I placed excised segments of five males into a conical glass vial containing 1 ml dichloromethane at 25°C for 1 hr. I then put five pieces of the filter paper into the vial to absorb all supernatant, after which, I 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, I 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, I 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, I 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.

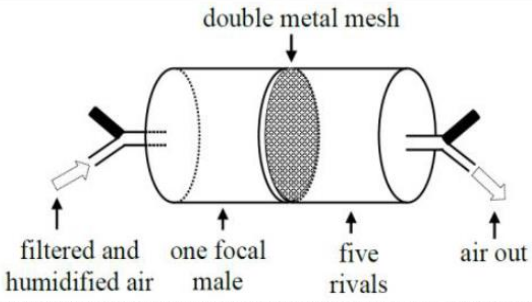
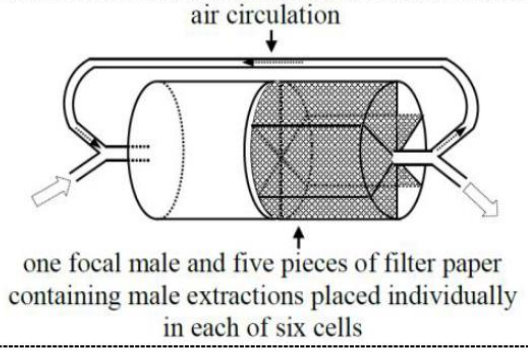
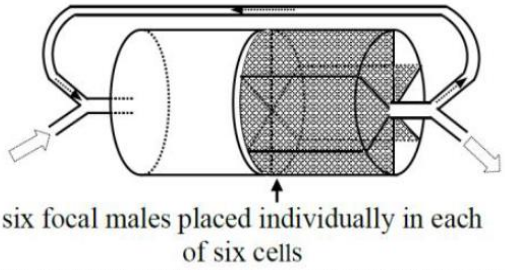
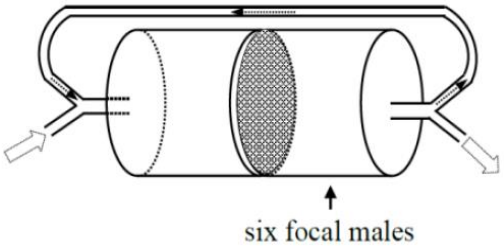
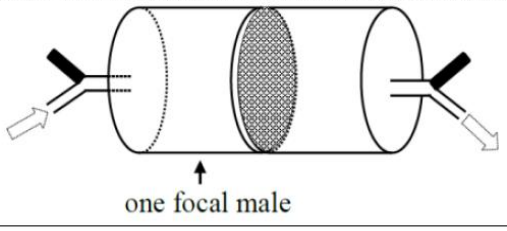
Treatment	Description	Pre-mating exposure device
+A	A focal male perceived only acoustic cue from rivals; one arm of each Y-tube was blocked with a cork.	
+C	A focal male perceived only chemical cue from rivals; one arm of each Y-tube was connected with a silicon tube to facilitate air circulation between the two chambers.	
+A+C	Focal males perceived both acoustic and chemical cues from rivals; one arm of each Y-tube was connected with a silicone tube to facilitate air circulation between the two chambers.	
+A+C+T	Focal males perceived acoustic, chemical and tactile cues from rivals; one arm of each Y-tube was connected with a silicon tube to facilitate air circulation between the two chambers.	
CONT	A focal male did not perceive any cue from rivals; one arm of each Y-tube was blocked with a cork.	

Figure 2.1 Treatments and devices used for premating exposure.

2.2.3 Sperm ejaculation and production

To test the function and impact of rival cues, I 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. I set the air speed to replace the air in all 15 mating chambers once per minute.

I introduced a 1-d-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. I 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 Koudelová & Cook (2001). I then introduced a 1-d-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. I dissected the dead focal male to count the number of eupyrene and apyrene sperm remaining in testes, seminal vesicle and vas deferens. I 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.

2.2.4 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, I 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, I also included the replicate identity as a random factor in the model. A CONTRAST statement was applied to perform the multiple comparisons between treatments.

I 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, I 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 my data showed that the influence of treatment on the number of sperm ejaculated disappeared after the fourth mating, I 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.

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

2.3 Results

2.3.1 Effects of the number of cues from rivals on focal males' lifetime sperm ejaculation and production

My 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 2.2A). 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 2.3A). Premating exposure to rivals also triggered males to ejaculate ($F_{4,79} = 34.34$, $P < 0.0001$) (Figure 2.2B) and produce ($F_{4,79} = 127.22$, $P < 0.0001$; Figure 2.3B) significantly more apyrene sperm in their lifetime than control males.

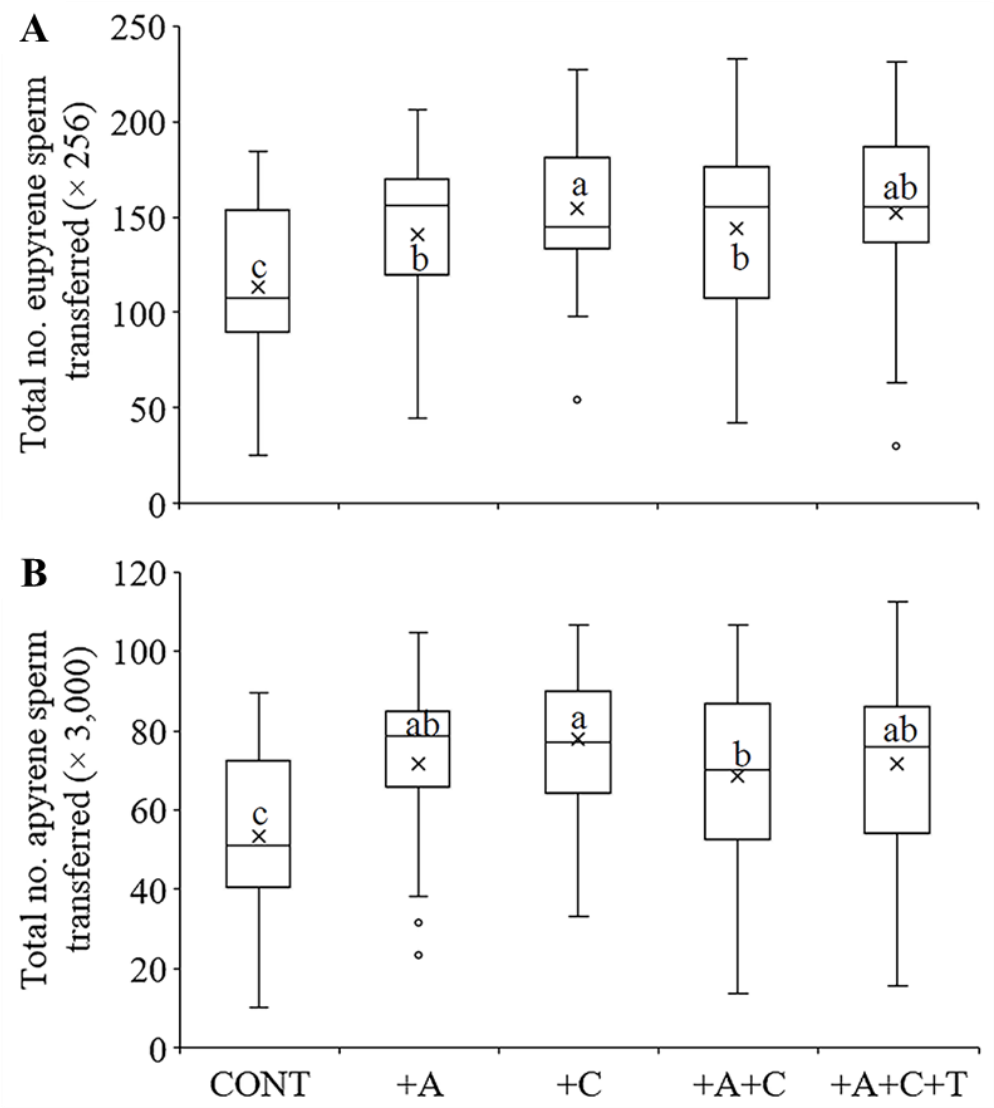


Figure 2.2 Mean (±*SE*) number of eupyrene (**A**) and apyrene (**B**) sperm ejaculated in E. kuehniella males' lifetime after a premating exposure to rival cues. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical and 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 '×' in a box show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum scores. Boxes with different letters are significantly different in mean between treatments ($P < 0.05$).

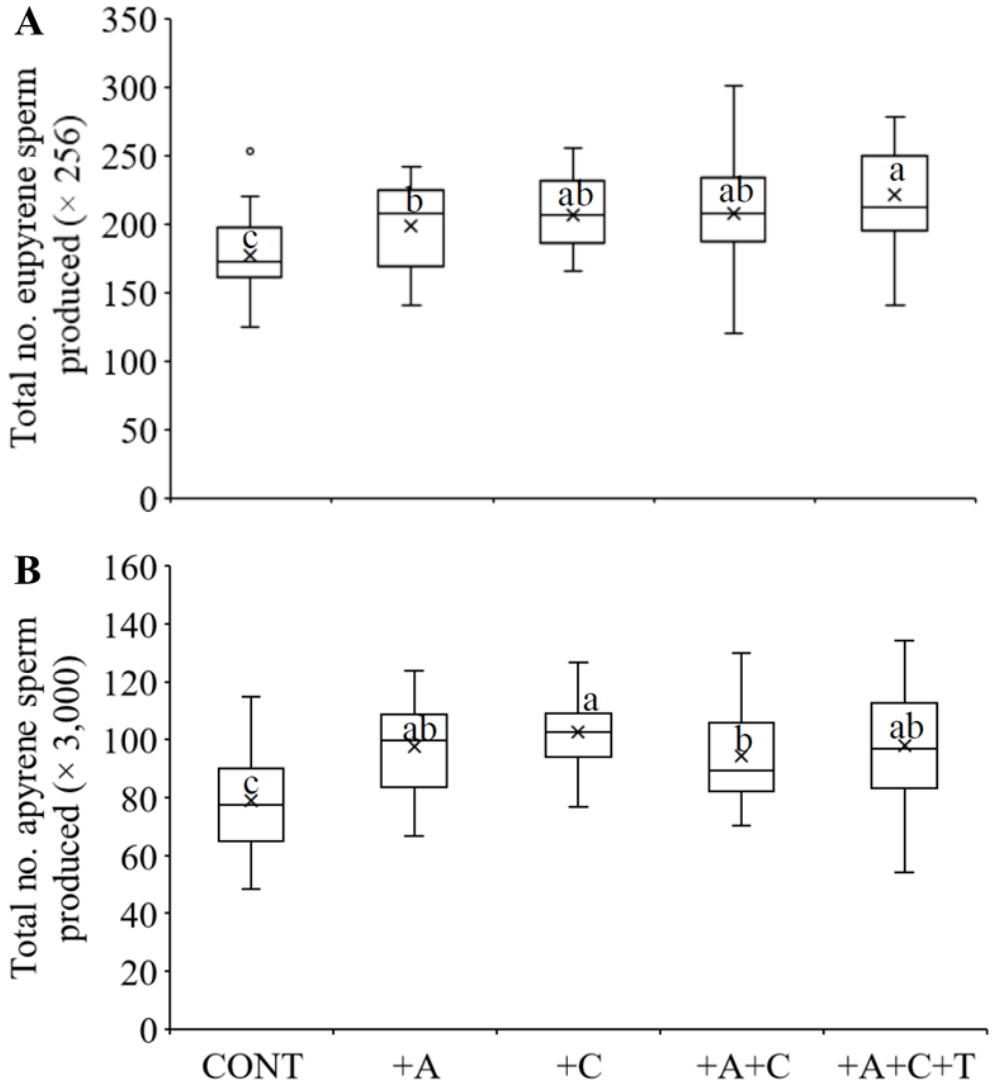


Figure 2.3 Mean (±*SE*) number of eupyrene (**A**) and apyrene (**B**) sperm produced in E. kuehniella males' lifetime after a premating exposure to rival cues. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical and 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 '×' in a box show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of maximum scores. Boxes with different letters are significantly different in mean between treatments ($P < 0.05$).

2.3.2 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 2.4). In comparison of slopes of regression lines, I 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 2.4A). A similar trend was also found in apyrene ejaculation over successive matings ($F_{4,101}$ = 12.46, P < 0.0001) but less obvious (Figure 2.4B).

Looking into all matings over focal males' lifetime, I found that the impact of rival exposure on sperm transfer disappeared after the fourth mating (P > 0.05). Following the analysis of how treatment affected the number of sperm ejaculated in the first four matings, I 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 2.5A). 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 2.5B–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 2.6B–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 2.6A).

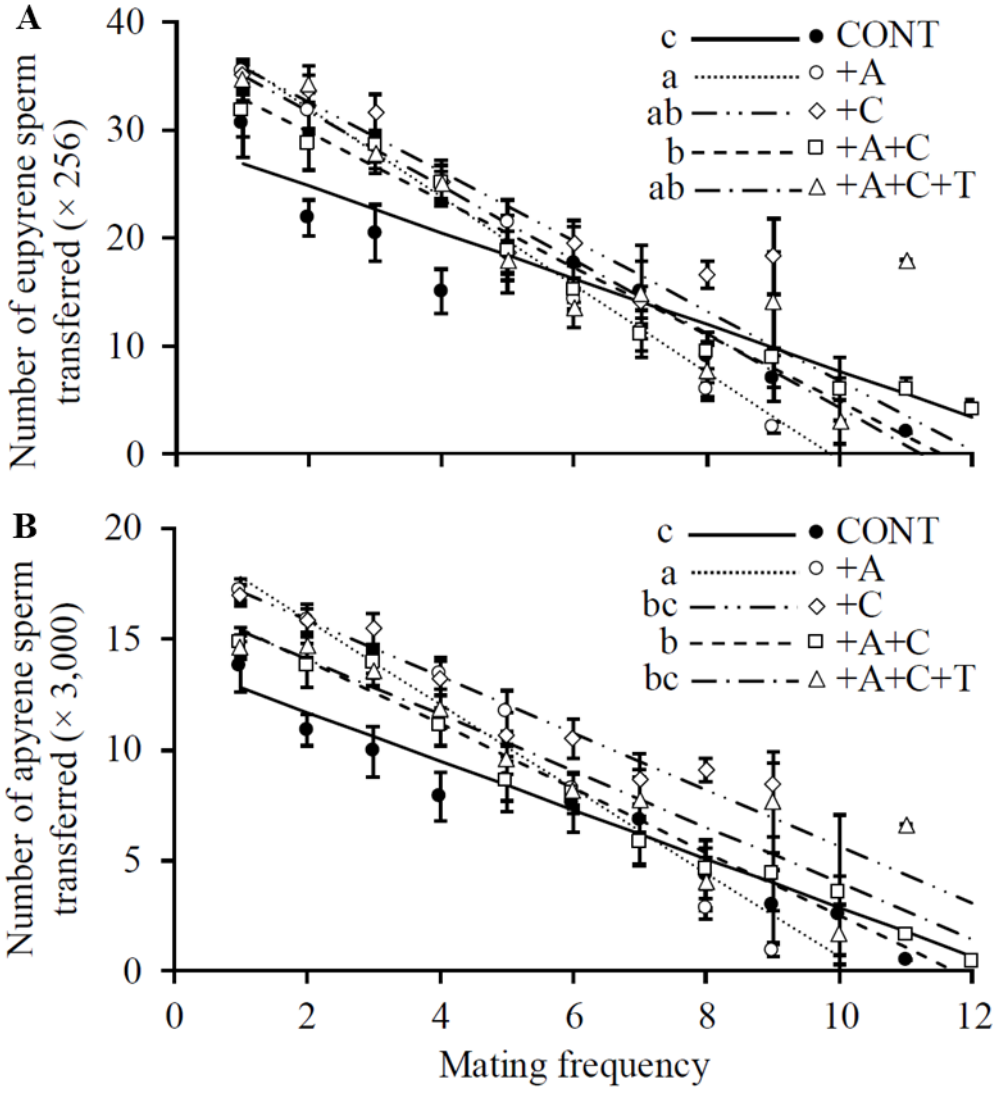


Figure 2.4 Number of eupyrene (**A**) and apyrene (**B**) sperm ejaculated by focal males in successive copulations after rival-cue exposure. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical and tactile cues. Treatments (lines) with the same letters are not significantly different in slope ($P > 0.05$). Raw data were subject to analyse but means (±*SE*) were presented.

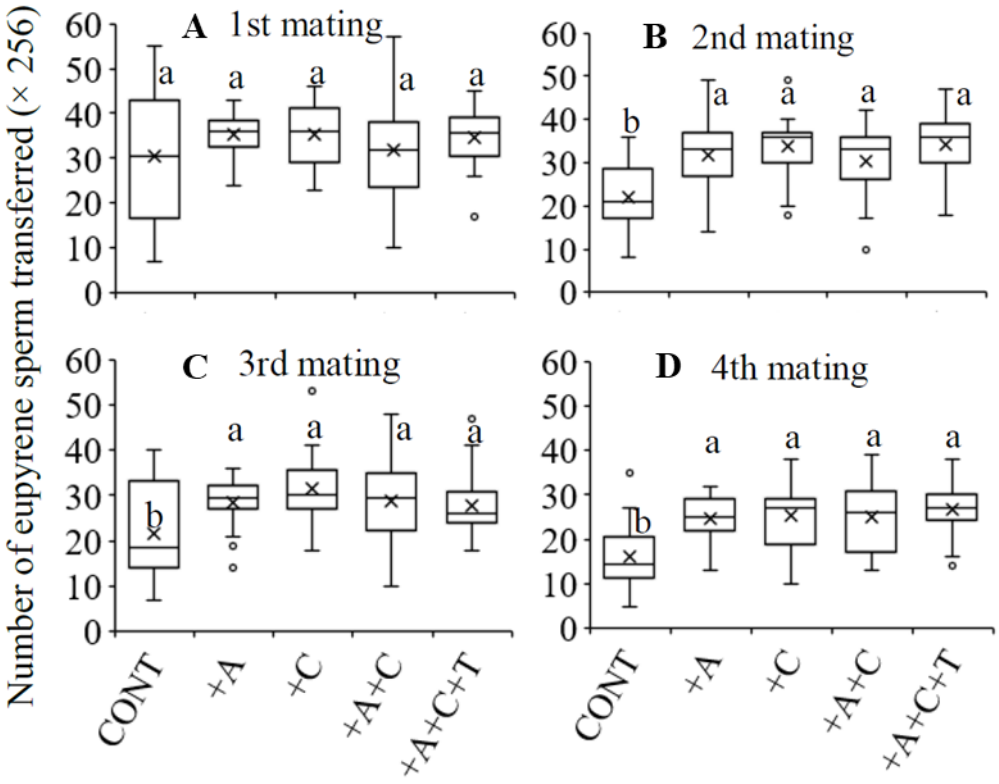


Figure 2.5 Mean (±*SE*) number of eupyrene sperm ejaculated in the first four matings (**A**–**D**, respectively) after a premating exposure to rival cues in male *E. kuehniella*. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, 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 '×' show the median score and means, respectively; the '⊥' and '⊤' 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 ($P < 0.05$).

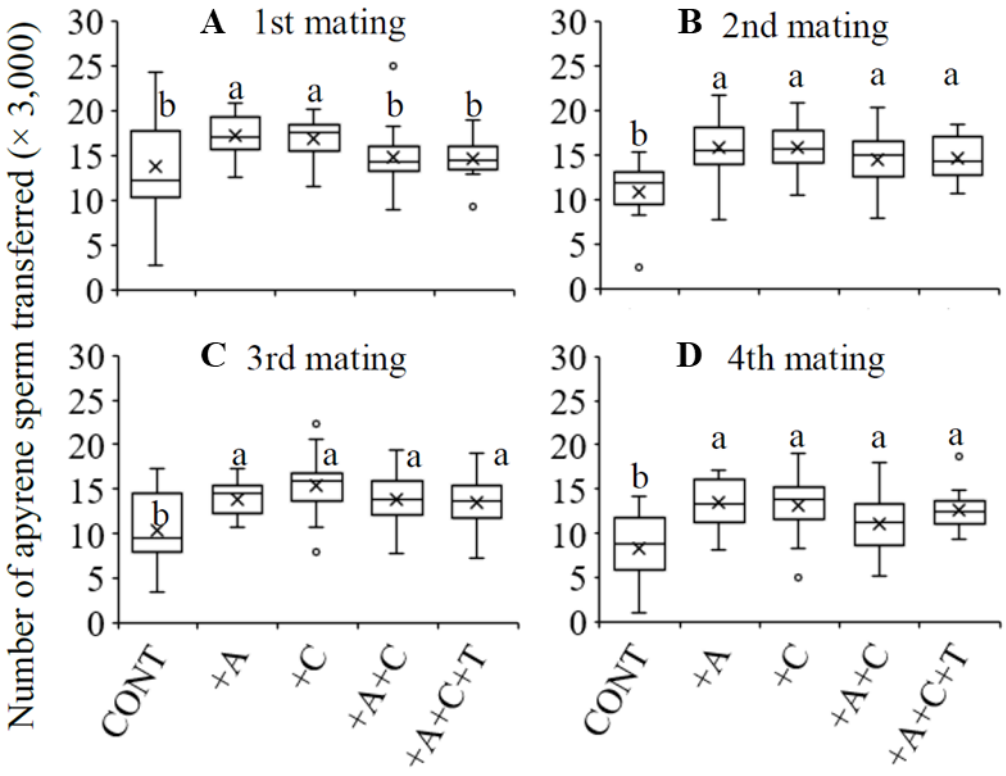


Figure 2.6 Mean (± *SE*) number of apyrene sperm ejaculated in the first four matings (**A**–**D**, respectively) after a premating exposure to rival cues in male *E. kuehniella*. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, 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 '×' show the median score and means, respectively; the '⊥' and '⊤' 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 ($P < 0.05$).

2.4 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 2.2). 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 et al., 2011a) 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 (Schneeberger & 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 (Cusumano et al., 2009; Allada & Chung, 2010). 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; Ponlawat & Harrington, 2007; Malawey et al., 2019). However, various studies suggest that lepidopteran males often stop producing eupyrene sperm after pupation (Chaudhury & Raun, 1966; Lachance & Olstad, 1988; Witalis & Godula, 1993; Friedländer, 1997; review in Friedländer et al., 2005; Mari et al., 2018). There are a few exceptions, though, for example, in the moths *Calpodes ethlius* (Lai-Fook, 1982), *A. grisella* (Fernandez-Winckler & da Cruz-

Landim, 2008) and *G. mellonella* (Bebas et al., 2018) and a butterfly *P. 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, I demonstrate that rival-exposed *E. kuehniella* adult males increased lifetime sperm production after exposure to rivals during the early adulthood (Figure 2.3). This finding strongly suggests that sperm competition risk can stimulate spermatogenesis during the adult stage in a lepidopteran species.

My data indicate that while a single cue causes an increase in eupyrene investment (Figures 2.2 and 2.3), combined cues (+A+C+T) seem to strengthen the response in eupyrene production (Figure 2.3A). Studies on other animals such as *Drosophila* spp. (see results in Bretman et al., 2011a; Maguire et al., 2015) and the spider *Schizocosa ocreata* (Uetz et al., 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 (Partan & Marler, 1999; Dore et al., 2018). However, there is no evidence that combined cues could enhance production of apyrene in their lifetime (Figure 2.3B), probably because apyrene play relatively minor roles in sperm competition (Konagaya & Watanabe, 2015; Thorburn et al., 2018; Mongue et al., 2019; Sakai et al., 2019).

I show that the number of sperm ejaculated by focal males decreased over successive matings in all treatments and the control (Figure 2.4). Because *E. kuehniella* adults do not feed, my 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, 2007; Vega-Trejo et al., 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 2.4). The faster decrease in sperm transfer in treatments could result from significantly more sperm expenditure during the first few matings (Figures 2.5 and 2.6). I suggest that both sperm priming and production are involved in the process, but the increase of spermatogenesis is not enough to fully compensate for that of sperm expenditure.

After 10-hr premating exposure (Figures 2.5 and 2.6) or 24-hr premating + lifetime exposure (Esfandi et al., 2020) 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., 2020). My findings support the notion that insects' brain has a long memory of an exposure to a socio-sexual environment (Dion et al., 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 this 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.

I 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.

My findings demonstrate that the focal males ejaculated similar number of eupyrenes in their first mating in all treatments and the control (Figure 2.5) while Esfandi et al. (2020) reveal that males ejaculated significantly more eupyrenes in their first mating after exposure to rival cues. I attribute the divergence of these two studies to the duration between rival cue detection and sperm ejaculation. In Esfandi et al. (2020) 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-h 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., Thorson & Riemann, 1977; Prosholdi, 1991), 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. (2020) 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 2.6) at the first mating was not as consistent as that of eupyrenes. The reasons behind are not clear.

In the present study, I 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. I conclude that (1) males raise their sperm allocation and production after detecting either acoustic or chemical cues from their rivals with combined cues somewhat strengthening such response, and (2) males can remember the sperm competition risk for most of their reproductive life following one premating exposure to rival cues.

CHAPTER 3

Larval Social Cues Influence Testicular Investment in *Ephestia kuehniella*

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.	
Student name:	Junyan Liu
Name and title of main supervisor:	Professor Qiao Wang
In which chapter is the manuscript/published work?	Chapter 3
What percentage of the manuscript/published work was contributed by the student?	80
Describe the contribution that the student has made to the manuscript/published work: The candidate conceived and designed the study, conducted laboratory work, and analysed the data. The candidate wrote the first draft of the manuscript, with changes and corrections completed with input from supervisors (co-authors).	
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(●) **The manuscript/published work is published or in press** Please provide the full reference of the research output: Liu JY, Zhang YJ, Zheng XL, He XZ, Wang Q. (2022) Larval social cues influence testicular investment in an insect, Current Zoology 68: 1–8. https://doi.org/10.1093/cz/zoab028	
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Student's signature: Junyan Liu	Main supervisor's signature: Digitally signed by Qiao Wang DN: cn=Qiao Wang, c=NZ, o=Massey University, ou=School of Agriculture and Environment, email=Q.Wang@massey.ac.nz Reason: I am approving this document Date: 2022.11.03 10:10:54 +13'00'
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1 Doctoral Research Committee July 2022

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*, I tested whether and how larval social environment affected sperm production, testis size and body weight. I exposed the male larvae to different juvenile socio-sexual cues and measured these traits. I 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. I conclude that the presence of larval social, but not sexual cues, is responsible for the increase of eupyrene production and decrease of testis size. I suggest that male larvae increase investment in fertile sperm cells and reduce investment in other testicular tissues in the presence of conspecific juvenile cues.

3.1 Introduction

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 behaviour and physiology to maximise 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 & Pizzari, 2010; Lüpold et al., 2020). Although larger males usually have more sperm (Pitnick, 1996; Hatala et al., 2018; Chung et al., 2019; Xu & Wang, 2020), the social environment experienced by juvenile males does not appear to affect their body size in some insects (Gage, 1995; Hosken & 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 two 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 & Wiklund, 2015; Simmons & 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., 2011a; Uzsak et al., 2014; Baker et al., 2019; Liu et al., 2020) and adjust their sperm investment accordingly (Wedell et al., 2002; Parker & 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 & 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 & Brooks, 2011; Gray & Simmons, 2013).

Because most resource allocation to traits making up the adult body (e.g., Oberlander, 1985; Nijhout & Emlen, 1998; Moczek & Nijhout, 2004; Rolff et al., 2019; Mirth et al., 2021) and immunity (e.g., Barnes & Siva-Jothy, 2000; Cotter et al., 2004; Triggs & 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 & Emlen, 1998; Simmons & Emlen, 2006; Luecke & 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 & Seal, 2001; Johnson et al., 2017) or ejaculate more sperm in the first mating (Gage, 1995; He & Miyata, 1997; McNamara et al., 2010) 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 & 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, I used a polygamous insect, *E. 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. *E. kuehniella* larval stage lasts 29–31 days and pupal stage takes 8–9 days, with larvae having six instars (instars 1–3 ≈ 14–15 days and instars 4–6 ≈ 15–16 days) (Brindley 1930; Liu, J.Y. personal observation). Adults of this species do not feed and thus all their resources are obtained during the larval stage (Norris & Richards, 1932). Females start producing sex pheromones at the pupal stage (Calvert & Corbet, 1973). Like most lepidopterans (reviewed in Swallow & Wilkinson, 2002), *E. kuehniella* males produce two types of sperm, larger fertile eupyrenes (nucleate) and smaller infertile apyrenes (anucleate) which can be easily distinguished (Garbini & Imberski, 1977; Koudelová & Cook, 2001). Prior to ejaculation, apyrene sperm bundles dissociate and become motile while eupyrene sperm remain in bundles (Koudelová & Cook, 2001; Liu, J.Y. personal observation). Both types of sperm migrate to the spermatheca but only eupyrenes can fertilize eggs (Friedländer & Gitay, 1972; Xu & Wang, 2010a). Apyrene sperm may delay the renewal of female receptivity (Cook & Wedell, 1999; Wedell et al., 2009), protect eupyrene sperm against a hostile female reproductive tract (Holman & 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, I 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, I prepared hundreds of larvae and reared them singly or in group with different sex ratios. I then weighed mature pupae, and upon emergence, dissected male adults, measured testis size, and counted the sperm in their testes. The

design allowed me 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.*

3.2 Materials and methods

3.2.1 Insect sampling and rearing

I collected *E. kuehniella* larvae by hand from chicken feed at Turks Poultry, Foxton, New Zealand. I allowed them to feed on their original food and develop to adults in the laboratory. I randomly selected and introduced about 300 newly emerged adults into a transparent plastic cage (28 cm length × 28 cm width × 24 cm height) lined with porous plastic sheets on the bottom for egg laying. I established a laboratory colony using larvae that hatched from these eggs. Briefly, I introduced 200 neonate larvae into a transparent plastic cylinder (8 cm diameter × 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. I covered the cylinder with two layers of cloth mesh. I maintained 10 cylinders of the colony, from which I randomly collected about 300 newly emerged adults and transferred them into the aforementioned plastic cage for egg laying. To generate an experimental line, I 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. I provided 0.25 g standard diet per larva in the experimental line. I kept the insect colony and experimental larvae at 25 ± 1°C, 60 ± 10% relative humidity, and 10:14 hr (dark:light) and carried out experiments under these environmental conditions.

3.2.2 Juvenile socio-sexual settings

Because sex can be determined through visible testes in male abdomens of the fourth instar larvae (Brindley, 1930; Liu, J.Y. personal observation), I randomly selected newly moulted fourth instar larvae from the experimental line and transferred them into glass vials (2 cm diameter × 7.5 cm height) to form three treatments (Figure 3.1A): (1)

SM – one male was maintained in a glass vial from the fourth instar larva to adult emergence; (2) 6M – six males were kept in a glass vial from the fourth instar larvae to adult emergence; and (3) 1M5F – one male and five 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. I only used insects from vials where all individuals successfully developed to adults for data collection. I 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 six males from each 6M vial. In total, I measured 32 adult males from treatment SM and 30 adult males for each of the other two treatments.

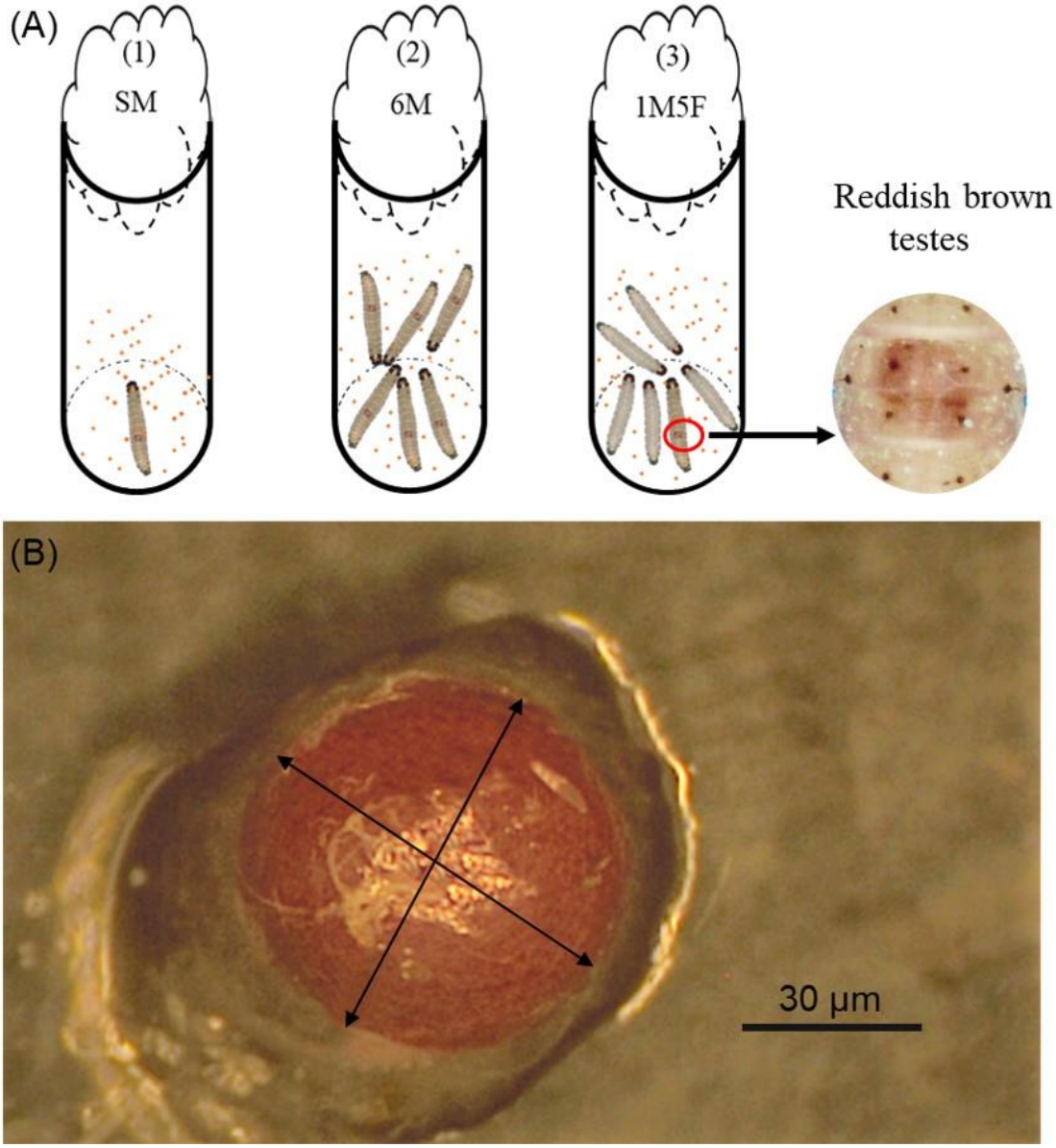

Figure 3.1 Treatment setups (**A**) and testis measurement (**B**) for *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, six males together from the fourth instar larvae to adult emergence, and 1M5F, one male and five females together from the fourth instar larvae to adult emergence.

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

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

Immediately after emergence, I individually transferred adult males into 2-ml transparent micro-centrifuge tubes, clearly labelled each tube, and killed them at –20°C in a freezer. I then individually dissected all males to extract their testes and measured testis volume under a stereomicroscope (Leica MZ12, Wetzlar, Germany) connected with a digital camera (Olympus SC30, Tokyo, 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.Y. personal observation), I determined its radius using the mean diameter from two measurements across the organ's central axis (Figure 3.1B; Raichoudhury, 1936; Gage, 1995) divided by two and calculated the testis volume (size) using the formula $4/3\pi r^3$, where $\pi = 3.14$ and r = radius of the testis.

3.2.4 Effects of juvenile socio-sexual settings on sperm production

After measurement of testis size, I 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 dissociate apyrenes. I counted the number of eupyrene bundles under a phase-contrast microscope (Olympus BX51, Tokyo, Japan) at 40× magnification and calculated the total number of eupyrenes as the total number of eupyrene bundles multiplied by 256 since each bundle contains 256 eupyrenes in *E. kuehniella* (Garbini & Imberski, 1977). I then thoroughly washed the sample off the cavity slide and diluted it with distilled water to 30 ml in a glass vial. I gently rotated the vial for about 30 s to allow even dispersal of apyrenes in the vial and then took eight 10-µl subsamples from the vial using a Gilson

autopipette. I placed these subsamples apart from each other on a microscope slide and allowed them to air dry. I counted the number of apyrene sperm of all eight subsamples under the phase-contrast microscope at 100× magnification and calculated the mean number per 10 μl as the sum of apyrene sperm in eight subsamples divided by eight. I then calculated the total number of apyrene sperm for each male as the mean number of apyrenes per 10 μl multiplied by the dilution factor (3,000) (Koudelová & Cook, 2001).

3.2.5 Statistical analysis

I calculated the residuals of data and tested the residual distribution (Shapiro–Wilk test, UNIVARIATE procedure) after fitting the data to a general linear model. I 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 the experimental design was pseudoreplicated, I analysed the data using a linear mixed-effects model (Millar & Anderson, 2004; Harrison et al., 2018) with treatment as a fixed factor and replicate nested into vial (male source) as a random factor (Davies & Gray, 2015; Harrison et al., 2018). I 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).

3.3 Results

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

My 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 3.2A). I 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 3.2B). 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 3.2B).

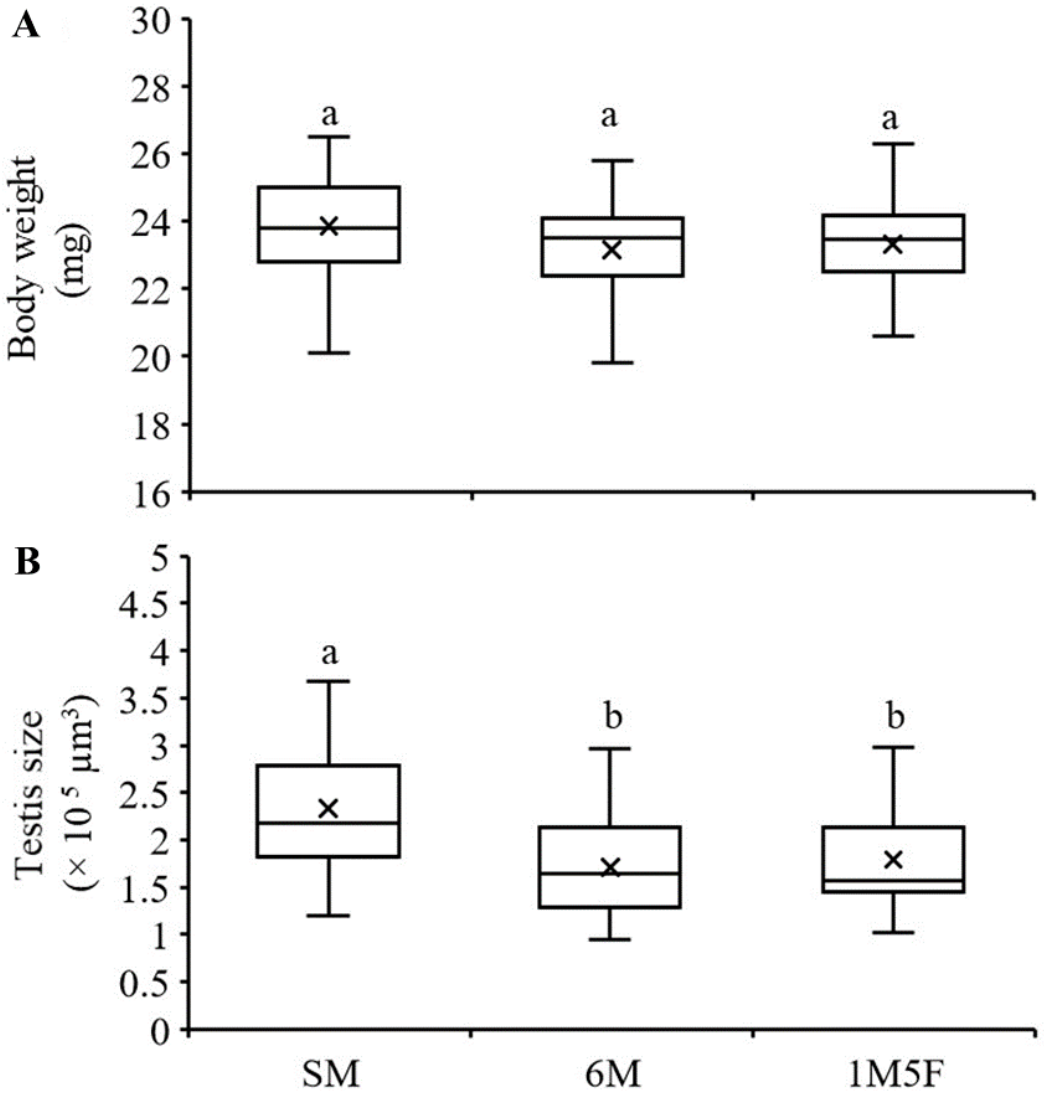

Figure 3.2 Effect of socio-sexual environment during immature stages on the body weight (**A**) and testis size (**B**) of *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, six males together from the fourth instar larvae to adult emergence, and 1M5F, one male and six 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 '×' and line in a box indicate the mean and median scores, respectively; the '⊤' and '⊥' are the upper and lower whiskers showing the maximum and minimum scores, respectively. For each parameter, boxes with different letters are significantly different ($P < 0.05$).

3.3.2 Effects of juvenile socio-sexual settings on sperm production

I 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 3.3A). However, testes in all treatments produced a similar number of apyrene sperm ($F_{2,29}$ = 1.47, P = 0.2458; Figure 3.3B). The number of eupyrene and apyrene produced did not vary with sex ratio during the immature stages (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 3.3).

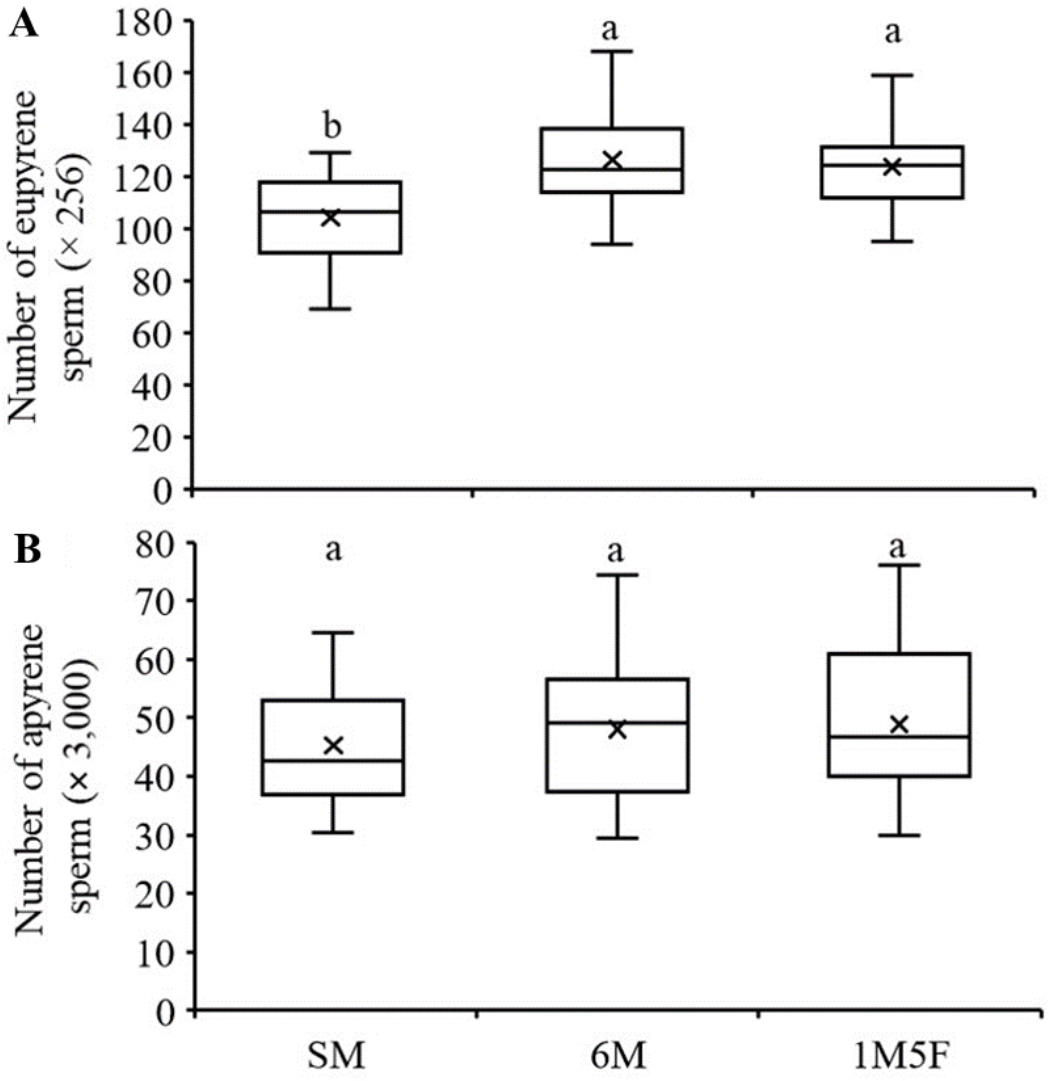

Figure 3.3 Effect of socio-sexual environment during immature stages on the total number of eupyrene (**A**) and apyrene (**B**) sperm in testes of *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, six males together from the fourth instar larvae to adult emergence, and 1M5F, one male and five 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 '×' and line in a box indicate the mean and median score, respectively; the '⊤' and '⊥' are the upper and lower whiskers showing the maximum and minimum scores, respectively. For each parameter, boxes with different letters are significantly different ($P < 0.05$).

3.4 Discussion

I 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 & Imberski, 1977) and other lepidopteran insects (Swallow & 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 & Miyata, 1997; McNamara et al., 2010) 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, the 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 & Gage, 1995; Thorburn et al., 2018; Esfandi et al., 2020) and the increased resource allocation to eupyrene production leaves less resource to produce more apyrenes. Furthermore, the last male sperm precedence is common in many insect species (Simmons, 2001) including *E. kuehniella* (Xu & Wang, 2010a). 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 & Wang, 2010a). 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 of 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 & Seal, 2001; Johnson et al., 2017). However, my data show that while 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., Verson, 1889; Nowock, 1973; Wolf, 1991; White-Cooper et al., 2009) and have functions other than sperm production (Simmons & Fitzpatrick, 2012; Ramm & 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 one trait may not be used for another, potential trade-offs between traits of different functions may occur (Nijhout & Emlen, 1998; Moczek & Nijhout, 2004; Luecke & Kopp, 2019). Based on the results from the present study and current knowledge about testicular components and functions, I 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 & 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 & Siva-Jothy, 2000; Cotter et al., 2004; Triggs & Knell, 2012) and pre-copulatory (Simmons & 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 & Pescador-Rubio, 2011), butterflies (Fitzgerald & Underwood, 1998) and sawflies (Flowers & 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, I propose that chemical, acoustic and tactile cues used by the larvae may provide reliable information about the future sperm competition levels, supporting Kasumovic & Brooks's (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 & 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. My 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, I have tested whether and how larval social cues affect sperm production, testis size and body weight in *E. kuehniella*. I 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. I conclude that the presence of non-sexual larval social cues is responsible for the increase of eupyrene production and decrease of testis size. I suggest that male larvae increase investment in fertile sperm cells and reduce investment in other testicular tissues in the presence of conspecific cues.

CHAPTER 4

Pupal Cues Increase Sperm Production but not Testis Size in *Ephestia kuehniella*

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.	
Student name:	Junyan Liu
Name and title of main supervisor:	Professor Qiao Wang
In which chapter is the manuscript/published work?	Chapter 4
What percentage of the manuscript/published work was contributed by the student?	80

Describe the contribution that the student has made to the manuscript/published work:

The candidate conceived and designed the study, conducted laboratory work, and analysed the data. The candidate wrote the first draft of the manuscript, with changes and corrections completed with input from supervisors (co-authors).

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Student's signature:	Junyan Liu	Main supervisor's signature:	Digitally signed by Qiao Wang DN: cn=Qiao Wang, c=NZ, o=Massey University, ou=School of Agriculture and Environment, email=Q.Wang@massey.ac.nz Reason: I am approving this document Date: 2022.11.03 10:11:25 +13'00'

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1 Doctoral Research Committee July 2022

Abstract

Theoretic and empirical studies show that social surroundings experienced by male insects during their larval or adult stage can influence their testicular investment in diverse ways. Although insect pupae do not feed and crawl, they can communicate using sex-specific and/or non-sex specific cues. Yet, it is unknown, in any insect, whether and how male pupae can fine-tune their resource allocation to sperm production and testis size in response to socio-sexual environment. I investigated this question using a moth, *Ephestia kuehniella*, which produces fertile eupyrene sperm and infertile apyrene sperm. I held male pupae individually or in groups with different sex ratios, and dissected adults upon eclosion, measured their testis size, and counted both types of sperm. I demonstrated that after exposure to conspecific pupal cues regardless of sex, male pupae increased production of eupyrenes and apyrenes at the same rate but kept testis size unchanged. I suggest that testis size is fixed after pupation because most morphological traits are formed during the larval stage, allowing little room for pupae to adjust testis size. Like adults, male pupae with fully grown testes have sufficient resources to produce more sperm of both types according to the perceived increase of sperm competition risk.

4.1 Introduction

Animals adjust their resource allocation strategies to maximise their reproductive fitness in response to socio-sexual environment (Dewsbury, 1982; Wedell et al., 2002; Dore et al., 2020). For example, male animals may invest more in sperm after they detect the presence of rivals to gain an advantage in sperm competition (Wedell et al., 2002; Bjork et al., 2007; Ramm & Stockley, 2009; Parker & Pizzari, 2010; Kelly & Jennions, 2011; Moatt et al., 2014; Firman et al., 2018; Liu et al., 2020). In insects, males fine-tune their sperm investment in response to sex specific cues experienced during the adult stage (Simmons et al., 2007; Xu & Wang, 2014; Larsdotter-Mellström & Wiklund, 2015; Lymbery et al., 2019; Fitzpatrick, 2020; Liu et al., 2020) or non-sex specific cues during the larval stage (Gage, 1995; He & Miyata, 1997; McNamara et al., 2010; McNamara & Simmons, 2017; Liu et al., 2022a). Although insect pupae do not feed and crawl, they can communicate with each other using species-specific acoustic (Hinton, 1948; Alexander, 1961; Downey, 1966; Travassos & Pierce, 2000; Álvarez et al., 2014; Dolle et al., 2018; Casacci et al., 2019; Lin et al., 2019) or chemical cues (Jefferson & Rubin, 1973; Feng & Roelofs, 1977; Tang et al., 1991; Choi et al., 2007; Kwadha et al., 2019). Furthermore, female pupae can release sex pheromones (Calvert & Corbet, 1973; Duthie et al., 2003; Estrada et al., 2010; Pontier & Schweisguth, 2015). These findings suggest that male pupae should be able to detect conspecific pupal cues representing the density and sex ratio of the local population, and thus future sperm competition risk. Yet, prior to the current study, nothing is known about whether and how insect pupae can adjust their sperm production in response to these cues.

Testes are a sperm production organ and their relative size or mass may be an indicator of sperm production. Evidence shows that male insect larvae, at growth and development stage, can adjust their testis size in response to conspecific larval cues regardless of sex. For example, with the increase of the larval density, testis size increases in some species, suggesting an increase of sperm production (sperm were not counted though) (Gage, 1995; Stockley & Seal, 2001; Johnson et al., 2017). In a study where testis size is measured and sperm are counted (Liu et al., 2022a), the male larvae

exposed to larval cues regardless of sex produce smaller testes but more fertile sperm. These discoveries suggest that in response to their social environment, male larvae are able to dedicate varying portions of testis volumes to spermatogenesis and other functions (Lüpold et al., 2020), resulting in potential trade-offs between traits of different functions (Nijhout & Emlen, 1998; Moczek & Nijhout, 2004; Luecke & Kopp, 2019; Liu et al., 2022a). However, there is no report that insects can alter their testis size in response to the socio-sexual environment experienced at the adult stage. This may be because most resource allocation to traits making up the adult body takes place during growth and development (Oberlander, 1985; Nijhout & Emlen, 1998; Moczek & Nijhout, 2004; Shingleton et al., 2007; Rolff et al., 2019; Mirth et al., 2021), leaving little room for adults to change their testis morphology. To date, it is not clear whether insect pupae can alter their testis size after exposure to different socio-sexual environments.

Here, I used a polygamous moth, *E. kuehniella*, as a model to investigate whether and how the socio-sexual environment during the pupal stage affected male investment in testis size and sperm production. Adults of this species do not feed so they acquire all resources via larval feeding (Norris & Richards, 1932; Esfandi et al., 2015). Pupal stage lasts about eight days (Brindley, 1930; Jacob & Cox, 1977; Liu et al., 2022a), during which time, females emit sex pheromones (Calvert & Corbet, 1973). Like most lepidopterans (Friedländer et al., 2005), *E. kuehniella* males produce two types of sperm, larger nucleated eupyrenes during the larval and pupal stages, and smaller anucleated apyrenes during the pupal stage (Garbini & Imberski, 1977). After mating, both types of sperm migrate to the sperm storage site (spermatheca) but only eupyrenes can fertilize eggs. Apyrenes may function to delay female remating (Cook & Wedell, 1999; Wedell et al., 2009) protect eupyrenes in female reproductive tract (Holman & Snook, 2008) or enable eupyrenes to migrate to the spermatheca (Sakai et al., 2019). More recent studies suggest that the role of apyrenes may be completed after both types of sperm arrive at the spermatheca (Konagaya et al., 2020; Hague et al., 2021). The apyrene to eupyrene ratio remains consistent under the food shortage during the larval stage (Gage & Cook, 1994) or environmental stress during the larval (Sait et al., 1998)

and pupal stages (Koudelová & Cook, 2001). However, *E. kuehniella* males increase the ratio after detecting rival cues during the adult stage (Liu et al., 2020) or reduce it following exposure to larval cues during the larval stage (Liu et al., 2022a). So far, it is still unclear whether the socio-sexual environment during the pupal stage affects the sperm production ratio.

Based on the theoretic framework and empirical evidence outlined above, I hypothesize that male pupae kept together with other male pupae should grow larger testes and produce more sperm with higher apyrene:eupyrene ratio than those maintained individually or with female pupae. To test this prediction, I individually reared hundreds of larvae under the same condition, starting from neonate larvae. I then transferred newly pupated pupae to experimental arenas and held male pupae individually or in groups with different sex ratios. Upon adult eclosion, I dissected them, measured their testis size, and counted both types of sperm. This is the first study to examine whether and how male insects adjust their testicular investment in response to their socio-sexual environment experienced during the pupal stage.

4.2 Materials and methods

4.2.1 Insects

I established a laboratory colony of *E. kuehniella* from thousands of larvae collected at Turks' Poultry, Foxton, New Zealand. I raised these larvae with their original food until adult eclosion in the laboratory. To standardize the colony, I randomly selected and confined about 300 newly eclosed adults (approx. 1:1 sex ratio; females with an ovipositor and males with a pair of claspers at the end of abdomen) in a transparent plastic cage (28 cm length × 28 cm width × 24 cm in height), lined with porous plastic sheets on the bottom for oviposition. I then randomly allocated 200 resultant neonate larvae to each of the 10 transparent plastic cylinders (8 cm diameter × 10 cm height), each filled with 50 g artificial diet (*ad libitum*) comprising of a 3.0:10.0:43.5:43.5 mixture of yeast, glycerine, maize meal, and whole meal wheat flour, respectively. I

covered the cylinder with a lid. I made a hole (3 cm diameter) in the middle of the lid and covered it with two layers of cloth mesh (2.8 apparatus per mm^2) for ventilation.

To generate an experimental line, I randomly collected 1,000 neonate larvae produced by adults from the above cylinders and reared them individually in 2-ml micro-centrifuge tubes, each with 0.25 g artificial diet for food and a ventilation hole in the lid made by an insect pin. I observed their pupation daily after the larvae reached the final (sixth) instar. The breeding colony and experimental line were kept and all experiments conducted at 25 ± 1 °C and 60 ± 10% relative humidity with photoperiod of 10:14 hr (dark:light).

4.2.2 Experimental setup and data collection

I randomly selected newly pupated pupae (male pupae with visible reddish testes in the abdomen) from the experimental line and transferred them into glass vials (2 cm diameter × 7.5 cm height) to create three treatments (Figure 4.1): (1) one male pupa in a vial (1M), (2) six male pupae in a vial (6M), and (3) one male pupa and five female pupae in a vial (1M5F). Pupae in treatments (2) and (3) were in close contact with each other. I plugged the glass vial opening with cotton wool and monitored adult emergence daily six days after transfer. All pupae from the vials successfully emerged. Immediately after eclosion, I individually transferred newly emerged male adults into micro-centrifuge tubes, clearly labelled them and placed them at -20 °C in a freezer. I considered all emerged males as replicates, i.e., the male from each 1M vial, the male from each 1M5F vial and all six males from each 6M vial. In total, I obtained 30 adult males (replicates) for each treatment.

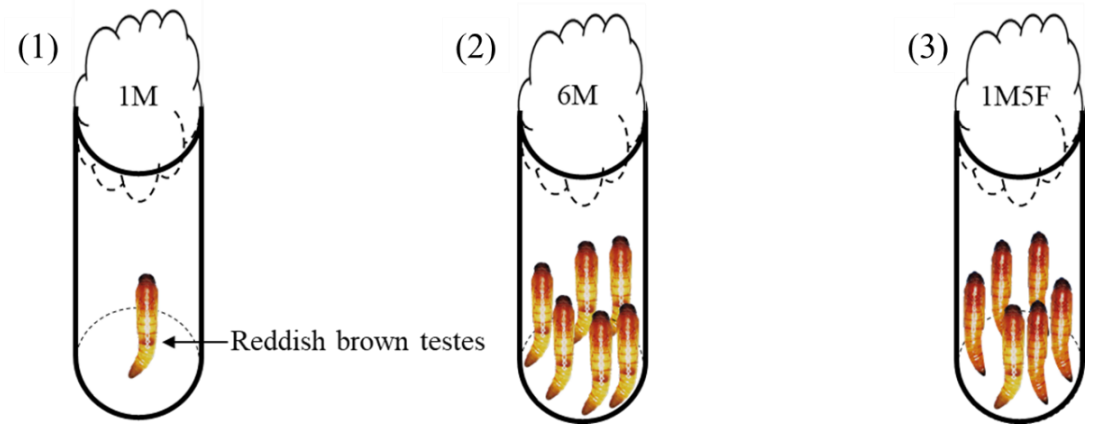

Figure 4.1 Experimental setup for the entire pupal stage of *E. kuehniella*: (1) 1M, one male, (2) 6M, six males together, and (3) 1M5F, one male and five females together.

I dissected all males, extracted their testes and measured testis volume with the aid of a stereomicroscope (Leica MZ12, Wetzlar, Germany) equipped with a digital camera (Olympus SC30, Tokyo, Japan) operated by the Olympus CellSens® software (GS-ST-V1.7, Tokyo, Japan). As *E. kuehniella* testes are fused into a spherical organ (Liu et al., 2022a), I calculated its volume using the sphere formula, $4/3\pi r^3$. I determined the *r* (radius) using the mean diameter from two measurements across the organ's central axis divided by two (Raichoudhury, 1936; Gage, 1995; Liu et al., 2022a). After volume measurement, I placed the testes into a drop of Belar saline solution on a cavity slide, tore them apart completely, gently rotated the slide, and counted the number of eupyrene and apyrene sperm under a phase-contrast microscope (Olympus BX51, Tokyo, Japan) according to Liu et al. (2022a).

4.2.3 Statistical analysis

Prior to statistical analyses, I fitted data to a general linear model to calculate their residuals and test residual distribution (Shapiro-Wilk test, UNIVARIATE procedure). Data on eupyrene number, apyrene number, and ln(x)-transformed testis size were normally distributed. Because the experimental design was pseudoreplicated, I employed a linear mixed-effects model (Millar & Anderson, 2004; Harrison et al., 2018) to analyse the data with treatment as a fixed factor and replicate nested into vial

(male source) as a random factor (Davies & Gray, 2015; Harrison et al., 2018; Amiri & Bandani, 2021; Liu et al., 2022a). I used a Tukey test for multiple comparisons between treatments. I analysed the relationship between eupyrenes and apyrenes by a general linear model (GLM procedure) and the slopes of linear lines by an analysis of covariance (ANCOVA) with treatment as the covariate in the model. The numbers of eupyrenes and apyrenes were ln(x)-transformed to achieve normal distribution of data before performing linear regression and ANCOVA. I performed the statistical analyses using SAS 9.4 (SAS Inc, USA).

4.3 Results

I demonstrate that males kept in groups (treatments 6M and 1M5F) produced significantly more eupyrene ($F_{2,29}$ = 26.31, P < 0.0001) and apyrene sperm ($F_{2,29}$ = 10.07, P = 0.0005) than those maintained singly (treatment 1M) (Figure 4.2A and B). Sex ratio did not significantly affect production of either eupyrene ($F_{1,29}$ = 3.66, P = 0.0658) or apyrene ($F_{1,29}$ = 3.19, P = 0.0847; Figure 4.2A and B). Testis size remained similar in all treatments ($F_{2,29}$ = 0.01, P = 0.9852; Figure 4.3).

My results show that the ratio of apyrene:eupyrene was about 5:1 with no significant difference between treatments ($F_{2,29}$ = 1.24, P = 0.3041). The numbers of eupyrenes and apyrenes were significantly positively correlated in all treatments ($F_{1,28}$ = 5.31, P = 0.0289 for 1M; $F_{1,28}$ = 16.65, P = 0.0003 for 1M5F; $F_{1,28}$ = 11.94, P = 0.0018 for 6M) but the slopes of regression lines were not significantly different ($F_{2,84}$ = 0.22, P = 0.7996; Figure 4.4).

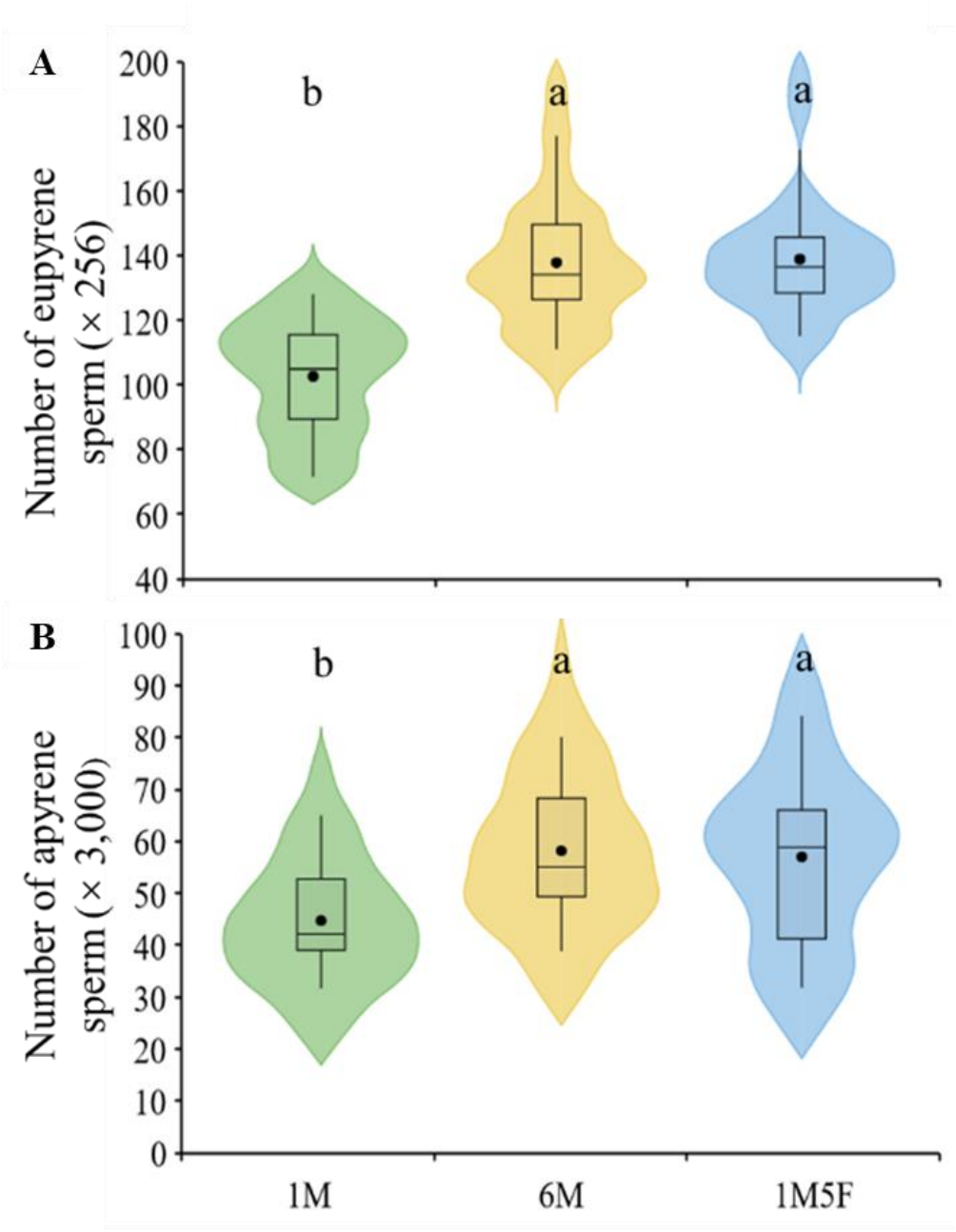

Figure 4.2 Effect of socio-sexual environment during the pupal stage on the number of eupyrene (**A**) and apyrene (**B**) sperm in testes of *E. kuehniella*. 1M, one male; 6M, six males together; 1M5F, one male and five females together. Each box plot shows the range between the first and third quartiles (black box), mean (black dot) and median scores (black lines); and 'violin' shapes show the shape of the distribution. Different letters on the top of the shapes denote significant differences between treatments ($P < 0.05$).

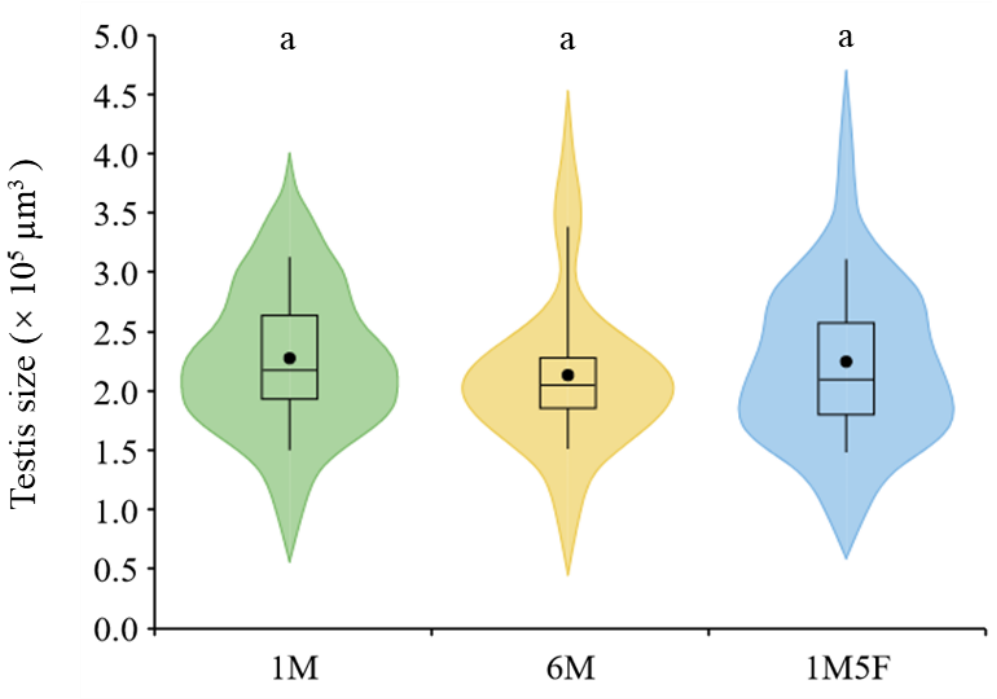

Figure 4.3 Effect of socio-sexual environment during the pupal stage on testis size of *E. kuehniella*. 1M, one male; 6M, six males together; 1M5F, one male and five females together. Each box plot shows the range between the first and third quartiles (black box), mean (black dot) and median scores (black lines); and 'violin' shapes show the shape of the distribution. The same letters on the top of the shapes denote no significant differences between treatments ($P > 0.05$).

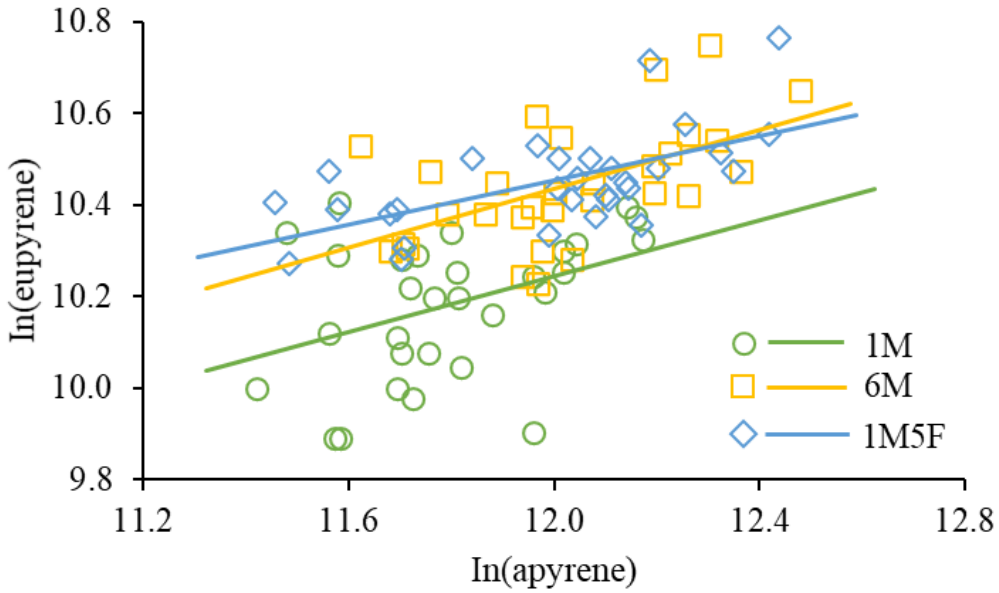

Figure 4.4 Relationship between the number of eupyrene and apyrene sperm produced. For 1M (one male), ln(eupyrene) = 6.58 + 0.31×ln(apyrene), R^2 = 0.1594; for 6M (six males together), ln(eupyrene) = 6.59 + 0.32×ln(apyrene), R^2 = 0.2990; and for 1M5F (one male and five females together), ln(eupyrene) = 7.54 + 0.24×ln(apyrene), R^2 = 0.3729.

4.4 Discussion

I demonstrate for the first time that male pupae of an insect increased sperm production after exposure to conspecific pupal cues regardless of sex (Figure 4.2). Previous studies report that male insect larvae also can increase their investment in sperm in the presence of non-sex specific larval cues (Gage, 1995; He & Miyata, 1997; McNamara et al., 2010; Liu et al., 2022a). These findings indicate that juvenile male insects can predict future sperm competition risks from cues of conspecific immature stages and subsequently adjust their sperm production (Gage, 1995; Allen et al., 2011; Kasumovic & Brooks, 2011; Gray & Simmons, 2013; Liu et al., 2022a). In lepidopteran insects, adults (Liu et al., 2020) and pupae (current study) adjust production of both fertile eupyrene and infertile apyrene sperm, while larvae only fine-tune production of eupyrene sperm (Liu et al., 2022a) in response to socio-sexual environment.

Furthermore, larvae either increase (Gage, 1995; Johnson et al., 2017) or reduce (Liu et al., 2022a) testis size in response to larval cues but pupae (Figure 4.3) and adults do not change their testis size under different socio-sexual situations. These discoveries suggest that resource allocation to sperm production and testis size differs depending on the life stages exposed to sperm competition environment.

The above diverse responses to social cues may be attributed to the fact that resource allocation to morphological traits and spermatogenesis takes place in different life stages. Evidence shows that most adult morphological traits are formed during the larval stage (Nijhout & Emlen, 1998; Moczek & Nijhout, 2004; Rolff et al., 2019; Mirth et al., 2021), allowing the larvae but not pupae and adults to adjust their testis size. Lepidopteran males produce most eupyrene sperm during the larval and pupal stages, most apyrene sperm during the pupal stage (Friedländer et al., 2005) and continue to produce both types of sperm during the adult stage (Liu et al., 2020). Therefore, male larvae can donate varying portions of testis volumes to spermatogenesis and other functions (Lüpold et al., 2020), and trade off testis size and apyrene sperm production to increase eupyrene sperm production in response to increasing sperm competition risk (Liu et al., 2022a). However, with fully grown testes adults and pupae have sufficient resources to increase production of both types of sperm in response to sperm competition environment.

In sperm-heteromorphic insects, the ayprene sperm often overwhelmingly outnumber the eupyrene sperm (Silberglied et al., 1984; Swallow & Wilkinson, 2002; Holman & Snook, 2008; Xu & Wang, 2014; Esfandi et al., 2020). Previous studies on *E. kuehniella* show that adult males increase the apyrene:eupyrene ratio in response to the presence of rivals (Liu et al., 2020) but male larvae reduce the ratio after exposed to larval cues (Liu et al., 2022a). These may be ascribed to the fact that spermatogenesis of apyrenes and eupyrenes occurs at different stages of insects (Swallow & Wilkinson, 2002; Friedländer et al., 2005) and they have different functions in reproduction (Holman & Snook, 2008; Wedell et al., 2009; Sakai et al., 2019; Hague et al., 2021), allowing adults to increase investment in apyrene and larvae to trade-off apyrene for more eupyrene. However, the current study on pupae demonstrates that the

apyrene:eupyrene ratio was about 5:1 with no significant difference between treatments. Furthermore, the numbers of eupyrenes and apyrenes were significantly positively correlated in all treatments with no significant difference in the slopes of regression lines (Figure 4.4). I suggest that in all life stages, males should strive to increase production of eupyrene sperm to ensure advantages in sperm competition (fertilization of more offspring) but also increase production of apyrene when they can (such as at the pupal and adult stages) to ensure successful arrival of eupyrene at the spermatheca.

Many studies reveal that insect larvae can communicate with each other using non-sex-specific cues (Corbet, 1971; Mudd, 1983; Crump et al., 1987; Fitzgerald & Underwood, 1998; Duthie et al., 2003; Flowers & Costa, 2003; Fitzgerald & Pescador-Rubio, 2011; Kojima et al., 2014; Dombrovski et al., 2017; Kwadha et al., 2019) and male larvae can adjust their testicular investment in response to these cues (Gage, 1995; Stockley & Seal, 2001; Johnson et al., 2017; Liu et al., 2022a). Although female pupae can produce sex pheromones in insects including my study species *E. kuehniella* (Calvert & Corbet, 1973; Duthie et al., 2003; Estrada et al., 2010; Pontier & Schweisguth, 2015), I have not found any indication that male pupae can respond to this sex specific cue and adjust sperm production accordingly (Figures 4.2 and 4.3). Because pupae were in close contact with each other in treatments (2) and (3), physical contact cues could also play a role in pupal response. These findings suggest that testicular investment in *E. kuehniella* juvenile males only responds to the presence of social (including contact), but not sexual cues, during their growth and development. An earlier study demonstrates that *E. kuehniella* adults can remember rival cues and increase sperm allocation for most of their reproductive life after the cues are removed (Liu et al., 2020). However, my findings on larval (Liu et al., 2022a) and pupal (current study) responses to social environment result from dissecting adults at emergence. Therefore, we still do not know whether different larval and pupal social exposures influence sperm allocation during their adult lifespan, which warrants further investigations.

In conclusion, this is the first report on testicular investment in response to the social environment during the pupal stage in an insect. I show that after exposure to pupal cues, male *E. kuehniella* pupae increase production of both eupyrene and apyrene sperm at the same rate but keep testis size unchanged. I suggest that testis size is fixed after pupation because resource allocation to most morphological traits occurs during the larval stage, allowing little room for pupae to adjust testis size. With fully grown testes, pupae can manipulate production of both types of sperm according to the sperm competition risk. Furthermore, sex specific cues such as sex pheromones do not affect sperm production.

CHAPTER 5

Juvenile Socio-Sexual Experience Determines Lifetime Sperm Expenditure and Adult Survival in *Ephestia kuehniella*

This chapter was published in *Insect Science* (2022).

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
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Name and title of main supervisor:	Professor Qiao Wang
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What percentage of the manuscript/published work was contributed by the student?	80

Describe the contribution that the student has made to the manuscript/published work:

The candidate conceived and designed the study, conducted laboratory work, and analysed the data. The candidate wrote the first draft of the manuscript, with changes and corrections completed with input from supervisors (co-authors).

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1 Doctoral Research Committee July 2022

Abstract

Male animals often adjust their sperm investment in response to sperm competition environment. To date, only a few studies have investigated how juvenile socio-sexual settings affect sperm production before adulthood and sperm allocation during the first mating. Yet, it is unclear whether juvenile socio-sexual experience (1) determines lifetime sperm production and allocation in any animal species; (2) alters the eupyrene:apyrene sperm ratio in lifetime ejaculates of any lepidopteran insects, and (3) influences lifetime ejaculation patterns, number of matings and adult longevity. Here I used a polygamous moth, *Ephestia kuehniella*, to address these questions. Upon male adult emergence from juveniles reared at different density and sex ratio, I paired each male with a virgin female daily until his death. I dissected each mated female to count the sperm transferred and recorded male longevity and lifetime number of matings. I demonstrate for the first time that males ejaculated significantly more eupyrenes and apyrenes in their lifetime after their young were exposed to juvenile rivals. Adult moths continued to produce eupyrene sperm, contradicting the previous predictions for lepidopterans. The eupyrene:apyrene ratio in the lifetime ejaculates remained unchanged in all treatments, suggesting that the sperm ratio is critical for reproductive success. Male juvenile exposure to other juveniles regardless of sex ratio caused significantly shorter adult longevity and faster decline in sperm ejaculation over successive matings. However, males from all treatments achieved similar number of matings in their lifetime. This study provides insight into adaptive resource allocation by males in response to juvenile social-sexual environment.

5.1 Introduction

Animals are expected to adjust their behaviour and physiology to gain a competitive edge in different socio-sexual environments (Kasumovic & Brooks, 2011; Acasuso-Rivero et al., 2019; Westneat et al., 2019). Various studies show that after adult males detect sperm competition risk, they raise their sperm expenditure for a higher paternity share (e.g., Parker, 1970; Gage, 1991; Simmons et al., 2007; Jarrige et al., 2015; Esfandi et al., 2020; Liu et al., 2020). To date, only a few studies have explored how male insects tailor their investment in sperm as a response to juvenile socio-sexual settings. For example, adults from juveniles exposed to higher density of conspecific juveniles regardless of sex ratio ejaculate more sperm in their first mating (Gage, 1995; He & Miyata, 1997; Yamane & Miyatake, 2005; McNamara et al., 2010) or have higher sperm counts at emergence (Liu et al., 2021, 2022a). Allen et al. (2011) report that after male juveniles are reared together, their adults transfer more sperm during the first mating. Yet, it is still unknown whether and how juvenile socio-sexual environment influences lifetime sperm production and allocation in any animal species. It is also unclear whether exposure to similar social-sexual settings by different juvenile stages results in diverse lifetime sperm expenditure in adults.

Most lepidopteran species produce two distinct spermatozoa, the nucleate eupyrenes (fertile) and anucleate apyrenes (infertile), and the most widely accepted notion is that spermatogenesis occurs in juvenile stages and eupyrene sperm production ends before adult emergence (Swallow & Wilkinson, 2002; Friedländer et al., 2005). However, it remains unclear whether juvenile socio-sexual situations affect spermatogenesis during the adult stage. Although the apyrene sperm cannot fertilize eggs, they assist eupyrene in migration from female bursa copulatrix to spermatheca (Sakai et al., 2019; Chen et al., 2020; Konagaya et al., 2020; Hague et al., 2021), protect eupyrene sperm against a hostile female reproductive tract (Holman & Snook, 2008) and help win sperm competition games (Cook & Wedell, 1999; Wedell et al., 2009; Mongue et al., 2019). Therefore, the eupyrene:apyrene ratio could be essential for reproductive success in males. Though, it is unknown whether the juvenile socio-sexual environment affects the sperm ratio in ejaculates transferred during the adult lifespan.

The number of sperm ejaculated by males should decrease over successive matings due to limited resources and aging (Wedell & Cook, 1999; Velde et al., 2011; Esfandi et al., 2015, 2020; Liu et al., 2020). However, it is not clear whether different juvenile experience alters lifetime ejaculation pattern. Because sperm production (Dewsbury, 1982; van Voorhies, 1992; Olsson et al., 1997; Pitnick et al., 2006; Hayward & Gillooly, 2011; Lemaître et al., 2020) and matings (Martin & Hosken, 2004; McNamara et al., 2008; Oliver & Cordero, 2009; Metzler et al., 2016; Mautz et al., 2019; Jehan et al., 2020) are costly, males may not be able to maintain maximal reproduction and longevity simultaneously (Kirkwood, 1977; Roff, 2002). Nevertheless, many studies show that the adult socio-sexual environment alters males' reproductive investment but not their longevity (e.g., Janowitz & Fischer, 2010; Moatt et al., 2013; Esfandi et al., 2015; Leech et al., 2019). To date, knowledge of how juvenile socio-sexual settings affect adult mating frequency and longevity is still lacking.

I used a polygamous moth, *E. kuehniella*, to investigate how juvenile socio-sexual environment influences lifetime sperm expenditure, mating frequency and survival in adult males. Under my experimental conditions, larval and pupal stages last about 29 and eight days, respectively. Adults do not feed, and all resources are acquired during the larval stage. Adults become sexually mature at emergence and start mating at the onset of the first scotophase (Xu et al., 2008). Adult males increase their sperm allocation after exposure to rival cues during early adulthood (Esfandi et al., 2020; Liu et al., 2020). Adults have more sperm of both types at emergence after their pupae are exposed to higher density of conspecifics (Liu et al., 2021) but if such exposure starts at the larval stage, only eupyrene sperm increases at emergence (Liu et al., 2022a), suggesting that larvae and pupae may respond to the same social context differently.

In the current study, I prepared thousands of larvae and manipulated larval-pupal and pupal density with varied sex ratios. Upon male adult emergence, I paired each male with a virgin female per day until his death. I dissected each mated female to count eupyrene and apyrene sperm transferred per mating and recorded males' lifetime mating frequency and longevity. This is the first study on how juvenile socio-sexual

experience affects male adult longevity, and lifetime sperm production and allocation, eupyrene:apyrene sperm ratio and mating frequency in an insect. Knowledge presented here provides novel understanding of adaptive resource allocation by males in response to juvenile social-sexual environment.

5.2 Materials and methods

5.2.1 Insects and environmental conditions

I collected *E. kuehniella* larvae from a poultry farm, in Foxton, New Zealand and reared them to adults with their original food (a mixture of wheat and maize flour) in 20 transparent plastic cylinders (10.0 cm height × 8.0 cm diameter). I randomly selected about 300 newly emerged adults (ca. 1:1 sex ratio) from all cylinders and introduced them into a transparent plastic cage (24.0 cm height × 28.0 cm length × 28.0 cm width) with a porous plastic sheet at the bottom for oviposition. I collected eggs by pulling out the sheet and replacing it with a new one once every day for 10 days and incubated the eggs in Petri dishes (1.5 cm height × 8.5 cm diameter). I then inoculated 200 neonate larvae to 50 g standard diet (*ad libitum*) (21.75 g whole meal wheat flour, 21.75 g maize meal, 5 g glycerine and 1.5 g yeast) in a plastic cylinder as mentioned above. I constantly maintained 20 such cylinders as the breeding colony for experimental insects.

I randomly transferred 300 newly emerged adults (ca. 1:1 sex ratio) from the colony to an aforementioned plastic cage for mating and subsequently letting females lay eggs in the cage. I then randomly collected 1,000 neonate larvae from the cage to establish an experimental line. I reared these larvae individually in 2-ml Eppendorf tubes, each of which held 0.25 g standard diet for food and pin holes in the lid for ventilation. To prepare virgin females for mating with focal males over the course of the experiment, I randomly collected about 1,000 female pupae from the breeding colony and individually housed them in the Eppendorf tubes until use for experiment. I maintained the colony and experimental insects and carried out all experiments at 25 ± 1°C and 60 ± 10% relative humidity under the photoperiod of 10:14 hr (dark:light).

5.2.2 Pre-adult socio-sexual settings for focal males

The sex of the fourth instar larvae and pupae can be determined by visible testes in males' abdomens (Figure 5.1; Liu et al., 2021). I randomly selected newly molted fourth-instar larvae (L) and newly pupated pupae (P) from the experimental line and transferred them into glass vials (7.5 cm height × 2.0 cm diameter) to create five socio-sexual enviroments for focal males (M) (Figure 5.1): (1) single male (SM-LP) – one male was kept in a glass vial with a 0.25 g standard diet from the fourth instar larva to adult emergence; (2) six males (6M-LP) – six males were raised in a glass vial with a 1.5 g standard diet from the fourth instar larvae to adult emergence; (3) one male and five females (1M5F-LP) – one male and five females (F) were reared in a glass vial with a 1.5 g standard diet from the fourth instar larvae to adult emergence; (4) six male pupae (6M-P) – six male pupae were maintained in a glass vial for the entire pupal stage until adult emergence, and (5) one male and five female pupae (1M5F-P) – one male and five females were put in a glass vial for the entire pupal stage until adult emergence. All glass vials were covered with wool cotton at the top.

For all treatments I monitored adult emergence hourly when the pupae turned dark brown (ca. 1 day before adult emergence). Immediately after males' eclosion, I individually transferred them into clean glass vials and clearly labelled all vials. To keep conditions consistent, I only used males from vials where all individuals emerged for data collection. I considered the male from each SM-LP, 1M5F-LP and 1M5F-P vial and all six males from each 6M-LP and 6M-P vial as focal males.

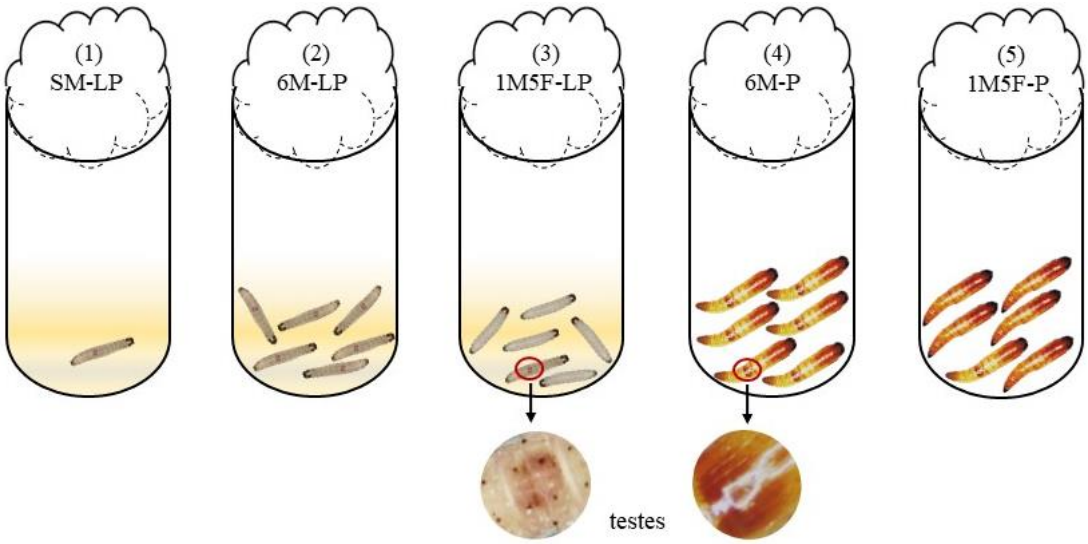

Figure 5.1 Socio-sexual environment treatments for *E. kuehniella* males before eclosion: (1) SM-LP, single male from the fourth instar larva to adult emergence; (2) 6M-LP, six males together from the fourth instar larvae to adult emergence; (3) 1M5F-LP, one male and five females together from the fourth instar larvae to adult emergence; (4) 6M-P, six male pupae together for the entire pupal stage until adult emergence; and (5) 1M5F-P, one male and five females together for the entire pupal stage until adult emergence.

5.2.3 Data collection

At the onset of the first scotophase following eclosion, I individually paired the focal males with 1-d-old virgin females randomly selected from the breeding colony, in transparent plastic cylinders (17.0 cm length × 6.5 cm diameter). Ten red light tubes (Sylvania, F36W/Red, Holland) 1.5 m above the cylinders were used for illumination. Because a male requires 24-hr recovery time to produce a full spermatophore again after each mating (Xu & Wang, 2009b), I randomly assigned another 1-d-old virgin female to the focal male in the cylinder at the onset of the next scotophase. This procedure was repeated until the death of the focal male. I monitored each mating pair once every 15 minutes until mating ended and immediately removed the mated female from the cylinder. I recorded mating frequency (lifetime number of matings) and longevity (duration between emergence and death) of each focal male. I considered

each focal male as a replicate. In total, I achieved 24, 23, 24, 22 and 24 replicates for treatments SM-LP, 6M-LP, 1M5F-LP, 6M-P, and 1M5F-P, respectively.

To record males' lifetime sperm allocation, I counted the number of sperm ejaculated by a male in each mating via dissecting all mated females from the above experiment and extracting the spermatophores out from their bursa copulatrix. In total I dissected 123, 130, 133, 136 and 142 females for SM-LP, 6M-LP, 1M5F-LP, 6M-P, and 1M5F-P, respectively. I placed the bursa copulatrix into a droplet of Belar saline solution on a cavity slide. Using two fine needles, I ruptured the spermatophore to release sperm under a stereomicroscope (Leica MZ12, Wetzlar, Germany). I then counted the number of bundles of eupyrene sperm under a phase-contrast microscope (Olympus BX51, Tokyo, Japan). I calculated the total number of eupyrene sperm as the total number of bundles multiplied by 256, the number of eupyrene sperm per bundle (Garbini & Imberski, 1977). Afterwards, the sample was thoroughly washed off the cavity slide and diluted in a glass vial with 30-ml distilled water. I gently rotated the vial for about 30 s to deliver even dispersal of apyrenes in the vial. I took eight 10-μl subsamples from the vial using a Gilson autopipette and placed them separately on a microscope slide. I counted the number of apyrene sperm of all eight subsamples under the phase-contrast microscope and calculated the mean number per 10 μl as the sum of apyrene sperm in eight subsamples divided by eight. I then calculated the total number of apyrene sperm for each mating as the mean number of apyrenes per 10 μl multiplied by the dilution factor (3,000) (Koudelová & Cook, 2001). The lifetime number of eupyrene and apyrene sperm ejaculated by a male adult is the sum of these sperm ejaculated in each mating.

5.2.4 Statistical analysis

I analysed all data using SAS 9.13 (SAS Institute Inc, USA) with a rejection level set at $P < 0.05$. Because the experimental design was pseudoreplicated, I analysed the mating frequency and lifetime number of sperm transferred by male adults using a linear mixed-effects model (MIXED procedure) (Millar & Anderson, 2004; Harrison et al., 2018), with treatment as a fixed factor and replicate nested into vial (male source)

as a random factor (Davies & Gray, 2015; Harrison et al., 2018). I applied a Tukey test in the model for multiple comparisons between treatments. A log-rank test (LIFETEST procedure) was applied to compare the survival probability of focal males between treatments. The relationship between the total number of eupyrene and apyrene ejaculated was analysed by a general linear model (GLM procedure) and an analysis of covariance (ANCOVA) was used to compare the slopes of regression lines between treatments (Liu et al., 2021). I used a linear mixed-effects model with repeated measures (MIXED procedure) to test how treatment affected males' sperm allocation in successive matings. I set treatment, mating frequency and their interaction as the fixed effects in the model with a subject effect of focal male in the statement of 'REPEATED / TYPE = cs SUBJECT = focal male' after the model. A CONTRAST statement was then applied to compare the slopes of regression lines of sperm ejaculation over successive matings between treatments. Because the moths used in the current study were from the same batch reared under the same conditions as in Liu et al. (2021, 2022a), I used a two-sample t test to compare the number of eupyrene and apyrene sperm and their ratio in lifetime ejaculates with those recorded at emergence (Liu et al., 2021, 2022a).

5.3 Results

5.3.1 Effect of socio-sexual environment during juvenile stages on lifetime sperm allocation

Males that were exposed to conspecific males during larval-pupal stages (6M-LP) or the pupal stage (6M-P) ejaculated significantly more eupyrene and apyrene sperm than those that were exposed to conspecific females during larval-pupal stages (1M5F-LP) or the pupal stage (1M5F-P) or reared singly during larval-pupal stages (SM-LP) ($F_{4,48}$ = 4.11, P = 0.0060 for eupyrene; $F_{4,48}$ = 4.58, P = 0.0033 for apyrene; Figure 5.2). The lifetime number of eupyrenes and apyrenes ejaculated was significantly positively correlated in all treatments ($P < 0.001$), with no significant difference in the slopes of regression lines between treatments ($F_{4,105}$ = 0.32, P = 0.8639; Figure 5.3).

Both eupyrene (Figure 5.4A) and apyrene (Figure 5.4B) sperm ejaculated declined significantly over successive matings ($P < 0.001$). However, both types of sperm ejaculated declined significantly faster in exposed males (6M-LP, 1M5F-LP, 6M-P, and 1M5F-P) than in unexposed ones (SM-LP) ($F_{4,635} = 4.73$, $P = 0.0009$ for eupyrene; $F_{4,635} = 7.96$, $P < 0.0001$ for apyrene) and there was no significant difference in slopes among exposed males (eupyrene: $F_{3, 514} = 0.63$, $P > 0.05$; apyrene: $F_{3, 514} = 0.96$, $P > 0.05$; Figure 5.4).

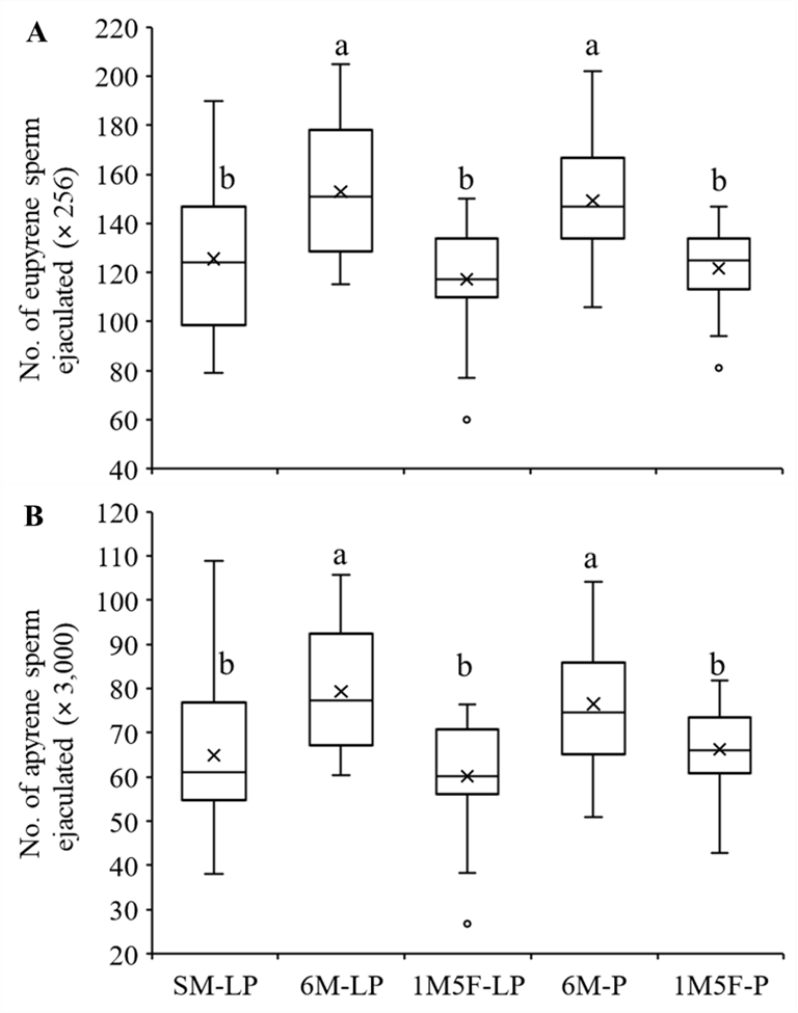

Figure 5.2 Effects of pre-adult socio-sexual environment on lifetime eupyrenes (**A**) and apyrenes (**B**) ejaculated by *E. kuehniella* males. SM-LP, single male from fourth instar larva to adult emergence; 6M-LP, six males together from fourth instar larvae to adult emergence; 1M5F-LP, one male and five females together from fourth instar larvae to adult emergence; 6M-P, six male pupae together for the entire pupal stage until adult emergence; and 1M5F-P, one male and five females together for the entire pupal stage until adult emergence. 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 '×' show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum scores. Boxes with different letters denote significant differences between treatments ($P < 0.05$).

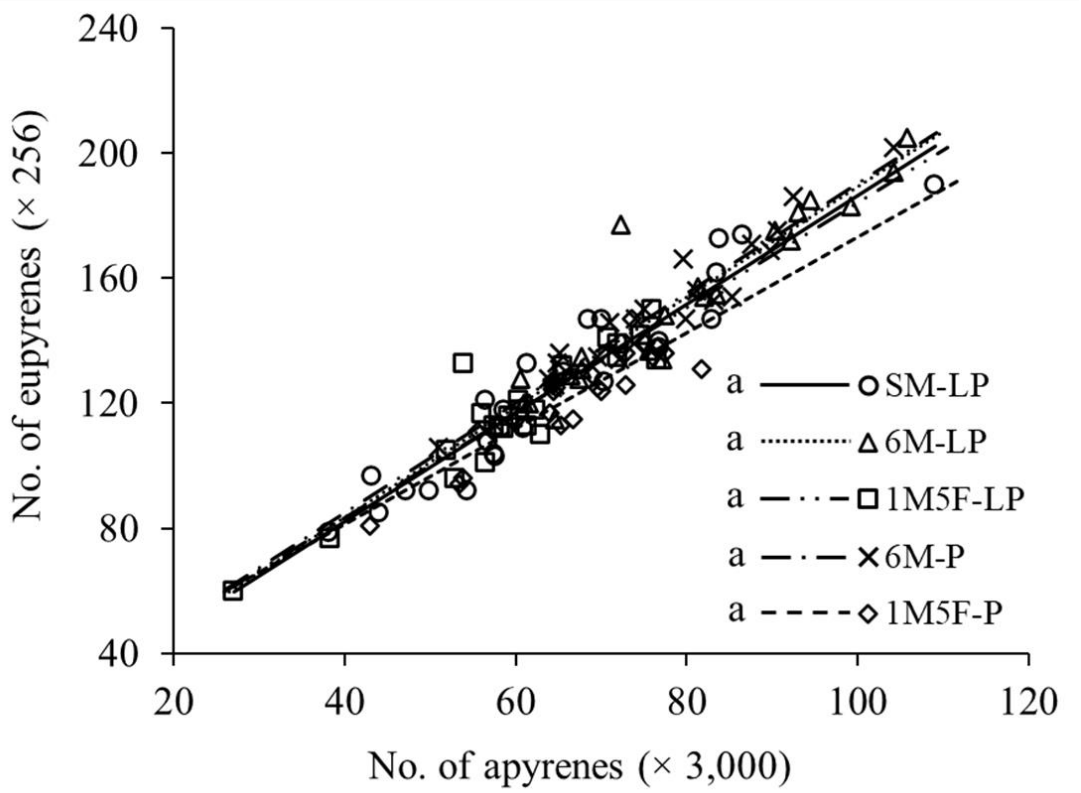

Figure 5.3 Relationship between the number of eupyrene and apyrene sperm ejaculated in *E. kuehniella* males' lifetime. For SM-LP, eupyrene = 12.31 + 1.74 × apyrene ($F_{1,22}$ = 204.10, $P < 0.001$); for 6M-LP, eupyrene = 13.17 + 1.76 × apyrene ($F_{1,20}$ = 130.51, $P < 0.001$); for 1M5F-LP, eupyrene = 16.13 + 1.68 × apyrene ($F_{1,21}$ = 126.29, $P < 0.001$); for 6M-P, eupyrene = 14.86 + 1.76 × apyrene ($F_{1,20}$ = 275.94, $P < 0.001$); and for 1M5F-P, eupyrene = 20.07 + 1.53 × apyrene ($F_{1,22}$ = 103.42, $P < 0.001$).

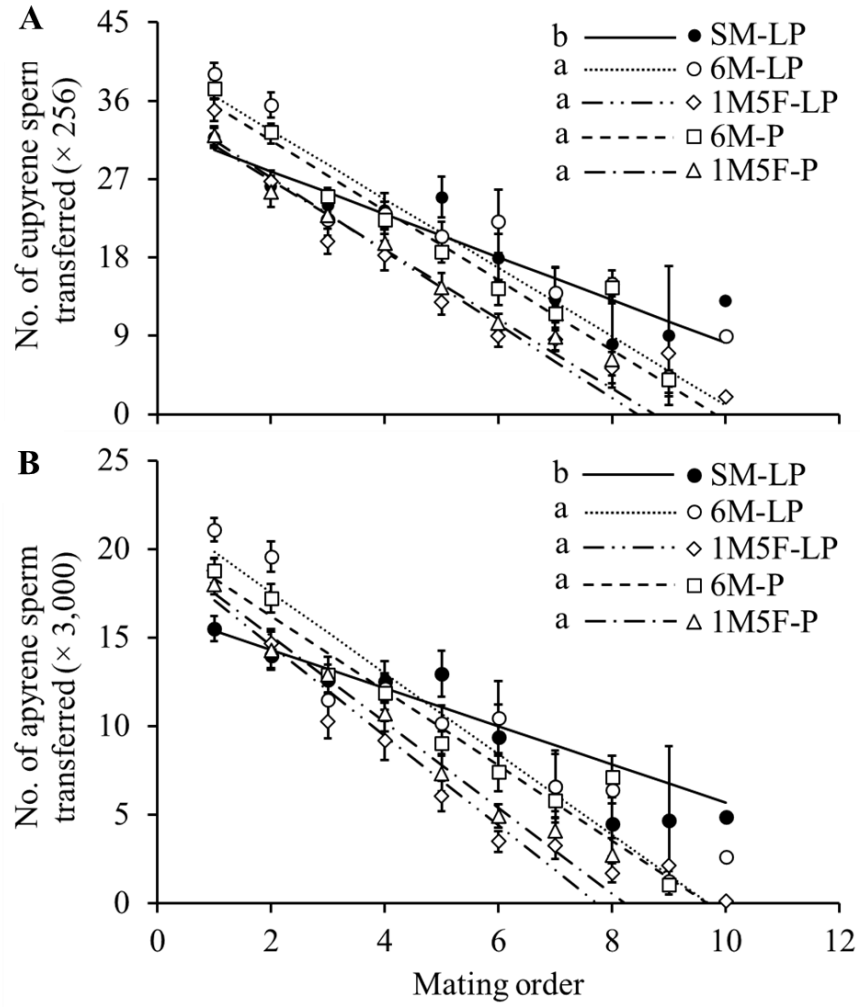

Figure 5.4 Effects of pre-adult social environment on eupyrenes (**A**) and apyrenes (**B**) ejaculated by *E. kuehniella* males in relation to mating order (MO). Eupyrene: for SM-LP, sperm = 32.84 - 2.46 × MO ($F_{1,98}$ = 56.27, P < 0.001); for 6M-LP, sperm = 40.51 - 3.95 × MO ($F_{1,108}$ = 201.38, P < 0.001); for 1M5F-LP, sperm = 35.67 - 4.23 × MO ($F_{1,110}$ = 251.93, P < 0.001); for 6M-P, sperm = 39.49 - 4.02 × MO ($F_{1,113}$ = 218.17, P < 0.001); and for 1M5F-P, sperm = 34.89 - 4.00 × MO ($F_{1,117}$ = 194.98, P < 0.001). Apyrene: for SM-LP, sperm = 16.48 - 1.08 × MO ($F_{1,98}$ = 33.38, P < 0.001); for 6M-LP, sperm = 22.11 - 2.28 × MO ($F_{1,108}$ = 157.73, P < 0.001); for 1M5F-LP, sperm = 19.62 - 2.54 × MO ($F_{1,110}$ = 237.20, P < 0.001); for 6M-P, sperm = 20.45 - 2.11 × MO ($F_{1,113}$ = 194.50, P < 0.001); and for 1M5F-P, sperm = 19.91 - 2.42 × MO ($F_{1,117}$ = 186.22, P < 0.001). Points and vertical lines represent means and standard errors, respectively.

5.3.2 Comparison between the number of sperm ejaculated in lifetime and that counted at emergence

In treatments 6M-LP, 6M-P and SM-LP, the lifetime number of eupyrene sperm ejaculated (Figure 5.2A) was significantly higher than that counted at emergence (t_{50} = 4.20, P = 0.0001 for 6M-LP; t_{50} = 2.52, P = 0.0159 for 6M-P; t_{54} = 3.09, P = 0.004 for SM-LP) (Liu et al., 2021, 2022a). The lifetime number of apyrene sperm ejaculated (Figure 5.2B) was also significantly higher than that measured at emergence in all five treatments (t_{50} = 8.61, P < 0.0001 for 6M-LP; t_{51} = 3.33, P = 0.0016 for 1M5F-LP; t_{50} = 5.58, P < 0.0001 for 6M-P; t_{52} = 3.21, P = 0.0023 for 1M5F-P; t_{54} = 5.15, P < 0.0001 for SM-LP) (Liu et al., 2021, 2022a). Furthermore, the apyrene:eupyrene ratio in lifetime ejaculates (6:1) (Figure 5.2) was significantly higher than that (5:1) at emergence (Liu et al., 2021, 2022a) (t_{50} = 7.74, P < 0.0001 for 6M-LP; t_{51} = 5.99, P < 0.0001 for 1M5F-LP; t_{50} = 6.03, P < 0.0001 for 6M-P; t_{52} = 6.54, P < 0.0001 for 1M5F-P; t_{54} = 3.80, P = 0.0004 for SM-LP).

5.3.3 Effect of socio-sexual environment during juvenile stages on mating frequency and longevity

Pre-adult socio-sexual exposure had no significant effect on the number of matings adult males achieved in their lifetime (mean ± SE = 5.13 ± 0.39, 5.91 ± 0.43, 5.78 ± 0.37, 6.18 ± 0.37, and 5.92 ± 0.30 for SM-LP, 6M-LP, 1M5F-LP, 6M-P, and 1M5F-P, respectively) ($F_{4,48}$ = 1.38, P = 0.2552). However, regardless of sex ratio, adult males that were exposed to conspecific individuals during larval-pupal stages or the pupal stage (6M-LP, 1M5F-LP, 6M-P, and 1M5F-P) lived significantly shorter than those that were reared singly (SM-LP) (x_4^2 = 21.44, P < 0.0001), and all exposed males had similar longevity (P > 0.05; Figure 5.5).

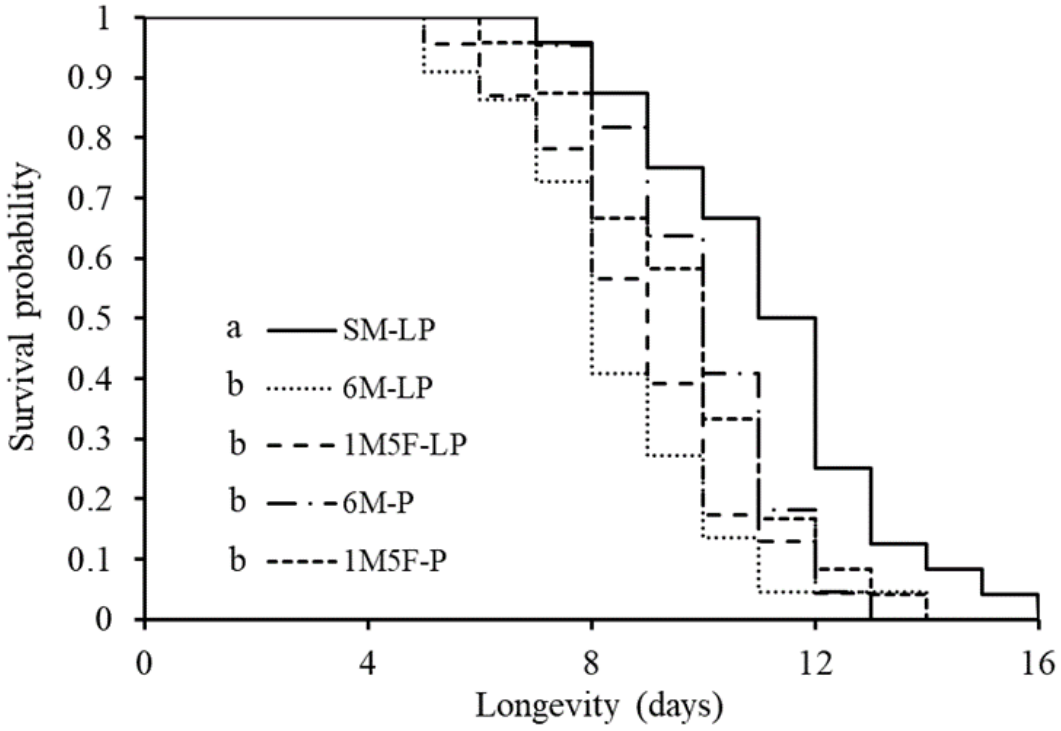

Figure 5.5 Effects of pre-adult socio-sexual environment on adult male longevity in *E. kuehniella*. SM-LP, single male from fourth instar larva to adult emergence; 6M-LP, six males together from fourth instar larvae to adult emergence; 1M5F-LP, one male and five females together from fourth instar larvae to adult emergence; 6M-P, six male pupae together for the entire pupal stage until adult emergence; and 1M5F-P, one male and five females together for the entire pupal stage until adult emergence. Lines with different letters are significantly different ($P < 0.05$).

5.4 Discussion

The present study shows that both juvenile stages of *E. kuehniella*, larvae and pupae, are sensitive to their socio-sexual environment and their experience affects lifetime sperm production and allocation and adult longevity but not eupyrene:apyrene ratio and mating frequency. I demonstrate for the first time that adult *E. kuehniella* males developing from juveniles reared with juvenile rivals transferred significantly more eupyrenes (Figure 5.2A) and apyrenes (Figure 5.2B) in their lifetime than those from juveniles raised solitarily or with juvenile mates. My findings provide strong evidence that the impact of socio-sexual environment during juvenile stages continues throughout the adult stage. Furthermore, the sperm allocation patterns remained the same following exposure either from late instar larval to pupal stages or just during the pupal stage (Figure 5.2). This suggests that the late juvenile stage is a critical period for building up the long-term memory of the pre-adult social environment in insects.

Using the same batch of moths reared under the same condition as Liu et al. (2021, 2022a), I show that the number of sperm ejaculated in lifetime (Figure 5.2) was significantly higher than that counted at emergence (Liu et al., 2021, 2022a) in *E. kuehniella*. These findings suggest that the production of both eupyrene and apyrene sperm continues during the adult stage in lepidopterans, contradicting previous perceptions (Swallow & Wilkinson, 2002; Friedländer et al., 2005). The sperm ratio (apyrene:eupyrene) in lifetime ejaculates (Figure 5.2) was also significantly higher than that at emergence (Liu et al., 2021, 2022a), supporting previous findings that apyrenes are cheaper to produce than eupyrenes (Silberglied et al., 1984; Cook & Gage, 1995). My study reveals that the sperm ratio in lifetime ejaculates remained the same regardless of treatments during juvenile stages (Figure 5.3), suggesting that the sperm ratio in ejaculates is critical for reproductive success.

Similar to previous findings (e.g., Wedell & Cook, 1999; Velde et al., 2011; Esfandi et al., 2020; Liu et al., 2020), I show that the number of sperm ejaculated by males significantly decreased over successive matings (Figure 5.4). These patterns fit the general prediction that males suffer from reduced quantity of their sperm with

(Dewsbury, 1982; Fricke & Maklakov, 2007; Vega-Trejo et al., 2019). However, the ejaculation of both eupyrene and apyrene sperm declined significantly faster over time in males whose juveniles were exposed to conspecific juveniles of any sex ratio than in those unexposed (Figure 5.4). Higher sperm production before emergence in the exposed males (Liu et al., 2021; 2022a) may exacerbate sperm senescence (Ball & Parker, 1996; Reinhardt, 2007; Pizzari et al., 2008) so that they are of greater urgency to expel the accumulated aged sperm in their reservoirs to gain reproductive fitness. This may result in ejaculation of more sperm in their first couple of matings, raising the starting points of the linear lines and leading to steeper slopes (Figure 5.4).

Adult *E. kuehniella* males had significantly shorter longevity after their juveniles were exposed to conspecific juveniles of any sex ratio as compared to those whose young were individually reared (Figure 5.5). Because most spermatogenesis occurs during juvenile stages (Friedländer et al., 2005; Liu et al., 2021, 2022a) and sperm production entails significant costs (Dewsbury, 1982; van Voorhies, 1992; Olsson et al., 1997; Pitnick et al., 2006; Hayward & Gillooly, 2011; Lemaître et al., 2020), I suggest that the increase of resource allocation to sperm production in the presence of conspecifics during juvenile stages (Liu et al., 2021, 2022a) causes the early death of male adults. I show that males in different treatments achieved the same number of matings in their lifetime, suggesting that the number of matings is ultimately important for maximal reproductive fitness regardless of juvenile experience in *E. kuehniella* males.

In conclusion, the present study provides the first evidence that adult *E. kuehniella* males ejaculate significantly more eupyrene and apyrene sperm in their lifetime after exposure to rivals during the larval-pupal or pupal stage. In contrary to previous predictions for lepidopterans, I show that adults continue to produce sperm of both types. Despite different lifetime sperm allocations among treatments, the apyrene:eupyrene ratio remains 6:1, implying that the sperm ratio in ejaculates is critical for reproductive success. While both types of sperm ejaculated decrease over successive matings in all treatments, the rate of decrease is faster in males exposed to conspecifics during juvenile stages. This may result from the fact that the exposed

males produce more sperm before emergence and ejaculate more in their first mating. Adults from juveniles exposed to conspecific juveniles of any sex ratio have shorter longevity probably because exposed juveniles allocate more resources to sperm production and trade off adult survival. Finally, all *E. kuehniella* males have similar number of matings in their lifetime regardless of whether their juveniles are exposed to conspecific juveniles or not. The knowledge generated here provides insight into adaptive resource allocation by males in response to social-sexual experience of different juvenile stages.

CHAPTER 6

Male Larvae Experience of Cues from Adult Rivals Alters Lifetime Sperm Investment Patterns in *Ephestia kuehniella*

This chapter was submitted to *Insect Science* for publication.

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Junyan Liu
Name and title of main supervisor:	Professor Qiao Wang
In which chapter is the manuscript/published work?	Chapter 6
What percentage of the manuscript/published work was contributed by the student?	80

Describe the contribution that the student has made to the manuscript/published work:

The candidate conceived and designed the study, conducted laboratory work, and analysed the data. The candidate wrote the first draft of the manuscript, with changes and corrections completed with input from supervisors (co-authors)

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1 Doctoral Research Committee July 2022

Abstract

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

6.1 Introduction

Animals often fine-tune their physiology and behaviour in response to their socio-sexual environment to gain a competitive advantage (Pigliucci, 2005; Bretman et al., 2011b; Kasumovic & Brooks, 2011; Taborsky, 2016; Dore et al., 2018). For example, adult males increase their investment in sperm for a higher paternity share when they perceive sperm competition risk (Parker, 1970; Wedell et al., 2002; Parker & Pizzari, 2010). Previous studies indicate that juvenile males also can detect their future sperm competition risk from their juvenile companions and adjust resource allocations accordingly (Gage, 1995; He & Miyata, 1997; Yamane & Miyatake, 2005; McNamara et al., 2010; Kasumovic & Brooks, 2011; Taborsky, 2016; Liu et al., 2021, 2022a, 2022b). In nature, conspecific young and adults may coexist regularly at a given time and space, adding adult cues to juvenile socio-sexual surroundings (Chapman et al., 2007; Nehring & Müller, 2009; Bjørnstad et al., 2016; Arbaiza-Bayona et al., 2022). To date, only a few studies have investigated the impact of adult males on resource allocations in juvenile male insects, including investment in testes (Bailey et al., 2010; Bretman et al., 2016) and ejaculation in the first mating (Gray & Simmons, 2013; Simmons & Lovegrove, 2017). Yet, it is still unknown whether juvenile sensitivity to adult male cues is stage dependent and whether these cues affect lifetime sperm production and ejaculation in any insect.

Sperm heteromorphic insects such as lepidopterans start producing larger nucleate eupyrene sperm and smaller anucleate apyrene sperm during juvenile stages (Garbini & Imberski, 1977; Friedländer et al., 2005). Eupyrenes fertilize eggs and apyrenes assist in success of sperm competition (Cook & Wedell, 1999; Wedell et al., 2009; Mongue et al., 2019) and fertilization (Holman & Snook, 2008; Sakai et al., 2019; Hague et al., 2021). So far, it is not clear whether and how juvenile males adjust their expenditure in these sperm of different functions according to the timing of their experience of adult males. Furthermore, changes in resource allocations may result in trade-offs between spermatogenesis and other life traits (Ramm & Stockley, 2009; Devigili et al., 2015; Simmons et al., 2017; Paschoal & Zara, 2022). However, previous studies suggest that juvenile social environment has little impact on juvenile survival

(Woodroffe & Macdonald, 2000; Berger et al., 2015; Cannarsa et al., 2015), body size at maturity (Gage, 1995; Lemaître et al., 2011; Bretman et al., 2016; Hobson et al., 2020), and resultant adults' mating frequency (Rutledge & Uetz, 2014; Liu et al., 2022b) and longevity (McNamara et al., 2010). Yet, none of these studies has explored the effect of juvenile experience on all these traits in any single species, making it difficult to determine whether adjustment of investment in sperm would alter resource allocation to all other traits.

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

In the current study, I exposed *E. kuehniella* larvae of different stages to adult males. I recorded their survival to adulthood, and dissected half of the treated males at emergence and measured their testis size and sperm number. I paired each of the remaining treated males with a virgin female daily and counted the number of sperm

ejaculated. I also recorded mating latency of the first mating, mating frequency and longevity of these paired males. This design allowed me to test stage-dependent sensitivity of juvenile males to adult male cues and subsequent resource allocations, providing insight into lifetime reproductive investment of juvenile males in response to adult cues.

6.2 Materials and methods

6.2.1 Insects and environmental conditions

I collected more than 2,000 *E. kuehniella* larvae together with their original food (a mixture of wheat and corn flour) from Turks Poultry, Foxton, New Zealand. I then transferred these into 20 transparent plastic cylinders (8 cm diameter × 10 cm length) covered with cotton gauze (2.8 apparatus per mm^2), each with 100 larvae and about 100 g of their original food, and maintained them in the laboratory. I randomly collected 300 newly eclosed adults (≈ 1:1 sex ratio) from all cylinders and introduced them into a transparent plastic cage (28 cm length × 28 cm width × 24 cm height), lined with a plastic sheet on the bottom for egg collection. I collected eggs daily for 10 days and incubated them in Petri dishes (8.5 diameter × 1.5 cm length). I inoculated 200 neonate larvae onto 50 g of standard diet (*ad libitum*), consisting of 3% yeast, 10% glycerine, 43.5% maize meal, and 43.5% whole meal wheat flour (Liu et al., 2020) in a cylinder mentioned above. I maintained 10 such cylinders as the laboratory colony. I kept the colony and conducted all experiments at 25 ± 1°C, 60 ± 10% relative humidity, and a photoperiod of 10:14 hr (dark:light). Under this condition, larval and pupal stages last about 29 and 8 days, respectively.

6.2.2 Treatments

I randomly selected 800 neonate larvae from the colony and evenly transferred 200 larvae into each of four above-mentioned cylinders with 50 g of standard diet. To determine whether and how *E. kuehniella* males adjusted their lifetime investment in reproduction and survival after their larvae of different stages were exposed to adult

males, I set up three treatments and one control as follows: (1) ELE (early larval exposure) – immediately after the transfer of neonate larvae, I introduced 10 adult males into the cylinder and allowed them to stay for five days, after which time, I removed all adults; (2) LLE (late larval exposure) – 15 days after the transfer of neonate larvae, I introduced 10 adult males into the cylinder and allowed them to stay for five days, after which time, I removed all adults; (3) CLE (complete larval exposure) – immediately after the transfer of neonate larvae, I introduced 10 adult males into the cylinder and replaced them with 10 new ones once every five days until pupation, after which time, I removed all adults, and (4) CON (control) – no adult males in the cylinder. All adult males used at the onset of exposure were newly emerged, virgin and randomly selected from the breeding colony. The cylinders were placed in four separate environment chambers (Percival Scientific I-36VL, Perry, the USA) with identical environmental conditions as the laboratory colony.

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

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

Immediately after eclosion, I randomly selected 30 newly emerged males (< 2 hr after eclosion) per day from each cylinder for six days (sampling time) and froze them at -20°C. I then dissected these males to extract their testes, and measured testis size with the aid of a stereomicroscope (Leica MZ12, Wetzlar, Germany) connected with imaging software (CellSens® GS-ST-V1.7, Olympus, Tokyo, Japan). Because the testis shape of this species is spherical (Nowock, 1973), I calculated its size as volume

$= 4/3\pi r^3$, where $\pi = 3.14$ and r = radius of the testis (Liu et al., 2021). I determined its radius r using the mean diameter from three measurements across the organ's central axis divided by two (Raichoudhury, 1936; Gage, 1995). I then quantified eupyrene and apyrene sperm using the methods detailed in Koudelová & Cook (2001) and Liu et al. (2022a). Briefly, I placed the testis into a drop of Belar saline solution on a cavity slide and tore it apart using a fine needle. To evenly disperse eupyrene sperm bundles and disassociate apyrenes, I gently rotated the solution for 30 s. I counted the number of eupyrene sperm bundles on the slide under a phase-contrast microscope (Olympus BX51, Tokyo, Japan) at 40× magnification and calculated the total number of eupyrenes as the total number of eupyrene bundles multiplied by 256 (each bundle has 256 eupyrene sperm). I then thoroughly flushed the sample off from the cavity slide into a glass vial and diluted it with 30-ml distilled water. I gently rotated the vial for 30 s to allow even dispersal of apyrenes in the vial and then pipetted eight 10-µl subsamples from the vial and dropped them separately onto a microscope slide. After air dry, I counted the number of apyrene sperm under the phase-contrast microscope at 100× magnification and calculated the total number of apyrene sperm for each male as the mean number of apyrenes per 10 µl multiplied by the dilution factor (i.e., 3000). Thirty males were tested for each treatment and control.

6.2.4 Mating latency, lifetime mating frequency and longevity

At the onset of the first scotophase, I randomly selected and individually paired 30 newly emerged males (< 2 hr of eclosion) from each treatment and control with 1-d-old-virgin females randomly selected from the breeding colony that had been singly housed in glass vials since the pupal stage. Each pair was confined in a mating chamber (transparent plastic cylinder, 6.5 cm diameter × 8.5 cm length) with the lid covered by cotton gauze (2.3 apparatus per mm^2). I monitored the chambers and observed the mating behaviour continuously until the end of each copulation under 10 red light tubes (Sylvania, F36W/Red, Holland) and recorded the mating latency (time between introduction of both sexes and their genital connection) of 28, 26, 27, and 28 males for ELE, LLE, CLE, and CON, respectively.

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

6.2.5 Lifetime sperm ejaculation

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

6.2.6 Statistical analysis

All analyses were carried out using SAS 9.13. Rejection level was set at $P < 0.05$. I used a generalised linear model (GENMOD procedure) followed by a CONTRAST statement to compare the difference in pupation and emergence rate between treatments as an estimate of juvenile survival. Data on the ln(x)-transformed testis size, number of sperm counted at emergence, square-rooted mating latency, total number of lifetime sperm ejaculated, and longevity were normally distributed (Shapiro-Wilk test, UNIVARIATE procedure), and thus analysed using a linear mixed-effect model (MIXED procedure) (Davies & Gray, 2015; Liu et al., 2021) followed by a Tukey test for multiple comparisons between treatments. Data on the body size and mating frequency were not normally distributed (Shapiro-Wilk test, UNIVARIATE procedure) and thus analysed using a generalised linear mixed models (GLMMIX procedure) with a Poisson distribution in the model followed by a Tukey test for multiple comparisons

between treatments. Because the experimental design was pseudoreplicated, I treated the treatment as a fixed factor and replicate nested into sampling time as a random factor in the models (Millar & Anderson, 2004; Harrison et al., 2018).

I used a two-sample *t* test to compare the difference between the number of sperm counted at emergence and lifetime number of sperm ejaculated (Figure 6.5). An exponential functional model (Archontoulis & Miguez, 2015) was used to fit the data on the cumulative percentage of eupyrene and apyrene sperm ejaculated over successive matings (Figure 6.6), i.e., cumulative percentage of sperm ejaculated = $a \times [1 - \exp(-b \times \text{mating order})]$, where a (= 1) is the maximum percentage of cumulative sperm ejaculated, and b is the increasing rate of sperm cumulation. I used the non-overlapped 83.4% confidence limits (83.4% CLs) of the cumulative sperm number to determine the statistical significance between treatments (Julious, 2004).

6.3 Results

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

I obtained 106, 94, 89 and 86 male pupae and 102, 90, 83, and 85 male adults from ELE, LLE, CLE and CONT, respectively, with no significant difference between treatments (for number of pupae, $x_3^2 = 4.67$, $P = 0.1975$; for number of adults, $x_3^2 = 3.96$, $P = 0.2653$). Body size (Figure 6.1A) and testis size (Figure 6.1B) were also not significantly different between treatments (for body size, $F_{3,266} = 0.60$, $P = 0.6145$; for testis size, $F_{3,87} = 0.31$, $P = 0.8192$). However, the number of eupyrene and apyrene sperm counted at emergence was significantly higher in LLE and CLE than in ELE and CON ($F_{3,87} = 15.59$, $P < 0.0001$ for eupyrenes; $F_{3,87} = 12.26$, $P < 0.0001$ for apyrenes; Figure 6.2). There was no significant difference in eupyrene count between LLE and CLE, or between ELE and CON ($P > 0.05$).

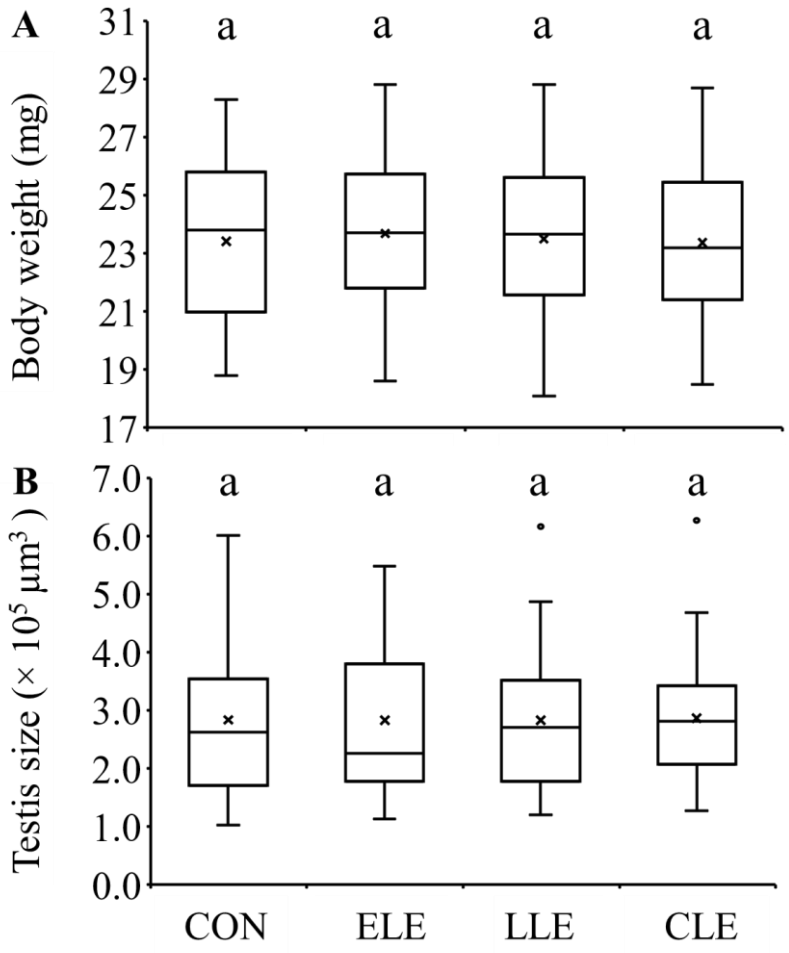

Figure 6.1 Effect of larval exposure to male adults on body size (**A**) at pupal stage and testis size (**B**) at emergence in *E. kuehniella*. CON, ELE, LLE and CLE denotes non-exposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and '×' in each box show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle; the dots are the outliers of maximum scores. The same letters on the top of the boxes indicate no significant differences between treatments ($P > 0.05$).

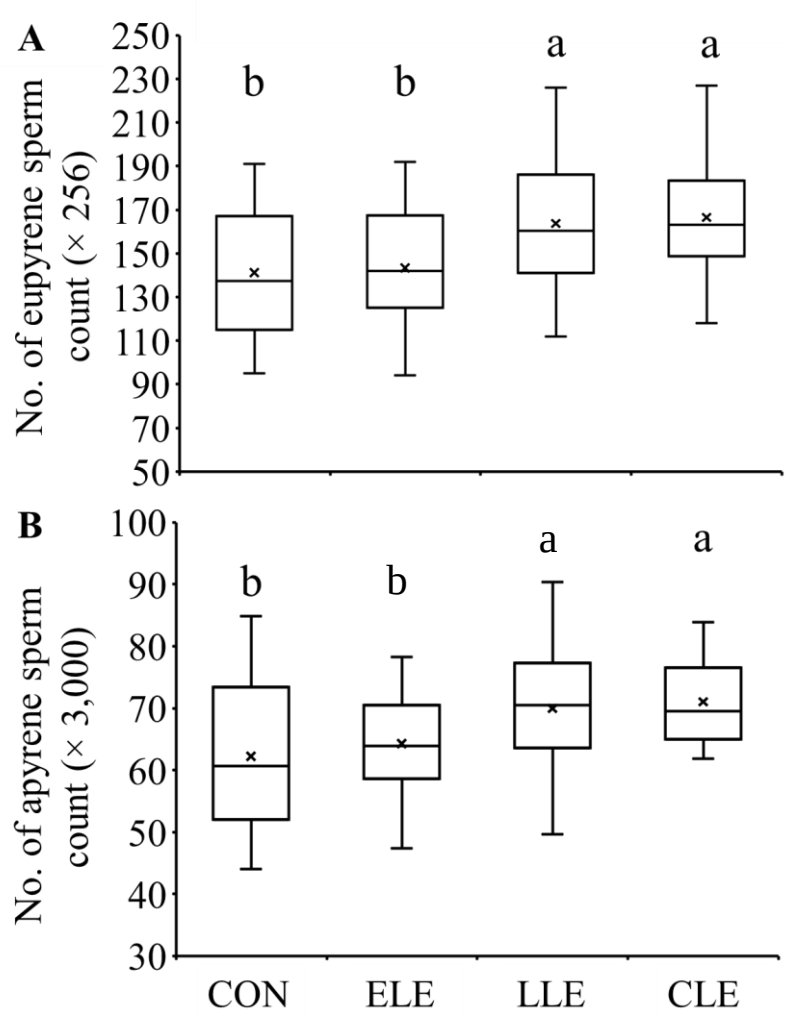

Figure 6.2 Effect of larval exposure to male adults on eupyrene count (**A**) and apyrene count (**B**) at emergence in *E. kuehniella*. CON, ELE, LLE and CLE denotes non-exposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and '×' in each box show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle. The different letters on the top of the boxes indicate significant differences between treatments ($P < 0.05$).

6.3.2 Mating latency, lifetime mating frequency and longevity

Males from LLE and CLE had significantly shorter mating latency than from ELE and CON ($F_{3,78}$ = 55.41, P < 0.0001; Figure 6.3). However, the presence of adult males during the larval stage had no significant effect on male mating frequency (mean ± *SE* = 6.25 ± 0.28, 5.62 ± 0.30, 5.81 ± 0.27, and 6.54 ± 0.24 times for ELE, LLE, CLE, and CON, respectively) ($F_{3,78}$ = 0.67, P = 0.5718) and longevity (mean ± *SE* = 9.93 ± 0.39, 8.77 ± 0.43, 9.37 ± 0.45 and 9.54 ± 0.34 days for ELE, LLE, CLE, and CON, respectively) ($F_{3,78}$ = 1.48, P = 0.2253).

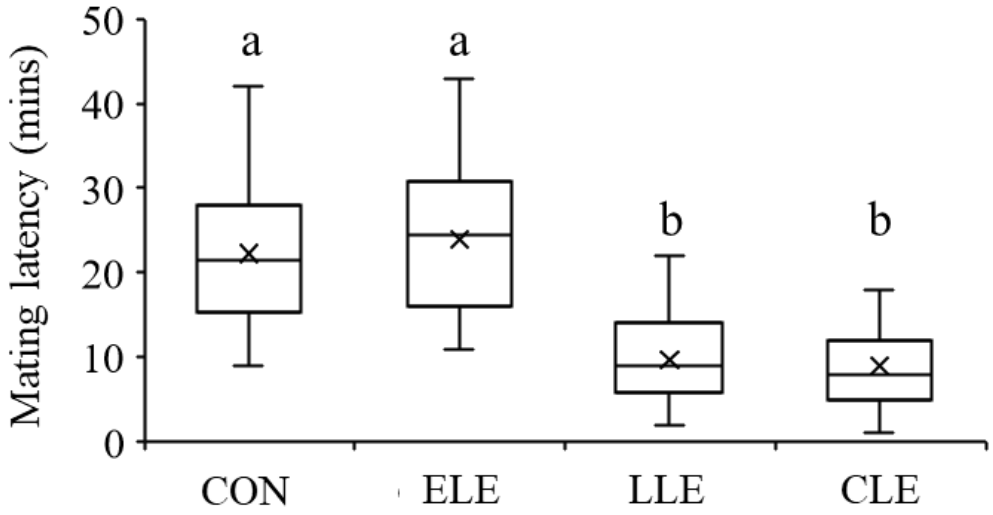

Figure 6.3 Effect of the presence of adult males during the larval stage on the first mating latency of *E. kuehniella*. CON, ELE, LLE and CLE denotes non-exposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and '×' in each box show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle. The different letters on the top of the boxes indicate significant differences between treatments (P < 0.05).

6.3.3 Lifetime sperm ejaculation

My findings indicate that males in all treatments ejaculated similar number of eupyrene ($F_{3,78}$ = 0.90, P = 0.4466; Figure 6.4A) and apyrene sperm ($F_{3,78}$ = 2.09, P = 0.1080; Figure 6.4B) in their lifetime. However, the lifetime number of eupyrene sperm ejaculated was significantly higher than that counted at emergence in ELE and CON (t_{56} = 2.29, P = 0.0260 for ELE; t_{56} = 2.93, P = 0.0049 for CON) while these were similar in LLE and CLE (t_{54} = -0.87, P = 0.3892 for LLE; t_{53} = -0.03, P = 0.9774 for CLE; Figure 6.5A). In all treatments, the lifetime number of apyrene sperm ejaculated was significantly higher than that measured at emergence (t_{56} = 9.27, P < 0.0001 for ELE; t_{54} = 8.64, P < 0.0001 for LLE; t_{53} = 10.30, P < 0.0001 for CLE; t_{56} = 8.65, P < 0.0001 for CON; Figure 6.5B). The cumulative percentage of both eupyrenes (Figure 6.6A) and apyrenes (Figure 6.6B) ejaculated over successive matings increased significantly faster in LLE and CLE than in ELE and CON (non-overlapping 83.4% CLs).

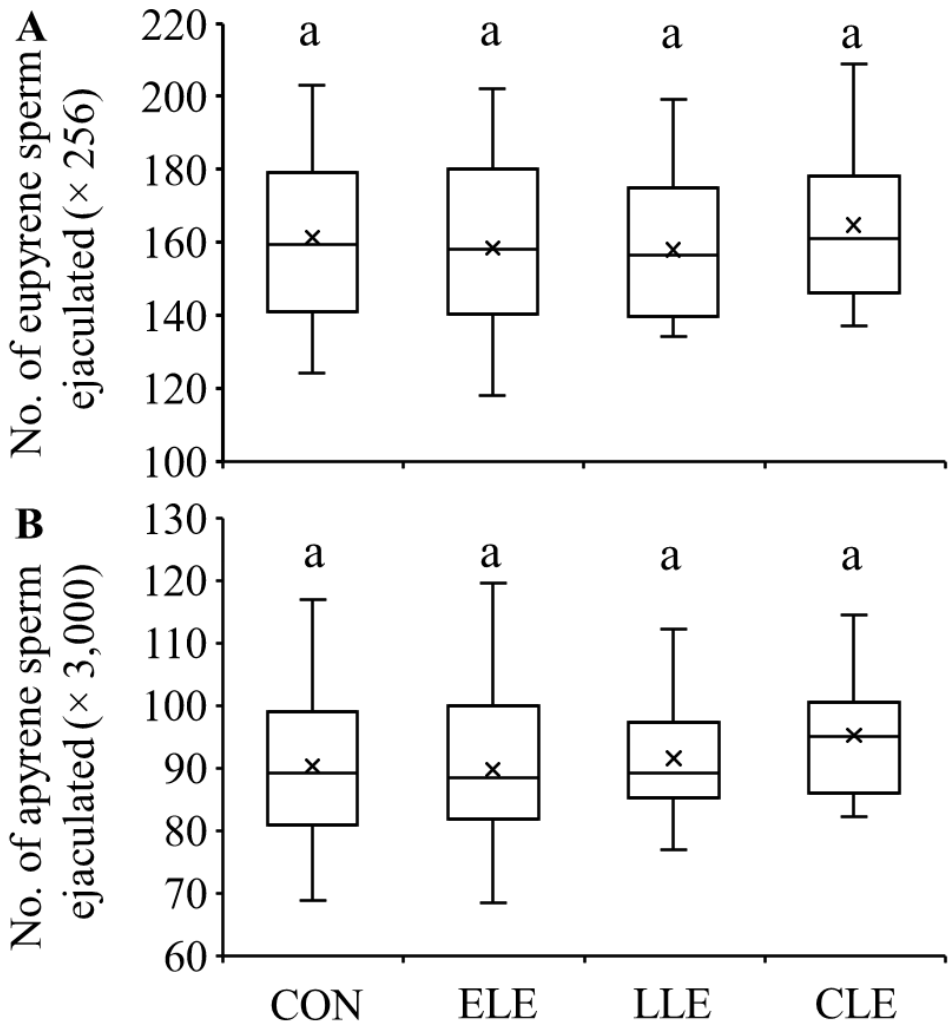

Figure 6.4 Effect of the presence of adult males during the larval stage on eupyrene (**A**) and apyrene (**B**) sperm ejaculated during the lifetime of *E. kuehniella*. CON, ELE, LLE and CLE denotes non-exposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. For each box plot, the lower and upper box lines indicate 25% and 75% of scores falling beyond the lower and upper quartiles, respectively; the line and '×' in each box show the median score and means, respectively; the '⊥' and '⊤' are the lower and upper whiskers representing scores outside the 50% middle. The same letters on the top of the boxes indicate no significant differences between treatments ($P > 0.05$).

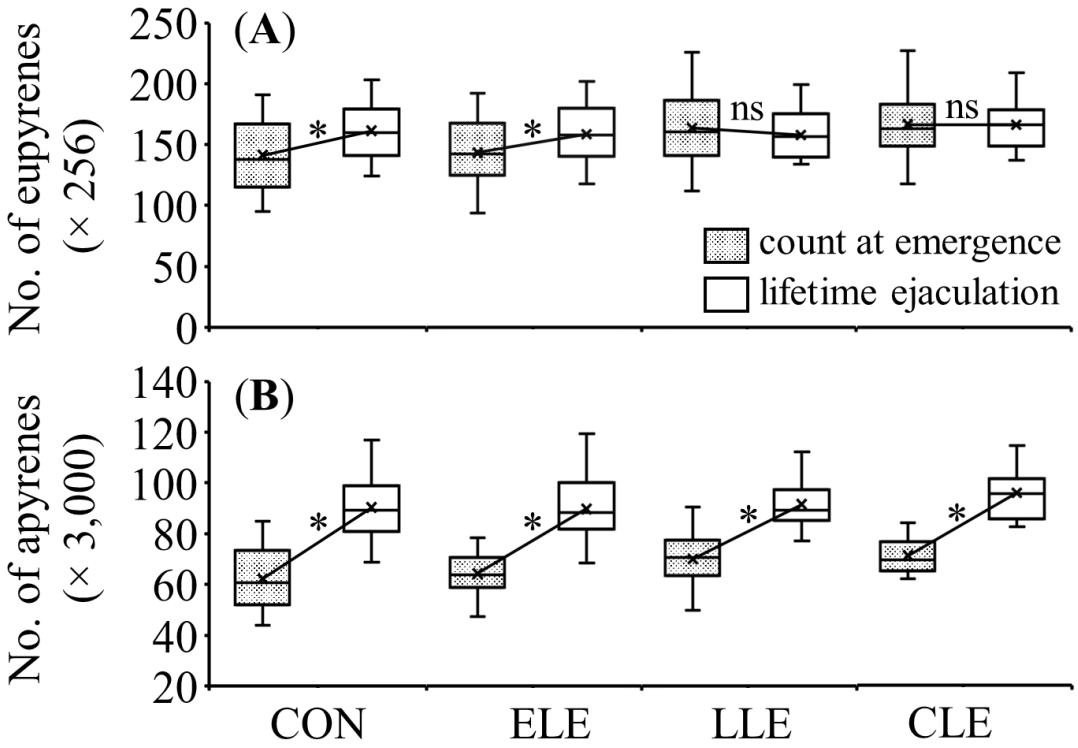

Figure 6.5 Effect of exposure to adult males during the larval stage on the number of eupyrenes (**A**) and apyrenes (**B**) counted at emergence and during lifetime ejaculation in *E. kuehniella*. CON, ELE, LLE and CLE denotes non-exposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. Each box plot shows the median line and the upper and lower quartiles, i.e., the range where 25% of scores fall above and 25% fall below the median; the line and '×' in each box indicate the median score and means, respectively; the '⊤' and '⊥' are the upper and lower whiskers showing the maximum and minimum scores, respectively. For each treatment, lines between boxes with '*' and 'ns' indicate significantly different ($P < 0.05$) and not significantly different ($P > 0.05$), respectively.

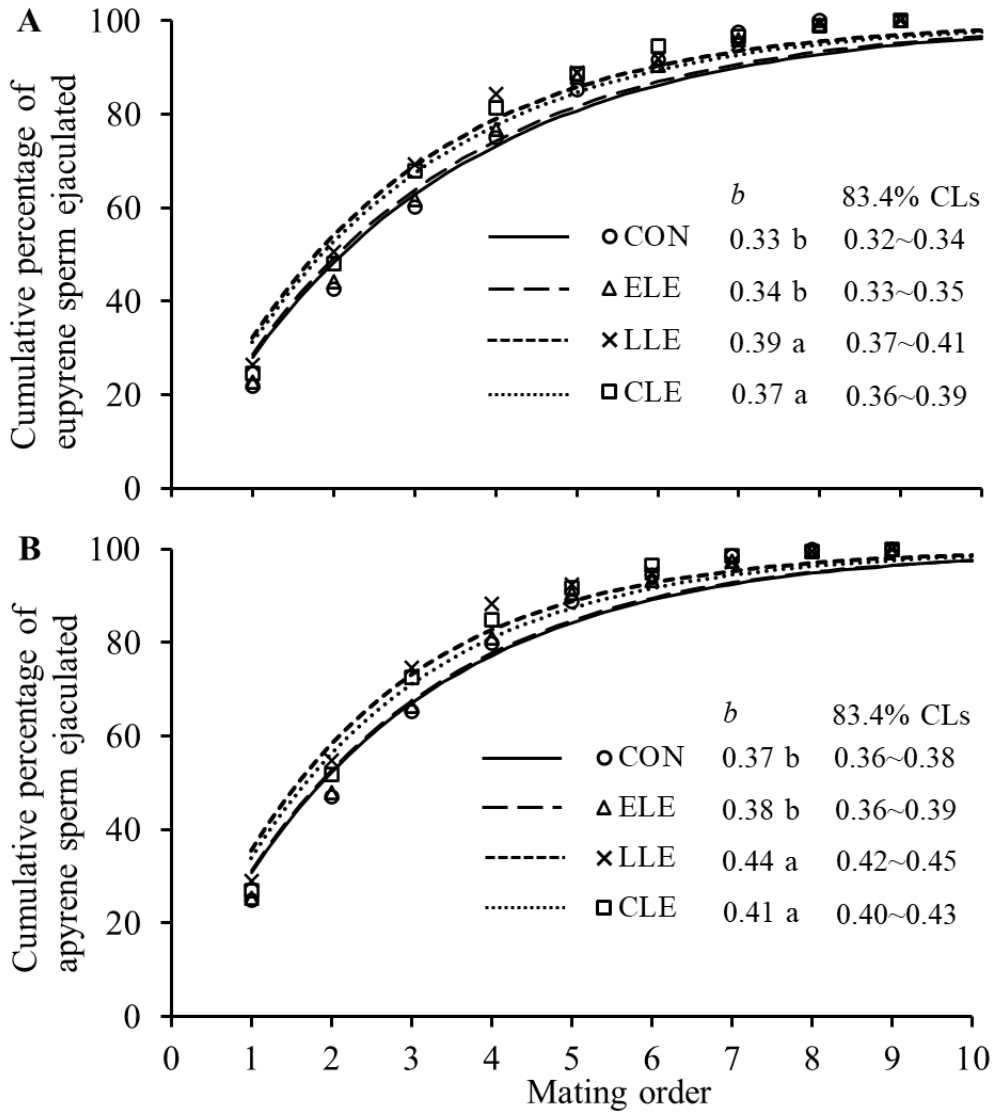

Figure 6.6 Effect of exposure to adult males during the larval stage on cumulative eupyrenes (**A**) and apyrenes (**B**) ejaculated by *E. kuehniella* males over successive matings. CON, ELE, LLE and CLE denotes non-exposure, exposure during the early larval stage, the late larval stage, and the complete larval stage, respectively. Cumulative percentage of sperm ejaculated = $a \times [1 - \exp(-b \times \text{mating order})]$, where a (= 1) is the maximum percentage of cumulative sperm ejaculated, and b is the increasing rate of sperm cumulation. The increasing rate b with different letters is significantly different (non-overlapping 83.4% CLs).

6.4 Discussion

The present study shows that after exposure to conspecific adult males during the late or entire larval stage, *E. kuehniella* males carried more eupyrenes (fertile and nucleate sperm) and apyrenes (infertile and anucleate sperm) at emergence (Figure 6.2) and had shorter mating latency (Figure 6.3). Adults from the larvae unexposed or only exposed to adult males during the early larval stage continued to produce eupyrenes after emergence while they kept on making apyrenes after emergence regardless of their larval experience (Figure 6.5). Compared to adults from the larvae unexposed or only exposed to adult males during the early larval stage, those from the larvae exposed during the late or entire larval stage transferred more eupyrenes and apyrenes in their early life (Figure 6.6). However, larval exposure to adult males had no effect on immature survival, body and testis size (Figure 6.1), and longevity, mating frequency and lifetime number of sperm ejaculated in resultant adults (Figure 6.4). These findings indicate that *E. kuehniella* larvae adjust their lifetime sperm production and ejaculation depending on whether and when they experience the cues from conspecific adult males.

Earlier studies have examined insect juvenile response to the cues of conspecific adult males but have not determined whether their sensitivity to these cues is stage dependent and whether these cues affect sperm production (Bailey et al., 2010; Gray & Simmons, 2013; Bretman et al., 2016; Simmons & Lovegrove, 2017). In the present study, I demonstrate for the first time that the late instar larvae of *E. kuehniella* could respond to sperm competition risk signalled by adult males, leading to higher sperm production before emergence (Figure 6.2) and shorter mating latency in their resultant adults (Figure 6.3). The lack of response to adult cues by younger larvae may be attributed to the fact that testes start forming their shape only when the larvae reach the fourth instar (about 15 days old) (Liu et al., 2022a, 2022b), allowing them to adjust their sperm production from this stage on. The shorter mating latency induced by adult male cues implies that intra-male competition risk also reduces mate selectivity by males.

Previous studies report that larval exposure to juvenile rivals increases eupyrene counts both at emergence (Liu et al., 2022a) and in lifetime ejaculates (Liu et al., 2022b) in *E. kuehniella*. However, the current study indicates that the higher sperm production during the juvenile stage in response to adult males (Figure 6.2) did not translate into greater sperm ejaculation during the lifetime of resultant adults (Figure 6.4). These findings reveal that male larvae respond to the cues from juvenile rivals and adult males differently. I suggest that the cues from juvenile rivals may signal the future sperm competition risk while those from adult males may indicate the immediate risk. Accordingly, after the larvae detect the immediate risk, they allocate all available resources for eupyrene production during the juvenile stage, whereas, if they perceive the future risk, they spread their resource allocations for eupyrene production across juvenile and adult stages.

Further comparison of sperm counts at emergence and in lifetime ejaculates reveals two clear patterns. First, whether adults could produce eupyrenes and apyrenes depended on their larval experience (Figure 6.5). These findings suggest that (1) the number of spermatogonia for producing eupyrenes may be limited (Witalis & Godula, 1993; Jarrige et al., 2015; Mari et al., 2018) so that the adults cannot manufacture more eupyrenes if their juveniles have used all of them, and (2) the number of cells for producing apyrenes may be less limited and their production may be cheaper (Silberglied et al., 1984; Cook & Gage, 1995; Liu et al., 2022b), allowing adults to produce more apyrene throughout their life regardless of their larval experience. Production of more apyrenes throughout lifetime may be important to gain advantages in sperm competition and fertilization success (Cook & Wedell, 1999; Holman & Snook, 2008; Wedell et al., 2009; Sakai et al., 2019; Hague et al., 2021). Second, adults carrying more sperm at emergence ejaculated more in their first few matings (Figure 6.6). I suggest that earlier ejaculation of more sperm may contribute to a greater reproductive success (Shackleton et al., 2005; Hosken et al., 2008; Wensing et al., 2017; Burke & Holwell, 2021) and support the sperm competition game model (Parker & Pizzari, 2010).

My study indicates that *E. kuehniella* did not trade off their survival, mating frequency, body size and testis size for sperm production in response to the cues from male adults during the larval stage (Figures 6.1 and 6.4). I suggest that under the rearing conditions with *ad libitum* food supply (also see Bhavanam et al., 2012; Liu et al., 2022a), the larvae have sufficient resources to adjust spermatogenesis without compromising juvenile survival, adult longevity, body size, and mating frequency, which are essential traits for male fitness (Honěk, 1993; Blanckenhorn, 2000; Komo et al., 2020; Kappeler, 2021). Although sperm production and testis size are positively correlated in some species (review in Vahed & Parker, 2012), various studies demonstrate that in response to sperm competition risk, testis size has no significant effect on sperm production in *E. kuehniella* (Liu et al., 2022a) and other animals (Byrne et al., 2002; Gay et al., 2009; Fitzpatrick et al., 2012; Bretman et al., 2016; Liao et al., 2019; Hobson et al., 2020). The lack of correlation between testis size and sperm production is probably because probably because animals can dedicate varying portions of testis volumes to spermatogenesis and other functions in response to sperm competition environment (Lüpold et al., 2020).

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

generated here provides insight into stage-dependent sensitivity of juvenile males to adult male cues and subsequent lifetime resource allocations.

CHAPTER 7

General Discussion

7.1 Introduction

During my PhD study, I carried out a series of experiments to investigate how males adjust their resource allocations according to the socio-sexual experience gained from adult and juvenile stages in *E. kuehniella*. In this chapter, I summarised my main findings, discussed their ecological implications, and recommended future studies.

7.2 Effects of conspecific rival cues on sperm production and allocation in adult males

Many studies show that adult males across animal taxa can adjust their investment in reproduction after detecting cues from conspecific rivals (e.g., Birkhead & Pizzari, 2002; Parker & Pizzari, 2010; Kelly & Jennions, 2011). In their studies on flies, Bretman et al. (2011a) and Maguire et al. (2015) demonstrate that males need to sense at least two types of cues from rivals before response to the sperm competition environment. However, in most mating systems it was largely unknown whether a single rival cue could elicit a response, whether combined cues could strengthen the response, and how these rival cues affected sperm production and allocation in a male's lifetime. Using *E. kuehniella*, I have carried out experiments to address these questions in Chapter 2.

I exposed adult males to a single (acoustic or chemical) cue or combined cues (acoustic + chemical, or acoustic + chemical + tactile) from their rivals before mating. I then examined exposed males' lifetime sperm production and allocation. My results demonstrate that following the detection of either a single or combined rival cues, males significantly increased the number of sperm ejaculated in their lifetime (Figure 2.2). This suggests that a single rival cue is sufficient to trigger a response by *E. kuehniella*

males to sperm competition environment, contrasting with previous findings in flies (Bretman et al., 2011a; Maguire et al., 2015). However, males exposing to combined cues produced more eupyrene sperm than those exposing to a single cue (Figure 2.3A). My findings imply that different types of rival cues may carry the same message and combined cues enhance receivers' response, perhaps due to synergistic impact (Partan & Marler, 1999; Dore et al., 2018).

My examination of sperm allocation in successive matings reveals that after 10-hr premating exposure to rival cues, *E. kuehniella* males allocated significantly more sperm in their first four matings (Figures 2.5 and 2.6). Therefore, the impact of sperm competition environment on *E. kuehniella* sperm allocation can last more than half of their reproductive life, supporting the notion that insects' brain has a long memory of an exposure to a socio-sexual environment (Dion et al., 2019). Because *E. kuehniella* is a short-lived species and its males can only mate a few times in their lifespan, there is limited room for them to reverse their resource allocation triggered by sperm competition levels earlier in life. Moreover, their sperm competition environment is unlikely to change rapidly because of their limited dispersal ability (Rees, 2004). It should thus be safe for them to maintain their response to the sperm competition level detected in the early adulthood.

7.3 Effects of juvenile social environment on sperm production and testis size in juvenile males

Insect larvae and pupae can communicate with each other using various non-sex and sex specific cues (Yack et al., 2001; Choi et al., 2007; Scott et al., 2010; Pontier & Schweisguth, 2015; Dolle et al., 2018). Yet, it was not fully clear whether and how larval and pupal social cues affected testicular investment during insects' growth and development. In Chapters 3 and 4, I explored these questions. I maintained juvenile males singly or with other juveniles of different sexes at the larval-pupal or pupal stage and measured testis size and counted sperm from testes upon adult emergence.

My results show that *E. kuehniella* adults that developed from group-reared larvae (Figure 3.3A) or pupae (Figure 4.2A) produced significantly more eupyrene than those from singly reared ones. These suggest that social cues at both larval and pupal stages could be indicative of sperm competition risk and males increase resource allocation to eupyrene production when their young are maintained in groups. Because many insects feature the last male sperm precedence (Simmons, 2001) including *E. kuehniella* (Xu & Wang, 2010a), production of more fertile eupyrene sperm during juvenile stages may increase males' reproductive output regardless of whether they mate with virgin or mated females.

My study demonstrates that *E. kuehniella* larvae only fine-tuned eupyrene production (Figure 3.3) while pupae adjusted both eupyrene and apyrene sperm (Figure 4.2) in response to social environment. Furthermore, under different juvenile socio-sexual settings, larvae (Figure 3.2B) but not pupae (Figure 4.3) could change testis size. These findings suggest that larvae and pupae may respond to the same social context differently and resource allocations to sperm production and testis size differ depending on the life stages exposed to sperm competition environment. The diverse responses to larval and pupal social cues may be attributed to the fact that resource allocations to morphological traits (Nijhout & Emlen, 1998; Mirth et al., 2021) normally take place from the early larval stage which is ahead of spermatogenesis (Swallow & Wilkinson, 2002; Friedländer et al., 2005).

7.4 Effects of adult rivals on sperm production in juvenile males

In nature, adult and juvenile males often co-exist, with adults providing juveniles with potential sperm competition risk (Kasumovic & Brooks, 2011; Lange et al., 2021). Bretman et al. (2016) demonstrate that insects' testis size changes after the larvae are reared with conspecific adult males. Yet, it was not clear whether juvenile sensitivity to adult male cues is stage dependent and whether these cues affect sperm production.

In Chapter 6, I exposed *E. kuehniella* to adult males at the early, late, or complete larval stage and then counted sperm produced upon male emergence. I demonstrate that

only the late larval stage was sensitive to adult cues and exposed males increased production of both eupyrenes and apyrenes (Figure 6.2). The lack of response to adult male cues by younger larvae may be attributed to the fact that testes start forming their shape only when the larvae reach the fourth instar (Figures 3.1 and 5.1), allowing them to adjust their sperm production from this stage on.

7.5 Effects of juvenile social cues on body size and survival in males

Earlier studies suggest that juvenile socio-sexual environment has little effect on insects' body size (Gage, 1995; McNamara et al., 2010; Allen et al., 2011; Bretman et al., 2016; Müller et al., 2016; Johnson et al., 2017; McNamara & Simmons, 2017; Gascoigne et al., 2021). Results from Chapter 3 and Chapter 6 support this view. I find that *E. kuehniella* body weight remained the same regardless of whether male larvae were group reared or not (Figure 3.2A) or whether they were exposed to adult males at different larval stages (Figure 6.1A). Furthermore, survival of juvenile males was similar between treatments. These discoveries indicate that under favourable rearing conditions, e.g., with *ad libitum* food supply and plentiful space, *E. kuehniella* larvae have sufficient resources to adjust spermatogenesis without sacrificing their juvenile survival and body size, which reflect overall fitness for animals (Honěk, 1993; Blanckenhorn, 2000; Komo et al., 2020; Kappeler, 2021).

7.6 Effects of juvenile social experience on adult reproductive performance and survival

Previous studies have only tested how insect adults allocate sperm in the first mating after their young are exposed to juvenile rivals (Gage, 1995; He & Miyata, 1997; Yamane & Miyatake, 2005; McNamara et al., 2010) or adult males (Gray & Simmons, 2013; Simmons & Lovegrove, 2017). Therefore, it was not clear whether and how these juvenile experiences affected adults' investment in reproduction over the lifetime, and in survival. I explored these questions in Chapter 5 and Chapter 6.

I demonstrate that *E. kuehniella* males developing from juveniles (larvae and pupae) reared with conspecific immature rivals ejaculated significantly more eupyrenes and apyrenes in their lifetime than those from juveniles raised solitarily or with immature females (Figure 5.2). The results suggest that the impact of socio-sexual environment during immature stages continues throughout the adult stage. Furthermore, the sperm allocation patterns remained the same following exposure either from late instar larval to pupal stages or just during the pupal stage (Figure 5.4), indicating that the late juvenile stage is a critical period for building up the long-term memory of the pre-adult social environment in insects.

Male larvae responded to the cues from juvenile rivals and adult males differently. Larval exposure to juvenile rivals increased eupyrene counts both at emergence (Figure 3.3A) and in ejaculates during lifetime (Figure 5.2A). However, the higher sperm counts at emergence (Figure 6.2) due to larval exposure to adult males did not translate into greater lifetime sperm ejaculation in adults (Figure 6.4). This difference may be because the cues from juvenile rivals signal the future sperm competition risk while those from adult males indicate the immediate risk. I suggest that larvae can adjust their eupyrene investment strategy in response to future and immediate sperm competition risk.

My results show that juvenile males exposed to conspecific juveniles (Figures 3.3 and 4.2) or to adult males (Figure 6.2) produced more sperm at emergence, and their resultant adults had shorter mating latency (in LLE and CLE males, Figure 6.3) and ejaculated more sperm in their first few matings (Figures 5.4 and 6.6). These findings may be attributed to the fact that greater sperm storage before emergence exacerbates sperm senescence (Ball & Parker, 1996; Reinhardt, 2007; Pizzari et al., 2008; Cattelan & Gasparini, 2021; Noguera, 2022) and males carrying more sperm may have higher motivation to discharge them from their reservoirs (Norris & Richards, 1933; Reinhardt, 2007; Pizzari et al., 2008; Cattelan & Gasparini, 2021), enhancing the reproductive fitness.

The exposure to juvenile conspecifics, regardless of sex ratio during immature stages, led to shorter longevity in male adults (Figure 5.5). This may be because most spermatogenesis occurs during immature stages (Figures 3.3 and 4.2) and sperm production entails significant costs (Dewsbury, 1982; Lemaître et al., 2020). As a result, the increase of resource allocation to sperm production in the presence of conspecifics during immature stages (Figures 3.3 and 4.2) causes the early death of male adults. Moreover, males with different juvenile social experiences (exposure to conspecific larvae, pupae or adult males) achieved the same number of matings in their lifetime, suggesting that the number of matings is ultimately important for maximal reproductive fitness regardless of juvenile experience in *E. kuehniella* males.

7.7 Effects of juvenile social experience on sperm production during the adult stage

Sperm competition theory predicts that males should increase sperm production after exposure to rivals because they may gain advantage in sperm competition (Wedell et al., 2002; Parker & Pizzari, 2010). In Lepidoptera, it is generally believed that eupyrene spermatogenesis stops after pupation (Chaudhury & Raun, 1966; Lachance & Olstad, 1988; Witalis & Godula, 1993; Friedländer et al., 2005; Jarrige et al., 2015; Mari et al., 2018). However, my results show that *E. kuehniella* adult males increased lifetime eupyrene sperm production after exposure to rivals during the early adulthood (Figure 2.3A) and the number of eupyrene sperm ejaculated in lifetime was higher than that measured at emergence after exposure to rivals during the juvenile stages (Figures 3.3A, 4.2A and 5.2A). These findings provide strong evidence that sperm competition risk can stimulate eupyrene spermatogenesis during the adult stage in a lepidopteran species.

My work indicates that adults continued to produce more apyrenes if they were exposed to rivals during the early adulthood (Figure 2.3B). Furthermore, regardless of males' juvenile social experience, the number of apyrenes or sperm ratio (apyrene:eupyrene) ejaculated in the lifetime (Figures 5.2 and 6.5) was higher than that

measured at emergence (Figures 3.3 and 4.2). These findings suggest that compared to eupyrenes, the number of cells for producing apyrenes may be less limited and their production may be cheaper (Silberglied et al., 1984; Cook & Gage, 1995). Because apyrene sperm play versatile roles in sperm competition and enhance fertilization success (Cook & Wedell, 1999; Friedländer et al., 2005; Holman & Snook, 2008; Konagaya et al., 2020; Hague et al., 2021), the ability to increase apyrene production during the adult stage helps maximise their reproductive fitness.

7.8 Conclusion

This thesis provides insights into the reproductive strategies employed by males of the Mediterranean flour moth *E. kuehniella* in response to various socio-sexual cues present at juvenile and adult stages. I demonstrate that adult males raise their sperm production and allocation after detecting either acoustic or chemical cues from their rivals with combined cues strengthening such response, and adult males can remember the sperm competition risk for most of their reproductive life following premating exposure to rival cues. I provide the first evidence that juvenile social cues from conspecific larvae, pupae or adult males also have lasting impacts on lifetime sperm production and allocation. The knowledge generated here contributes to broadening our understanding of how polygamous animals in general and *E. kuehniella* in particular calibrate their reproductive investment in response to social environmental heterogeneity.

7.9 Recommendations for future research

The experiments in my thesis have focused on how the social experience from different life stages influences males' adjustment in sperm quantity. It would be of interest to understand whether and how the experience affects sperm quality, such as sperm motility, viability, morphological characteristics, and metabolic rates, together with seminal fluid composition (Morrow & Gage, 2000; Snook, 2005; Wigby et al., 2009; Sirot, 2019; Ramm, 2020; Polak et al., 2021). By doing so, we would learn if there would be trade-offs between sperm counts and other sperm traits/performance,

knowledge of which would advance our understanding of adaptive resource allocations by males in response to social environment.

My study demonstrates that under ideal conditions, such as *ad libitum* food supply during growth and development, the adjustment of sperm production and allocation has little impact on other crucial life-history traits (e.g., juvenile survival, body size, and mating frequency). However, it is unclear if trade-offs between these traits occurs under food-stressed conditions. Further studies on this question are warranted. Furthermore, I have carried out experiments under environmental conditions optimal to *E. kuehniella.* However, thermal variations affect animal reproduction and survival (Deutsch et al., 2008; Iglesias-Carrasco et al., 2020; Rodrigues et al., 2022; Ristyadi et al., 2022). It may thus be worth testing how males respond to socio-sexual environment under gradual warming conditions due to climate change.

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Appendix: Published Papers from PhD Study

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RESEARCH ARTICLE

Functional Ecology

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.

KEYWORDS
Ephestia kuehniella, multimodal composite signal, rival detection, socio-sexual surroundings, sperm allocation, sperm production

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-Ramírez, Zamora-Álvarez, & Martinez, 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 (Acquistapace, 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, & Alfthan, 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; Garbaczewska, Billeter, & Levine, 2013; Jarrige, Riemann, Goubault, & Schmoll, 2015; Price, Lizé, Marcello, & Bretman, 2012; Ullah, Sugimoto, Kongchuensin, Konvipasruang, & Gotoh, 2017; Wigby 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, Iwabuchi, Reddy, & Mitsuhashi, 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 spermatophore 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

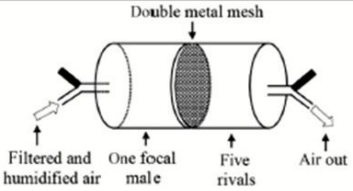
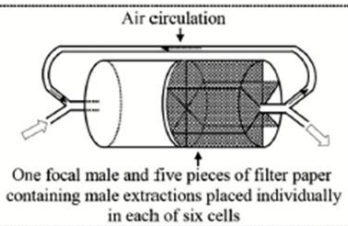
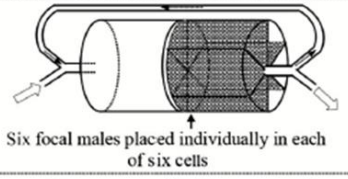
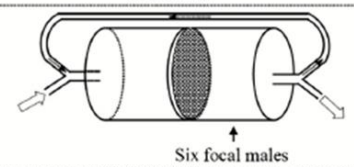
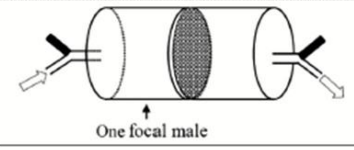
Treatment	Description	Pre-mating exposure device
+A	A focal male perceived only acoustic cue from rivals; one arm of each Y-tube was blocked with a cork	
+C	A focal male perceived only chemical cue from rivals; one arm of each Y-tube was connected with a silicon tube to facilitate air circulation between the two chambers	
+A+C	Focal males perceived both acoustic and chemical cues from rivals; one arm of each Y-tube was connected with a silicone tube to facilitate air circulation between the two chambers	
+A+C+T	Focal males perceived acoustic, chemical and tactile cues from rivals; one arm of each Y-tube was connected with a silicon tube to facilitate air circulation between the two chambers	
CONT	A focal male did not perceive any cue from rivals; one arm of each Y-tube was blocked with a cork	

FIGURE 1 Treatments and devices used for premating exposure

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 × 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 gently 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

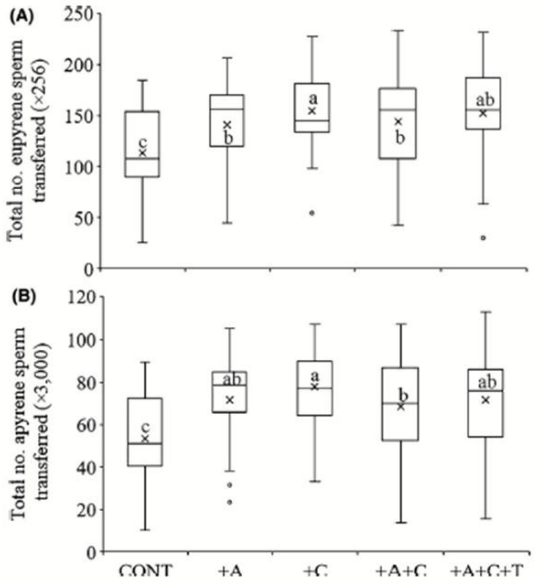


FIGURE 2 Mean (±*SE*) number of eupyrene (A) and apyrene (B) sperm ejaculated in *Ephestia kuehniella* males' lifetime after a premating exposure to rival cues. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical and 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 '×' in a box show the median score and means, respectively; the '⊥' and 'T' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum or maximum scores. Boxes with different letters are significantly different in mean between treatments ($p < 0.05$)

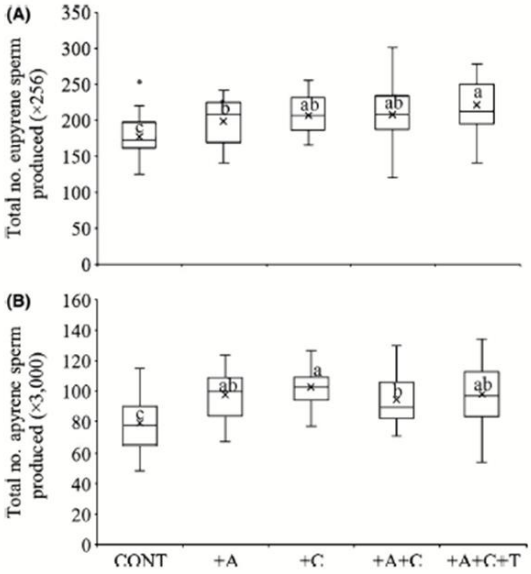


FIGURE 3 Mean (±*SE*) number of eupyrene (A) and apyrene (B) sperm produced in *Ephestia kuehniella* males' lifetime after a premating exposure to rival cues. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical and tactile 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 '×' in a box show the median score and means, respectively; the '⊥' and 'T' are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum or maximum scores. Boxes with different letters are significantly different in mean between treatments ($p < 0.05$)

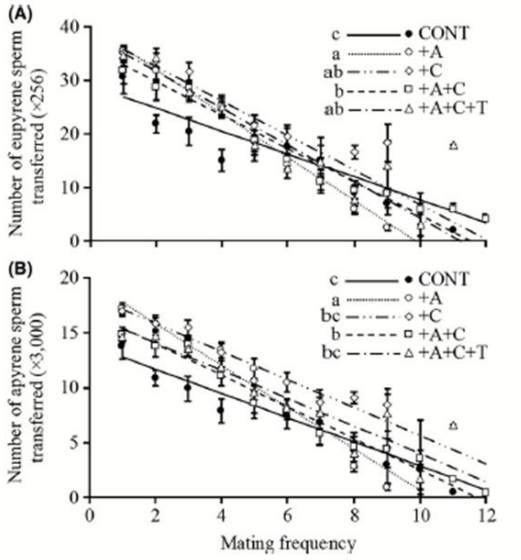


FIGURE 4 Number of eupyrene (A) and apyrene (B) sperm ejaculated by focal males in successive copulations after rival-cue exposure. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, chemical and tactile cues. Treatments (lines) with the same letters are not significantly different in slope ($p > 0.05$). Raw data were subject to analyse but means (±*SE*) were presented

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

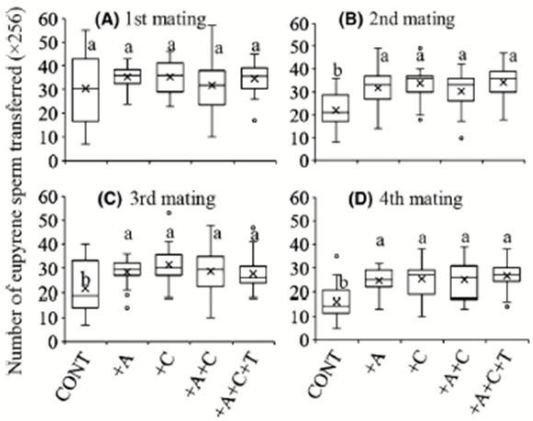


FIGURE 5 Mean (±*SE*) number of eupyrene sperm ejaculated in the first four matings (A–D, respectively) after a premating exposure to rival cues in male *Ephestia kuehniella*. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, 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 '×' show the median score and means, respectively; the '⊥' 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 ($p < 0.05$)

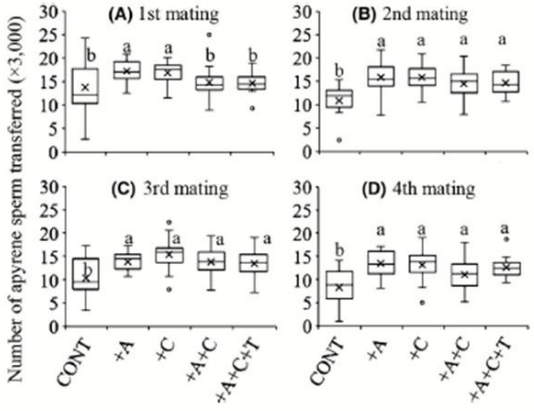


FIGURE 6 Mean (±*SE*) number of apyrene sperm ejaculated in the first four matings (A–D, respectively) after a premating exposure to rival cues in male *Ephestia kuehniella*. CONT = no rival cue; +A = acoustic cue; +C = chemical cue; +A+C = both acoustic and chemical cues; +A+C+T = combined acoustic, 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 '×' show the median score and means, respectively; the '⊥' and 'I' 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 (Schneeberger & 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-Winckler & 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, Sorenson, & 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 this 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.

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AUTHORS' CONTRIBUTIONS

J.L., X.Z.H. and Q.W. 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.

DATA AVAILABILITY STATEMENT

All data used for this paper are archived on Dryad Digital Repository https://doi.org/10.5061/dryad.zgmsbcc7d (Liu, Zhang, Zheng, He, & Wang, 2020).

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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, *Ephestia 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. *Ephestia kuehniella* larval stage lasts 29–31 days and pupal stage takes 8–9 days, with larvae having 6 instars (instars 1–3 ≈ 14–15 days and instars 4–6 ≈ 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 × 28 cm width × 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 × 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 ± 1°C,

60 ± 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 × 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) 6 M—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 6 M 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 π=3.14 and r = radius of the testis.

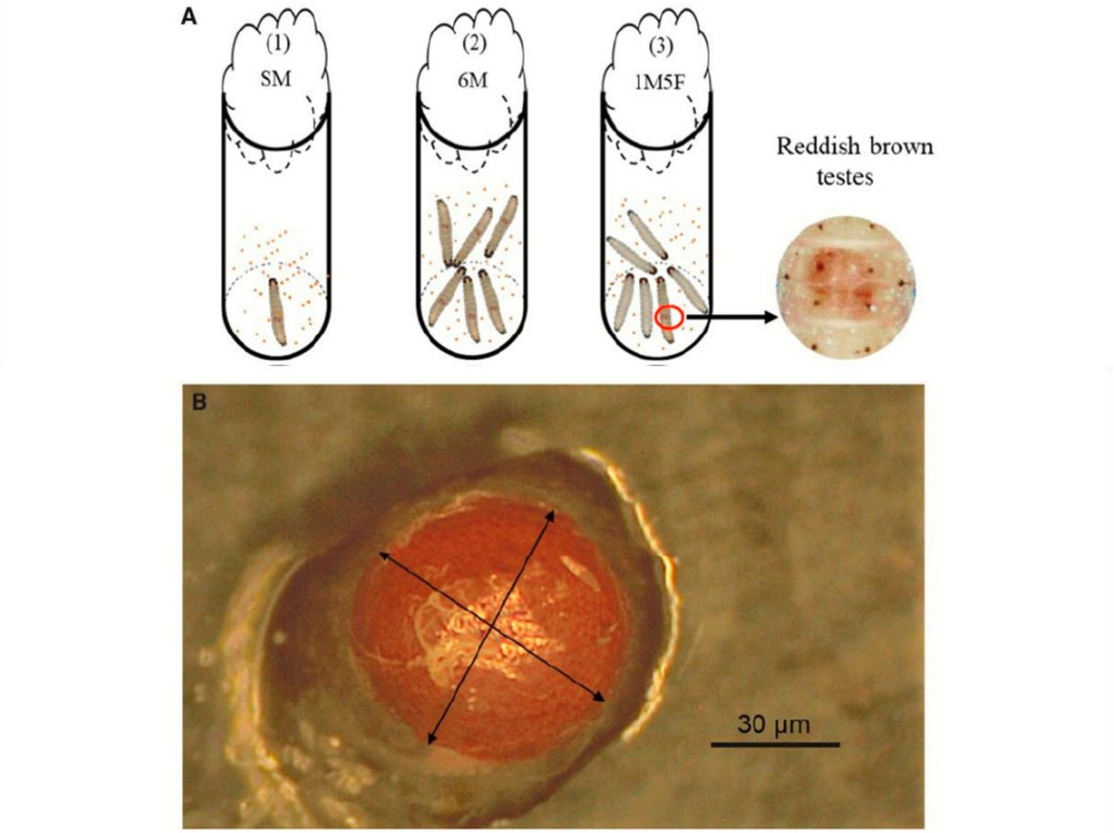

Figure 1. Treatment setups (A) and testis measurement (B) for *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6 M, 6 males together from the fourth instar larvae to adult emergence, and 1M5F, 1 male and 5 females together from the fourth instar larvae to adult emergence.

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-μL 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 ×100 magnification and calculated the mean number per 10 μL 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 μL 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

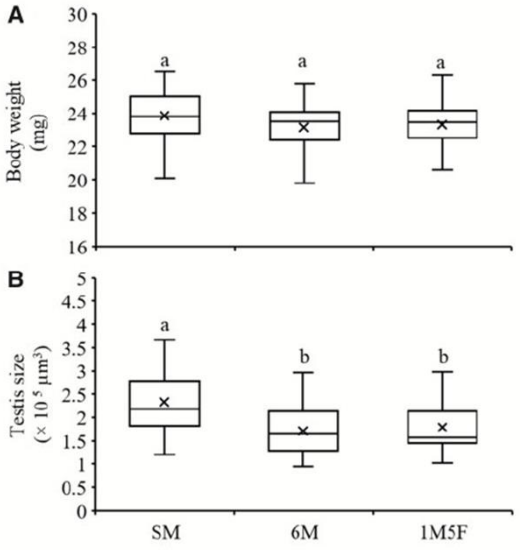

Figure 2. Effect of socio-sexual environment during immature stages on the body weight (A) and testis size (B) of *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, 6 males together from the fourth instar larvae to adult emergence, and 1M5F, 1 male and 5 females together 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 "×" 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

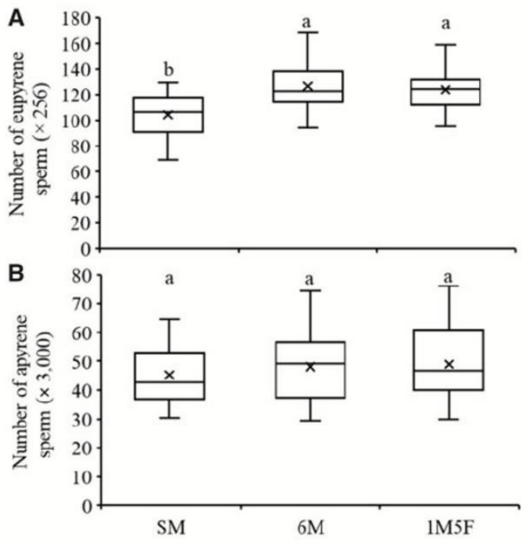

Figure 3. Effect of socio-sexual environment during immature stages on the total number of eupyrene (A) and apyrene (B) sperm in testes of *E. kuehniella*. SM, single male from the fourth instar larva to adult emergence; 6M, 6 males together from the fourth instar larvae to adult emergence, and 1M5F, 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 '×' 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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Article

Pupal Cues Increase Sperm Production but Not Testis Size in an Insect

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Simple Summary: Animals adjust their resource allocation strategies to maximize their reproductive benefit under dynamic socio-sexual environments. For example, male insect adults increase their testicular investment with the perceived increase of rivals to gain a competitive advantage in fathering offspring. To date, it is not clear whether insect pupae, which do not feed and crawl, can fine-tune their investment in sperm and testis size according to their social-sexual settings. This knowledge is vital to understanding how male insects respond to their surroundings experienced at different life stages. Using a moth which produces both fertile and unfertile sperm, we demonstrated for the first time that after detecting cues from conspecific pupae regardless of sex, male pupae increased production of both types of sperm at the same rate but kept testis size unchanged. Because most morphological traits are formed during the larval stage in insects, testis size may be fixed after pupation, allowing little room for the pupae to adjust testis size with social changes. Like adults, male pupae with fully grown testes have sufficient resources to produce more sperm of both types according to the perceived increase of sperm competition risk.

Abstract: Theoretic and empirical studies show that social surroundings experienced by male insects during their larval or adult stage can influence their testicular investment in diverse ways. Although insect pupae do not feed and crawl, they can communicate using sex-specific and/or non-sex specific cues. Yet, it is unknown, in any insect, whether and how male pupae can fine-tune their resource allocation to sperm production and testis size in response to socio-sexual environments. We investigated this question using a moth, *Ephestia kuehniella*, which produces fertile eupyrene sperm and unfertile apyrene sperm. We held male pupae individually or in groups with different sex ratios, and dissected adults upon eclosion, measured their testis size, and counted both types of sperm. We demonstrated that after exposure to conspecific pupal cues regardless of sex, male pupae increased production of eupyrenes and apyrenes at the same rate but kept testis size unchanged. We suggest that testis size is fixed after pupation because most morphological traits are formed during the larval stage, allowing little room for pupae to adjust testis size. Like adults, male pupae with fully grown testes have sufficient resources to produce more sperm of both types according to the perceived increase in sperm competition risk.

Keywords: spermatogenesis; sperm competition; testes; socio-sexual environment

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1. Introduction

Animals adjust their resource allocation strategies to maximize their reproductive fitness in response to socio-sexual environments [1–3]. For example, male animals may invest more in sperm after they detect the presence of rivals to gain an advantage in sperm competition [2,4–10]. In insects, males fine-tune their sperm investment in response to sex specific cues experienced during the adult stage [10–16] or non-sex specific cues during the

larval stage [17–21]. Although insect pupae do not feed and crawl, they can communicate with each other using species-specific acoustic [22–29] or chemical cues [30–34]. Furthermore, female pupae can release sex pheromones [35–38]. These findings suggest that male pupae should be able to detect conspecific pupal cues representing the density and sex ratio of the local population, and thus future sperm competition risk. Yet, prior to the current study, nothing is known about whether and how insect pupae can adjust their sperm production in response to these cues.

Testes are a sperm production organ and their relative size or mass may be an indicator of sperm production. Evidence shows that male insect larvae, in the growth and development stage, can adjust their testis size in response to conspecific larval cues, regardless of sex. For example, with the increase in larval density, testis size increases in some species, suggesting an increase of sperm production (sperm were not counted though) [17,39,40]. In a study where testis size is measured and sperm are counted [19], the male larvae exposed to larval cues, regardless of sex, produce smaller testes but more fertile sperm. These discoveries suggest that in response to their social environment, male larvae are able to dedicate varying portions of testis volumes to spermatogenesis and other functions [41], resulting in potential trade-offs between traits of different functions [19,42–44]. However, there is no report that insects can alter their testis size in response to the socio-sexual environment experienced at the adult stage. This may be because most resource allocation to traits making up the adult body takes place during growth and development [42,43,45–48], leaving little room for adults to change their testis morphology. To date, it is not clear whether insect pupae can alter their testis size after exposure to different socio-sexual environments.

Here, we used a polygamous moth, *Ephestia kuehniella*, as a model to investigate whether and how the socio-sexual environment during the pupal stage affects male investment in testis size and sperm production. Adults of this species do not feed so they acquire all resources via larval feeding [49,50]. The pupal stage lasts about eight days [19,51,52], during which time, females emit sex pheromones [36]. Like most lepidopterans [53], *E. kuehniella* males produce two types of sperm, larger nucleated eupyrenes during the larval and pupal stages, and smaller anucleated apyrenes during the pupal stage [54]. After mating, both types of sperm migrate to the sperm storage site (spermatheca) but only eupyrenes can fertilize eggs. Apyrenes may function to delay female remating [55,56], protect eupyrenes in the female reproductive tract [57], or enable eupyrenes to migrate to the spermatheca [58]. More recent studies suggest that the role of apyrenes may be completed after both types of sperm arrive at the spermatheca [59,60]. The apyrene-to-eupyrene ratio remains consistent under food shortage during the larval stage [61] or environmental stress during the larval [62] and pupal stages [63]. However, *E. kuehniella* males increase the ratio after detecting rival cues during the adult stage [10] or reduce it following exposure to larval cues during the larval stage [19]. So far, it is still unclear whether the socio-sexual environment during the pupal stage affects the sperm production ratio.

Based on the theoretic framework and empirical evidence outlined above, we hypothesize that male pupae kept together with other male pupae should grow larger testes and produce more sperm with higher apyrene-eupyrene ratio than those maintained individually or with female pupae. To test this prediction, we individually reared hundreds of larvae under the same condition, starting from neonate larvae. We then transferred newly pupated pupae to experimental arenas and held male pupae individually or in groups with different sex ratios. Upon adult eclosion, we dissected them, measured their testis size, and counted both types of sperm. This is the first study to examine whether and how male insects adjust their testicular investment in response to their socio-sexual environment experienced during the pupal stage.

2. Materials and Methods

2.1. Insects

We established a laboratory colony of *E. kuehniella* from thousands of larvae collected at Turks' Poultry, Foxton, New Zealand. We raised these larvae with their original food

until adult eclosion in the laboratory. To standardize the colony, we randomly selected and confined about 300 newly eclosed adults (approx. 1:1 sex ratio; females with an ovipositor and males with a pair of claspers at the end of abdomen) in a transparent plastic cage (28 cm in length and width and 24 cm in height), lined with porous plastic sheets on the bottom for oviposition. We then randomly allocated 200 resultant neonate larvae to each of the 10 transparent plastic cylinders (8 cm in diameter and 10 cm height), each filled with 50 g artificial diet (ad libitum) comprising of a 3.0:10.0:43.5:43.5 mixture of yeast, glycerine, maize meal, and whole meal wheat flour, respectively. We covered the cylinder with a lid. We made a hole (3 cm diameter) in the middle of the lid and covered it with two layers of cloth mesh (2.8 apparatus per mm^2) for ventilation.

To generate an experimental line, we randomly collected 1000 neonate larvae produced by adults from the above cylinders and reared them individually in 2.0-mL micro-centrifuge tubes, each with 0.25 g artificial diet for food and a ventilation hole in the lid made by an insect pin. We observed their pupation daily after the larvae reached the final (sixth) instar. The breeding colony and experimental line were kept and all experiments conducted at 25 ± 1 °C and 60 ± 10% RH with a photoperiod of 10:14 h (dark:light).

2.2. Experimental Setup and Data Collection

We randomly selected newly pupated pupae (male pupae with visible reddish testes in the abdomen) from the experimental line and transferred them into glass vials (2 cm in diameter and 7.5 cm height) to create three treatments (Figure 1): (1) one male pupa in a vial (1M), (2) six male pupae in a vial (6M), and (3) one male pupa and five female pupae in a vial (1M5F). Pupae in treatments (2) and (3) were in close contact with each other. We plugged the glass vial opening with cotton wool and monitored adult emergence daily six days after transfer. All pupae from the vials successfully emerged. Immediately after eclosion, we individually transferred newly emerged male adults into micro-centrifuge tubes, clearly labelled them and placed them at −20 °C in a freezer. We considered all emerged males as replicates, i.e., the male from each 1M vial, the male from each 1M5F vial, and all six males from each 6M vial. In total, we obtained 30 adult males (replicates) for each treatment.

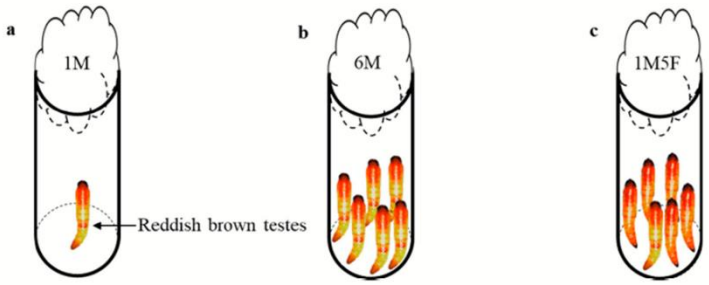

Figure 1. Experimental setup for the entire pupal stage of *E. kuehniella*: (a) 1M, one male, (b) 6M, six males together, and (c) 1M5F, one male and five females together.

We dissected all males, extracted their testes, and measured testis volume with the aid of a stereomicroscope (Leica MZ12, Germany) equipped with a digital camera (Olympus SC30, Tokyo, Japan) operated by Olympus CellSens® software (GS-ST-V1.7, Tokyo, Japan). As *E. kuehniella* testes are fused into a spherical organ [19], we calculated its volume using the sphere formula, $4/3\pi r^3$. We determined the r (radius) using the mean diameter from two measurements across the organ's central axis divided by two [17,19,64]. After volume measurement, we placed the testes into a drop of Belar saline solution on a cavity slide, tore them apart completely, gently rotated the slide, and counted the number of eupyrene and apyrene sperm under a phase-contrast microscope (Olympus BX51, Tokyo, Japan) according to Liu et al. [19].

2.3. Statistical Analysis

Prior to statistical analyses, we fitted data to a general linear model to calculate their residuals and test residual distribution (Shapiro-Wilk test, UNIVARIATE procedure). Data on eupyrene number, apyrene number, and ln(x)-transformed testis size were normally distributed. Because the experimental design was pseudoreplicated, we employed a linear mixed-effects model [65,66] to analyze our data with treatment as a fixed factor and replicate nested into vial (male source) as a random factor [19,66–68]. We used a Tukey test for multiple comparisons between treatments. We analyzed the relationship between eupyrenes and apyrenes by a general linear model (GLM procedure) and the slopes of linear lines by an analysis of covariance (ANCOVA) with treatment as the covariate in the model. The numbers of eupyrenes and apyrenes were ln(x)-transformed to achieve normal distribution of data before performing linear regression and ANCOVA. We performed the statistical analyses using SAS 9.4 (SAS, Inc, Cary, NC, USA).

3. Results

We demonstrate that males kept in groups (treatments 6M and 1M5F) produced significantly more eupyrene ($F_{2,29} = 26.31$, $p < 0.0001$) and apyrene sperm ($F_{2,29} = 10.07$, $p = 0.0005$) than those maintained singly (treatment 1M) (Figure 2a,b). Sex ratio did not significantly affect production of either eupyrene ($F_{1,29} = 3.66$, $p = 0.0658$) or apyrene ($F_{1,29} = 3.19$, $p = 0.0847$) (Figure 2a,b). Testis size remained similar in all treatments ($F_{2,29} = 0.01$, $p = 0.9852$) (Figure 3).

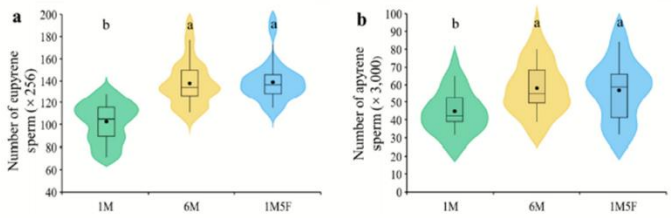

Figure 2. Effect of socio-sexual environment during the pupal stage on the number of eupyrene (a) and apyrene (b) sperm in testes of *E. kuehniella*. 1M, one male; 6M, six males together; 1M5F, one male and five females together. Each box plot shows the range between the first and third quartiles (black box), mean (black dot) and median scores (black lines); and 'violin' shapes show the shape of the distribution. Different letters on the top of the shapes denote significant differences between treatments ($p < 0.05$).

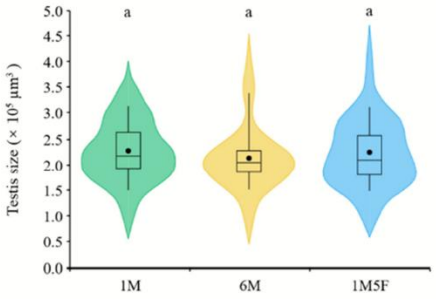

Figure 3. Effect of socio-sexual environment during the pupal stage on testis size of *E. kuehniella*. 1M, one male; 6M, six males together; 1M5F, one male and five females together. Each box plot shows the range between the first and third quartiles (black box), mean (black dot) and median scores (black lines); and 'violin' shapes show the shape of the distribution. The same letters on the top of the shapes denote no significant differences between treatments ($p > 0.05$).

Our results show that the ratio of apyrene:eupyrene was about 5:1 with no significant difference between treatments ($F_{2,29}$ = 1.24, p = 0.3041). The numbers of eupyrenes and apyrenes were significantly positively correlated in all treatments ($F_{1,28}$ = 5.31, p = 0.0289 for 1M; $F_{1,28}$ = 16.65, p = 0.0003 for 1M5F; $F_{1,28}$ = 11.94, p = 0.0018 for 6M) but the slopes of regression lines were not significantly different ($F_{2,84}$ = 0.22, p = 0.7996) (Figure 4).

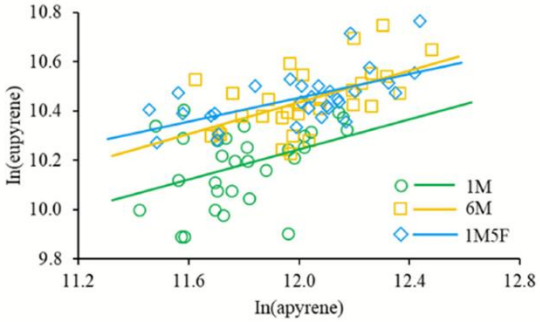

Figure 4. Relationship between the number of eupyrene and apyrene sperm produced. For 1M (one male), ln(eupyrene) = 6.58 + 0.31 × ln(apyrene), R^2 = 0.1594; for 6M (six males together), ln(eupyrene) = 6.59 + 0.32 × ln(apyrene), R^2 = 0.2990; and for 1M5F (one male and five females together), ln(eupyrene) = 7.54 + 0.24 × ln(apyrene), R^2 = 0.3729.

4. Discussion

We demonstrate for the first time that male pupae of an insect increased sperm production after exposure to conspecific pupal cues regardless of sex (Figure 2). Previous studies report that male insect larvae also can increase their investment in sperm in the presence of non-sex specific larval cues [17,19–21]. These findings indicate that juvenile male insects can predict future sperm competition risks from cues of conspecific immature stages and subsequently adjust their sperm production [17,19,69–71]. In lepidopteran insects, adults [10] and pupae (current study) adjust production of both fertile eupyrene and infertile apyrene sperm, while larvae only fine-tune production of eupyrene sperm [19] in response to socio-sexual environments. Furthermore, larvae either increase [17,40] or reduce [19] testis size in response to larval cues but pupae (Figure 3) and adults do not change their testis size under different socio-sexual situations. These discoveries suggest that resource allocation to sperm production and testis size differs depending on the life stages exposed to sperm competition environment.

The above diverse responses to social cues may be attributed to the fact that resource allocation to morphological traits and spermatogenesis takes place in different life stages. Evidence shows that most adult morphological traits are formed during the larval stage [42,43,47,48], allowing the larvae but not pupae and adults to adjust their testis size. Lepidopteran males produce most eupyrene sperm during the larval and pupal stages, most apyrene sperm during the pupal stage [53] and continue to produce both types of sperm during the adult stage [10]. Therefore, male larvae can donate varying portions of testis volumes to spermatogenesis and other functions [41], and trade off testis size and apyrene sperm production to increase eupyrene sperm production in response to increasing sperm competition risk [19]. However, with fully grown testes, adults and pupae have sufficient resources to increase production of both types of sperm in response to sperm competition environment.

In sperm-heteromorphic insects, the ayprene sperm often overwhelmingly outnumber the eupyrene sperm [12,16,57,72,73]. Previous studies on *E. kuehniella* show that adult males increase the apyrene-eupyrene ratio in response to the presence of rivals [10] but male larvae reduce the ratio after being exposed to larval cues [19]. These may be ascribed to the fact that spermatogenesis of apyrenes and eupyrenes occurs at different stages of insects [53,73] and they have different functions in reproduction [56–60], allowing adults

to increase investment in apyrene and larvae to trade-off apyrene for more eupyrene. However, the current study on pupae demonstrates that the apyrene-eupyrene ratio was about 5:1 with no significant difference between treatments. Furthermore, the numbers of eupyrenes and apyrenes were significantly positively correlated in all treatments with no significant difference in the slopes of regression lines (Figure 4). We suggest that in all life stages, males should strive to increase production of eupyrene sperm to ensure advantages in sperm competition (fertilization of more offspring) but also increase production of apyrene when they can (such as at the pupal and adult stages) to ensure successful arrival of eupyrene at the spermatheca.

Many studies reveal that insect larvae can communicate with each other using non-sex-specific cues [34,35,74–81] and male larvae can adjust their testicular investment in response to these cues [17,19,39,40]. Although female pupae can produce sex pheromones in insects including our study species *E. kuehniella* [35–38], we have not found any indication that male pupae could respond to this sex specific cue and adjust sperm production accordingly (Figures 2 and 3). Because pupae were in close contact with each other in treatments (2) and (3), physical contact cues could also play a role in pupal response. These findings suggest that testicular investment in *E. kuehniella* juvenile males only responds to the presence of social (including contact), but not sexual cues, during their growth and development. An earlier study demonstrates that *E. kuehniella* adults can remember rival cues and increase sperm allocation for most of their reproductive life after the cues are removed [10]. However, our findings on larval [19] and pupal (current study) responses to social environments result from dissecting adults at emergence. Therefore, we still do not know whether different larval and pupal social exposures influence sperm allocation during their adult lifespan, which warrants further investigation.

5. Conclusions

This is the first report on testicular investment in response to the social environment during the pupal stage in an insect. We show that after exposure to pupal cues, male *E. kuehniella* pupae increase production of both eupyrene and apyrene sperm at the same rate but keep testis size unchanged. We suggest that testis size is fixed after pupation because resource allocation to most morphological traits occurs during the larval stage, allowing little room for pupae to adjust testis size. With fully grown testes, pupae can manipulate production of both types of sperm according to the sperm competition risk. Furthermore, sex specific cues such as sex pheromones do not affect sperm production.

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ORIGINAL ARTICLE

Juvenile socio-sexual experience determines lifetime sperm expenditure and adult survival in a polygamous moth, *Ephestia kuehniella*

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Abstract Male animals often adjust their sperm investment in response to sperm competition environment. To date, only a few studies have investigated how juvenile sociosexual settings affect sperm production before adulthood and sperm allocation during the first mating. Yet, it is unclear whether juvenile sociosexual experience (1) determines lifetime sperm production and allocation in any animal species; (2) alters the eupyrene : apyrene sperm ratio in lifetime ejaculates of any lepidopteran insects, and (3) influences lifetime ejaculation patterns, number of matings and adult longevity. Here we used a polygamous moth, *Ephestia kuehniella*, to address these questions. Upon male adult emergence from juveniles reared at different density and sex ratio, we paired each male with a virgin female daily until his death. We dissected each mated female to count the sperm transferred and recorded male longevity and lifetime number of matings. We demonstrate for the first time that males ejaculated significantly more eupyrenes and apyrenes in their lifetime after their young were exposed to juvenile rivals. Adult moths continued to produce eupyrene sperm, contradicting the previous predictions for lepidopterans. The eupyrene : apyrene ratio in the lifetime ejaculates remained unchanged in all treatments, suggesting that the sperm ratio is critical for reproductive success. Male juvenile exposure to other juveniles regardless of sex ratio caused significantly shorter adult longevity and faster decline in sperm ejaculation over successive matings. However, males from all treatments achieved similar number of matings in their lifetime. This study provides insight into adaptive resource allocation by males in response to juvenile sociosexual environment.

Key words juvenile experience; Lepidoptera; mating frequency; sperm allocation; sperm production; sperm ratio

Introduction

Animals are expected to adjust their behavior and physiology to gain a competitive edge in different sociosexual environments (Kasumovic & Brooks, 2011; Acasuso-Rivero *et al*., 2019; Westneat *et al*., 2019). Various studies show that after adult males detect sperm competition risk, they raise their sperm expenditure for a higher paternity share (e.g., Parker, 1970; Gage, 1991; Simmons *et al*., 2007; Jarrige *et al*., 2015; Esfandi *et al*., 2020; Liu *et al*., 2020). To date, only a few studies have explored how male insects tailor their investment in sperm as a response to juvenile sociosexual settings. For example, adults from juveniles exposed to higher density of conspecific juveniles regardless of sex ratio ejaculate more sperm in their first mating (Gage, 1995; He & Miyata, 1997; Yamane &

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Miyatake, 2005; McNamara *et al*., 2010) or have higher sperm counts at emergence (Liu *et al*., 2021, 2022). Allen *et al*. (2011) report that after male juveniles are reared together, their adults transfer more sperm during the first mating. Yet, it is still unknown whether and how juvenile sociosexual environment influences lifetime sperm production and allocation in any animal species. It is also unclear whether exposure to similar sociosexual settings by different juvenile stages results in diverse lifetime sperm expenditure in adults.

Most lepidopteran species produce two distinct spermatozoa, the nucleate eupyrenes (fertile) and anucleate apyrenes (unfertile), and the most widely accepted notion is that spermatogenesis occurs in juvenile stages and eupyrene sperm production ends before adult emergence (Swallow & Wilkinson, 2002; Friedländer *et al*., 2005). However, it remains unclear whether juvenile sociosexual situations affect spermatogenesis during the adult stage. Although the apyrene sperm cannot fertilize eggs, they assist eupyrene in migration from female bursa copulatrix to spermatheca (Sakai *et al*., 2019; Chen *et al*., 2020; Konagaya *et al*., 2020; Hague *et al*., 2021), protect eupyrene sperm against a hostile female reproductive tract (Holman & Snook, 2008), and help win sperm competition games (Cook & Wedell, 1999; Wedell *et al*., 2009; Mongue *et al*., 2019). Therefore, the eupyrene : apyrene ratio could be essential for reproductive success in males. Though, it is unknown whether the juvenile sociosexual environment affects the sperm ratio in ejaculates transferred during the adult lifespan.

The number of sperm ejaculated by males should decrease over successive matings due to limited resources and aging (Wedell & Cook, 1999; Velde *et al*., 2011; Esfandi *et al*., 2015, 2020; Liu *et al*., 2020). However, it is not clear whether different juvenile experience alters lifetime ejaculation pattern. Because sperm production (Dewsbury, 1982; Van Voorhies, 1992; Olsson *et al*., 1997; Pitnick *et al*., 2006; Hayward & Gillooly, 2011; Lemaître *et al*., 2020) and matings (Martin & Hosken, 2004; McNamara *et al*., 2008; Oliver & Cordero, 2009; Metzler *et al*., 2016; Mautz *et al*., 2019; Jehan *et al*., 2020) are costly, males may not be able to maintain maximal reproduction and longevity simultaneously (Kirkwood, 1977; Roff, 2002). Nevertheless, many studies show that the adult sociosexual environment alters males' reproductive investment but not their longevity (e.g., Janowitz & Fischer, 2010; Moatt *et al*., 2013; Esfandi *et al*., 2015; Leech *et al*., 2019). To date, knowledge of how juvenile sociosexual settings affect adult mating frequency and longevity is still lacking.

We used a polygamous moth, *Ephestia kuehniella*, to investigate how juvenile sociosexual environment influences lifetime sperm expenditure, mating frequency and survival in adult males. Under our experimental conditions, larval and pupal stages last about 29 and 8 days, respectively. Adults do not feed, and all resources are acquired during the larval stage. Adults become sexually mature at emergence and start mating at the onset of the first scotophase (Xu *et al*., 2008). Adult males increase their sperm allocation after exposure to rival cues during early adulthood (Esfandi *et al*., 2020; Liu *et al*., 2020). Adults have more sperm of both types at emergence after their pupae are exposed to higher density of conspecifics (Liu *et al*., 2021) but if such exposure starts at the larval stage, only eupyrene sperm increases at emergence (Liu *et al*., 2022), suggesting that larvae and pupae may respond to the same social context differently.

In the current study, we prepared thousands of larvae and manipulated larval–pupal and pupal density with varied sex ratios. Upon male adult emergence, we paired each male with a virgin female per day until his death. We dissected each mated female to count eupyrene and apyrene sperm transferred per mating and recorded males' lifetime mating frequency and longevity. This is the first study on how juvenile sociosexual experience affects male adult longevity, and lifetime sperm production and allocation, eupyrene : apyrene sperm ratio and mating frequency in an insect. Knowledge presented here provides novel understanding of adaptive resource allocation by males in response to juvenile sociosexual environment.

Materials and methods

Insects and environmental conditions

We collected *E. kuehniella* larvae from a poultry farm, in Foxton, New Zealand and reared them to adults with their original food (a mixture of wheat and maize flour) in 20 transparent plastic cylinders (10.0 cm height × 8.0 cm diameter). We randomly selected about 300 newly emerged adults (ca. 1 : 1 sex ratio) from all cylinders and introduced them into a transparent plastic cage (24.0 cm height × 28.0 cm length × 28.0 cm width) with a porous plastic sheet at the bottom for oviposition. We collected eggs by pulling out the sheet and replacing it with a new one once every day for 10 days and incubated the eggs in Petri dishes (1.5 cm height × 8.5 cm diameter). We then inoculated 200 neonate larvae to 50 g standard diet (ad libitum) (21.75 g whole meal wheat flour, 21.75 g maize meal, 5 g glycerine, and 1.5 g yeast) in a plastic cylinder as mentioned above. We constantly maintained 20 such cylinders as the breeding colony for experimental insects.

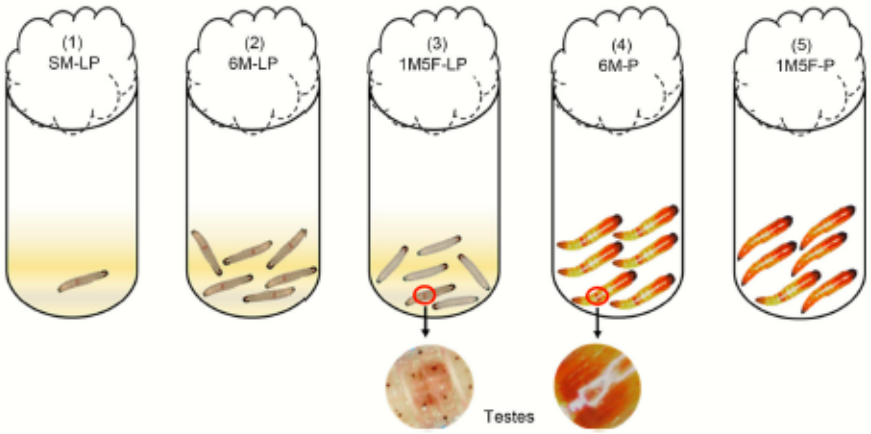

Fig. 1 Socio-sexual environment treatments for *E. kuehniella* males before eclosion: (1) SM-LP, single male from the fourth instar larva to adult emergence; (2) 6M-LP, six males together from the fourth instar larvae to adult emergence; (3) 1M5F-LP, one male and five females together from the fourth instar larvae to adult emergence; (4) 6M-P, six male pupae together for the entire pupal stage until adult emergence; and (5) 1M5F-P, one male and five females together for the entire pupal stage until adult emergence.

We randomly transferred 300 newly emerged adults (ca. 1 : 1 sex ratio) from the colony to an aforementioned plastic cage for mating and subsequently letting females lay eggs in the cage. We then randomly collected 1000 neonate larvae from the cage to establish an experimental line. We reared these larvae individually in 2-mL Eppendorf tubes, each of which held 0.25 g standard diet for food and pinholes in the lid for ventilation. To prepare virgin females for mating with focal males over the course of the experiment, we randomly collected about 1000 female pupae from the breeding colony and individually housed them in the Eppendorf tubes until use for experiment. We maintained the colony and experimental insects and carried out all experiments at 25 ± 1 °C and 60% ± 10% RH under the photoperiod of 10 : 14 h (dark : light).

Pre-adult socio-sexual settings for focal males

The sex of the fourth instar larvae and pupae can be determined by visible testes in males' abdomens (Fig. 1; Liu *et al*., 2021). We randomly selected newly molted fourth-instar larvae (L) and newly pupated pupae (P) from the experimental line and transferred them into glass vials (7.5 cm height × 2.0 cm diameter) to create five socio-sexual enviroments for focal males (M) (Fig. 1): (1) single male (SM-LP)—one male was kept in a glass vial with a 0.25 g standard diet from the fourth instar larva to adult emergence; (2) six males (6M-LP)—six males were raised in a glass vial with a 1.5 g standard diet from the fourth instar larvae to adult emergence; (3) one male and five females (1M5F-LP)—one male and five females (F) were reared in a glass vial with a 1.5 g standard diet from the fourth instar larvae to adult emergence; (4) six male pupae (6M-P)—six male pupae were maintained in a glass vial for the entire pupal stage until adult emergence, and (5) one male and five female pupae (1M5F-P)—one male and five females were put in a glass vial for the entire pupal stage until adult emergence. All glass vials were covered with wool cotton at the top.

For all treatments, we monitored adult emergence hourly when the pupae turned dark brown (ca. 1 day before adult emergence). Immediately after males' eclosion, we individually transferred them into clean glass vials and clearly labeled all vials. To keep conditions consistent, we only used males from vials where all individuals emerged for data collection. We considered the male from each SM-LP, 1M5F-LP and 1M5F-P vial and all six males from each 6M-LP and 6M-P vial as focal males.

Data collection

At the onset of the first scotophase following eclosion, we individually paired the focal males with 1-day-old

virgin females randomly selected from the breeding colony, in transparent plastic cylinders (17.0 cm length × 6.5 cm diameter). Ten red light tubes (Sylvania, F36W/Red, Holland) 1.5 m above the cylinders were used for illumination. Because a male requires 24-h recovery time to produce a full spermatophore again after each mating (Xu & Wang, 2009), we randomly assigned another 1-day-old virgin female to the focal male in the cylinder at the onset of the next scotophase. This procedure was repeated until the death of the focal male. We monitored each mating pair once every 15 min until mating ended and immediately removed the mated female from the cylinder. We recorded mating frequency (lifetime number of matings) and longevity (duration between emergence and death) of each focal male. We considered each focal male as a replicate. In total, we achieved 24, 23, 24, 22, and 24 replicates for treatments SM-LP, 6M-LP, 1M5F-LP, 6M-P, and 1M5F-P, respectively.

To record males' lifetime sperm allocation, we counted the number of sperm ejaculated in each mating via dissecting all mated females from the above experiment and extracting the spermatophores out from their bursa copulatrix. In total we dissected 123, 130, 133, 136, and 142 females for SM-LP, 6M-LP, 1M5F-LP, 6M-P, and 1M5F-P, respectively. We placed the bursa copulatrix into a droplet of Belar saline solution on a cavity slide. Using two fine needles, we ruptured the spermatophore to release sperm under a stereomicroscope (Leica MZ12, Wetzlar, Germany). We then counted the number of bundles of eupyrene sperm under a phase-contrast microscope (Olympus BX51, Tokyo, Japan). We calculated the total number of eupyrene sperm as the total number of bundles multiplied by 256, the number of eupyrene sperm per bundle (Garbini & Imberski, 1977). Afterward, the sample was thoroughly washed off the cavity slide and diluted in a glass vial with 30-mL distilled water. We gently rotated the vial for about 30 s to deliver even dispersal of apyrenes in the vial. We took eight 10-μL subsamples from the vial using a Gilson autopipette and placed them separately on a microscope slide. We counted the number of apyrene sperm of all eight subsamples under the phase-contrast microscope and calculated the mean number per 10 μL as the sum of apyrene sperm in eight subsamples divided by eight. We then calculated the total number of apyrene sperm for each mating as the mean number of apyrenes per 10 μL multiplied by the dilution factor (3000) (Koudelová & Cook, 2001). The lifetime number of eupyrene and apyrene sperm ejaculated by a male adult is the sum of the sperm ejaculated in each mating.

Statistical analysis

We analyzed all data using SAS 9.13 (SAS Institute Inc, USA) with a rejection level set at $P < 0.05$. Because the experimental design was pseudoreplicated, we analyzed the mating frequency and lifetime number of eupyrene and apyrene sperm transferred by male adults using a linear mixed-effects model (MIXED procedure) (Millar & Anderson, 2004; Harrison *et al*., 2018), with treatment as a fixed factor and replicate nested into the vial (male source) as a random factor (Davies & Gray, 2015; Harrison *et al*., 2018). We applied a Tukey's test in the model for multiple comparisons between treatments. A log-rank test (LIFETEST procedure) was applied to compare the survival probability of focal males between treatments. The relationship between the total number of eupyrene and apyrene ejaculated was analyzed by a general linear model (GLM procedure) and an analysis of covariance (ANCOVA) was used to compare the slopes of regression lines between treatments (Liu *et al*., 2021). We used a linear mixed-effects model with repeated measures (MIXED procedure) to test how treatment affected males' sperm allocation in successive matings. We set treatment, mating frequency and their interaction as the fixed effects in the model with a subject effect of focal male in the statement of "REPEATED / TYPE = cs SUBJECT = focal male" after the model. A CONTRAST statement was then applied to compare the slopes of regression lines of sperm ejaculation over successive matings between treatments. Because the moths used in the current study were from the same batch reared under the same conditions as in Liu *et al*. (2021, 2022), we used a two-sample t test (TTEST procedure) to compare the number of eupyrene and apyrene sperm and their ratio in lifetime ejaculates with those recorded at emergence (Liu *et al*., 2021, 2022).

Results

Effect of socio-sexual environment during juvenile stages on lifetime sperm allocation

Males that were exposed to conspecific males during larval–pupal stages (6M-LP) or the pupal stage (6M-P) ejaculated significantly more eupyrene and apyrene sperm than those that were exposed to conspecific females during larval–pupal stages (1M5F-LP) or the pupal stage (1M5F-P) or reared singly during larval–pupal stages (SM-LP) ($F_{4,48} = 4.11$, $P = 0.006$ for eupyrene;

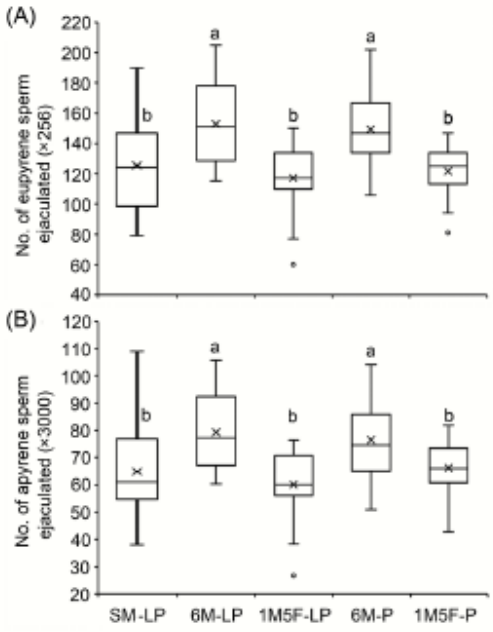

Fig. 2 Effects of preadult socio-sexual environment on lifetime eupyrenes (A) and apyrenes (B) ejaculated by *E. kuehniella* males. SM-LP, single male from fourth instar larva to adult emergence; 6M-LP, six males together from fourth instar larvae to adult emergence; 1M5F-LP, one male and five females together from fourth instar larvae to adult emergence; 6M-P, six male pupae together for the entire pupal stage until adult emergence; and 1M5F-P, one male and five females together for the entire pupal stage until adult emergence. 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 "×" show the median score and means, respectively; the "⊥" and "T" are the lower and upper whiskers representing scores outside the 50% middle; the circles are the outliers of minimum or maximum scores. Boxes with different letters denote significant differences between treatments ($P < 0.05$).

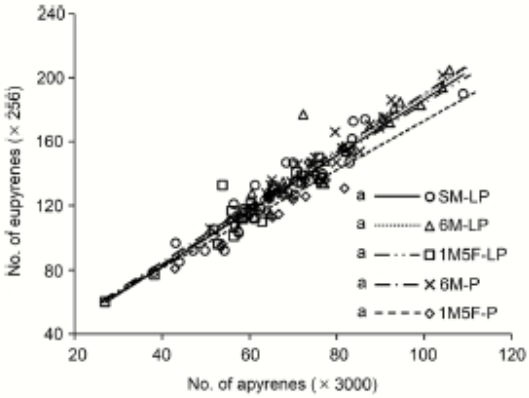

Fig. 3 Relationship between the number of eupyrene and apyrene sperm ejaculated in *E. kuehniella* males' lifetime. For SM-LP, eupyrene = 12.31 + 1.74 × apyrene ($F_{1,22} = 204.10$, $P < 0.001$); for 6M-LP, eupyrene = 13.17 + 1.76 × apyrene ($F_{1,20} = 130.51$, $P < 0.001$); for 1M5F-LP, eupyrene = 16.13 + 1.68 × apyrene ($F_{1,21} = 126.29$, $P < 0.001$); for 6M-P, eupyrene = 14.86 + 1.76 × apyrene ($F_{1,20} = 275.94$, $P < 0.001$); and for 1M5F-P, eupyrene = 20.07 + 1.53 × apyrene ($F_{1,22} = 103.42$, $P < 0.001$).

$F_{4,48} = 4.58$, $P = 0.003$ for apyrene) (Fig. 2). The lifetime number of eupyrenes and apyrenes ejaculated was significantly positively correlated in all treatments ($P < 0.001$), with no significant difference in the slopes of regression lines between treatments ($F_{4,105} = 0.32$, $P = 0.864$) (Fig. 3).

Both eupyrene (Fig. 4A) and apyrene (Fig. 4B) sperm ejaculated declined significantly over successive matings ($P < 0.001$). However, both types of sperm ejaculated declined significantly faster in males exposed to conspecifics (6M-LP, 1M5F-LP, 6M-P, and 1M5F-P) than in ones unexposed to conspecifics (SM-LP) ($F_{4,635} = 4.73$, $P < 0.001$ for eupyrene; $F_{4,635} = 7.96$, $P < 0.001$ for apyrene) and there was no significant difference in slopes among exposed males (eupyrene: $F_{3,514} = 0.63$, $P > 0.05$; apyrene: $F_{3,514} = 0.96$, $P > 0.05$) (Fig. 4).

Comparison between the number of sperm ejaculated in lifetime and that counted at emergence

In treatments 6M-LP, 6M-P, and SM-LP, the lifetime number of eupyrene sperm ejaculated (Fig. 2A) was significantly higher than that counted at emergence ($t_{50} = 4.20$, $P < 0.001$ for 6M-LP; $t_{50} = 2.52$, $P = 0.016$ for 6M-P; $t_{54} = 3.09$, $P = 0.004$ for SM-LP) (Liu *et al.*, 2021, 2022).The lifetime number of apyrene sperm ejaculated (Fig. 2B) was also significantly higher than that measured at emergence in all five treatments ($t_{50} = 8.61$, $P < 0.001$ for 6M-LP; $t_{51} = 3.33$, $P = 0.002$ for 1M5F-LP; $t_{50} = 5.58$, $P < 0.001$ for 6M-P; $t_{52} = 3.21$, $P = 0.002$ for 1M5F-P; $t_{54} = 5.15$, $P < 0.001$ for SM-LP) (Liu *et al.*, 2021, 2022). Furthermore, the apyrene : eupyrene ratio in lifetime ejaculates (6 : 1) (Fig. 2) was significantly higher than that (5 : 1) at emergence (Liu *et al.*, 2021, 2022) ($t_{50} = 7.74$, $P < 0.001$ for 6M-LP; $t_{51} = 5.99$, $P < 0.001$ for 1M5F-LP; $t_{50} = 6.03$, $P < 0.001$ for 6M-P;

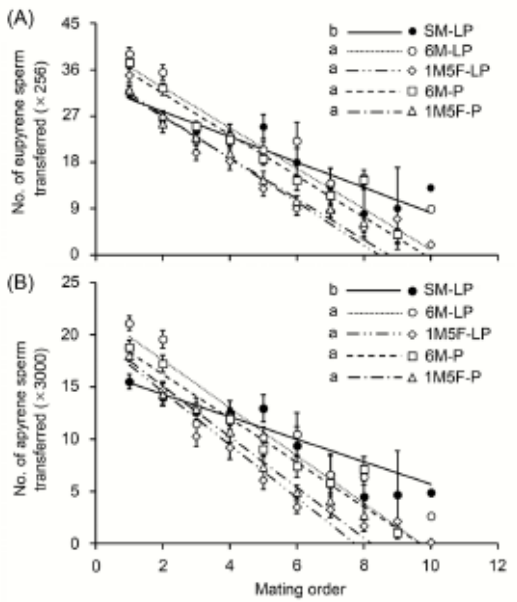

Fig. 4 Effects of preadult social environment on eupyrenes (A) and apyrenes (B) ejaculated by *E. kuehniella* males in relation to mating order (MO). Eupyrene: for SM-LP, sperm = 32.84 – 2.46 × MO ($F_{1,98}$ = 56.27, $P < 0.001$); for 6M-LP, sperm = 40.51 – 3.95 × MO ($F_{1,108}$ = 201.38, $P < 0.001$); for 1M5F-LP, sperm = 35.67 – 4.23 × MO ($F_{1,110}$ = 251.93, $P < 0.001$); for 6M-P, sperm = 39.49 – 4.02 × MO ($F_{1,113}$ = 218.17, $P < 0.001$); and for 1M5F-P, sperm = 34.89 – 4.00 × MO ($F_{1,117}$ = 194.98, $P < 0.001$). Apyrene: for SM-LP, sperm = 16.48 – 1.08 × MO ($F_{1,98}$ = 33.38, $P < 0.001$); for 6M-LP, sperm = 22.11 – 2.28 × MO ($F_{1,108}$ = 157.73, $P < 0.001$); for 1M5F-LP, sperm = 19.62 – 2.54 × MO ($F_{1,110}$ = 237.20, $P < 0.001$); for 6M-P, sperm = 20.45 – 2.11 × MO ($F_{1,113}$ = 194.50, $P < 0.001$); and for 1M5F-P, sperm = 19.91 – 2.42 × MO ($F_{1,117}$ = 186.22, $P < 0.001$). Points and vertical lines represent means and standard errors, respectively.

$t_{52} = 6.54$, $P < 0.001$ for 1M5F-P; $t_{54} = 3.80$, $P = 0.001$ for SM-LP).

Effect of socio-sexual environment during juvenile stages on mating frequency and longevity

Preadult sociosexual exposure had no significant effect on the number of matings adult males achieved in their lifetime (mean ± *SE* = 5.13 ± 0.39, 5.91 ± 0.43, 5.78 ± 0.37, 6.18 ± 0.37, and 5.92 ± 0.30 for SM-LP, 6M-LP, 1M5F-LP, 6M-P, and 1M5F-P, respectively) ($F_{4,48}$ = 1.38, $P = 0.255$). However, regardless of sex ratio, adult

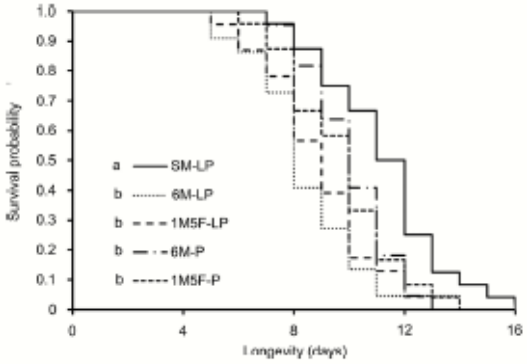

Fig. 5 Effects of preadult socio-sexual environment on adult male longevity in *E. kuehniella*. SM-LP, single male from fourth instar larva to adult emergence; 6M-LP, six males together from fourth instar larvae to adult emergence; 1M5F-LP, one male and five females together from fourth instar larvae to adult emergence; 6M-P, six male pupae together for the entire pupal stage until adult emergence; and 1M5F-P, one male and five females together for the entire pupal stage until adult emergence. Lines with different letters are significantly different ($P < 0.05$).

males that were exposed to conspecific individuals during larval–pupal stages or the pupal stage (6M-LP, 1M5F-LP, 6M-P, and 1M5F-P) lived significantly shorter than those that were reared singly (SM-LP) ($x^2_4 = 21.44$, $P < 0.001$), and all exposed males had similar longevity ($P > 0.05$) (Fig. 5).

Discussion

The present study shows that both juvenile stages of *E. kuehniella*, larvae and pupae, are sensitive to their socio-sexual environment and their experience affects lifetime sperm production and allocation and adult longevity but not eupyrene : apyrene ratio and mating frequency. We demonstrate for the first time that adult males developing from juveniles reared with juvenile rivals transferred significantly more eupyrenes (Fig. 2A) and apyrenes (Fig. 2B) in their lifetime than those from juveniles raised solitarily or with juvenile mates. Our findings provide strong evidence that the impact of sociosexual environment during juvenile stages continues throughout the adult stage. Furthermore, the sperm allocation patterns remained the same following exposure either from late instar larval to pupal stages or just during the pupal stage (Fig. 2). This suggests that the late juvenile stage is a critical period for building up the long-term memory of the preadult social environment in insects.

Using the same batch of moths reared under the same conditions as in Liu *et al*. (2021, 2022), we show that the number of sperm ejaculated in lifetime (Fig. 2) was significantly higher than that counted at emergence (Liu *et al*., 2021, 2022) in *E. kuehniella*. These findings suggest that the production of both eupyrene and apyrene sperm continues during the adult stage in lepidopterans, contradicting previous perceptions (Swallow & Wilkinson, 2002; Friedländer *et al*., 2005). The sperm ratio (apyrene : eupyrene) in lifetime ejaculates (Fig. 2) was also significantly higher than that at emergence (Liu *et al*., 2021, 2022), supporting previous findings that apyrenes are cheaper to produce than eupyrenes (Silberglied *et al*., 1984; Cook & Gage, 1995). Our study reveals that the sperm ratio in lifetime ejaculates remained the same regardless of treatments during juvenile stages (Fig. 3), suggesting that the sperm ratio in ejaculates is critical for reproductive success.

Similar to previous findings (e.g., Wedell & Cook, 1999; Velde *et al*., 2011; Esfandi *et al*., 2020; Liu *et al*., 2020), we show that the number of sperm ejaculated by males significantly decreased over successive matings (Fig. 4). These patterns fit the general prediction that males suffer from reduced quantity of their sperm with age (Dewsbury, 1982; Fricke & Maklakov, 2007; Vega-Trejo *et al*., 2019). However, the ejaculation of both eupyrene and apyrene sperm declined significantly faster over time in males whose juveniles were exposed to conspecific juveniles of any sex ratio than in those unexposed (Fig. 4). Higher sperm production before emergence in the exposed males (Liu *et al*., 2021, 2022) may exacerbate sperm senescence (Ball & Parker, 1996; Reinhardt, 2007; Pizzari *et al*., 2008) so that they are of greater urgency to expel the accumulated aged sperm in their reservoirs to gain reproductive fitness. This may result in ejaculation of more sperm in their first couple of matings, raising the starting points of the linear lines and leading to steeper slopes (Fig. 4).

Adult *E. kuehniella* males had significantly shorter longevity after their juveniles were exposed to conspecific juveniles of any sex ratio as compared to those whose young were individually reared (Fig. 5). Because most spermatogenesis occurs during juvenile stages (Friedländer *et al*., 2005; Liu *et al*., 2021, 2022) and sperm production entails significant costs (Dewsbury, 1982; Van Voorhies, 1992; Olsson *et al*., 1997; Pitnick *et al*., 2006; Hayward & Gillooly, 2011; Lemaître *et al*., 2020), we suggest that the increase of resource allocation to sperm production in the presence of conspecifics during juvenile stages (Liu *et al*., 2021, 2022) causes the early death of male adults. We show that males in different treatments achieved the same number of matings in their lifetime, suggesting that the number of matings is ultimately important for maximal reproductive fitness regardless of juvenile experience in *E. kuehniella* males.

In conclusion, the present study provides the first evidence that adult *E. kuehniella* males ejaculate significantly more eupyrene and apyrene sperm in their lifetime after exposure to rivals during the larval–pupal or pupal stage. In contrary to previous predictions for lepidopterans, we show that adults continue to produce sperm of both types. Despite different lifetime sperm allocations among treatments, the apyrene : eupyrene ratio remains 6 : 1, implying that the sperm ratio in ejaculates is critical for reproductive success. While both types of sperm ejaculated decrease over successive matings in all treatments, the rate of decrease is faster in males exposed to conspecifics during juvenile stages. This may result from the fact that the exposed males produce more sperm before emergence and ejaculate more in their first mating. Adults from juveniles exposed to conspecific juveniles of any sex ratio have shorter longevity probably because exposed juveniles allocate more resources to sperm production and trade off adult survival. Finally, all *E. kuehniella* males have similar number of matings in their lifetime regardless of whether their juveniles are exposed to conspecific juveniles or not. The knowledge generated here provides insight into adaptive resource allocation by males in response to sociosexual experience of different juvenile stages.

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Disclosure

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

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